



Review

# The regulation of neuroendocrine function: Timing is everything

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## Abstract

Hormone secretion is highly organized temporally, achieving optimal biological functioning and health. The master clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus coordinates the timing of circadian rhythms, including daily control of hormone secretion. In the brain, the SCN drives hormone secretion. In some instances, SCN neurons make direct synaptic connections with neurosecretory neurons. In other instances, SCN signals set the phase of “clock genes” that regulate circadian function at the cellular level within neurosecretory cells. The protein products of these clock genes can also exert direct transcriptional control over neuroendocrine releasing factors. Clock genes and proteins are also expressed in peripheral endocrine organs providing additional modes of temporal control. Finally, the SCN signals endocrine glands via the autonomic nervous system, allowing for rapid regulation via multisynaptic pathways. Thus, the circadian system achieves temporal regulation of endocrine function by a combination of genetic, cellular, and neural regulatory mechanisms to ensure that each response occurs in its correct temporal niche. The availability of tools to assess the phase of molecular/cellular clocks and of powerful tract tracing methods to assess connections between “clock cells” and their targets provides an opportunity to examine circadian-controlled aspects of neurosecretion, in the search for general principles by which the endocrine system is organized.

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## Circadian aspects of reproduction

The importance of circadian (about a day) timing in hormone production/secretion has been known since the 1950s when Everett and Sawyer determined that a stimulatory signal occurring during a narrow temporal window on the afternoon of proestrus is necessary for induction of ovulation later that night (Everett and Sawyer, 1950). Such close temporal organization is important for successful reproduction, as numerous hormone-dependent behavioral and physiological processes must be coordinated. If optimal temporal relationships are disrupted, pronounced deficits in fertility can result. For example, ovulation, behavioral estrus, fertilization, and pregnancy maintenance require a specific temporal pattern of hormone secretion in spontaneous ovulators such as rats, hamsters, and mice (Blaustein et al., 1994; McEwen et al., 1987; Mong et al., 2003). Prior to behavioral estrus, rising levels of estrogen both trigger a precisely timed preovulatory surge in luteinizing hormone (LH) and stimulate the production of brain progesterone receptors in preparation for progesterone effects on neurons. The timing of progesterone receptor regulation relative to estrogen ensures that behavioral receptivity is coordinated with the time of ovulation, thereby increasing the likelihood of pregnancy (Hansen et al., 1979). Following ovulation, a prolactin surge is necessary to support the corpus luteum to maintain progesterone secretion necessary for pregnancy and its maintenance (Egli et al., 2004). This sequence is under circadian control.

In the present review, we summarize evidence indicating that the timing of endocrine secretions is coordinated by a brain “clock” located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Fig. 1). Over the last decade, there have been substantial, rapid advances in our understanding of the circadian modulation of brain and peripheral organ activity. Current data indicate that a neural signal from the SCN is necessary for the circadian timing of hormone secretion, while a diffusible signal is sufficient to modulate non-endocrine events such as daily behavioral activities. The identification of core clock genes, clock-controlled genes (CCG), and their localization in neurosecretory cells (Kriegsfeld et al., 2003; Olcese et al., 2003), the pituitary gland (Shieh, 2003; Von Gall et al., 2002) and a number of peripheral endocrine glands (Bittman et al., 2003; Morse et al., 2003; Zylka et al., 1998) each provide new opportunities for evaluating the loci and mechanisms of temporal gating of hormone secretion.

We review evidence that hormone secretion is regulated not only by the feedback loops long studied by endocrinologists but also by the SCN and SCN-derived temporal signals acting directly on neurosecretory cells, on the autonomic nervous system, and on clock genes and clock-controlled genes. To this end, we present an abbreviated introduction to the core negative feedback loop controlling cellular circadian clock function to provide a basis for understanding how time can be tracked within a cell and to set the foundation for understanding the possible role of clock genes in endocrine regulation. For detailed summaries of research on cellular/molecular clock genes and proteins, the reader is referred to the following reviews (Albrecht, 2004; Ashmore and Sehgal, 2003; Du and

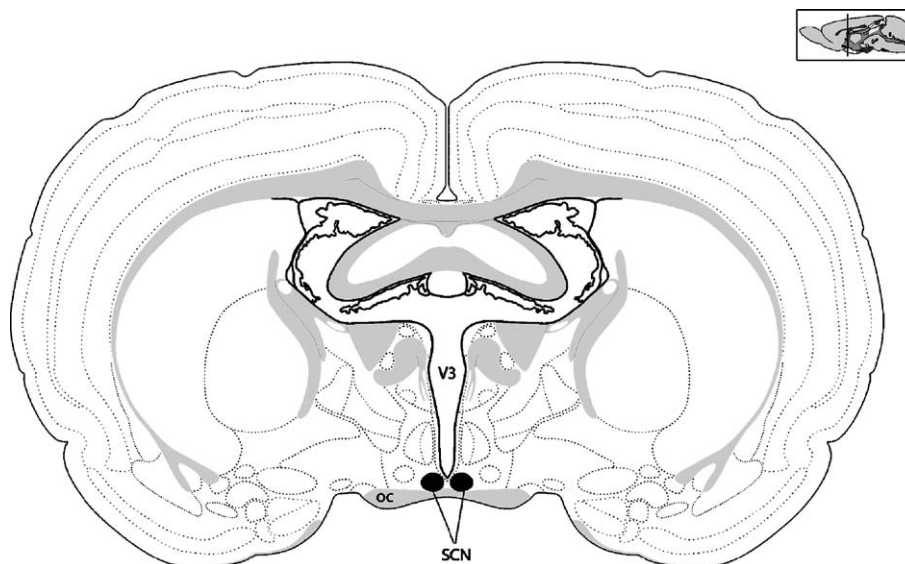


Fig. 1. The mammalian circadian clock is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. The SCN pictured here in this schematic is a coronal section through a rodent brain. The SCN is situated at the base of the brain directly above the optic chiasm (oc) and surrounding the third ventricle (V3). The sagittal schematic in the upper right corner depicts the approximate rostral–caudal location depicted in the coronal section.

Tong, 2002; Duffield, 2003; Gachon et al., 2004; Glossop and Hardin, 2002; Green, 2003; Hastings et al., 2003; Liu, 2003; Okamura, 2003a,b; Okamura et al., 2002; Piggins, 2002; Roenneberg and Merrow, 2003; Schibler and Sassone-Corsi, 2002; Schultz and Kay, 2003; Zordan et al., 2003).

### Circadian control of endocrine secretions

Biological events typically exhibit marked, predictable cycles ranging in time from seconds to years. In the endocrine system, virtually every factor measured to date shows a circadian (endogenous) or diurnal (driven) rhythm. These alterations are achieved by modulation of pulse amplitude (i.e., amount of hormone released), pulse frequency (i.e., rate of hormone release), or by a combination of both of these processes. For example, studies in male rhesus macaques in which animals were sampled at 20-min intervals in an LD cycle revealed diurnal rhythms in luteinizing hormone (LH), testosterone, prolactin, and cortisol (Plant, 1981). Likewise, studies in rats, Syrian hamsters, and humans indicate circadian variation in gonadotropins and gonadal steroids around the onset of puberty (Andrews and Ojeda, 1981; Boyar et al., 1976; de la Iglesia et al., 1999; Jakacki et al., 1982; Smith and Stetson, 1980). It has been suggested that diurnal variation in hormone concentrations may simply be modulated by sleep. However, sleep reversal (subjects sleep during the day rather than night) and sleep interruption do not affect the daily pattern of most hormones, confirming regulation by an endogenous clock independent of sleep (Desir et al., 1982; Kapen et al., 1974; Van Cauter and Refetoff, 1985), although interactions between sleep and the circadian system exist (Kriegsfeld et al., 2002a). The present review highlights the current understanding of circadian system regulation of endocrine rhythms via multiple means of modulation and proposes a novel mechanism of hierarchical control beginning with the master brain clock. For a complete description of daily hormone secretion patterns, the reader is referred to the following reviews (Gore, 1998; Hastings, 1991; Kriegsfeld et al., 2002a; Turek and Van Cauter, 1994).

