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Expression of the circadian clock gene *Period 1* in neuroendocrine cells: an investigation using mice with a *Per1::GFP* transgene

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Abstract

The circadian clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus regulates daily temporal organization in behaviour and neuroendocrine function. The molecular basis for circadian rhythm generation is an interacting transcriptional/translational feedback loop comprised of several ‘clock genes’ and their respective protein products. Clock genes are expressed not only in the SCN but also in numerous other locations throughout the brain, including regions rich in neuroendocrine cells. In order to investigate whether neuroendocrine cells function as autonomous oscillators, we used female transgenic mice in which an unstable, degradable jellyfish green fluorescent protein (GFP) gene is driven by a mouse *Period 1* (*Per1*) gene promoter. Mice were injected (s.c.) with fluorogold (FG) in order to label neuroendocrine cells and brain sections were double-labelled for either FG and *Per1* mRNA (labelled by GFP immunostaining) or FG and PER1 protein using fluorescence immunocytochemistry. Mice were killed during either the day or night. Neuroendocrine cells contained *Per1* mRNA and PER1 protein in several brain regions with the greatest proportion of double-labelled cells occurring in the arcuate nucleus (Arc). The number of neuroendocrine cells labelled was not affected by the stage of the estrous cycle. Fewer FG-labelled cells expressed *Per1* message and protein during the night compared to the day. In the Arc, staining for tyrosine hydroxylase revealed that neuroendocrine cells expressing *Per1* message and protein were dopaminergic. Together, these findings suggest that neuroendocrine cells contain the molecular machinery necessary to oscillate independently. It remains to be determined whether these cells actually function as autonomous oscillators or whether these rhythms are driven by signals from the SCN. These findings also indicate that the endocrine system represents an opportunity to study the interactions between central (SCN and neuroendocrine cells) and peripheral circadian (endocrine gland) oscillators.

Keywords

clock gene; dopamine; hormone; SCN; suprachiasmatic nucleus; TIDA

Introduction

Precise temporal coordination among numerous neuroendocrine and behavioural events is necessary for organisms to function optimally and maintain homeostasis. For example, in anticipation of waking, cortisol levels rise to mobilize energy resources while insulin levels increase in advance of food intake (Van Cauter, 1990; Czeisler & Klerman, 1999; Gillette & Tischkau, 1999; Kriegsfeld *et al.*, 2002). The daily orchestration of these various neuroendocrine, behavioural and other physiological events is regulated by a circadian clock located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (Turek *et al.*, 1984; Van Cauter, 1990; Czeisler & Klerman, 1999; Gillette & Tischkau, 1999).

Lesions of the SCN abolish circadian rhythms in behaviour and physiology (Moore & Eichler, 1972; Stephan & Zucker, 1972). Transplantation of fetal SCN tissue restores rhythms in several behavioural measures but does not restore neuroendocrine rhythms (Lehman *et al.*, 1987; Ralph *et al.*, 1990; Meyer-Bernstein *et al.*, 1999). As the transplanted SCN does not re-establish many of its normal neural connections with the host brain (Canbeyli *et al.*, 1991; Lehman *et al.*, 1998), this finding suggests that SCN neural output may be critical in the regulation of circadian endocrine rhythms. In agreement with this hypothesis, semicircular knife cuts caudal to the SCN abolish estrous cyclicity but not rhythms in drinking behaviour in rats (Nunez & Stephan, 1977). Additionally, horizontal knife cuts abolish estradiol-induced daily surges in luteinizing hormone (LH), yet leave circadian locomotor behaviour intact (Watts *et al.*, 1989). Finally, surgical isolation of the SCN abolishes the photoperiodic gonadal response but not circadian locomotor behaviour in Syrian hamsters (Hakim *et al.*, 1991). Taken together, these findings suggest that behavioural rhythms can be regulated by a diffusible signal from the SCN to behavioural effector areas, whereas rhythms in neuroendocrine function require neural output (Silver *et al.*, 1996; Kramer *et al.*, 2001; Cheng *et al.*, 2002).

