there are hundreds of genes controlled by the circadian clock (Storch et al., 2002; Panda et al., 2002). Importantly, these clock-controlled genes (*ccgs*) and their temporal patterns are different among different tissues. Thus, it should be kept in mind that the time signals derived from the clock's loops are read differently by different cell types.

An important question is how the time information generated by the cell clock is transmitted to the *ccgs* that represent the output of the clock. Two routes are proposed: The 1st goes directly from the central loop of the mammalian clock to the *ccgs*. Clock control of vasopressin expression is an example of this route: Transcription of the vasopressin gene is activated by CLOCK-BMAL1 heterodimers and repressed by the mPER, mTIM, and mCRY proteins through an E-box in its promoter region (Jin et al., 1999). This type of clock gene regulation will provide genes that possess an E-box (CACGTG, CACGTT) with strong oscillatory characteristics. The 2nd route is indirect and regulates the antagonistic effects of PAR proteins (DBP, HLF, TEF) and E4BP4 (Mitsui et al., 2001) on the D-box (Fig. 1). In this system, PAR proteins activate the transcription of target genes by binding to the specific sequence RTTAYGTAAY (R, purine; Y, pyrimidine) during the day, and E4BP4 suppresses transcription of these target genes during the night (Mitsui et al., 2001). E4BP4 and the PAR proteins may switch back and forth in turning target gene transcription on and off (Fig. 1). In fact, it is known that by directly binding to their promoters, DBP activates the transcription of some genes in the liver, for example, albumin, cholesterol 7α hydroxylase, and cytochrome P450 (Lavery et al., 1999).

CELL CLOCK REGULATES THE TIMING OF MITOSIS IN LIVER REGENERATION

Since the life span of each cell is limited, cell growth and mitosis are required to maintain organ or tissue function. There is substantial evidence that circadian rhythms affect the timing of cell divisions in vivo. Day-night variations in both the mitotic index and DNA synthesis were found in oral mucosa (Bjarnason and Jordan, 2000), corneal epithelium (Scheving et al., 1974), and bone marrow (Smaaland, 1996). Since some of these mitotic rhythms were shown to persist in constant darkness, it was concluded that they might be under control of an endogenous clock. These studies used histochemical techniques and normal physiological conditions, so the mitotic cells composed only a few percentage of the cells examined. A suitable system with a high proportion of dividing cells is required to apply biochemical techniques to study the molecular links between regeneration and the circadian clock.

The liver provides a suitable organ since it is known to undergo vigorous regeneration after incomplete surgical removal. Although the cell cycle period overall is several months in unoperated animals, a 2/3 partial hepatectomy (PH) induces the large majority of the remaining, preexisting hepatocytes to divide, and the regeneration speed is so rapid that liver mass is restored within 7 days (Allison, 1986; Michalopoulos and DeFrances, 1997). Moreover, expression of *mPer1* and *mPer2* oscillates vigorously in the liver (Fig. 2A). *BMAL1* mRNA also cycles but in antiphase to the *mPers*, while *clock* mRNA levels stay flat (as they do in SCN). The temporal profiles and the vigorousness of the expression of clock genes were not altered after PH. Thus, the mouse liver is very suitable for analyzing the molecular connection between the circadian clock and the cell cycle.

Matsuo et al. (2003) compared the rate and timing of liver regrowth after PH in mice housed in a 12:12 LD cycle, when PH was performed in the morning at lights-on (ZT 0) or in the afternoon (ZT 8). S-phase kinetics, represented by the incorporation of bromodeoxyuridine, was similar in both ZT 0–operated and ZT 8–operated animals (Fig. 2B). However, there was an 8-h delay in the M-phase (cells entering mitosis) when PH was performed at ZT 0 (Fig. 2C), as compared to ZT 8. This indicated that the timing of the hepatectomy determines the timing of entry into M-phase for these regenerating cells. To determine the impact of circadian clocks on the cell cycle, *Cry1*$^{-/-}$ *Cry2*$^{-/-}$ mice, which have nonfunctioning clocks in all tissues, both central and peripheral, were subjected to PH (Okamura et al., 1999). In these mice, mitosis was severely impaired, and liver regeneration was severely blunted (Matsuo et al., 2003).

DNA arrays and Northern blots were used to characterize the molecular differences in M-phase entry. Of the 68 cell cycle–related genes, 11 showed a clear difference in expression between PH at ZT 8 compared to ZT 0, with 3 genes (*cyclin B1*, *cdc2*, and *wee1*) showing a remarkable difference (Matsuo et al., 2003). *Cyclin B1* and *cdc2* were positively correlated, and *wee1*, the gene for a kinase that inhibits mitosis by inactivating CDC2/cyclin B, was negatively correlated to

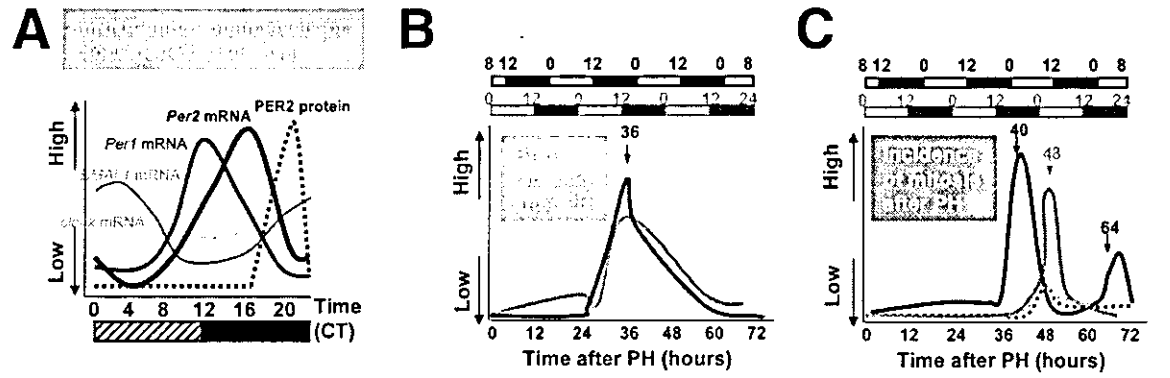

Figure 2. Expression of clock genes and regeneration in liver after partial hepatectomy (PH). (A) Schematic representation of the circadian expression profiles of *mPer1*, *mPer2*, *mClock*, and *mBMAL1* mRNA in the liver. (B) Time course of the number of hepatocytes that have incorporated bromodeoxyuridine (BrdU) after PH at ZT 8 (black) and at ZT 0 (gray). (C) Time course of the number of mitotic hepatocytes after PH at ZT 8 (black) and at ZT 0 (gray). Dotted line indicates the mitoses in *Cry*-deficient mice after PH at ZT 8 (black) and at ZT 0 (gray). (B) and (C) are redrawn from Matsuo et al. (2003), Copyright 2003 AAAS, Science 302:255-259.

M-phase. This is interesting since all these genes are cell cycle regulators governing G2 to M transition (see below), and these expression profiles correlate well with M-phase progression.

In the livers of normal mice, there were clear circadian rhythms of *wee1* expression, with very low levels at CT 0/CT 4 and high levels at CT 8/CT 12/CT 16 (Fig. 3A, left). WEE1 phosphorylates the cyclin-dependent kinase CDC2, a key regulator of G2 to M transition (Fig. 3B). To allow entry into mitosis, CDC2 has to be dephosphorylated by CDC25, a protein phosphatase, and it is the competition between the activities of WEE1 and CDC25 that determines the phosphorylation status of CDC2 (and hence the propensity of cells to enter mitosis). Regenerating hepatocytes display a strong circadian oscillation in WEE1 levels, as do normal liver cells. Only when WEE1 levels are low (normally in the morning) can CDC25 phosphatase successfully antagonize the action of WEE1.

Levels of *wee1* were always high in *Cry*-deficient mice, whereas levels were always low in *Clock* mutant mice (*Clock/Clock*). The normal or mutant expression profiles of *wee1* resembled those of genes such as *dbp* that are controlled by the clock via E-box elements. Indeed, there are 3 E-boxes (CACGTG) in the 5'-upstream region of *wee1* that were, furthermore, activated by CLOCK/BMAL1 and suppressed by PER2, PER3, CRY1, and CRY2. When these E-box regions in *wee1* were mutated, stimulation of transcription by CLOCK/BMAL1 was decreased (Fig. 3A, right). These results suggest that *wee1* transcription is regulated directly by the core feedback loop through its E-box elements. Changes in transcription of *wee1* are reflected at the protein level, influence CDC2 activity levels, and are negatively correlated with the mitotic peak (Matsuo et al., 2003). Thus, in *Cry*-deficient mice, *wee1* mRNA, WEE1 protein levels, WEE1 kinase activity, and p-CDC2(Tyr15) were all very high after PH, which accounts for the low activity of the CDC2 kinase in *Cry*-deficient mice.

These findings, taken together, demonstrate that the circadian clock controls the G2 to S transition via the regulation of WEE1 (Fig. 3B). A recent work on zebra fish development and zebra fish cell proliferation in vitro (Dekens et al., 2003) also showed that the circadian clock strongly influences the timing of DNA replication (S-phase). A high incidence of lymphoma in arrhythmic mice lacking the PER2 protein may shed light on the association of circadian rhythms and cell growth from another perspective (Fu et al., 2002). Perhaps the inactivation of *mPer2* leads to derepression of *Myc* expression, resulting in uncontrolled cell growth and tumor formation.