### Endocrine influences on the circadian system

Not only does the SCN regulate endocrine rhythms but hormones also feed back to the SCN, presumably to “fine-tune” the temporal pattern of endocrine secretion given current conditions. For example, high-affinity melatonin receptors are localized to the SCN, and administration of melatonin can alter SCN phase (Dubocovich et al., 1996; Hastings et al., 1997; Lewy et al., 1992; Slotten et al., 1999; Vanecek and Watanabe, 1999). In addition, exogenous melatonin alters the phase of SCN electrical activity measured in a slice preparation *in vitro* in a manner predicted by the phase response curve (McArthur et al., 1991). The sensitivity of the SCN to melatonin may be a function of daily variation in the density of melatonin binding sites within the SCN (Schuster et al., 2001). In humans, melatonin administration causes phase delays during late night or early morning and phase advances in late morning to early afternoon (Lewy and

Sack, 1997). This finding has significant implications for shift workers, jet lag, and the blind.

Estrogen has pronounced effects on circadian activity rhythms, likely through both direct effects on the SCN and indirect mechanisms. In females, estrogen modulates both period and activity consolidation, suggesting actions on the circadian clock rather than transient effects on SCN targets. Cycling female hamsters and rats show a phase advance in locomotor activity on the day of estrous (scalloping), when estradiol levels are highest, and continuous administration of estradiol in silastic capsules shortens the free-running period of ovariectomized hamsters (Morin et al., 1977). When hamsters are maintained in constant light, the normally stable activity phase frequently splits into two activity components that stabilize approximately 12 h apart. Continuous administration of estradiol in silastic capsules to ovariectomized hamsters prevents these changes (Morin, 1980).

Direct effects of estrogen on the circadian clock are suggested by the expression of  $\alpha$  and  $\beta$  estrogen receptors in the SCN across mammals, including humans (Gundlach et al., 2000; Kruijver and Swaab, 2002; Su et al., 2001). These receptors may be important for its normal development and synchronization to the environment (Abizaid et al., 2004; Gundlach et al., 2000). In humans, the presence of estrogen receptor expression, along with sex differences in SCN structure, suggests that estrogen may act on the SCN during development (Hofman et al., 1988, 1996; Kruijver and Swaab, 2002). Indirect effects in estrogen on the circadian clock are indicated by studies in which simultaneous injection of anterograde and retrograde tract tracers into the SCN reveal that ER $\alpha$ -expressing cells in the preoptic area, amygdala, BNST, and arcuate provide input to the SCN, but the SCN does not project directly to these ER $\alpha$ -expressing cells (de la Iglesia et al., 1999).

In males, testosterone also affects consolidation of locomotor activity rhythms. Extended exposure to short day lengths induces a decrease in testicular size and a decline in plasma testosterone concentrations in male hamsters (Ellis and Turek, 1983). Following testicular regression (or after castration), there is an increase in lability of activity onset, an expansion of the daily activity duration, with a decrease in wheel revolutions per cycle; testosterone replacement prevents these changes (Morin and Cummings, 1981). Testosterone may act through SCN androgen receptors. To date, androgen receptors have been identified in the SCN of several species (Clancy et al., 1994; Fernandez-Guasti et al., 2000; Kashon et al., 1996; Michael and Rees, 1982; Rees and Michael, 1982). Alternatively, testosterone may exert its effects through conversion to estradiol, which may act either directly on receptors in the SCN (e.g., Shughrue et al., 1997), or indirectly in ER-expressing cells in other brain areas that, in turn, communicate with the SCN (de la Iglesia et al., 1999). In rats, conversion of testosterone to estradiol may be important for the activity-stimulating effects of testosterone (Roy and Wade, 1975). Estradiol is nearly 100 times as effective as testosterone at increasing activity, while dihydrotestosterone (a non-aromatizable androgen) has no effect on wheel-running activity of rats (Roy and Wade, 1975). Taken together, these

findings suggest that the conversion of testosterone to estrogen, by aromatase, may be important for the effects of testosterone on circadian rhythms.

#### *Identification of a brain “clock”: from tissue to gene*

A highly localized brain clock in mammals was first suggested in 1972, with the demonstration that lesions ablating the SCN abolish circadian rhythmicity in adrenal corticoid secretion and locomotor behavior (Moore and Eichler, 1972; Stephan and Zucker, 1972). SCN-lesioned animals continue to show the full range of normal behaviors, but their temporal organization is lost and never recovers, irrespective of how early in development the lesions are performed (Mosko and Moore, 1979). The initial conclusion that the SCN serves as a brain master clock has been confirmed in the subsequent 30 years by converging lines of research involving *in vivo*, *ex vivo*, and *in vitro* studies carried out in many different laboratories. For example, transplants of donor SCN tissue into the brains of arrhythmic, SCN-lesioned hosts restore circadian rhythmicity in behavior (Lehman et al., 1987; Ralph et al., 1990). Importantly, rhythms are restored with the period of the donor SCN, indicating that the transplanted tissue does not act by restoring host brain function but that the “clock” is contained in the transplanted tissue. Further evidence that clock function is contained within the SCN comes from studies demonstrating that circadian rhythms in neural firing rate persist in isolated SCN tissue maintained in culture (Green and Gillette, 1982; Groos and Hendriks, 1982; Shibata et al., 1982). An excellent overview of these studies in historical perspective is available (Weaver, 1998).

While the foregoing work demonstrated that the SCN tissue as a whole served as a clock, the finding that circadian rhythmicity is a property of *individual* SCN neurons set the stage for the next breakthrough. The demonstration that dispersed, cultured SCN cells exhibit circadian rhythms of electrical activity indicated that circadian timing is a cellular property rather than an emergent property of a neural network (Welsh et al., 1995). These studies allowed for the exploration and subsequent discovery of the cellular molecular machinery responsible for circadian function.

Within a cell, circadian rhythms are produced by an autoregulatory transcriptional/translational negative feedback loop that takes approximately 24 h, whereas the general mechanism for circadian oscillations at the cellular level is common among organisms, the components comprising the feedback loop differ. For the purpose of clarity, only the core mammalian feedback loop is described. To date, it is thought that two proteins, CLOCK and BMAL1, bind to one another and drive the transcription of messenger RNA (mRNA) of the *Period* (*Per*) and *Cryptochrome* (*Cry*) genes by binding to the E-box (CACGTG) domain on these gene promoters. Three *Period* (*Per1*, *Per2*, and *Per3*) and two *cryptochrome* genes (*Cry1* and *Cry2*) have been identified. The mRNA for these genes is translated into PER and CRY proteins in the cytoplasm of the cell over the course of the day. Throughout the day, these proteins build up within the cytoplasm, and when they reach

high enough levels, they form hetero- and homo-dimers. These newly formed dimers then feed back to the nucleus where they bind to the CLOCK:BMAL1 protein complex to turn off their own transcription (Fig. 2). Numerous other “clock” genes and regulatory enzymes have been identified but will not be reviewed for the sake of brevity. Future studies on the specific genes and their interactions that result in circadian timekeeping at the cellular level will likely yield exciting new information on other regulatory elements and their interactions.