Circadian rhythms within the SCN are a cell-autonomous property produced by an autoregulatory transcriptional/translational feedback loop composed of several 'clock' genes and their protein products. The proteins CLOCK and BMAL1 provide positive transcriptional drive in this feedback loop, while the protein products of the *Period* gene (PER1, PER2, and PER3) in combination with the products of the *Cryptochrome* gene (CRY1 and CRY2) provide negative feedback (Welsh *et al.*, 1995; Gekakis *et al.*, 1998; Sangoram *et al.*, 1998; Griffin *et al.*, 1999; Jin *et al.*, 1999; Kume *et al.*, 1999). Clock genes are also present and rhythmic in numerous extra-SCN brain regions (Yamazaki *et al.*, 2000; Abe *et al.*, 2002) as well as in peripheral organs (Damiola *et al.*, 2000; Yamazaki *et al.*, 2000; Hara *et al.*, 2001). However, the precise function of these extra-SCN clock genes and the means by which the SCN regulates clock gene expression in these brain regions and peripheral organs has not been elucidated.

The SCN projects monosynaptically to numerous brain regions containing neuroendocrine cells, including the preoptic area (POA), paraventricular nucleus of the hypothalamus (PVH), anterior hypothalamus (AH), and the arcuate nucleus (Arc) (Stephan *et al.*, 1981; Watts & Swanson, 1987; Watts *et al.*, 1987; Kalsbeek *et al.*, 1993; Morin *et al.*, 1994). Imaging studies of isolated brain regions from transgenic rats using a luciferase reporter driven by the *mPer1* promoter established that average *Per1*-luciferase expression is rhythmic in tissue segments from these same SCN target regions (Abe *et al.*, 2002); the specific cell type and neurochemical phenotype of these averaged rhythmic cell populations remains to be determined. Additionally, cultured fibroblasts and peripheral organs contain clock genes, express circadian rhythms *in vitro*, and are presumably reset daily by the SCN *in vivo* (Balsalobre *et al.*, 1998; Balsalobre *et al.*, 2000; Yamazaki *et al.*, 2000). These findings suggest that, like fibroblasts and peripheral organs, numerous brain cell types may

contain clock genes, yet require a periodic signal to temporally coordinate the combined rhythmicity of a population of autonomous oscillators. This possibility could account for the loss of circadian endocrine rhythms after transection of SCN output fibres or SCN lesions.

The present study was undertaken to determine if neuroendocrine cells contain the *mPer1* gene, one of the core circadian clock components. Because the pattern of circadian endocrine rhythms is critical for females in the organization of the hormonal milieu necessary for successful ovulation and mating, female mice were investigated in these studies. Transgenic mice with a degradable green fluorescent protein (GFP) reporter driven by a *Per1* gene promoter were used (Kuhlman *et al.*, 2000). This mouse model allowed for the indirect detection of *Per1* mRNA in neuroendocrine cells using double-label fluorescence immunocytochemistry (ICC) permitting both conventional and confocal microscopy. The results of the present investigation indicate that several neuroendocrine cell populations show daily variation in *Per1* mRNA and PER1 protein, particularly neuroendocrine dopaminergic neurons in the arcuate nucleus.

Materials and methods

Experiment 1

Animals and housing—Twelve heterozygous transgenic mice were used in the present experiment (a generous gift of Dr Douglas McMahon, University of Kentucky). All animals were housed in translucent polypropylene cages (48 × 27 × 20 cm) and provided with access to food and water *ad libitum* for the duration of the study. Animals were maintained in a colony room with a 24 h light–dark cycle (LD 12: 12). The rooms were maintained at 23 ± 1 °C. All animals were allowed a minimum of a 1-week acclimation period prior to the onset of the experiment. All animal research in this report was approved by Columbia University's Animal Care and Use Committee.