EVOLUTIONARY LINK BETWEEN CIRCADIAN CLOCK AND CELL CYCLE

Why do genes crucial to the cell cycle need to couple with the circadian clock? The cell cycle is surely essential for all organisms, and circadian rhythms have been found in all eukaryotes and some prokaryotes. Since both kinds of cycle have long histories and

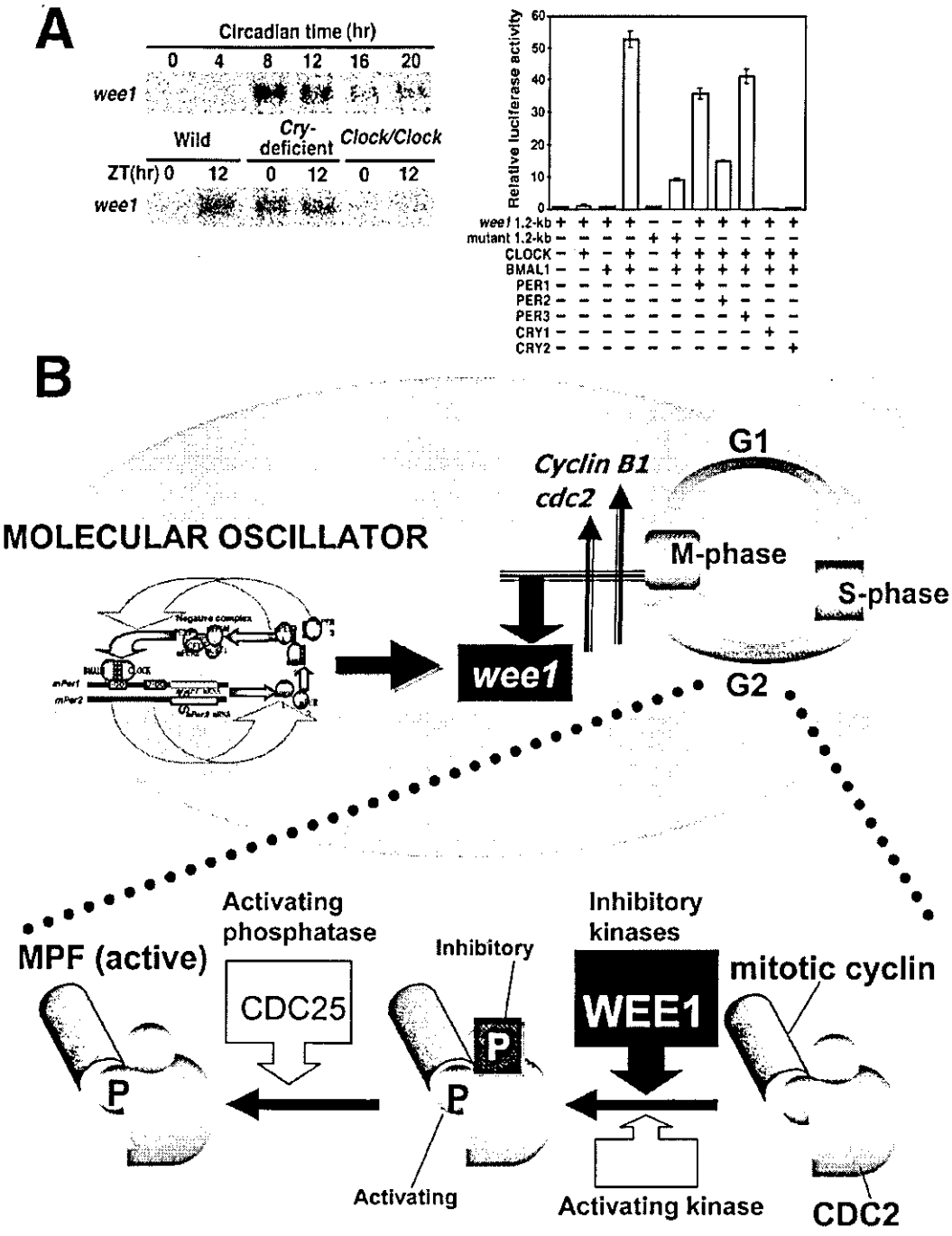

Figure 3. Control of cell cycle by cell clock. (A) Left: Circadian fluctuation of *wee1* transcripts in wild-type mice (upper). Note the steady-state increase in *Cry*-deficient mice and the decrease in *Clock/Clock* mice (lower). Right: Transcriptional regulation of the mouse *wee1* gene by clock genes. Reporter plasmids containing the 1.2-kb mouse *wee1* 5′-upstream region, including the 3 E-boxes (*wee1* 1.2-kb) or mutated E-boxes (all 3 E-boxes were mutated to 5′-CTGCAG-3′; mutant 1.2 kb), were used for the transcriptional assay. Presence (+) or absence (–) of the reporter and expression plasmids is shown. Each value represents the mean ± SEM. (B) Schematic representation showing the link between the circadian clock and the cell cycle (upper). Role of CDC2, mitotic cyclin, WEE1, and CDC25 in making active mitosis promoting factor (MPF) is depicted at the bottom. (A) is from Matsuo et al. (2003).

are linked, it may be worth speculating on their possible evolutionary relationship (see also Tauber et al., 2004 [this issue]). Pittendrigh (1966) suggested early on that the LD cycle induced by the rotation of the earth was the "historical cause (selective agent) of circadian oscillations" and that these oscillations were an adaptation for dealing with the noxious effects of light on gene induction and replication. Paietta (1982) hypothesized that oxygen radicals produced by the photooxidative process are the noxious agents. He speculated that organisms developed protective pigments, such as the carotenoids, to shield themselves from these photooxidative effects and/or concentrated and segregated the cellular processes that might be hindered by light period to the dark period. By the time eukaryotes developed, phasing of cell cycle processes to avoid light-induced hazards may have locked in the association of circadian clock genes and cell cycle genes.

However, about 2 to 3 billion years have now passed since the formation of the magnetic field around the earth, and about 1 billion years have now passed since the formation of ozone layers and the accumulation of oxygen to the present concentration in the air. Multicellular animals have evolved for at least 0.6 billion years under relatively reduced exposure to ultraviolet and other hazardous, high-energy solar rays. The various pigments that have developed have probably also reduced the hazard of photooxidative processes stimulated by solar light. These developments might have relieved evolutionary pressure to protect the S-phase (DNA replication) from high-energy environmental light, so that the S-phase need no longer be linked to the circadian clock in mammals. One can imagine, however, that the process of cell division (M-phase), the most complex and thus the most energy-consuming process of the cell cycle, must still be performed when the internal environment of the body is optimal.

CLOCK GENES IN DIFFERENTIATING TISSUE

If the cell cycle is intimately linked to the circadian clock, then it should be interesting to examine clock genes in rapidly replicating and differentiating tissues such as gonads. Insect reproductive tissue displays cyclic production of core circadian clock proteins, and the clock controls sperm release (Giebultowicz and Riemann, 1990). Moreover, clock gene mutants of *Drosophila* displayed reduced fertility (Beaver et al., 2002). In mammals, testis is the organ with the highest expression level of *mPer1*, and so a strong circadian influence on spermatogenesis might be expected. Two groups (Morse et al., 2003; Alvarez et al., 2003) upset this expectation when they found that *mPer1* and *Clock* genes in mice testis are expressed constantly, with *mPer1* mRNA and mPER1 protein levels staying high and *Clock* mRNA and CLOCK protein levels staying low. On the other hand, Morse et al. (2003) have reported that expression of *mPer1* mRNA is developmentally regulated; it is expressed only in stage VII-IX spermatids. *Clock* transcripts are also developmentally regulated but are primarily found in spermatogonia and early-stage spermatocytes. Thus, *Clock* expression occurs at an earlier stage in spermatogenesis than does *mPer1* expression.

If CLOCK is usually so low, how does mPER1 reach such high levels? The finding that *mPer1* knockout mice do not show any change in spermatogenesis (Morse et al., 2003) strongly supports the idea that the large amount of *mPer1* expression in testis is not required for clock functions—indeed, that it may be "accidental" and may not have any specific consequences. Morse et al. (2003) speculated that *mPer* is kept high by a nonclock mechanism: induction by CREM activation of the CRE site on the *mPer1* promoter. In contrast to *mPer1*, *mPer2* is unusually low in the testis (Takumi et al., 1998). This finding supports the idea of a nonclock role for *mPer2* as well as *mPer1* since, in most tissues, *mPer1* and *mPer2* levels vary together, cycling in phase with each other. Why is it, then, that circadian clock genes in testis, which is the most proliferative organ in the body, do not link to spermatogenesis? Alvarez et al. (2003) proposed, based on the observation that clock gene expression is flat in 2 organs (testis and thymus) whose cells are constantly differentiating, that the circadian cycle is suspended while cells are undergoing differentiation.

Then what is happening in other rapidly growing tissues? During somite genesis in mouse embryos, it is known that a pair of somites buds off from the presomitic mesoderm every 2 h, suggesting that somite segmentation is controlled by a biological clock with a 2-h cycle. The novel bHLH *Hes* genes show cyclic expression (every 2 h) in the somite-forming process (Hirata et al., 2004). This cycling is cell autonomous and depends on negative autoregulation of *Hes* transcription and ubiquitin/proteasome–mediated deg-

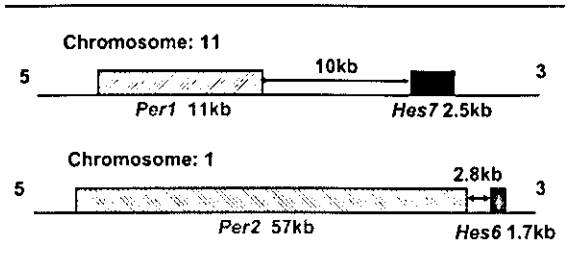

Figure 4. Gene alignment of *mPer1* and *Hes7* and of *mPer2* and *Hes6*. Note *Hes7* locates 10 kb downstream of *Per1* in mouse chromosome 11, and *Hes6* locates 2.8 kb downstream of *Per2* in mouse chromosome 1.

radation of HES proteins (Hirata et al., 2002). The somite clock *Hes7* gene is located just downstream of *mPer1* gene (Bessho et al., 2003; Sakaki, 2004). Interestingly, the *Hes6* and *mPer2* genes are also close to each other, only 2.8 kb apart (Fig. 4). In the developing brain, it is known that neuronal stem cells express *Hes1* and *Hes6* genes abundantly in the proliferating and differentiating stages, but their expressions were faint after maturation of neurons. In contrast, *Per1* and *Per2* genes are expressed after completion of neurogenesis and increased as the neurons grew (Sladek et al., 2004).