Discovery of the genes regulating circadian rhythmicity led to breakthroughs identifying clock genes and their protein products in numerous sites, including extra-SCN brain loci and in the periphery (Abe et al., 2002; Balsalobre et al., 1998; Kriegsfeld et al., 2003; Yamazaki et al., 2000). These findings, in turn, led to questions about the unique nature of the master oscillator in the SCN, the functional significance of extra-SCN oscillators, and mechanisms of coordination of these widely dispersed clocks.

To compare cellular mechanisms of clock gene expression in the SCN and in the periphery, embryonic fibroblasts from wild-type and (behaviorally arrhythmic) *Cry*<sup>-/-</sup> mice were used (Yagita et al., 2001). Clock properties of cell lines derived from peripheral cells of each strain were similar to those of the strain-specific SCN, supporting the conclusion of common core clock gene function in all tissues (Yagita et al., 2001). It remains controversial, however, whether peripheral oscillators are similar to those of the SCN in their ability to sustain endogenous rhythmicity for long durations (Balsalobre et al., 1998; Yoo et al., 2004).

When a tissue, either SCN or peripheral, loses coherent rhythmicity, it is important to determine whether this is due to dampening of rhythms in individual cells or to loss of synchrony among a population of cells in the tissue. Use of *Per1*-luciferase transgenic animals indicates that rhythms in peripheral tissues damp then disappear over time due to uncoupling (desynchronization) among oscillators that retain their individual rhythms (Nagoshi et al., 2004; Welsh et al., 2004). Presumably peripheral clock cells normally get phase information (directly or indirectly) from the SCN to synchronize individual oscillators to each other. In this view, the SCN sets the phase of peripheral circadian clocks daily, coordinating the activity of tissues and organs of the body relative to one another, thereby maintaining homeostasis.

### **Circadian output and orchestration of endocrine function**

#### *Diffusible signals controlling behavioral rhythms*

Rhythmic electrical activity and oscillation of clock genes within the SCN neurons ultimately lead to rhythmicity in the whole organism. Compelling evidence for a diffusible output signal derives from neural tissue transplantations in which the SCN from a fetal donor is implanted into the third ventricle of an adult, SCN-lesioned host. As mentioned previously, these grafts restore activity-related behaviors such as locomotor, drinking, and gnawing rhythms (Lehman et al., 1987; Ralph et al., 1990; Silver et al., 1990). That a diffusible signal is

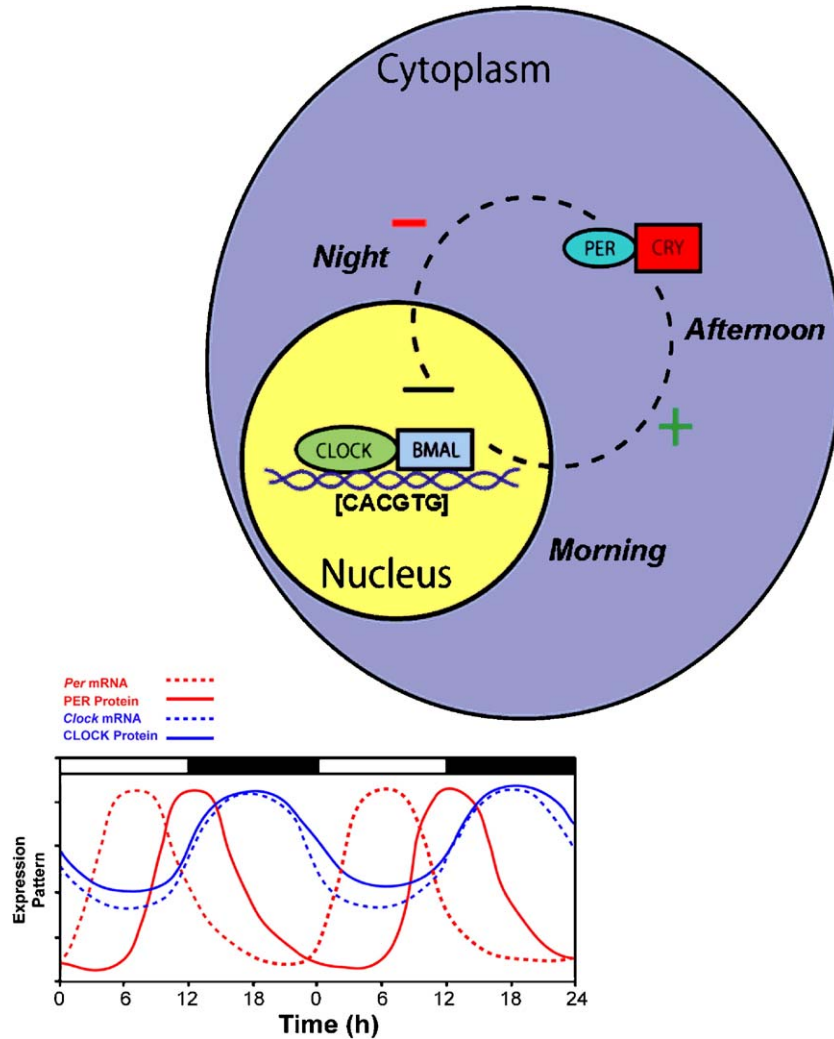


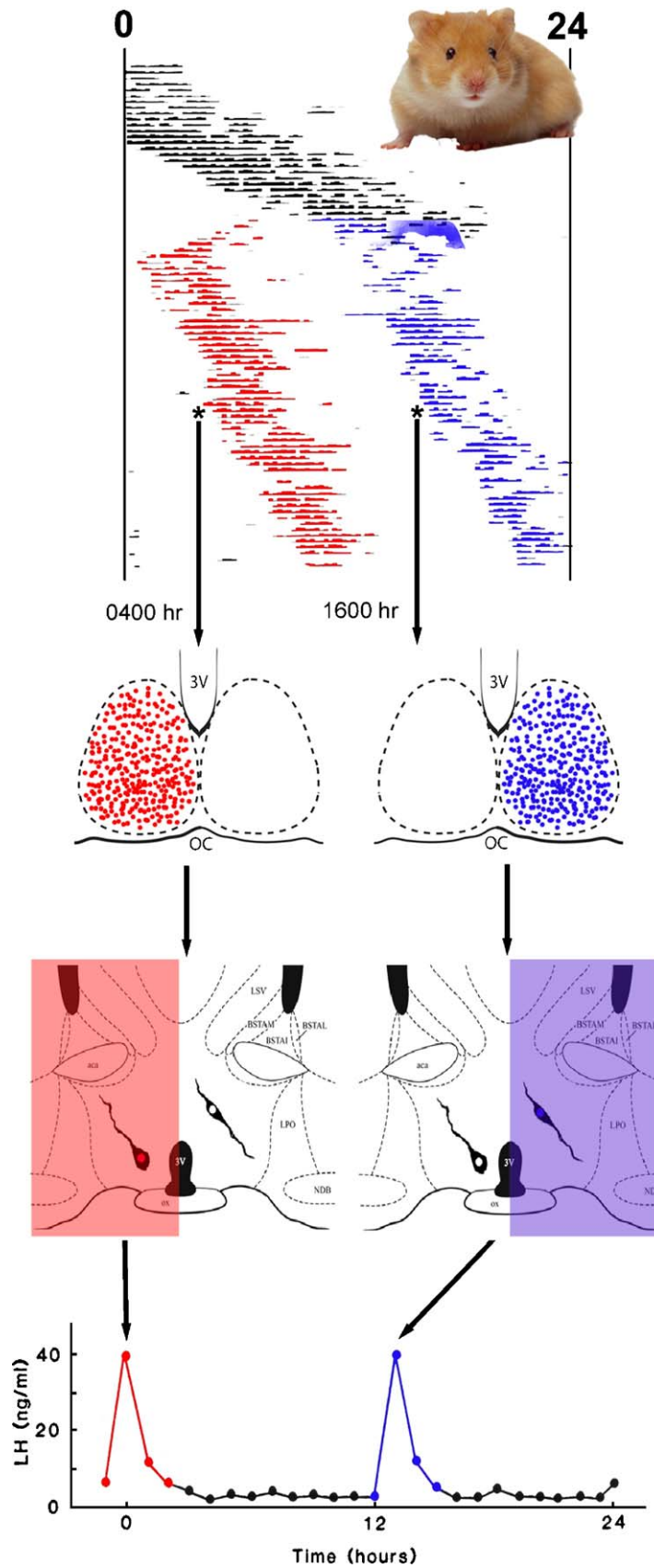
Fig. 2. A simplified model of the intracellular mechanisms responsible for mammalian circadian rhythm generation. The process begins when CLOCK and BMAL1 proteins dimerize to drive the transcription of the *Per* (*Per1*, *Per2*, and *Per3*) and *Cry* (*Cry1* and *Cry2*) genes. In turn, *Per* and *Cry* are translocated to the cytoplasm and translated into their respective proteins. Throughout the day, PER and CRY proteins rise within the cell cytoplasm. When levels of PER and CRY reach a threshold, they form heterodimers, feed back to the cell nucleus and negatively regulate CLOCK:BMAL1 mediated transcription of their own genes. This feedback loop takes approximately 24 h, thereby leading to an intracellular circadian rhythm.