Experimental manipulation and tissue collection—In order to label neuroendocrine cells in the CNS, all animals were injected (s.c.) with 0.05 cc of 4% fluorogold (FG; Fluorochrome, Inc., Denver, CO) in 0.9% saline and killed 5 days later with an injection of sodium pentobarbital (see below). Because FG does not cross the blood–brain barrier, this procedure only labels cells in the brain that come into contact with fenestrated capillaries (Merchenthaler, 1991). One set (*n* = 4) of the animals was killed during the day at *Zeitgeber* time 10 (ZT10; 10 h after light onset) without regard to the stage of the estrous cycle. GFP protein peaks at approximately ZT7.5–ZT8 (D. McMahon, personal communication) while PER1 protein is elevated between ZT10 and ZT16 (Hastings *et al.*, 1999). Thus, ZT10 was chosen as a time point during which both GFP and PER1 could be investigated in the same animal. Because activation or appearance of clock components may be important for the onset of estrous behaviour, a second set (*n* = 4) of the females was killed during vaginal proestrus (i.e. behavioural estrus). Finally, because previous studies in *Per1*-luciferase rats have reported that maximal clock gene expression in some extra-SCN brain regions is in antiphase (i.e. 12 h out of phase) with expression in the SCN (Abe *et al.*, 2002), an additional set of animals (*n* = 4) was killed during the night at ZT22.

To collect the brains, animals were deeply anaesthetized with sodium pentobarbital (200 mg/kg) and perfused transcardially with 50 mL of 0.9% saline followed by 100 mL of 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were postfixed in 4% paraformaldehyde for 2 h at 4 °C, then cryoprotected in 20% sucrose in 0.1 M PBS overnight at 4 °C. Coronal sections (30 µm) were cut on a cryostat and collected into 0.1 M PBS. The brain sections were processed as free-floating sections. Every fourth section was processed for FG and a different protein (i.e. GFP or PER1).

Double-label GFP::Per1 fluorescence immunocytochemistry—For simultaneous visualization of GFP and FG, every 4th, 30 μ m section was double-labelled (4 brains for each condition; ZT10, $n=4$; ZT10 vaginal proestrus, $n=4$; ZT22, $n=4$) using fluorescence immunocytochemistry. For visualization of GFP, sections were washed in PBS, incubated in 0.5% H_2O_2 , and then incubated in normal goat serum in 0.1% Triton X-100 (PBT) for 1 h. Sections were then incubated for 48 h at 4 °C in rabbit anti-GFP (Molecular Probes, Eugene, OR) diluted at 1: 800 000 with 0.1% PBT. Following incubation in anti-GFP, brains were incubated for 1 h in biotinylated goat anti-rabbit (1: 300; Vector, Burlingame, CA), followed by incubation in avidin–biotin–horseradish peroxidase complex (ABC Elite Kit, Vector, Burlingame, CA). Brains were then incubated in a biotinylated tyramide solution (0.6%) for 30 min. This protocol allowed for the amplification of the highly diluted anti-GFP antibody required for double-labelling with two antibodies generated in the same species. Cells were then labelled using Cy-2 conjugated to streptavidin (Jackson Laboratories, West Grove, PA) as the fluorophore. Following labelling for GFP, sections were incubated for 48 h in a rabbit anti-FG antibody (Chemicon, Temecula, California) diluted 1: 2000 with 0.1% PBT. FG cells were labelled with Cy-2 donkey anti-rabbit (Jackson Laboratories, West Grove, PA) as the secondary antibody/fluorophore. Sections were mounted onto gelatin-coated slides and coverslips were applied following dehydration in a graded series of alcohols. For control experiments, one antibody was eliminated (i.e. GFP or FG) and all other steps were followed as previously described.

Double-label PER1 protein fluorescence immunocytochemistry—To determine if *Per1* mRNA seen in neuroendocrine cells is being translated into a biologically significant protein, one series of 30 μ m sections from the brains of all animals from each condition were stained for PER1 protein and FG. Immunocytochemistry was performed as described above for GFP and FG. PER1 was the first antibody labelled using a rabbit anti-PER1 antibody (1: 50 000; generous gift of Dr Stephen Reppert and Dr David Weaver, University of Massachusetts, Worcester, MA). PER1 labelling was subsequently amplified using the biotinylated tyramide protocol as described above. Staining for FG was also performed as described above. For control experiments, one antibody was eliminated (i.e. PER1 or FG) and all other steps were followed as previously described.