One speculative explanation for such segregation of gene expression involves genome-segregating DNA elements. In the study of the chicken β-globulin gene, insulator DNA elements were found to protect the transcribed region from outside regulatory influences (Recillas-Targa et al., 2002). These elements are present near chromatin domain boundaries or at sites where they prevent inappropriate activation of a promoter by a nearby heterogeneous enhancer. In another example, the DNA-binding protein CTCF, which acts on a chromatin "insulator," regulates imprinting of the mammalian Igf2 and H19 genes in a methylation-sensitive manner (Bell and Felsenfeld, 2000; Hark et al., 2000). If such genome-segregating elements exist between the *Per* and *Hes* genes, one could imagine that the "24-h slow" circadian clock is switched off in developing tissue, allowing the "2-h rapid" clock to work. After morphogenesis, the 2-h rapid clock involving *Hes* is switched off, and the 24-h slow circadian clock involving *mPer* genes is switched on to adapt to the day-night environmental cycle. This scheme might apply to other rapidly differentiating tissues, although the expression dynamics of the *Hes* genes are not known for most organs. The silencing of *Per* genes in differentiating tissue could explain the enigma of why *wee1*-mediated G2 to M gating is not essential for coordinating cell division. Also, it would explain why embryogenesis seem to be perfectly normal in clockless mutant mice.

INTERACTIONS BETWEEN SCN AND PERIPHERAL CELL CLOCKS

Results of gene array studies suggest that a number of fundamental cell functions are controlled by the core circadian oscillator, a cell clock. Such studies cannot tell us whether the signals generated by the cell clock are transmitted to other cells or to other organs, or are restricted to the cells in which they are made. In *Drosophila* and zebra fish, most peripheral cells that are oscillating have the capacity to oscillate independently, and they are all entrained directly by light (Stanewsky et al., 1998; Whitmore et al., 2000). Independent mammalian clock gene oscillations have been observed in a variety of organs, although peripheral clocks are not entrained directly by light (Yamazaki et al., 1999). Entrainment signals for mammalian peripheral clocks are not fully understood, but it is known that liver is phase reset by restricted feeding (Damiola et al., 2000). This feeding-related entrainment is strong and is observed in SCN-lesioned animals, which indicates that it is independent of the central clock (Hara et al., 2001).

We will first address whether signals are transmitted from cell to cell in the SCN and then to the periphery. Yamaguchi et al. (2003) monitored the oscillation of cellular *mPer1* gene expression in slice cultures of the SCN taken from transgenic mice carrying the luciferase gene driven by the *mPer1* promoter. These SCN cells showed robust transcription rhythms with a period length of ~24 h, with several hundreds of cells expressing *mPer1* genes synchronously. Moreover, the individual oscillatory cells are arranged topographically: The phase leader with a shorter period length is located in the dorsomedial periventricular part of the SCN. A protein synthesis inhibitor (cycloheximide) sets all the cell clocks to the same phase, and following withdrawal, intrinsic interactions among cell clocks reestablish the stable program of gene expression across the assemblage. Tetrodotoxin, which blocks action potentials, not only desynchronizes the cell population but also suppresses the level of clock gene expression, demonstrating that neuronal network properties dependent on action potentials play a dominant role in both establishing cellular synchrony and

maintaining spontaneous oscillations across the SCN. Thus, the cell-rhythm oscillation generated by the core clock oscillatory loop is coupled and amplified by the ordered cell-cell communications in the SCN.

In view of the intercellular communication of cell clocks in the SCN, peripheral tissues were checked for synchrony among neighboring cells. Using *mPer1*-promoter-luciferase transgenic mice, Yamazaki et al. (2000) recorded rhythmic luciferase activity in peripheral organs (lung, liver, etc.) for only a few cycles. In later experiments using *mPer2*-luciferase knock-in animals, instead of *mPer1*, sustained rhythms in various peripheral tissues were observed for more than 20 cycles in culture (Yoo et al., 2004), a duration similar to those obtained with SCN (Yamazaki et al., 2000; Asai et al., 2001). The existence of these sum rhythms might indicate the existence of synchronizing mechanisms in peripheral tissue. Absent obvious synaptic or neurohumoral connections, the mechanisms of cell clock communication within peripheral tissues are completely unknown at present. One possibility is that the development of gap junctions among hepatic cells, or among the smooth muscle cells, might be related to clock cell synchronization.

The dominance of the SCN clock over peripheral oscillators was demonstrated by Pando et al. (2002) using grafts from animals with genetically altered period lengths. When embryonic fibroblasts from *Per1*$^{-/-}$ mice, which can cycle but have a much shorter period than do wild-type cells, were implanted into wild-type mice, the fibroblasts took on the period length of the host. When embryonic fibroblasts of the severely arrhythmic *Clock*(c/c) genotype were used, however, the transplant did not take up the host's rhythm, suggesting that the central clock is unable to drive clock-defective peripheral cells. Based on their cultured ex vivo studies, Yoo et al. (2004) strongly supported this view and suggested that the SCN is not the body's rhythm generator and driver but is the body's rhythm synchronizer, at least for these peripheral clock rhythms. The relationship between SCN and non-SCN clocks at the behavioral level, however, differs markedly. In contrast to results from the in vitro studies using peripheral tissues or embryonic fibroblasts, Sujino et al. (2003) reported, astonishingly, that SCN grafts from wild-type mice into mice that were "doubly arrhythmic," due to being both SCN lesioned and *Cry1/Cry2* double-knockout mice, restored circadian locomotor activities. This rhythm emergence strongly suggests that non-SCN peripheral clocks are unnecessary for generation and expression of behavioral rhythms and that SCN is the rhythm generator for brain rhythms.

Transplantation studies have shown that grafts from the cerebral cortex cannot restore rhythms to SCN-ablated animals (Sujino et al., 2003). Destruction of the SCN abolishes rhythms in clock gene expression in the liver in vivo (Sakamoto et al., 1998). The SCN is unique in having strong clock gene oscillations, with strong outputs linking to hypothalamic autonomic nuclei, which, in turn, connect to peripheral autonomic systems and the adrenal gland (Buijs and Kalsbeek, 2001; Balsalobre et al., 2000; Terazono et al., 2003). Thus, the mammalian circadian system displays a complex hierarchical structure of cell clocks in various tissues, with the SCN cell clocks at the top (Fig. 5).

What about the ability of peripheral cell clocks to influence other organs? It appears that if the ability exists, it will be mediated by secretory substances such as hormones. It is well known that glucocorticoid is secreted from the adrenal gland in circadian fashion. Since there is a clear circadian rhythm of *Per1* and *Per2* expression in this organ, it would appear that there are cell clocks in the adrenal cells. Recently, Balsalobre et al. (2000) reported that the glucocorticoid hormone analog dexamethasone, known to induce circadian gene expression in cultured rat-1 fibroblasts, can induce phase shifts in the rhythms of circadian gene expression in the liver, kidney, and heart. Thus, glucocorticoids are a candidate for an output of "adrenal cell clocks" that can govern the cell clocks in other organs. However, the rhythm of glucocorticoid secretion itself is strictly regulated by the SCN, since lesioning the SCN abolished the rhythm of glucocorticoid secretion (Moore and Eichler, 1972). Current thought predicts that the SCN entrains the peripheral clock that generates the circadian rhythm of glucocorticoid secretion, as it does other rhythms in peripheral organs, but it may still turn out that the SCN drives the glucocorticoid secretion rhythm without any intervening peripheral clocks. Future studies, using tissue-specific conditional knockouts of the cell clocks in peripheral organs, will clarify the specific roles of cell clocks in these organs and the effects of hormones from one organ on clock function in other organs. Moreover, they will most likely determine whether the SCN is the body's rhythm generator and driver, the body's rhythm synchronizer, or both, depending on the tissue.

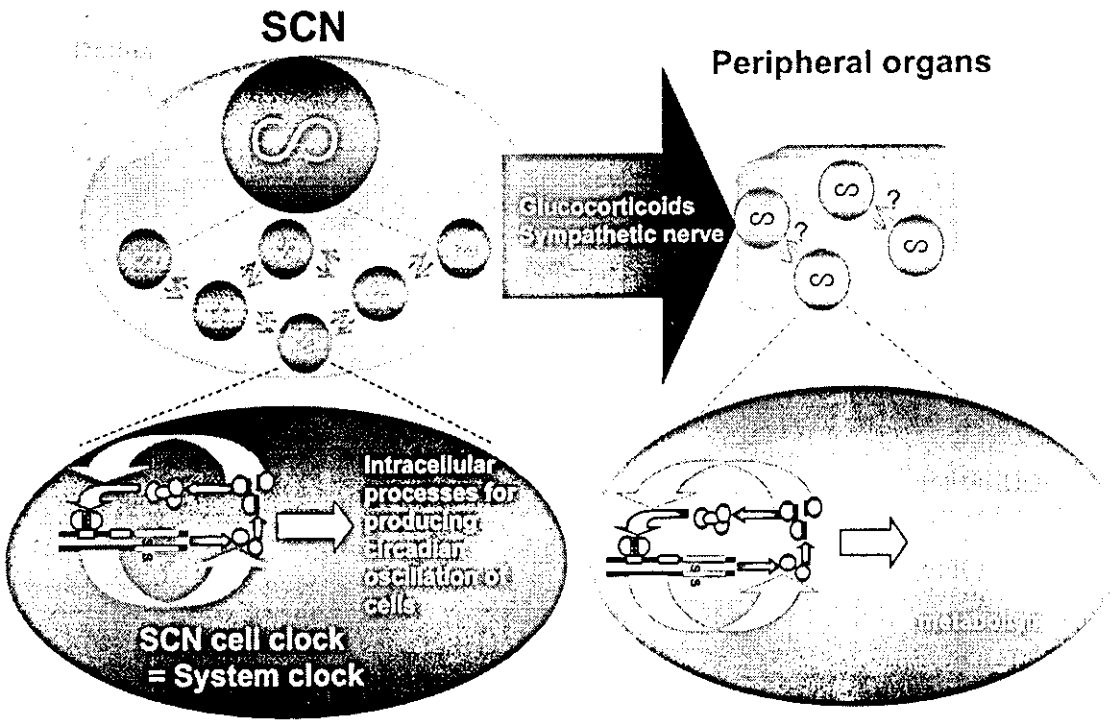

Figure 5. Molecular core oscillator and cell clocks in the SCN and peripheral organs. The molecular oscillator is common to both SCN and peripheral clocks. The SCN clock is the only clock known (with confidence) to regulate other clocks in other organs at the system level. Outputs of SCN regulate behavioral rhythms via non-SCN brain clocks and peripheral clocks via feeding activity, glucocorticoids, and the autonomic nervous system. In peripheral organs, a variety of cell clocks oscillate to regulate cellular events.