sufficient to restore locomotor rhythmicity in SCN-lesioned hosts was demonstrated by encapsulating donor SCN tissue in a membrane that prevented neural outgrowth while allowing the diffusion of signals between graft and host (Silver et al., 1996).

One candidate diffusible signal is prokineticin-2 (PK2) (Cheng et al., 2002). This protein is expressed rhythmically in the SCN, and its receptor is present in all major SCN targets (Cheng et al., 2002, 2005). Likewise, PK2 administration during the night (when levels are low) inhibits wheel running behavior. Whether or not this signal normally operates in a diffusible manner and/or is released synaptically requires further examination. A second candidate diffusible signal is transforming growth factor- $\alpha$  (TGF- $\alpha$ ) (Kramer et al., 2001). As with PK2, TGF- $\alpha$  is expressed rhythmically in the SCN, and its administration inhibits wheel-running behavior. The receptor for TGF- $\alpha$  is also expressed in the SPVZ, the major target of the SCN. The degree which TGF- $\alpha$  is released in a diffusible manner under normal conditions

is not known. Studies in which the contribution of neural efferents and diffusible signals can be distinguished are necessary to begin to answer this question.

Although it is intriguing to speculate on the role of these signals in communicating information from the SCN, the problem of unequivocally identifying an endogenous, physiologically relevant diffusible SCN signal is complex and parallel in scope to the task faced by Sir Geoffrey Harris' in providing evidence for hypothalamic control over pituitary function in the 1950s. The necessary and sufficient criteria to confirm the existence of a diffusible signal in a fluid volume have been summarized previously (Nicholson, 1999). First, the removal or replacement of the signaling substance must result in a change in the response being controlled. The substance should be present or increased, or both, in a well-defined temporal relationship to the response (and similarly declines when the response disappears). In addition, evidence must be obtained that a fluid compartment is the conduit for a diffusible or



transported signal. The signal must have access to and enter the compartment where the fluid dynamics and turnover in the compartment should allow appropriate movement of the signal. While PK2 and TGF- $\alpha$  meet some of these criteria, further research is necessary to clarify the role of these molecules in communicating circadian information.

#### *Neural control of neurosecretory factors*

In contrast to behavioral rhythms (e.g., locomotion, drinking, gnawing), endocrine rhythms require *neural* projections from the SCN to endocrine targets; endocrine rhythms are abolished after knife cuts severing SCN efferents (Hakim et al., 1991; Nunez and Stephan, 1977) and are not restored in SCN-lesioned transplanted animals (Meyer-Bernstein et al., 1999; Nunez and Stephan, 1977; Silver et al., 1996), presumably due to inadequate neural innervation of the host brain by the graft. Further evidence for a neural SCN output signal regulating hormone secretion is seen in studies of female hamsters. When housed in constant light, the activity of a subset of hamsters “splits” into two separate activity bouts within a 24-h interval. These split females display two daily LH surges, each approximately half the concentration of a single surge in a non-split female (Swann and Turek, 1985) (Fig. 3). Under normal conditions, both halves of the bilaterally symmetrical SCN are active in synchrony. In ovariectomized, estrogen-implanted split hamsters killed during one of their activity bouts, however, activation of the SCN occurs on one side of the brain (monitored by FOS expression) but not on the other, suggesting that each half of the SCN can control an activity bout (de la Iglesia et al., 2000). Remarkably, FOS activation in GnRH neurons was only seen on the side of the brain in which SCN FOS expression occurred (de la Iglesia et al., 2003). These findings suggest that the precise timing of the LH surge is derived from a neural signal originating in the SCN and communicated to ipsilateral GnRH neurons, as a diffusible output signal would reach both sides of the brain. Importantly, some hypothalamic sites are activated ipsilaterally, while others are activated either ipsilaterally or bilaterally in the split animal, again supporting the notion of multiple SCN output pathways (Tavakoli-Nezhad and Schwartz, 2005; Yan et al., 2005).

Neural output from the SCN has been extensively investigated in rats and hamsters using tract tracing techniques (Kalsbeek et al., 1993; Kriegsfeld et al., 2004; Leak and Moore, 2001; Morin et al., 1994; Stephan et al., 1981; Watts and Swanson, 1987; Watts et al., 1987). Importantly, many of these monosynaptic projections target brain regions containing neuroendocrine cells producing hypothalamic releasing hormones (Fig. 4). Direct projections have been traced from the SCN to the medial preoptic area (MPOA), supraoptic nucleus (SON), anteroventral periven-

tricular nucleus (AVPV), the paraventricular nucleus (PVN), the dorsomedial nucleus of the hypothalamus (DMH), and the lateral septum and the arcuate (Arc). The SCN also projects to the pineal through a multisynaptic pathway (Klein, 1985; Klein et al., 1983). There is abundant evidence for direct neural SCN control of neuroendocrine cell populations (Buijs et al., 1998, 2003; Egli et al., 2004; Gerhold et al., 2001; Horvath, 1997; Horvath et al., 1998; Kalsbeek and Buijs, 2002; Kalsbeek et al., 1996a,b, 2000; Kriegsfeld et al., 2002a, b; Van der Beek et al., 1993, 1997b; Vrang et al., 1995). Because these cell populations can regulate neurochemicals that are secreted into the CSF (Reiter and Tan, 2002; Skinner and Caraty, 2002; Skinner and Malpoux, 1999; Tricoire et al., 2003) or general circulation, SCN-derived signals can control widespread systems in the brain and body.

Together, the findings summarized above suggest several possibilities: behavioral rhythms may be controlled by a diffusible signal(s), while endocrine rhythms may require neural output. Alternatively, behavioral and endocrine rhythms can both be supported by diffusible signals, but the threshold for supporting behavioral rhythms is lower. Finally, behavioral rhythms are controlled by both neural and diffusible signals, and either can maintain rhythmic function, while endocrine rhythms can only be supported via neural connections. Definitive identification of biologically significant endogenous diffusible signal(s) and the precise mode of SCN control is a current line of inquiry.