Light microscopy—GFP or PER1 proteins and FG double-label was investigated using a Nikon Eclipse E800 microscope. Sections were examined using the standard wavelengths for Cy-2 (488 nm) and Cy-3 (568 nm). Every 4th section (for each protein) from the medial septum to the caudal aspect of the anterior hypothalamus was assessed, and those areas expressing GFP or PER1 were investigated more extensively for coexpression with FG using confocal microscopy (see below). Areas identified as having double-labelled cells were digitally captured at 200 x in 8 bit greyscale using a cooled CCD camera (SPOT; Morrel, Meville, NY). Each label was captured as a single image without moving the position of the stage or plane of focus between captures. Images were superimposed digitally using SPOT software (Morrel, Meville, NY). Brain areas were examined for double-labelling using conventional light microscopy and were scanned at the confocal level for quantification.

Confocal microscopy—Brain sections used for light microscopy were also used for the confocal scans in order to characterize FG and GFP or FG and PER1 double-labelling. Regions of the brain with putative double-label identified at the light level were scanned at 400 $\times$ using confocal microscopy. Cells were observed under a Zeiss Axiovert 100TV fluorescence microscope (Carl Zeiss, Thornwood, NY) with a Zeiss LSM 410 laser scanning confocal attachment. The sections were excited with an Argon-Krypton laser using the standard excitation wavelengths for Cy-2 and Cy-3. Stacked images were collected as 1.0

μm multitract optical sections (with sequential excitation by each laser to avoid ‘cross-talk’ between the two wavelengths). Using the LSM 3.95 software (Zeiss), red and green images of the sections were superimposed. Neuroendocrine cells in a given brain region were examined through their entirety in $1.0\ \mu\text{m}$ steps. The number of scans through each brain region was a function of the rostral–caudal width of a particular population of cells within a nucleus, and therefore differed for each brain region. The following brain areas expressing double-labelled cells were investigated (other brain regions did not have double-labelled cells, see Results section): anterodorsal preoptic nucleus (ADP; one brain section per animal per label), anteroventral periventricular nucleus (AVPV; 1 brain section per animal per label), PVH, (three brain sections per animal per label), and Arc (three brain sections per animal per label). Counts from the brains of three animals from each experimental condition [for GFP labelling ZT10 ($n=3$), ZT10 proestrus ($n=3$), ZT22 ($n=3$); for PER1 labelling, ZT10 ($n=3$), ZT10 proestrus ($n=3$), ZT22 ($n=3$)] were quantified at the confocal level.

Experiment 2

Treatment and immunocytochemistry—Because the Arc contains a large number of dopaminergic neuroendocrine cells, and this region shows robust rhythms of *Per1*-luciferase expression (Abe *et al.*, 2002), one series of $30\ \mu\text{m}$ sections from the brains of animals from Experiment 1 was stained for GFP or PER1 protein as well as tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine production. Immunocytochemistry was performed on 4 additional animals as described in Experiment 1. In alternate sections, brains were labelled for either GFP and TH (TH from Diasorin Inc; diluted 1: 5000) or PER1 and TH as described in Experiment 1. For control experiments, one antibody was eliminated and all other steps were followed as previously described.

Statistics—All reported analyses and statistics were performed on data collected at the confocal level. For each brain region within an animal, results were averaged over individual brain sections to yield a single value for each brain region for each animal. Total cell count data for Experiments 1 and 2 were analysed using 2×3 (time $\times$ brain region) analyses of variance (ANOVA). Because percentage data from Experiments 1 and 2 were nonparametric, all percentage data was analysed using 2×3 (time $\times$ brain region) analyses of variance on ranks. Data from Experiment 2 were also nonparametric percentage data analysed using a 2×2 (time $\times$ staining type) ANOVA on ranks. Group comparisons were made following a significant main effect using the Tukey HSD test. Results were considered significant when $P < 0.05$.