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A Missense Variation in Human *Casein Kinase I Epsilon* Gene that Induces Functional Alteration and Shows an Inverse Association with Circadian Rhythm Sleep Disorders

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Recent studies have shown that functional variations in clock genes, which generate circadian rhythms through interactive positive/negative feedback loops, contribute to the development of circadian rhythm sleep disorders in humans. Another potential candidate for rhythm disorder susceptibility is casein kinase I epsilon (CKIε), which phosphorylates clock proteins and plays a pivotal role in the circadian clock. To determine whether variations in *CKIε* induce vulnerability to human circadian rhythm sleep disorders, such as delayed sleep phase syndrome (DSPS) and non-24-h sleep–wake syndrome (N-24), we analyzed all of the coding exons of the human *CKIε* gene. One of the variants identified encoded an amino-acid substitution S408N, eliminating one of the putative autophosphorylation sites in the carboxyl-terminal extension of CKIε. The N408 allele was less common in both DSPS ($p=0.028$) and N-24 patients ($p=0.035$) compared to controls. When DSPS and N-24 subjects were combined, based on an *a priori* prediction of a common mechanism underlying both DSPS and N-24, the inverse association between the N408 allele and rhythm disorders was highly significant ($p=0.0067$, odds ratio = 0.42, 95% confidence interval: 0.22–0.79). *In vitro* kinase assay revealed that CKIε with the S408N variation was ~1.8-fold more active than wild-type CKIε. These results indicate that the N408 allele in *CKIε* plays a protective role in the development of DSPS and N-24 through alteration of the enzyme activity.
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INTRODUCTION

In mammals, including humans, circadian cycles of approximately 24 h are observed in behavior and physiology, including cycles of sleep, hormone secretion, and core body temperature. The master circadian pacemaker is localized in the hypothalamic suprachiasmatic nucleus (SCN). Clock genes, *Per1/2/3*, *Cry1/2*, *Bmal1*, and *CLOCK* are expressed in the SCN and produce a nearly 24 h cycle through interacting positive/negative feedback loops (Harmer *et al*, 2001; Reppert and Weaver, 2002). BMAL1 and CLOCK proteins bind to E-box elements and activate transcription of *Per* and *Cry* genes. As the PERs and CRYs are translated, they enter the nucleus and inhibit BMAL1/CLOCK-driven transcription in the negative feedback loop. The circadian pacemaker is synchronized (entrained) to the 24 h day, primarily by the environmental light/dark cycle.

Certain human sleep disorders, designated circadian rhythm sleep disorders, are attributed to the disruption of the circadian timing system (Weitzman *et al*, 1981; Campbell *et al*, 1999; Wijnen *et al*, 2002). Patients with circadian

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rhythm sleep disorders, such as delayed sleep phase syndrome (DSPS), advanced sleep phase syndrome (ASPS), and non-24-h sleep–wake syndrome (N-24), fail to adjust their sleep/wake cycle to the daily schedule required for social life. Despite normal sleep architecture, sleep onset and offset are persistently delayed (DSPS) or advanced (ASPS) compared to the societal norm. N-24 patients suffer from daily delays of sleep onset and offset times, with the consequence of progressive cycling through the 24 h environmental day. The pathogenesis of DSPS and N-24 is not yet known, but several possible mechanisms have been proposed: reduced sensitivity of the oscillator to photic entrainment, a prolonged intrinsic period beyond the range of entrainment to 24 h day, and abnormal coupling of the sleep/wake cycle to the circadian rhythm (Weitzman *et al*, 1981; Campbell *et al*, 1999; Uchiyama *et al*, 2000). It is estimated that 0.13% (in Japan) (Yazaki *et al*, 1999), 0.17% (in Norway) (Schrader *et al*, 1993), and 0.7% (in USA) (Ando *et al*, 1995) of the general population suffer from DSPS, while the prevalence of N-24 is lower. Genetic factors reportedly confer predisposition to ASPS and DSPS (Ancoli-Israel *et al*, 2001; Jones *et al*, 1999; Reid *et al*, 2001).

Analysis of animals and humans with altered circadian rhythms demonstrated that casein kinase I epsilon (CKIε) (and presumably its most closely related homolog, CKIδ) plays a crucial role in regulating the circadian pacemaker (Eide and Virshup, 2001). CKIε (and CKI δ) phosphorylates PER proteins, leading to their destabilization and relocalization (Takano *et al*, 2000; Vielhaber *et al*, 2000; Keesler *et al*, 2000; Akashi *et al*, 2002; Camacho *et al*, 2001). CKIε/δ have long carboxyl-terminal (C-terminal) extensions, which can be autophosphorylated, with the consequence of autoinhibition of kinase activity (Graves and Roach, 1995; Cegielska *et al*, 1998). *Double-time* (*dbt*) gene, a Drosophila homolog of mammalian *CKIε*, was shown to alter or ablate circadian rhythm when functionally mutated (Price *et al*, 1998). In hamsters, a point mutation in *CKIε* that decreases kinase activity causes the semidominant short-period *tau* phenotype (Ralph and Menaker, 1988; Lowrey *et al*, 2000). A recent report showed that, in humans, familial ASPS can be induced by a *Per2* S662G mutation, which reduces CKIε-induced phosphorylation of the PER2 protein (Toh *et al*, 2001). We have reported that a *Per3* gene haplotype, in which one of the variations lies close to the CKIε target site and presumably alters PER3 protein phosphorylation, is significantly associated with DSPS (Ebisawa *et al*, 2001). These results suggest the possibility that human *CKIε* (h*CKIε*) gene may also be involved in susceptibility to circadian rhythm sleep disorders.

Accordingly, we set out to screen the complete coding region of the *CKIε* gene, as well as adjacent exon–intron boundaries for the presence of genetic variants in circadian rhythm sleep disorder patients and controls.

MATERIALS AND METHODS

Subjects

In all, 98 DSPS patients (60 males; 38 females; mean age: 27.1 ± 9.1 years) and 39 N-24 patients (29 males; 10 females; mean age: 26.9 ± 8.4 years) were recruited. Diagnosis was assigned by a trained psychiatrist according to the International Classification of Sleep Disorders (ICSD1990) criteria. All of the patients were unrelated, except for two sibling pairs, of which each consisted of a patient with DSPS and a patient with N-24. In a combined analysis of DSPS and N-24, two of the DSPS subjects with siblings of N-24 were excluded from the DSPS/N-24 group to avoid an increase in the Type I error rate. Neither of the sibling pairs carried the S408N variation. Another three patients with DSPS had relatives with probable DSPS, who were not involved in this study, and another patient with N-24 had a first-degree relative with severe insomnia. In all, 138 healthy subjects were recruited as controls (81 males; 57 females; mean age: 32.1 ± 8.6 years). Control individuals were free from sleep disorders or psychoses. All of the study subjects were sighted. In total, 59 DSPS patients, 36 N-24 patients, and 107 control subjects of the study population were reported previously (Iwase *et al*, 2002), while the others were newly recruited for this study. In order to minimize the effect of the population stratification, which may cause false results, all of the study subjects were Japanese and recruited in mainland Japan. The controls were geographically matched to the patients. Written informed consent was obtained from the subjects. The protocol was approved by the ethics committee of Saitama Medical School and the participating institutes.

Blood samples were drawn by venipuncture and genomic DNAs were prepared from leukocytes using QIAamp DNA Blood Maxi Kit or QIAGEN Blood & Cell Culture DNA Midi Kit (QIAGEN, Hilden, Germany).

DNA Analysis

Polymerase chain reaction/single-strand conformation polymorphism (PCR-SSCP) analysis was used to screen for variations in all coding exons of the *CKIε* gene. Fluorescein-labelled primers to amplify each of the coding exons and adjacent exon–intron junctions were derived from the genomic structure determined by alignment of the cDNA and genomic sequence of hCKIε (AB024597 and AL020993, respectively) (Table 1). PCR was performed in a total volume of 50 μl containing 100 ng DNA, 0.5 μM of each primer, 1 × PCR buffer II, 0.2 mM dNTPs, 1.5 mM Mg^{2+}, and 1.25 U of AmpliTaq Gold DNA Polymerase. Conditions for PCR were preincubation at 95°C for 9 min to denature the DNA and to activate the polymerase, followed by 45 cycles at 95°C for 20 s, 63–68°C for 45 s, and 72°C for 1 min, with a subsequent final extension step at 72°C for 10 min.