#### *Neural SCN output and estrus regulation*

SCN control of the rodent estrous cycle has been investigated extensively (Barbacka-Surowiak et al., 2003), providing an excellent model system for investigations of circadian and neuroendocrine interactions. The SCN sends projections directly to GnRH neurons in female rodents (Horvath et al., 1998; Van der Beek et al., 1997a). These efferents express the SCN peptide, vasoactive intestinal polypeptide (VIP). GnRH neurons particularly important for the regulation of the estrous cycle are activated at the time of proestrus and receive SCN input (van der Beek et al., 1994). Also, sex differences in the daily expression of SCN VIP mRNA are seen in rats, with females exhibiting a peak 12 h out of phase with that of males (Krajnak et al., 1998). Presumably, the signal regulating the estrous cycle is sexually dimorphic, thereby lending further support for VIP regulation of estrus. Furthermore, antisense oligonucleotides directed against VIP lead to a delayed and attenuated LH surge (Harney et al., 1996), reminiscent of that seen in middle-aged rats (Gore, 1998). Sex differences also exist in the pattern of projections from the SCN (Horvath et al., 1998; Van der Beek et al., 1997a,b). These sex differences in SCN projections upon GnRH cells, and in the production of

Fig. 3. Circadian control of gonadotropin secretion. Syrian hamsters normally exhibit one consolidated bout of activity every 24 h. Under conditions of constant light, the hamsters activity splits into two components separated by about 12 h. In one ingenious study (de la Iglesia et al., 2003), the investigators killed animals prior to each of the activity bouts (see asterisk on activity records). Brains were analyzed for FOS activity in the SCN and in neurons of the GnRH neuronal system. Splitting behavior resulted in only one half of the SCN being active during a given time of day. GnRH was only activated (expressed FOS) on the side of the brain in which the SCN was active. Given that ovariectomized, estrogen-implanted hamsters with split behavior experience two LH surges (Swann and Turek, 1985), we are speculating that the neural mechanism underlying this phenomenon can be explained by differential left–right activation of the GnRH system at two times of day.

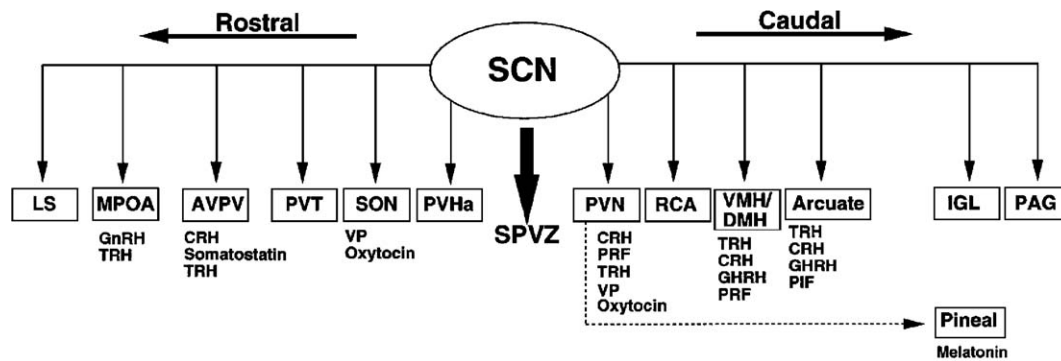


Fig. 4. Efferent projections of the rodent SCN to its targets in the brain. Below each target area is a list of neuroendocrine cells that lie in that region of the brain and could potentially be regulated by direct projections from the SCN. Solid lines represent monosynaptic projections, while the dotted line represents a multisynaptic projection to the pineal gland. The pronounced overlap between neuroendocrine cells and SCN efferent terminals, combined with reports demonstrating direct neuronal projections from the SCN to neuroendocrine cells (e.g., GnRH and CRH cells), provides suggestive evidence for a global mechanism of circadian hormonal regulation. Adapted from Kriegsfeld et al. (2002a) with permission.

neurochemicals in SCN cells projecting to the GnRH system, may underlie the absence of an LH surge in males.

In addition to direct connections to GnRH neurons, the SCN projects extensively to the anteroventral periventricular nucleus (AVPV), a brain region associated with the induction of the preovulatory LH surge (Le et al., 1997; Levine, 1997). The cells to which the SCN projects are estrogen-responsive (Watson et al., 1995), suggesting that the AVPV may be an important integration point for circadian and steroidal signals. Given the widespread projections of the SCN throughout the CNS, along with extensive input to the GnRH system, the potential for additional indirect modulation of the reproductive axis is considerable.

Vasopressin, another SCN peptide important in the regulation of the estrous cycle, is synthesized and released in a circadian manner. SCN vasopressin is rhythmically secreted with a peak during a sensitive time window prior to the LH surge (Kalsbeek et al., 1996a,b). Vasopressin administration into the MPOA induces an LH surge in SCN-lesioned, ovariectomized rats treated with estradiol (Palm et al., 2001a, b). Electrical stimulation of the MPOA and VP administration into the MPOA induces an LH surge in SCN-lesioned rats (Palm et al., 1999). Finally, in co-cultures of POA and SCN tissue, the rhythm of GnRH release is in phase with the rhythm of VP release, but not with that of VIP (Funabashi et al., 2000). Paradoxically, Brattleboro rats incapable of synthesizing vasopressin are fertile, although abnormal estrous cyclicity and reduced fertility have been noted (Boer et al., 1982). Taken together, these data indicate a potential role for VP in inducing the LH surge, although it is likely not the sole mediator.

Positive feedback effects of estrogen serve a permissive role in initiating the LH surge upon the arrival of the signal from the circadian pacemaker (Barbacka-Surowiak et al., 2003; Levine, 1997); implants of an anti-estrogen into the POA block the LH surge (Petersen and Barraclough, 1989). This dependence on estrogen ensures the maturation of the follicle during the time of the surge, while the circadian dependence ensures that receptive behavior coincides with ovulation. It remains unclear, however, how these two signals converge at the cellular level to allow integration at the appropriate time of day. One possibility is that

estrogen alters neurochemical secretion by the SCN or the signaling efficacy of these chemicals via second messenger systems and kinases (Chappell and Levine, 2000; Levine, 1997). An alternative hypothesis is that estrogen stimulates ligand-independent progesterone receptor production, and the timed neuronal signal acts on progesterone receptors (Chappell et al., 2000; Levine, 1997; Levine et al., 2001). This scheme suggests that the effects of estrogen are integrated with the SCN signal at the level of progesterone receptors to ensure that the GnRH system is sensitive to the daily signal only during the preovulatory estrogen surge. The fact that few estrogen receptors have been localized to GnRH neurons (Shivers et al., 1983) suggests that estrogen acts to produce progesterone in neurons upstream of the GnRH system and hints at important, unidentified, SCN projections to these upstream components. Given the potential importance of estrogen/progesterone receptor expressing neurons upstream of the GnRH system, it will be interesting to establish the means by which the circadian system communicates with and modulates these regulatory elements.

The importance of a functional molecular clock in driving GnRH cells is seen in mice with a mutant form of the *Clock* gene. These mice have long, irregular estrous cycles and fail to exhibit an LH surge following estradiol treatment. Furthermore, this mutation also leads to an increase in fetal reabsorption during pregnancy and a decline in full-term parturition (Miller et al., 2004). These deficits are associated with a decline in mid-term levels of progesterone, suggesting abnormal secretion patterns of prolactin (Miller et al., 2004). Together, these findings highlight the importance of the circadian system in regulating the temporal pattern of hormone secretion necessary for mating, pregnancy, and its maintenance, although results using mutant models must be interpreted cautiously until converging approaches consistently support these conclusions.