Results

Experiment 1

Characterization of FG and *Per1::GFP* and PER1 labeling—Following s.c. injection of FG, neuroendocrine cells in the medial septum (MS), diagonal band of Broca (DBB), ADP, POA, AVPV and hypothalamic areas including the PVH, dorsomedial hypothalamus (DMH), supraoptic nucleus (SON), Arc were labelled by the FG antibody.

Comparisons of the expression of GFP in the SCN at two different times of day indicated that, during the day, GFP and PER1 protein expression were extensive throughout the rostral–caudal extent of the SCN, whereas staining was virtually absent during the night (Fig. 1). In addition to the SCN, the expression of GFP and PER1 was also observed in the following brain regions that contained neuroendocrine cells: septal areas, the DBB, ADP, POA, AVPV, PVH, DMH and Arc. The total number of GFP cells seen was significantly reduced in the Arc at night compared to the day ($P < 0.05$; Fig. 2, top), while there were no

significant differences seen in the ADP and PVH with regard to the time that the animal was killed. Results for PER1 staining are presented below.

In order to determine whether or not *Per1* mRNA expression occurred within neuroendocrine cells, all brain regions in which the FG and GFP labelling was observed (i.e. MS, DBB, ADP, POA, AVPV, PVH, DMH, and Arc), were first examined at the light microscopic level. Double-labelled cells were observed at the light level only in the ADP, PVH and Arc during both the day and night. The percentage of GFP cells double-labelled for FG was quantified in 1 μm confocal scans (Fig. 2, bottom). Double-labelling was confirmed and quantified at the confocal microscopic level in the ADP, PVH and Arc (Figs 2 and 3). Confocal scans of the POA and AVPV failed to reveal any double-labelled cells. As there were no significant differences between animals in proestrus vs. animals at random stages of the reproductive cycle ($P > 0.05$ for each brain region), data were collapsed over reproductive stage within each time point. The percentage of GFP cells also labelled with FG was reduced in the Arc at night compared to the day ($P < 0.05$). A similar trend, that did not reach statistical significance was observed in the ADP and PVH ($P > 0.05$).

To establish whether *Per1* mRNA was being translated into protein in neuroendocrine cells, PER1 expression was examined. PER1 was localized to the same brain regions listed for GFP but PER1-ir cells were greater in number than GFP-ir cells. Overall, the mean number of PER1-ir cells was higher during the day than at night ($P < 0.05$), with significant differences in the PVH and Arc ($P < 0.05$ in each case; Fig. 4). As with GFP, PER1 protein expression was seen in FG cells in the ADP, PVH, and Arc at both the light and confocal microscope levels (Fig. 3). The percentage of double-labelled PER1-ir was significantly reduced in the Arc at night compared to the day ($P < 0.05$), with a similar trend that did not reach significance in the ADP and PVH ($P > 0.05$ in each case; Fig. 4). PER1-ir in cells in the AVPV was not consistent among animals. In one animal, about 25% of the cells (of the 69 cells evaluated at the confocal level) in the AVPV contained PER1 protein. Staining was absent in the AVPV of other animals during the day and night.

Experiment 2

The results of Experiment 1 indicated that a large number of neuroendocrine cells in the Arc expressed both *Per1* mRNA and protein. The rostral Arc contains neuroendocrine dopaminergic neurons that project to the anterior pituitary (tuberohypophyseal dopaminergic neurons; THDA) (Bjorklund *et al.*, 1973), while more caudal aspects of the Arc contain neuroendocrine dopaminergic neurons that project to the infundibulum (tuberoinfundibular dopaminergic neurons; TIDA) (Fuxe, 1964); both populations of cells regulate prolactin secretion (Ben-Jonathan, 1985). To determine if the cell phenotype of GFP- and PER1-expressing FG cells labelled was dopaminergic, TH cells were visualized in the Arc. A subset of TH cells was labelled with both GFP and PER1 proteins, with the latter marker revealing more cells ($P < 0.05$; Figs 5 and 6). Significantly more TH cells were labelled with PER1 during the day than the night ($P < 0.05$; Fig. 6). A similar trend that did not reach statistical significance was seen with GFP labelling ($P > 0.05$).