SSCP electrophoresis was carried out on a denaturing gel in a DSQ-500S DNA sequencer (Shimadzu, Kyoto, Japan) basically as described (Ebisawa *et al*, 2001). Briefly, 1 μl of PCR products were mixed with 19 μl of formamide buffer (90% formamide, 5 mM EDTA, 10 mg/ml Blue dextran), heated at 80°C for 7 min, and 1.5 μl of the sample mixture was electrophoresed on a 0 or 5% glycerol SSCP Gel at 20°C, according to the manufacturer's protocol. Genomic DNAs in which variants were detected by SSCP were amplified using primers that encompass the SSCP-amplified region, and purified using QIAquick PCR Purification Kit (QIAGEN). Sequence reactions were performed on both strands using internal primers and the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) according to the protocol of the

Table 1 Primers Used for SSCP Analysis of the Human *CKIε* Gene

Exon number	Primer name	Sequence (5′–3′)	Fragment size	Annealing temperature (°C)
1	1F	CCA CGT CGC TGA CCC TCA TGT TCC	234	68
	1R	GCC CCT GGA GCC ACA TTC TGA CTT C		
2	2F	CAC ACG CCA GAT CTC AGA AAT GCT TAG TGG	266	63
	2R	CTG TGC TCA TGG CTG CCC ACC G		
3	3F	CTG CCT GCC TCT GAC CCC TGA C	264	63
	3R	GGC AGG AGG CAG GGC TGG TAT C		
4	4F	CTG CCT GGC CCA GAG TGC TAG GCA AG	335	68
	4R	AGT GGC CCC GGG TGC ACA CTG C		
5	5F	CCC AGA GGA TGA GTT AGG GGC CTG AGT G	306	68
	5R	GCC TCA CCT TTC CCT TAG ACA GTG CCT C		
6	6F	GTG GCT AGG ACA GTG CTG GCT GCA G	310	68
	6R	CCA GCT CAC TCT GGC CCT CTG AGT C		
7	7F	CTG GCC TCT GGG GCT GAC TGG TG	271	68
	7R	CTG AAC CCA GCC CAC TGC CTG AGT C		
8	8F	GAC TCA GGC AGT GGG CTG GGT TCA G	267	63
	8R	CTC AGT TCT GAG GCC CAG AGG GAC TG		
9	9F	ATC GCC AGC GGC TAA GGG ACT TGA C	241	63
	9R	CCC ACC CCT CCA CAA CAC ATT GGT C		

F or R in the primer names indicate the forward or reverse orientation of each primer.

manufacturer, and detected by an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). One of the PCR-amplified fragments in which a deletion was detected by direct sequence analysis was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), and multiple isolates were sequenced on both strands. To determine the frequency of the S408N variant, all of the samples were amplified by PCR using 9F and 9R primers in Table 1 and subjected to either SSCP and/or denaturing high-performance liquid chromatography (DHPLC) analysis, followed by sequencing reactions as described above.

For DHPLC analysis, PCR products were denatured at 98°C for 30 s and 95°C for 7 min, followed by gradual reannealing from 95 to 15°C over 40 min. The crude PCR products (5–7 μl) were then injected into a DNASep column and separated through a 13.5–15.75% acetonitrile gradient at 61°C using a WAVE DNA Fragment Analysis System (Transgenomic, Omaha, NE, USA).

Purification of Recombinant Proteins

The partial cDNAs encoding mouse PER1 (mPER1) (amino acids 547–799), rat PER2 (rPER2) (486–793), and mouse PER3 (mPER3) (367–880) fragments, which correspond to the CKIε-binding regions (Takano, A *et al*, unpublished observation), were subcloned into pGEX4T-3 or pGEX6P-1 vector (Pharmacia, Peapack, NJ, USA) for the production of glutathione-*S*-transferase (GST)-fused recombinant proteins. The partial fragments of PERs were used for *in vitro* kinase assay, because it is practically impossible to obtain enough amount of intact full-length PER proteins due to their instability when expressed in *Escherichia coli*. The PER fragments we used correspond to the CKIε-binding domains which contain the phosphorylation sites; therefore, they can be properly used for *in vitro* kinase assay of CKIεs to compare the kinase activity against PERs.

The S408N substitution was introduced into the rat CKIε (rCKIε) cDNA by site-directed mutagenesis using PCR, generating CKIε-S408N. The amino-acid sequence of rCKIε is identical to that of hCKIε, except for two amino acids. Neither of the two amino acids is a phosphoacceptor residue. The expression constructs encoding GST-fused wild-type rCKIε (GST-CKIε-WT) and CKIε-S408N (GST-CKIε-S408N) were prepared using pGEX4T-3 (for α-casein) or pGEX6P-1 (for GST-PERs) vector. *Escherichia coli* (*E. coli*) strain BL21 (DE3) was transformed with the expression plasmids and the fusion proteins expressed were purified with glutathione sepharose 4B (Pharmacia) according to the manufacturer's protocol. GST-CKIε proteins were easily degraded, therefore, for the use in kinetic analysis

against α-casein, the fusion proteins were further purified by immunoprecipitation with the specific antibody against the C-terminal end of rCKIε to remove the contamination of partially degraded recombinant rCKIε (Takano *et al*, 2000). To perform *in vitro* kinase assay using GST-fused PER fragments as substrates, GST tag was removed from GST-CKIε using PreScission protease (Amersham) to discriminate phosphorylated GST-PERs and autophosphorylated rCKIε on electrophoretic mobility.

In Vitro Kinase Assay and Kinetic Analysis

Kinase reactions were performed in buffer containing 45 mM Tris-HCl, pH 7.4, 9 mM $MgCl_2$, 0.9 mM β-mercaptoethanol, 40 μM ATP, 74 kBq of [γ-^{32}P], kinase and α-casein or GST-PER in a final volume of 20 μl. Approximately 40 ng of the immunoprecipitated GST-CKIε (for α-casein), 2 pmol of rCKIε (for GST-mPER1 and GST-rPER2), or 10 pmol of rCKIε (for GST-mPER3) was added to the reaction mixture. Varying concentrations of α-casein (0–100 μM), or 20 pmol of GST-mPER1, GST-rPER2, or GST-mPER3 protein, was used as a substrate. The amount of rCKIε, GST-CKIε, or GST-PER used in each reaction was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie brilliant blue staining using bovine serum albumin as a standard, revealing that the difference in the amount of rCKIε or GST-CKIε in each experiment was smaller than 7.3% of the wild type. The kinase reactions for α-casein were allowed to proceed at 37°C for 10 min, because the enzyme activity was linear with time for up to 20 min (data not shown). Reactions were terminated by addition of 20 μl SDS-PAGE sample buffer. A part of the reaction mixture was subjected to electrophoresis on 12% (for α-casein) or 7.5% (for GST-PERs) polyacrylamide gels, and [^{32}P] incorporation into the substrates was determined by a BAS-2000 image analyzer. When α-casein was used as a substrate, the data were presented as a double-reciprocal plot and V_{max} and K_m were obtained using computer software (Kaleida Graph, Abelbeck Software).

Statistical Analysis

Departure from Hardy–Weinberg equilibrium was tested using a χ^2 goodness-of-fit test. The allele and genotype frequencies were compared by means of Fisher's exact test. All *p*-values reported are two-tailed. Correction for multiple testing for the analyses in the previous studies was not performed since a considerable number of subjects were newly recruited for this study, which was conducted with a pre-established hypothesis (Perneger, 1998). Unpaired *t*-test was performed to compare the amounts of incorporated [^{32}P] into GST-PER by CKIε-WT and CKIε-S408N.

RESULTS

Using PCR-SSCP and subsequent sequencing of the PCR-amplified fragments, all of the coding exons and flanking exon–intron boundaries of the *CKIε* gene were screened for sequence variations. In an initial screen of 35 genomic DNA samples (17 of DSPS and 18 of N-24), three sequence variants were identified (Table 2). One single-nucleotide variation (51C>T) was located in exon 1, another variation (1223G>A) in exon 9, and one intronic deletion of 4 bp (77-63_77-60delGGCG) resided upstream of exon 2. The 1223G>A exonic variation predicted an amino-acid substitution, S408N. S408 is located in the C-terminal extension of the CKIε and is conserved in CKIεs of humans, hamsters, mice, rats, and *Xenopus laevis*, as well as in CKIδs of humans and rats. Previous studies demonstrated that the C-terminal extensions of mammalian CKIε (and CKIδ) can be autophosphorylated, inhibiting the kinase activity (Graves and Roach, 1995; Cegielska *et al*, 1998), and that S408 is one of the putative phosphoacceptor residues (Gietzen and Virshup, 1999). Therefore, the S408N variation is likely to eliminate one of the autophosphorylation sites, resulting in decreased autophosphorylation and increased enzyme activity. The 51C>T exonic variation resulted in synonymous substitution. Neither the 51C>T variation nor the intronic deletion (77-63_77-60delGGCG) appeared to affect known splice sites or to create better splice donor/acceptor consensus sequences, based on visual examination of the sequence context, so functional alterations appeared unlikely (Burset *et al*, 2000). Therefore, we focused on the S408N variation for further analysis.

The frequency of the S408N variation was analyzed in a total of 137 circadian rhythm sleep disorder patients and 138 control subjects. Allele and genotype distributions are shown in Tables 3 and 4. No significant deviation from Hardy–Weinberg equilibrium was detected for the variation either in patients or in controls. The distribution analysis resulted in the detection of an additional silent sequence variation (1263A>G) in the 3′-untranslated region of the

Table 2 Sequence Variations Identified in the Human *CKIε* Gene

DNA polymorphism	Location	Amino-acid substitution
51C>T	Exon 1	None
77-63_77-60delGGCG	Intron 1	None
1223G>A	Exon 9	S408N
1263A>G	Exon 9 (3′-untranslated region)	None

Variations were named basically according to den Dunnen and Antonarakis (2001). Nucleotide numbers refer to the human CKIε cDNA sequence (AB024597) with the A of the ATG start codon denoted as 1. The S408N variation was submitted to DDBJ (http://www.ddbj.nig.ac.jp/, Accession no. AB080742).