Not only does the SCN regulate GnRH during the estrous cycle, but other aspects of the estrous cycle are also regulated by the SCN. During proestrus, rising levels of estradiol reach a critical point and trigger the release of prolactin at a specific time of day. This release of prolactin is dependent upon the estradiol-induced increase in tuberoinfundibular dopaminergic

(TIDA) neuron activity (Neill et al., 1971). Administration of an estradiol antiserum on the morning of diestrus-2 blocks the proestrous surge of prolactin (Neill et al., 1971). The proper timing of prolactin is achieved via SCN projections to TIDA neurons in the arcuate (Horvath, 1997). Additionally, TIDA neurons rhythmically express the clock gene, *Per1*, providing a potential additional means of temporal control (Kriegsfeld et al., 2003)—and see below). SCN lesions result in an abolition of a daily prolactin rhythm (Mai et al., 1994) and abolish the preovulatory prolactin surge (Pan and Gala, 1985). Given the importance of prolactin timing in maintaining the corpus luteum, these findings further suggest an essential role for the circadian clock in reproduction and may explain the increased fetal reabsorption in *Clock* mutant mice described above (Miller et al., 2004).

It has been well established that environmental factors act to fine-tune endogenous regulation of the reproductive cycle. For example, the timing of the prolactin surge is regulated by the phase of the light–dark (LD) cycle. A change in the LD cycle leads to predictable changes in the timing of the estrogen-induced prolactin surge and the mating-induced prolactin surge (Blake, 1976; Pieper and Gala, 1979). Thus, environmental time of day information is transmitted to the circadian system to precisely coordinate reproductive events relative to local time. Cervically stimulated prolactin surges have a free-running cycle in ovariectomized, estradiol-treated rats held in constant conditions. This pattern is abolished after ablation of the SCN (Bethea and Neill, 1980). As with the LH surge, this finding suggests that both the timing and production of the prolactin surge require a signal from the circadian clock. The means by which environmental stimuli other than light (e.g., social signals, local conditions, nutrition, etc.) are integrated into this system to fine-tune the timing of hormonal and behavioral events are unknown and represent an opportunity for exploration.

As with estrous behavior, the preovulatory LH surge occurs at regular 4- or 5-day intervals in rats, on the day of proestrus at a specific time of day coupled to the LD cycle (Colombo et al., 1974). Interestingly, despite the fact that the temporal pattern of SCN neural activity is similar in nocturnal and diurnal rodents (Smale et al., 2003), with a peak during the day and a trough at night, the timing of the preovulatory LH surge is reversed (Mahoney et al., 2004; McElhinny et al., 1999). Although the mechanisms by which this reversal occurs remain elusive, comparisons between diurnal and nocturnal species may provide insight into how circadian control is accomplished in humans (i.e., a diurnal species). Studies in rhesus initially indicated that the LH surge could be induced at any circadian phase in primates (Knobil, 1974). However, frequent urinary LH monitoring of women with regular menstrual cycles suggests a pronounced influence of circadian timing on the preovulatory LH surge, with most exhibiting the LH surge between midnight and 8:00 AM (Cahill et al., 1998; Edwards, 1981) (Fig. 5). Of 155 regular cycles monitored, 146 surges occurred during this 8-h time window (Cahill et al., 1998). Given that the daily timing of the LH surge in women can be unmasked under carefully monitored and controlled conditions, it is likely that the

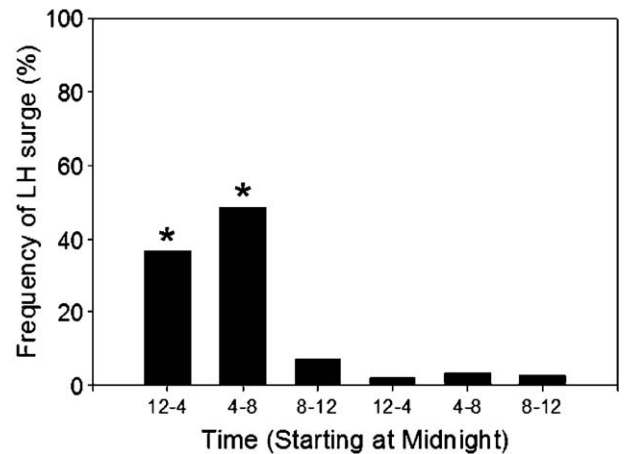


Fig. 5. Frequency of onset of the LH surge by time of day. A total of 155 cycles from 35 women were monitored. The graph represents the percentage of preovulatory LH surges occurring during each time interval. Adapted from Cahill et al. (1998).

human ovulatory cycle also requires interactions between the circadian and reproductive systems.

#### Direct and indirect transcriptional control as a clock output

The circadian system exerts a widespread influence over numerous bodily functions. DNA microarray studies in mice indicate that ~5–9% of the genome, excluding genes involved in the core clock loop, are under circadian control (Akhtar et al., 2002; Panda et al., 2002; Storch et al., 2002). However, these so-called clock-controlled genes (CCGs) differ among tissues, with any two tissues likely sharing less than 10% of CCGs under circadian control. Together, these findings suggest that circadian control is ubiquitous throughout the body, and tissue-specific processes may be controlled by differential activation of downstream genes in individual systems.

#### Direct transcriptional control

CCGs maintain a predictable phase relationship with the core clock genes (Ueda et al., 2002), indicating that the CCGs are either directly or indirectly regulated via the circadian transcriptional machinery. The expression of some CCGs is directly controlled by the CLOCK:BMAL1 heterodimer binding to an E-box enhancer (CACGTG) in their promoter (Jin et al., 1999). An example of direct transcriptional control by the circadian system is seen with vasopressin regulation. Vasopressin is present in the SCN where it acts locally to regulate rhythm generation (Mihai et al., 1994a,b). It cannot be the sole regulatory factor, as the SCN of Brattleboro rats maintains rhythms in electrical activity (Ingram et al., 1998), and these animals exhibit only slight disruptions in rhythm amplitude and entrainability (Brown and Nunez, 1989; Murphy et al., 1993, 1996). In addition to a role within the nucleus, SCN, vasopressin-expressing neurons signal distant hypothalamic targets and SCN vasopressin signaling has been implicated in the control of estrus (Buijs et al., 2003a,b; Kalsbeek et al., 1996a,

b; Mihai et al., 1994a,b; Palm et al., 2001a,b). The SCN rhythm in vasopressin (a rhythm that is not present in other vasopressinergic cell population—see below) is dependent on an E-box element in the 5' flanking region of the vasopressin gene to which the CLOCK:BMAL complex binds. The vasopressin rhythm is abolished in mutant mice with aberrant *Clock* gene expression (Jin et al., 1999; Silver et al., 1999).

E-box elements in the promoter have the potential for direct control by circadian clock genes, but this alone is not sufficient. In the SON (unlike the SCN), vasopressin is dependent on osmotic balance and is not rhythmic (Jin et al., 1999). While expression of *Clock* is robust in the SCN and SON, *Bmal1* is expressed robustly only in the SCN and is barely detectable in the SON. These findings indicate some of the conditions necessary for direct control of genes regulating neuroendocrine function by the circadian clock transcriptional machinery.

A gene with an E-box in its promoter region is a potential target for direct control by the transcriptional machinery of the circadian clock. We screened hypothalamic and pituitary endocrine factors for E-box elements in the promoter of their published gene sequences to determine their potential for this means of control (Table 1). We found that some releasing hormones (TSH, GHRH, and vasopressin) do have E-box enhancers. We have shown in the adult mouse brain that a subset of neuroendocrine cells in the preoptic area, paraventricular nucleus of the hypothalamus, and the arcuate nucleus express the clock gene *Per1* (Kriegsfeld et al., 2003), suggesting that this direct transcriptional regulation likely plays a key role in the organization of endocrine timing.