Discussion

The present results are the first to reveal that specific neuroendocrine cell populations (i.e. the ADP, PVH, and Arc) express *Per1*, with several exhibiting a diurnal rhythm. These findings extend previous studies examining isolated brain regions from *Per1*-luciferase rats, showing that mean *Per1* expression is rhythmic in brain regions outside of the SCN (e.g. Abe *et al.*, 2002). Overall, *Per1* expression in neuroendocrine cells showed a diurnal rhythm paralleling that of the SCN. In a number of brain regions (septum, DBB, POA, AVPV, and DMH), both neurosecretory neurons and *Per1*-expressing neurons were seen, but no double-

labelled cells were detected. In contrast, in other brain regions (ADP, PVH, Arc), neuroendocrine cells expressed *Per1* mRNA and protein. Additionally, neuroendocrine dopaminergic neurons in the Arc exhibited a diurnal rhythm in *Per1* expression. Dopamine neurons in the Arc are the major regulators of prolactin secretion (Fuxe, 1964; Bjorklund *et al.*, 1973; Ben-Jonathan, 1985), suggesting that daily rhythms in prolactin secretion may be regulated locally by a circadian mechanism in dopaminergic neurosecretory cells. Together with data from previous studies of SCN regulation of neuroendocrine rhythms, the findings reveal the hierarchical organization by which circadian endocrine rhythms may be regulated by SCN input.

In neuroendocrine cells, *Per1* mRNA and protein exhibit the same diurnal pattern of expression as the SCN. Most hormones exhibit a circadian rhythm but, importantly, the phase of peak production and secretion differs among hormonal systems (reviewed in Kriegsfeld *et al.*, 2002). That the general pattern of *Per1* expression in several neuroendocrine cell populations in the present investigation mirrored that of the SCN (i.e. high during the day and low at night) suggests that differences in the rhythmic pattern among hormonal systems may ultimately be regulated at the level of the target endocrine gland. Further studies are necessary to determine if this daily rhythm will persist in populations of neuroendocrine cells (or in individual cells) in the absence of the SCN or if input from the main circadian pacemaker is necessary to sustain this rhythm *in vivo*.

Because dopamine is the only catecholamine expressed in the Arc, the present results with tyrosine hydroxylase staining suggest that circadian clock function in neuroendocrine dopaminergic neurons in this brain region are important for the regulation of reproduction in females. Female rodents exhibit a surge of prolactin on the afternoon of proestrus (Butcher *et al.*, 1974; Smith *et al.*, 1975). As mentioned above, the primary regulator of prolactin secretion is dopamine (Ben-Jonathan, 1985), and the SCN projects to dopamine neurons in the Arc (Gerhold *et al.*, 2001). Lesions of the SCN abolish the proestrus surge of prolactin, resulting in constant basal release of this hormone (Palm *et al.*, 2001). One SCN neurohormone, vasopressin, is secreted in a circadian fashion with low levels at night and high levels during the day (Schwartz *et al.*, 1983). Administration of vasopressin into the 3rd ventricle inhibits prolactin release in a time-dependent fashion (Palm *et al.*, 2001). Together, these findings suggest that projections from the SCN communicate critical timing information to dopaminergic neurons in the Arc, but time-dependent sensitivity to this SCN signal may be regulated or gated locally by clocks within these dopaminergic cells.