Table 3 Frequency of the S408N Variant in Patients and Controls

		Allele frequency		
	n	N408 (%)	S408 (%)	*p*-value
Control	276	34 (12.3)	242 (87.7)	
DSPS	196	12 (6.1)	184 (93.9)	0.028[a]
N-24	78	3 (3.8)	75 (96.2)	0.035[b]
DSPS/N-24	270	15 (5.6)	255 (94.4)	0.0067[c]

[a]Odds ratio (OR) = 0.46, 95% confidence interval (CI): 0.23–0.92.
[b]OR = 0.28, 95% CI: 0.085–0.95.
[c]OR = 0.42, 95% CI: 0.22–0.79.

Table 4 Genotype Distribution of the S408N Variant in Patients and Controls

		Genotype				
	n	N/N (%)	N/S (%)	S/S (%)	N/N+N/S (%)	*p*-value
Control	138	4 (2.9)	26 (18.8)	108 (78.3)	30 (21.7)	
DSPS	98	1 (1.0)	10 (10.2)	87 (88.8)	11 (11.2)	0.038[a]
N-24	39	0 (0)	3 (7.7)	36 (92.3)	3 (7.7)	0.061[b]
DSPS/N-24	135	1 (0.7)	13 (9.6)	121 (89.6)	14 (10.3)	0.013[c]

The frequency of the N408 allele carrier is shown as (N/N+N/S). Odds ratio (OR) and 95% confidence interval (CI) are for (N/N+N/S) *vs* S/S.
[a]OR = 0.46, 95% CI: 0.22–0.96.
[b]OR = 0.3, 95% CI: 0.086–1.04.
[c]OR = 0.42, 95% CI: 0.21–0.83.
Two of the DSPS subjects, who had siblings with N-24, were excluded from the combined DSPS/N-24 group to avoid an increase in the Type I error rate. Neither of the sibling pairs carried the S408N variation.

CKIε gene, which was located 40 bp downstream of S408N polymorphic site (Table 2). One of the DSPS patients and two of the N-24 patients were heterozygous for the 1263A > G variation, while it was not detected in the control individuals. However, the frequency of the 1263A > G variation was too low to establish whether the variation affects the development of DSPS and N-24.

The N408 allele was significantly less frequent in DSPS ($p = 0.028$) and in N-24 ($p = 0.035$) than in control subjects (Table 3). The frequency of the N408-allele carrier was also significantly lower in DSPS subjects ($p = 0.038$) compared to controls, while the difference in carrier frequency between N-24 subjects and controls showed a similar tendency but did not come to statistical significance ($p = 0.061$) (Table 4). N-24 patients often suffer from DSPS during the course of the illness (Kamgar-Parsi *et al*, 1983; Oren and Wehr, 1992; McArthur *et al*, 1996), and reportedly share some of the physiological characteristics of DSPS, such as prolonged interval between natural wake time and the core body temperature trough (Uchiyama *et al*, 2000) or melatonin midpoint (Shibui *et al*, 1999; Uchiyama *et al*, 2002). These observations led to an *a priori* prediction that DSPS and N-24 are essentially the same disorder expressed with different degrees of severity (Weitzman *et al*, 1981; Campbell *et al*, 1999; Regestein and Monk, 1995). Indeed, when DSPS and N-24 subjects were combined, highly significant inverse associations were found between the N408 variant and DSPS/N-24 in both allele frequency ($p = 0.0067$, odds ratio (OR) = 0.42, 95% confidence interval (CI): 0.22–0.79) and carrier frequency ($p = 0.013$, OR = 0.42, 95% CI: 0.21–0.83), suggesting that the N408 allele protects against the development of DSPS/N-24. Our sample size had a 78% power to detect this effect of the S408N allele at a significance level of $p = 0.05$.

We next considered whether the S408N variation induces a functional alteration in CKIε, as expected from the location of the substitution. To determine whether the N408 variation in CKIε affects kinase activity *in situ*, phosphorylation of PER1 was assayed in transfected COS-7 cells by pulse-chase analysis. COS-7 cells were co-transfected with expression plasmids encoding mPer1 and either wild-type rCKIε or rCKIε with the S408N substitution. The transfected cells were pulse-labeled with [^{35}S]methionine for 1 h and chased for 0–6 h. After the chase period, cells were lysed and mPER1 protein expressed in COS-7 cells was immunoprecipitated using anti-mPER1 antibody. The immunoprecipitates were electrophoresed, and [^{35}S]-labeled mPER1 was detected. rCKIε with the S408N substitution induced a more pronounced mobility shift and reduced mPER1 protein level at 6 h post-pulse, which was indistinguishable from the effects induced by wild-type rCKIε. These results indicate that, *in situ*, wild-type rCKIε and rCKIε with the S408N substitution induce similar levels of phosphorylation and subsequent instability of the mPER1 protein (data not shown).

Previous reports suggested that subsets of autophosphorylation sites in CKIε are dephosphorylated in HEK293 and NIH3T3 cells by endogenous phosphatases, thus activating CKIε activity (Gietzen and Virshup, 1999; Rivers *et al*, 1998). Therefore, it is possible that rCKIεs transfected into COS-7 cells are dephosphorylated, consequently masking the effect of the S408N substitution on kinase activity.

To test this hypothesis, we performed *in vitro* kinase assays of GST-CKIε with or without the S408N substitution, using α-casein as a substrate. Recombinant GST-CKIε proteins were expressed in *E. coli*, purified with glutathione sepharose 4B, and immunoprecipitated with the C-terminus-specific antibody for CKIε to remove partially degraded protein. The kinetic analysis was performed using varying substrate concentrations (0–100 μM). As expected, GST-CKIε-S408N exhibited higher kinase activity than GST-CKIε-WT (Figure 1a). The data were represented as a double-reciprocal plot (Figure 1b). GST-CKIε-S408N showed significantly increased V_{max} (181% of GST-CKIε-WT) and a slightly decreased apparent K_m (78% of GST-CKIε-WT) against casein. To investigate whether the S408N substitution in CKIε causes higher enzyme activity on endogenous clock components, *in vitro* kinase assays using GST-PERs as substrates were also performed. To distinguish the phosphorylated GST-PERs from autophosphorylated CKIεs, GST tags were removed from recombinant CKIεs. As shown in Figure 2, CKIε-S408N incorporated more [^{32}P] into PER1, PER2, and PER3 fragments, respectively, than CKIε-WT did.

DISCUSSION

CKIε is one of the seven isoforms of CKI, designated α, β, γ1–3, δ, and ε (Eide and Virshup, 2001). Activity of CKIε

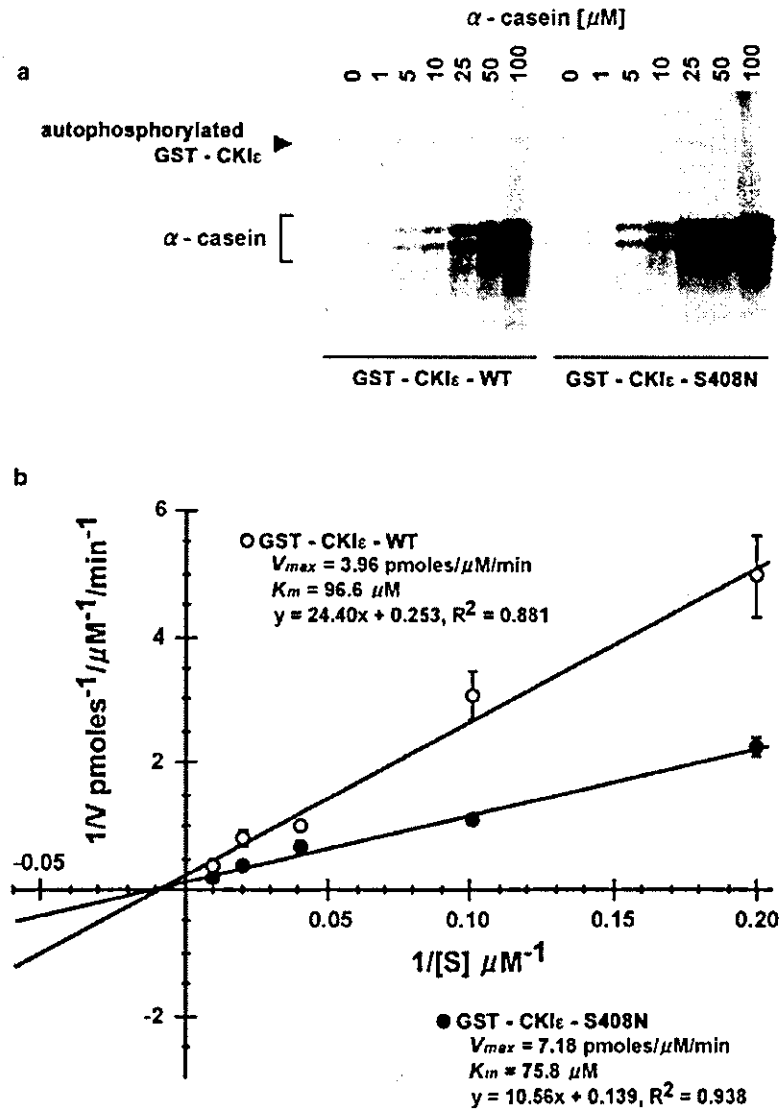

Figure 1 Kinetic analysis of recombinant GST-CKIε for α-casein. (a) Assays were performed with various concentrations of α-casein in the presence of GST-CKIε-WT or CKIε-S408N. Autophosphorylation of GST-CKIε-WT and CKIε-S408N that were only slightly visible in this figure were readily apparent when increased amounts of recombinant enzymes were used (data not shown). (b) Double-reciprocal plot of the data derived from the kinase assay performed with various concentrations of α-casein. Open and closed circles indicate the results for GST-CKIε-WT and CKIε-S408N, respectively. Calculated V_{max} and K_m of GST-CKIε-WT and CKIε-S408N are shown in the upper and the lower parts of the plot, respectively (means ± standard errors (SE) from three independent experiments).