#### Indirect transcriptional control

In studies of the liver, it has been demonstrated that D-element binding protein (DBP) is another CCG under direct transcriptional regulation by the core circadian feedback loop (Ripperger et al., 2000). Importantly, DBP binds to other gene promoters to temporally regulate their transcription. This process is important in the control of hepatic metabolic

Table 1  
List of mammalian neuroendocrine factors containing an E-box (CACGTG) enhancer in their promoter

Gene	E-box (#)	Reference
Hypothalamic factors:		
CRH	No	(Muglia et al., 1994)
GHRH	Yes (1)	(Laird, 2001—direct submission)
GnRH-I	No	(Hayflick et al., 1989)
GnRH-II	No	(White et al., 1998)
Ghrelin	No	(Kanamoto et al., 2004)
Oxytocin	No	(Hara et al., 1990)
Vasopressin	Yes (1)	(Hara et al., 1990; Jin et al., 1999)
TRH	No	(Satoh et al., 1996)
Pituitary:		
POMC (ACTH, MSH)	No	(Drouin et al., 1985)
FSH	No	(Kumar, 1994—direct submission)
GH	No	(Das et al., 1996)
LH	No	(Kaiser et al., 1998)
Prolactin	No	(Gubbins et al., 1980)
TSH (beta subunit)	Yes (3)	(Croyle and Maurer, 1984)

processes; DBP activates the transcription of albumin, cholesterol 7 $\alpha$  hydroxylase, and cytochrome P450 (Lavery et al., 1999). This second order system can provide ubiquitous control via the circadian system and temporal control of key enzymatic pathways involved in hormone production.

A process similar to hepatic regulation by DBP may modulate the reproductive axis, as the gene for GnRH does not have an E-box but appears to be under circadian control. A series of studies using GT1 cells, an immortalized line of GnRH cells, demonstrated the rhythmic expression of numerous circadian clock genes (Chappell et al., 2003; Gillespie et al., 2003; Olcese et al., 2003). Importantly, GnRH release occurs episodically approximately every 90 min in most species, and GT1 cells also express this ultradian pattern of GnRH production. When clock genes are disrupted in GT1 cells, not only is the daily rhythm disrupted, but both pulse frequency and amplitude are also dramatically altered (Chappell et al., 2003). These findings suggest that, in addition to 24-h cycles, clock genes expressed within neuroendocrine cells may regulate the episodic pattern of hormone secretion outside of the circadian range, even when the regulated neuroendocrine gene lacks an E-box enhancer. While these data are intriguing, a direct link between clock genes and ultradian rhythmicity awaits further supporting evidence.

While the forgoing studies using embryonic cell lines provide insights into cellular mechanisms, how these data generalize to functioning in vivo needs to be determined, as immortalized cell lines may exhibit properties different from those of the living animal. In addition, cell lines lack the innumerable regulatory systems that act directly or indirectly on GnRH cells in vivo. To address the question of whether or not GnRH cells in adult mice express *Per1* message, we used mice with a green fluorescent protein (GFP) reporter driven by *Per1* promoter. In these mice, GFP expression was observed in neurons near to GnRH-immunoreactive cells, but the two proteins were not co-localized. We have confirmed these negative findings using double-label immunofluorescence in mice and rats using several different antibodies against *Per1* and GnRH (Kriegsfeld and Silver, unpublished data). We conclude that there are key differences between clock gene expression between cultured GT1 cells and GnRH neurons in the brains of adult animals.

#### System-level control and coordination of endocrine function

The task in understanding the orchestration of hormonal systems is best understood by recognizing that most processes in the body exhibit a circadian rhythm, and that activity in various systems exhibits different phases (i.e., peak and trough times) relative to each other. One explanation for how this feat is accomplished suggests that the SCN secretes one neurochemical to control each rhythmic process at its appropriate phase. In this view, the SCN secretes numerous substances, each precisely timed. Alternatively, control may be accomplished by the specificity of SCN projections and combinations of transmitter release at each target (Kalsbeek and Buijs, 2002). As an alternative hypothesis, we have suggested that SCN timing

signals have different consequences at each targeted effector system (Kriegsfeld et al., 2003).

According to this view, a small number of rhythmic SCN signals must be differentially interpreted by a large number of targets to accomplish precise phase control by the SCN over an ensemble of rhythmic processes. Different targets respond differentially to the same message based on time of day (or local conditions), with some systems responding maximally to a signal(s) at a particular time of day, while another system might respond to this same signal(s) with inhibition. This hypothesis requires that target systems of the SCN have a mechanism for keeping time.

Seasonal as well as circadian timing are dependent upon the SCN. Several hypotheses have been proposed to account for how the SCN and its targets track time on a seasonal basis (Carr et al., 2003; Hofman, 2004; Lincoln et al., 2003). In the SCN of Syrian and Siberian hamsters, photoperiod alters the *duration* of clock and clock-controlled gene expression, while the *amplitude* of gene expression is influenced by photoperiod in the pars tuberalis (Johnston et al., 2003; Messenger et al., 2000). In sheep, however, the relative timing of clock genes is altered by photoperiod in the pars tuberalis, providing a mechanism of temporal encoding and downstream control (Hazlerigg et al., 2004; Lincoln et al., 2002, 2003, 2005). These correlational results are intriguing and suggest that phase and/or amplitude of clock and CCGs in SCN brain targets and endocrine glands may predict their responsiveness to upstream signals on a daily schedule.

Numerous systems display time-gated sensitivity to stimuli, with the same stimulus or neurochemical producing different effects at different times of day, suggesting temporal control at effector sites rather than passive regulation by the SCN. The preoptic area, for example, exhibits robust clock gene expression (Palm et al., 2001a,b; Tei et al., 1997; Yamamoto et al., 2001). Importantly, stimulation of the POA of ovariectomized rats with the SCN peptide, vasopressin, induces an LH surge during the second portion of the light period, but not the first (Palm, 2001 #122-001), indicating important temporal control at the level of the POA.

#### *Clocks in the neuroendocrine system*

The fact that time-keeping machinery is functional in numerous central neurosecretory cells (Kriegsfeld et al., 2003) and peripheral endocrine tissues (Bittman et al., 2003; Morse et al., 2003; Shieh, 2003; Von Gall et al., 2002; Zylka et al., 1998) represents a mechanism by which SCN targets can anticipate the reception of SCN signals and respond based upon local needs and time of day. In addition, because peripheral systems are controlled hierarchically by multiple upstream components, temporal modification in each “link” along the hypothalamo–pituitary–endocrine gland axis could provide additional control over daily patterns of individual rhythms. At the top of this circadian hierarchy of control, the SCN sends signals to neuroendocrine cells and tissues to maintain synchronization among cellular oscillators in phenotypically distinct neuroendocrine cell populations. Although capable of oscillating

independently, these neuroendocrine cells respond to periodic SCN input to maintain a synchronized rhythm in the local cell population. Such mechanism could account for the loss of coherent circadian endocrine rhythms following lesions or transection of outputs from the SCN (Meyer-Bernstein et al., 1999; Moore and Eichler, 1972; Nunez and Stephan, 1977). Loss of coherence among elements would result in a blunted overall output and absence of rhythmicity when individual cells drift out of phase with each other.