In all cases, GFP expression in neuroendocrine cells was lower than that of PER1. This discrepancy is most likely due to the different time course of GFP and PER1 translation following the transcription of their respective genes, with the former peaking approximately ZT7.5–ZT8 (McMahon, personal communication) while the latter is elevated between ZT10–ZT16 (Hastings *et al.*, 1999). The present study investigated *Per1* expression during both the day (ZT10) and the night (ZT22). Insertion of the *Per1::GFP* transgene may not insure transcription in all cell types. For example, adjacent chromatin could silence the transgene in some cells or cell types (Walters *et al.*, 1995; Festenstein *et al.*, 1996; Graubert *et al.*, 1998; Festenstein & Kioussis, 2000). Thus, GFP staining in the present study may underestimate the number of neuroendocrine cells expressing *Per1*. Nonetheless, results with PER1 protein expression, demonstrating the same basic pattern of findings in the present study, indicate that GFP provides a useful tool for evaluating which populations of neuroendocrine cells express *Per1*.

The results of the present study suggest a hierarchical organization by which some circadian endocrine rhythms are generated. This theoretical model is based on the assumption that neuroendocrine cells containing clock genes can oscillate independently, although the

present findings do not rule out the possibility that *Per1* is driven by the SCN in some neuroendocrine cells. At the top of this hierarchy, oscillator cells within the SCN communicate rhythmic information via neural efferents to putative ‘slave’ oscillators in neuroendocrine cells. Although capable of oscillating independently, these neuroendocrine cells require periodic SCN input to maintain a synchronized rhythm in the local cell population. Such a mechanism could account for the loss of circadian endocrine rhythms following lesions or transection of outputs from the SCN (Moore & Eichler, 1972; Nunez & Stephan, 1977; Meyer-Bernstein *et al.*, 1999). Coordinated rhythmic output from neuroendocrine cells can then be communicated to the pituitary, which also contains circadian clock genes (Messenger *et al.*, 2001; von Gall *et al.*, 2002). Finally, rhythmic information from the pituitary can be communicated humorally to target glands in the periphery that themselves contain clock genes (Zylka *et al.*, 1998; Tong *et al.*, 2002). This hierarchy, with multiple levels of circadian regulation, may allow the anticipation of the reception of specific daily signals at each level of the axis. Consistent with this logic, multisynaptic projections from the SCN to several endocrine glands have been identified using viral tracers (Buijs *et al.*, 1998; Buijs *et al.*, 1999; Gerendai *et al.*, 2000; Kalsbeek *et al.*, 2000), suggesting a mechanism by which the SCN could coordinate timing among glands to optimize responses to endocrine signals (Fig. 7).

A hierarchical organization requires that the SCN communicate directly or indirectly with neuroendocrine cells containing clock genes. There is substantial evidence of direct projections from the SCN to neuroendocrine cells. The SCN projects monosynaptically to corticotropin-releasing hormone (CRH) and spinal-projecting cell populations in the PVH (Buijs *et al.*, 1993; Vrang *et al.*, 1995; Teclemariam-Mesbah *et al.*, 1997). Likewise, direct SCN projections to neuroendocrine dopamine neurons in the Arc have also been identified (Gerhold *et al.*, 2001). Expression of the clock gene, *Per1*, has been shown in both the PVH and Arc (Asai *et al.*, 2001; Takahashi *et al.*, 2001; Abe *et al.*, 2002); *Per1* is rhythmically expressed in the Arc (Abe *et al.*, 2002) and exhibits a stress-induced increase in the PVH (Takahashi *et al.*, 2001; Abe *et al.*, 2002). *Per1* has also 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 in the present study. In addition, results from the present study extend these previous findings by showing a diurnal rhythm in *PER1* protein in the PVH and Arc, providing evidence for the rhythmic translation of *Per1* mRNA into its functional protein product in discrete brain regions.

In summary, results from the present study along with previous findings demonstrate that the neuroendocrine axis is hierarchically organized with circadian clock gene regulation at multiple levels of the hypothalamo–pituitary–endocrine gland axis. Circadian regulation at multiple levels of the neuroendocrine system suggests that this redundancy is required to anticipate the reception of daily signals from each higher level of the axis. This organization suggests that the endocrine system provides an ideal system by which to study the significance of the interactions between central and peripheral oscillators in circadian regulation.