(and the closely related CKIδ) is regulated in part by autophosphorylation of the C-terminal extension (Eide and Virshup, 2001; Graves and Roach, 1995; Cegielska *et al*, 1998). *In vitro*, CKIε is highly autophosphorylated, which inhibits enzyme activity (Gietzen and Virshup, 1999; Rivers *et al*, 1998). Both dephosphorylation by phosphatase treatment and removal of the C-terminal domain reactivate the kinase (Graves and Roach, 1995; Cegielska *et al*, 1998). In a site-directed mutagenesis study, eight amino acids in the C-terminal domain were identified as probable autophosphorylation sites, including serine-408 (Gietzen and Virshup, 1999). Therefore, the amino-acid change from serine-408 to asparagine (S408N) in CKIε, which was found in this study, is likely to eliminate one of the autophosphorylation sites, and is expected to reactivate part, but not all, of the kinase activity. Indeed, in our *in vitro* kinase assay with α-casein, recombinant GST-CKIε with the S408N substitution purified from *E. coli* exhibited a moderate (1.8-fold) elevation of specific activity compared to that of wild-type GST-CKIε, while a previous study showed that a mutant CKIε, in which all of the putative autophosphorylation sites are disrupted, was eight-fold more active than wild-type CKIε (Gietzen and Virshup, 1999). The moderate elevation of CKIε activity by S408N substitution was

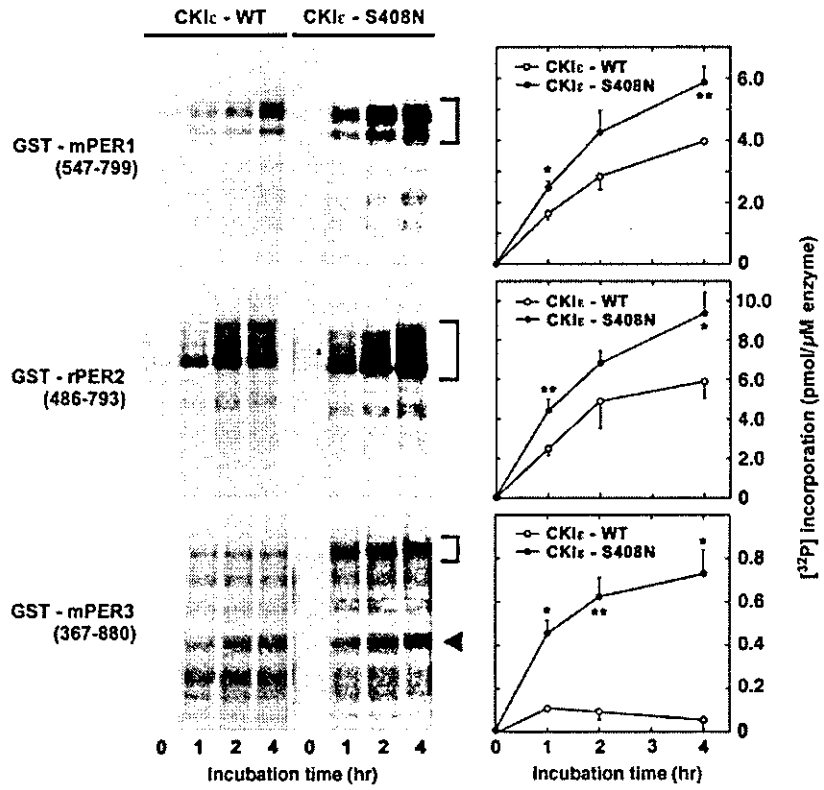

Figure 2 *In vitro* kinase assay of recombinant CKIε using GST-fused PER fragments as substrates. GST-mPER1 (amino acids 547–799) (top panels), GST-rPER2 (486–793) (middle panels), or GST-mPER3 (367–880) (bottom panels) fragment was incubated with recombinant CKIε-WT or CKIε-S408N for the indicated duration and analyzed by 7.5% polyacrylamide gels as described in Materials and methods. Representative autoradiograms are shown. Angle brackets indicate the phosphorylated GST-PER fragments. Arrowhead indicates autophosphorylated CKIε (left panels). Incorporated [^{32}P] was quantified and normalized to the total amount of kinase used (means ± SE from three to five independent experiments). Statistically significant differences in [^{32}P] incorporation induced by CKIε-WT and CKIε-S408N are shown by asterisks (*$p<0.05$, **$p<0.01$) (right panels).

identically observed in the *in vitro* kinase assay with each of the three subtypes of PER proteins, which are endogenous substrates for CKIε. It is intriguing that CKIε-S408N induced more phosphorylation of PER3 than CKIε-WT did, because we have previously reported that a *Per3* gene haplotype, which presumably alters PER3 protein phosphorylation, is significantly associated with DSPS (Ebisawa *et al*, 2001). However, it should be noted that we observed much less phosphorylation of PER3 compared with that of PER1 or PER2, which is consistent with the previous reports showing CKIε-induced phosphorylation of PER3 (Takano *et al*, 2000; Akashi *et al*, 2002) and unstable interaction of PER3 with CKIε in the absence of PER1 (Akashi *et al*, 2002; Lee *et al*, 2004). We could not find any elevation of enzyme activity in pulse-chase analysis *in situ*, presumably because of dephosphorylation by endogenous phosphatases as described in 'Results', or because the analysis was insufficiently sensitive to detect a moderate difference of activity.

The *tau* mutation in hamster *CKIε* decreases kinase activity by as much as eight-fold (Lowrey *et al*, 2000), whereas the S408N variant in h*CKIε* results in only 1.8-fold change (an increase) in the activity. This difference might explain the reason why the N408 allele of h*CKIε* induces a significant but modest effect (~2-fold reduction in the risk to develop DSPS/N-24), compared with the *tau* mutation in hamster *CKIε*, which causes a semidominant short-period phenotype (Ralph and Menaker, 1988).

Studies in flies and mammals suggest that CKIε binds to and phosphorylates PER proteins, leading to instability and intracellular relocalization of the PERs (Takano *et al*, 2000; Vielhaber *et al*, 2000; Keesler *et al*, 2000; Akashi *et al*, 2002). Mutant *CKIε* in the Syrian Golden hamster is deficient in PER phosphorylation (Lowrey *et al*, 2000). *Per2* S662G mutation in a reported familial ASPS cause hypophosphorylation by CKIε (Toh *et al*, 2001). In both cases, the PER protein(s) seems to undergo delayed degradation and accelerated accumulation, leading to hastened nuclear entry and shortened circadian period. In contrast, in flies with dbt^{L} or dbt^{ar} (long-period alleles of *dbt*, the Drosophila homolog of *CKIε*), it is likely that delayed phosphorylation and increased nuclear stability of PER protein slow the rate of PER elimination from the nucleus and lengthen circadian rhythm (Price *et al*, 1998; Rothenfluh *et al*, 2000). Therefore, hypophosphorylation of PER protein appears to cause different phenotypes depending on the subcellular localization of the stabilized PERs. hCKIε with an S408N substitution appears more active than wild type only when

the protein is autophosphorylated. A recent study suggests that the autophosphorylation level of CKIε, in neuroblastoma N2a cells, is dynamically regulated through transient dephosphorylation and subsequent phosphorylation, thus regulating the kinase activity (Liu *et al*, 2002). Additionally, in clock-relevant cells, CKIε intracellular localization is under circadian control (Lee *et al*, 2001); therefore, it is possible that a dynamic autophosphorylation/dephosphorylation cycle could differentially regulate CKIε activity at different subcellular locations in pacemaker cells. The S408N variation of h*CKIε* might alter circadian rhythmicity through increased phosphorylation and decreased stability of PER protein; the expected phenotypic consequences, however, would differ depending on the levels of CKIε autophosphorylation in each subcellular location. It will be of interest to investigate the autophosphorylation status of CKIε-S408N and to clarify its functional role in circadian clock machinery.

Although a significant inverse association was observed between the N408 variant and DSPS/N-24, 10.3% of the patients carried the N408 allele, indicating that DSPS/N-24 is genetically heterogeneous and multiple genes affect susceptibility to the development of DSPS/N-24.

The 1263A>G variation in the 3′-untranslated region of h*CKIε* was detected only in three of the rhythm disorder subjects, but not in controls. A larger sample size will be necessary to ascertain its relevance to DSPS/N-24.

Owing to the potential role of CKIε in the circadian rhythm, all of the coding exons in h*CKIε* gene were screened for variations in circadian rhythm sleep disorder patients and controls. We found a missense variation S408N, for the first time, which eliminates one of the putative autophosphorylation sites in hCKIε and confers 1.8-fold higher enzyme activity *in vitro*. There was a significant difference in the frequency of N408 allele between controls and DSPS or N-24, respectively, with an excess of N408 allele in controls. When considering the whole sample of circadian rhythm sleep disorders (DSPS/N-24), we found a highly significant inverse association between N408 allele and DSPS/N-24 ($p=0.0067$, OR = 0.42, 95% CI: 0.22–0.79). These results indicate that the N408 allele of the h*CKIε* gene is a marker for decreased risk of DSPS/N-24. S408N variation would also be useful to investigate other disorders related to disturbed circadian rhythm or interindividual differences of circadian rhythmicity in apparently normal subjects (Johansson *et al*, 2003). Our results will yield a new insight into the mechanism of DSPS/N-24 and raise a question in the role of CKIε autophosphorylation on mammalian clock functioning.

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Should thrombolysis be given to a stroke patient refusing therapy due to profound anosognosia?