If the SCN coordinates cell populations, why do individual cells need their own clocks? According to this view, clocks in SCN target populations provide responsiveness to local conditions and temporal fine-tuning in a local population, not possible with a single driving master clock. These local clocks could selectively respond depending on time of day and appropriately drive the expression of cell/tissue-dependent CCGs that act as output to affect target systems or regulate local conditions. Coordinated rhythmic output from neuroendocrine cells can then be communicated to the pituitary, which also exhibits circadian clock gene expression indicating a zone for further temporal modification (Messenger et al., 2000). Finally, rhythmic information from the pituitary can be communicated humorally to target glands in the periphery that themselves express clock genes (Bittman et al., 2003; Zylka et al., 1998). As mentioned previously, multisynaptic projections from the SCN to several endocrine glands have been identified using viral tracers (Buijs et al., 1998, 1999; Gerendai and Halasz, 2000; Kalsbeek et al., 2000). These connections provide a mechanism for the SCN to coordinate peripheral cellular oscillators to optimize responses to slower endocrine signals. Several lines of evidence support the notion that neural communication from the SCN to the periphery is responsible for the timing of clock gene expression in target organs and glands (Guo et al., 2005; Shibata, 2004; Terazono et al., 2003).

#### *The top of the hierarchy: neural SCN output*

An organization of this type requires that the SCN communicate (directly or indirectly) with neuroendocrine cells expressing clock genes. There is substantial evidence of direct projections from the SCN to neuroendocrine cells (Buijs et al., 1993; Horvath et al., 1998; Kriegsfeld et al., 2002a,b; Teclemariam-Mesbah et al., 1997; Van der Beek et al., 1997a, b; Vrang et al., 1995). Expression of the clock gene, *Per1*, is rhythmically expressed in the Arc (Abe et al., 2002) and exhibits a stress-induced increase in the PVH (Abe et al., 2002; Takahashi et al., 2001). *Per1* has been localized to CRH-ir cells in the PVH (Takahashi et al., 2001), while the neurochemical phenotype of *Per1*-expressing cells in the Arc has been identified as dopaminergic providing a potential mechanism of control of prolactin rhythms and the preovulatory prolactin surge (Kriegsfeld et al., 2003).

#### *Hormonal and neural communication to glands*

Relative to the neural communication by the SCN to neuroendocrine cells, hormonal communication is slow.

However, by using the bloodstream as a route of communication, hormones modulated by the circadian system can communicate rhythmic information throughout the body. Additional temporal control occurs at target glands and organs by using circadian clock machinery to modulate responsiveness to hormonal signals. If this means of temporal control is implemented at peripheral targets, necessary alterations in the timing of organ/gland responsiveness due to changes in local conditions can be communicated rapidly to hormone-sensitive targets to adjust the timing of their circadian clocks. Given that numerous organs (e.g., liver, pancreas) and endocrine glands (e.g., testes, adipose tissue, adrenal gland) investigated to date receive autonomic innervation from the SCN (Bamshad et al., 1998; Bartness et al., 2001; Buijs et al., 1999, 2003; Kalsbeek et al., 2000; Olcese et al., 2003), these multisynaptic connections may provide a rapid means of clock resetting in peripheral tissues to ensure proper reception of slower diffusible communication (Fig. 6). A role of autonomic control in peripheral clock resetting comes from investigations in which manipulations of autonomic connections to liver reset clock gene expression in this organ (Terazono et al., 2003).

In summary, global clock resetting may be accomplished via hormonal signals, as glucocorticoids can adjust the phase of peripheral circadian clock genes (Balsalobre et al., 2000). Whereas the role of hormones other than glucocorticoids in resetting peripheral oscillators has not been investigated, the marked effects of hormones on circadian function reviewed herein suggest a potentially crucial role for endocrine factors in orchestrating this multisynaptic arrangement.

## Conclusions and perspectives

In general, we are not aware of the precision in the timing and coordination of numerous events in our bodies, unless it is disrupted (e.g., jet lag). However, processes as fundamental as the timing of sleep and its coordination with feelings of hunger are a manifestation of numerous physiological and biochemical events that change systematically and predictably over the course of the day. Given the numerous salient time cues in the environment, one might intuit that these daily changes are passive responses to environmental change. However, as reviewed here, daily rhythms are endogenously generated and are synchronized to external time cues in order to ensure that bodily processes are carried out at the appropriate, optimal time of day or night.

Because most brain and bodily processes require a significant amount of time to achieve appropriate regulation the body must anticipate these changes and prepare accordingly in advance. For example, genomic actions of steroid hormones can take several hours to have their effects, and these hormones must be secreted prior to the time during which the behavior is best performed. Likewise, timed peak and trough hormone secretion may be required to prevent receptor down-regulation and desensitization. Because generating new receptors requires significant metabolic energy and time, episodic hormone secretion may be required to allow for adequate receptor turnover. Thus, an endogenous time-keeping system is necessary to anticipate environmental change and initiate internal adjustments in advance of the

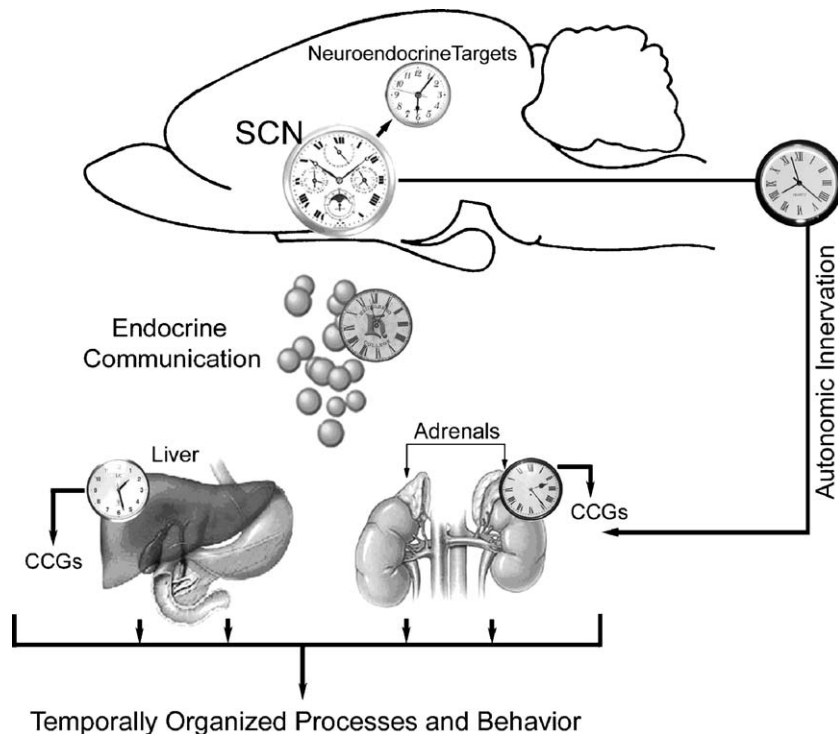


Fig. 6. Overall organization of the circadian system. This organization is based on the postulation that rhythmic system physiology is controlled by a combination of neural and diffusible signals originating from the SCN. In this view, specific systems may be differentially regulated by SCN signals via local clocks allowing for more specific responsiveness based upon local needs and time of day (see text for additional details).

appropriate environmental time in order to coordinate innumerable bodily processes.

The present overview shows how the circadian system controls the timing of hormone secretion using a number of mechanisms, including direct transcriptional and SCN neural control of neurosecretory factors, and control of glands by hormones, clock genes, and autonomic innervation. Because hormones can have a widespread influence over physiology and behavior, and provide a means by which circadian information can be communicated systemically, it is important to determine how these rhythms are regulated. Not only are hormones modulated by the circadian system, but hormonal feedback to the SCN also influences circadian function (Dubocovich et al., 1996; Ellis and Turek, 1983; Hastings et al., 1997; Jechura et al., 2003; Labyak and Lee, 1995; Lewy and Sack, 1997; Morin et al., 1977). Together, these mechanisms controlling endocrine timing entail regulatory actions by the circadian system and provide extensive opportunities for empirical investigations of behaviorally relevant systems.

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