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Abbreviations

AH anterior hypothalamus

ADP	anterodorsal preoptic nucleus
Arc	arcuate nucleus
AVPV	anteroventral periventricular nucleus
DBB	diagonal band of Broca
DMH	dorsomedial hypothalamus
FG	fluorogold
GFP	green fluorescent protein
ICC	immunocytochemistry
MS	medial septum
POA	preoptic area
PVH	paraventricular nucleus of the hypothalamus
SCN	suprachiasmatic nuclei
SON	supraoptic nucleus
TH	tyrosine hydroxylase
THDA	tuberohypophyseal dopaminergic neurons

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Fig. 1. Photomicrographs of GFP, (panels A and B) and PER1, (panels C and D) immunoreactive neurons in the SCN of animals killed during the day (ZT10, panels A and C) and during the night (ZT22, panels B and D).

Fig. 2.

Mean (+SEM) number of GFP cells in the ADP, PVH and Arc in animals that were killed during the day and night (ZT10 and ZT22, respectively; top panel). Mean (+SEM) percentage of GFP cells that are expressed in FG-positive neurons. Cells were evaluated in the ADP, PVH, and Arc in animals that were killed during the day and night (ZT10 and ZT22, respectively; bottom panel). *Significantly less than animals killed during the day within the same brain region ($P < 0.05$).

Fig. 3. Photomicrographs showing 1 μ m confocal optical scans through the ADP (top panels), paraventricular nucleus of the hypothalamus (PVH; middle panels), and the arcuate nucleus (Arc; bottom panels) from animals killed during the day and night (ZT10 and ZT22, respectively). Brains were double-labelled for GFP or PER1 protein (green) and FG (red) using immunofluorescence. Arrows for GFP staining in the ADP in row 1 point to cells for which the red channel is shown separately in the insert. Arrows for PER1 staining in the ADP in row 1 point to representative double-labelled cells in the insert. V3, 3rd ventricle. Arrows in rows 2 and 3 point to cells that are displayed as separate red and green channels.

Fig. 4. Mean (+SEM) number of PER1-ir cells in the ADP, PVH, and Arc in animals that were killed during the day and night (ZT10 and ZT22, respectively; top panel). Mean (+SEM) percentage of PER1-labelled nuclei that were expressed in FG-positive neurons (bottom panel). Cells were evaluated in the ADP, PVH, and Arc in animals that were killed during the day and night (ZT10 and ZT22, respectively). *Significantly less than animals killed at ZT10 within the same brain region ($P < 0.05$).

Fig. 5. Photomicrographs showing a 1 μ m confocal optical scan through the arcuate nucleus of animals killed during the day (ZT10; left) and night (ZT22; right). Brains were double-labelled for either GFP or PER1 protein (green) and TH (red) using immunofluorescence. Arrows in each figure point to cells that are displayed as separate red and green channels

Fig. 6.
Percentage of TH-ir cells containing GFP or PER1 in animals killed during either the day or night (ZT10 and ZT22, respectively).

Fig. 7.

Theoretical model based on the present and previous studies suggesting a means by which circadian regulation of neuroendocrine function may be hierarchically organized such that each level of the axis can anticipate signals from other levels of the axis. In order for neuroendocrine rhythms to be synchronized to local environmental time, retinal input is first transmitted to the SCN to synchronize the endogenous oscillators in this nucleus. Given the present findings of a core clock component in neuroendocrine neurons, and previous studies showing direct SCN innervation of these neurons, the 2nd link in this hierarchy is a direct neural connection from the SCN to potentially autonomous oscillating neuroendocrine cells which theoretically have rhythms of different periods and phases until coordinated by SCN input. This SCN input synchronizes rhythms among independent cells and sets their phase in these brain regions. In turn, rhythmic neurohormone secretion communicates to downstream targets of the neuroendocrine axis (some of which contain clock genes).