Jeffrey M. Katz, MD; and Alan Z. Segal, MD

A 70-year-old woman was told by a neighbor that her speech was slurred. She was also having difficulty standing. She was brought to the emergency room within 45 minutes of symptom onset stating "they said I had a stroke, but I didn't." She had a history of hypertension, hypercholesterolemia, and stage IV non-small cell lung cancer. Her examination was notable for a severe anosognosia, dysarthria, a right gaze preference, and left homonymous hemianopia, hemiparesis, hemihypesthesia, and visual, tactile, and spatial neglect. Her NIH Stroke Scale score was 15. Head CT was unremarkable, and she had no contraindications for IV thrombolysis.

Preparations were made for the administration of tPA, at approximately 2 hours after symptom onset. The patient, however, vehemently refused therapy because she did not believe she was having a stroke. Attempts were made to contact the patient's family, but this did not prove possible until after the 3-hour time window had elapsed. Upon discharge to our acute rehabilitation facility, the patient required maximal assist for transfers, remained hemiparetic, and had persistent neglect and hemianopia.

Although the administration of tPA does not require written informed consent, the risks and benefits of this therapy must be explained to the patient and family to the greatest extent possible.[1] According to the American Academy of Neurology (AAN) position paper,[2] tPA may be given without consent, if considered an accepted standard of care, in keeping with the doctrine of emergency treatment and implied consent. This would apply in particular to patients who are unable to speak due to acute aphasia. Our patient differs in that she was interactive and talkative, and was actively refusing to be treated. Because her anosognosia prevented her from properly comprehending the nature of her medical situation, she was only partially competent to make this decision.[3] Her lack of complete competency, however, still may not warrant treating her against her will. tPA has long-term benefits but also carries a trade-off of short term risk (including potentially fatal intracerebral hemorrhage). Under the pressure of a tense emergency situation, some patients may not be willing to accept this risk even without anosognosia complicating the discussion.

Since up to half of all stroke cases might involve a portion of the right middle cerebral artery and produce a component of anosognosia, we would hypothesize that our case is not entirely unusual. In an effort to expand the number of patients eligible for tPA, while ensuring compliance with valid informed consent, we would propose that the AAN consider updating its guidelines to incorporate anosognosia.

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CSF hypocretin-1 (orexin-A) levels in childhood narcolepsy and neurologic disorders

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Hypersomnia and cataplexy expression in childhood narcolepsy are often different from adult cases, making diagnosis difficult.[1] The Multiple Sleep Latency Test (MSLT) for demonstrating hypersomnia and sleep-onset REM periods has not been standardized for children under age 8[2] and has limited value in pediatrics.

CSF hypocretin-1 measurements were established as a new diagnostic tool for narcolepsy–cataplexy in adults[3,4] but has not yet been evaluated in children.[4] We measured CSF hypocretin-1 levels in 132 children with various neurologic disorders (including six narcoleptic children) to evaluate the diagnostic value of CSF hypocretin measures for childhood narcolepsy.

Patients and methods. We analyzed previously collected data, gathered from 1992 to 2003 for diagnostic and research purposes, including CSF samples (n = 132, including 14 cases previously reported), collected from patients (under age 20 from seven Japanese hospitals) with neurologic disorders (the core experimental protocol was approved at Akita University in August 2002; the local ethical committee approved the use of CSF samples). Patients were categorized based on the diagnosis determined by individual clinical records (table). Patients without complete records or definite diagnosis were excluded. Narcolepsy was diagnosed by the criteria of the International Classification of Sleep Disorders.[5]

Hypocretin-1 was measured by direct radioimmunoassay of CSF stored at −80 °C (detection limit 40 pg/mL).[7] As there was no difference in the mean CSF hypocretin level between children and adults,[7] the levels were defined as low (<110 pg/mL), intermediate (≥110 to ≤200 pg/mL), and normal (>200 pg/mL).[3] The low value represents 30% of the mean value of normal adult CSF hypocretin and has the best sensitivity/specificity ratio for diagnosing adult narcolepsy.[3]

Results. Low CSF hypocretin-1 levels were observed in all six narcoleptic subjects (mean age 9.7 years; 6 to 16 years) (see the table; see also table E-1 on the *Neurology* Web site at www.neurology.org). All narcoleptic subjects had positive human leukocyte antigen DR2 markers. The duration of hypersomnia (DH) was 1 to 20 months prior to the CSF sampling. In two of these patients (DH: 1 and 2 months), the clinical diagnosis of narcolepsy was not clear at the time of the CSF sampling. Nevertheless, they later exhibited cataplexy, a typical symptom of narcolepsy.

In four neurologic categories, Guillain–Barré syndrome (GBS) (6/6), acute disseminated encephalomyelitis (ADEM) (2/7), brain tumor (2/4), and head trauma (3/3), 13 children had low to intermediate hypocretin-1 levels (see the table; also see table E-2 on the *Neurology* Web site).

All GBS subjects in this study showed reduced CSF hypocretin levels. Only one case (151 pg/mL) exhibited short sleep latency (<1 minute) by a two-nap test after the recovery of the neurologic symptoms. Two ADEM cases (102 and 146 pg/mL) presented transient sleepiness associated with bilateral hypothalamic lesions on MRI. Two subjects with head trauma and two with brain tumor reported sleepiness, and these subjects together with one subject with head trauma without sleepiness had reduced levels.

Intermediate hypocretin-1 levels were also found in some neuropediatric-specific conditions, such as Prader–Willi syndrome

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Table CSF hypocretin-1 levels in various neurologic disorders

Diagnosis	n	Low, <110 pg/mL	Intermediate, 110–200 pg/mL	Mean hypocretin-1 level (range), pg/mL
Narcolepsy (EDS with/without cataplexy, all are DR2 positive)	6	6	0	(L–79)
MSL ≦8 min + ≧2 SOREMPs	4	4	0	<40
MSL ≦8 min + no SOREMPs	2	2	0	<40, 79
Other primary hypersomnia (recurrent hypersomnia, idiopathic hypersomnia)	5	0	0	263 (232–292)
CNS infection (meningitis, encephalitis, cerebellitis)	22	0	2	282 (156–423)
Autoimmune and postinfectious disease	18	3	5	202 (L–366)
GBS	6	2	4	
ADEM	7	1	1	
Others (MS, CIDP, myelopathy)	5	0	0	
Head trauma (subdural hematoma, diffuse axonal injury, contusion)	3	1	2	132 (56–192)
Brain tumor (hypothalamic tumor, thalamic tumor)	4	1	1	175 (102–257)
Malignancy without CNS invasion (leukemia, lymphoma)	12	0	0	297 (232–364)
Psychological/psychiatric status (depression, hysteria)	3	0	0	303 (265–345)
CNS malformations (migration disorder, brain anomaly)	8	0	0	270 (223–383)
Chromosome aberration (PWS, tuberous sclerosis, Sturge–Weber syndrome)	5	0	1	233 (192–310)
Epilepsy or mental retardation of unknown origin (epilepsy, mental retardation, infantile spasms)	19	0	2	286 (124–372)
Perinatal asphyxia and trauma (cerebral palsy)	2	0	0	307 (304–310)
Metabolic or degenerative diseases (NPC, mitochondria encephalopathy, leukoencephalopathy, spinocerebellar degeneration)	6	0	1	307 (142–461)
Chronic CNS infection (SSPE)	2	0* (2)	0	313 (311–315)
Epileptic encephalopathy (progressive myoclonic encephalopathy, Lafora disease, Rasmussen encephalopathy)	3	0	0	290 (215–348)
Motor unit disease (congenital myotonic dystrophy, spinal muscular atrophy, congenital myopathy)	4	0	0	307 (265–338)
Cerebral hypertension (idiopathic cerebral hypertension, hydrocephalus)	2	0	0	320 (280–360)
Transient neurologic conditions (suspected meningitis but negative culture, migraine)	8	0	1	279 (195–338)
Total	132	11* (13)	15	

When patients received multiple CSF taps, the values during the most representative phase of the disease are reported.

* Undetectably low levels under interferon-α treatment.

L = low levels; EDS = excessive daytime sleepiness; MSL = mean sleep latency; SOREMPs = sleep-onset REM periods; GBS = Guillain–Barré syndrome; ADEM = acute disseminated encephalomyelitis; MS = multiple sclerosis; CIDP = chronic inflammatory demyelinating polyneuropathy; PWS = Prader–Willi syndrome; NPC = Niemann–Pick type C; SSPE = subacute sclerosing panencephalitis.

(PWS) (1/1), infantile spasms due to birth trauma of unknown origin (2/3), Niemann–Pick type C (NPC) (1/2), CNS infection (2/22), and febrile convulsion (1/3). None of these patients showed hypersomnia, but the NPC case with intermediate hypocretin level (147 pg/mL) presented cataplectic-like episodes.

Discussion. Five subjects in four diagnostic categories (GBS, ADEM, brain tumor, and head trauma) showed low hypocretin levels. Partial impairments of hypocretin systems secondary to hypothalamic damage may be responsible for decreased hypocretin levels (and some rare hypersomnia cases). Clinical symptoms and other diagnostic findings (such as MRI) are useful in differentiating these cases from narcolepsy, so low hypocretin levels in these diseases do not confound the diagnostic value for narcolepsy.

High percentages of low levels of GBS and ADEM are interesting because they may suggest immune-mediated damage of hypocretin neurons. A similar mechanism may also be involved in hypocretin-deficient idiopathic narcolepsy. Intermediate levels were seen in a neonatal case of PWS prior to the appearance of hypersomnia and obesity. PWS may thus be a model for congenital dysfunction/developmental failure of the hypocretin system. Similarly, the NPC case with cataplectic-like episodes may be a model for acquired deterioration of the hypocretin system by accumulation of lipids in the brain structures responsible for the induction of cataplexy.

There may be some false negatives in the presumed nonnarcoleptic group, as this group did not receive the same series of