

A Molecular Mechanism Regulating Rhythmic Output from the Suprachiasmatic Circadian Clock

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Summary

We examined the transcriptional regulation of the clock-controlled arginine vasopressin gene in the suprachiasmatic nuclei (SCN). A core clock mechanism in mouse SCN appears to involve a transcriptional feedback loop in which CLOCK and BMAL1 are positive regulators and three *mPeriod* (*mPer*) genes are involved in negative feedback. We show that the RNA rhythm of each *mPer* gene is severely blunted in *Clock/Clock* mice. The vasopressin RNA rhythm is abolished in the SCN of *Clock/Clock* animals, leading to markedly decreased peptide levels. Luciferase reporter gene assays show that CLOCK-BMAL1 heterodimers act through an E box enhancer in the vasopressin gene to activate transcription; this activation can be inhibited by the mPER and mTIM proteins. These data indicate that the transcriptional machinery of the core clockwork directly regulates a clock-controlled output rhythm.

Introduction

A brain pacemaker driving circadian rhythms in mammals resides in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (Klein et al., 1991; Reppert and Weaver, 1997). The SCN sense changes in ambient lighting through both direct and indirect retina-to-SCN neural pathways. In this way, light synchronizes (entrains) the SCN and its driven output rhythms to the 24 hr day. An important feature of SCN organization is that it is a multioscillatory system with the entire clockwork residing in single neurons (Welsh et al., 1995; Liu et al., 1997). Interactions among these "clock cells" in the whole SCN serve to synchronize individual circadian clocks to generate coordinated circadian outputs. These outputs ultimately control a vast array of circadian rhythms in physiology and behavior.

A clear view of a molecular clock mechanism within SCN neurons is beginning to emerge (reviewed in Reppert, 1998). The central clockwork appears to involve an intracellular transcriptional/translational feedback loop, similar to the one described for the clock genes *period* (*per*) and *timeless* (*tim*) in *Drosophila melanogaster*. In the fly, the positive transcriptional regulation of *per* and *tim* is controlled by two basic helix-loop-helix (bHLH)/PAS proteins, dCLOCK and dBMAL1, which heterodimerize and bind to CACGTG E box enhancers (Hao et al., 1997; Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998). As the levels of PER and TIM rise, they are phosphorylated (Kloss et al., 1998; Price et al., 1998), form heterodimers, and then translocate to the nucleus. Once in the nucleus, PER and TIM act as negative regulators of their own transcription by interfering with CLOCK-BMAL1-mediated transcription (Darlington et al., 1998), perhaps by directly interacting with CLOCK (Lee et al., 1998). Thus, the rhythmic expression of *per* and *tim* constitutes a core feedback loop in the fly clock that is driven by both positive and negative elements.

Positive transcriptional elements of a mammalian feedback loop also involve CLOCK and BMAL1, which were first cloned (Hogenesch et al., 1997; Ikeda and Nomura, 1997; King et al., 1997) and characterized (Hogenesch et al., 1998; Gekakis et al., 1998) in mammals. The negative elements of a mammalian loop appear to involve a family of three *per* homologs (Albrecht et al., 1997; Shearman et al., 1997; Sun et al., 1997; Tei et al., 1997; Takumi et al., 1998a, 1998b; Zylka et al., 1998a). Each mouse (*m*)*Per* gene encodes a protein with a protein dimerization PAS domain, and they all appear to be clock relevant as the RNA levels of each are rhythmic in the SCN. As in the fly, mammalian CLOCK-BMAL1 heterodimers bind E box elements in the 5' flanking region of the *mPer1* gene to activate transcription (Gekakis et al., 1998). Homodimeric and heterodimeric interactions between the mPER proteins may be important for their nuclear translocation and for completing the negative limb of a mammalian clock feedback loop (Zylka et al., 1998b). Recently, a mouse (*m*)*Tim* homolog has been cloned, but its role in SCN clock function has not yet been clearly defined (Sangoram et al., 1998; Zylka et al., 1998b). Transcriptional feedback loops have emerged as a common mechanism for circadian clocks across phylogeny, including clocks in cyanobacteria (Ishiura et al., 1998), fungi (reviewed in Dunlap, 1998), and plants (Schaffer et al., 1998; Wang and Tobin, 1998).

An important goal of research in the field of circadian biology is defining the ways in which a central clock mechanism ultimately regulates the temporal variations in diverse biological processes (Loros, 1995). Indeed, nothing is known in mammals about the molecular mechanisms linking the circadian clock to output genes that control or modulate rhythms in physiology and behavior. Even in the fly and fungus *Neurospora crassa*, several clock-controlled genes (CCGs) have been identified, but none have been directly linked to a central clock mechanism (Loros and Dunlap, 1991; Van Gelder et al., 1995; Bell-Pedersen et al., 1996; Rouyer et al.,

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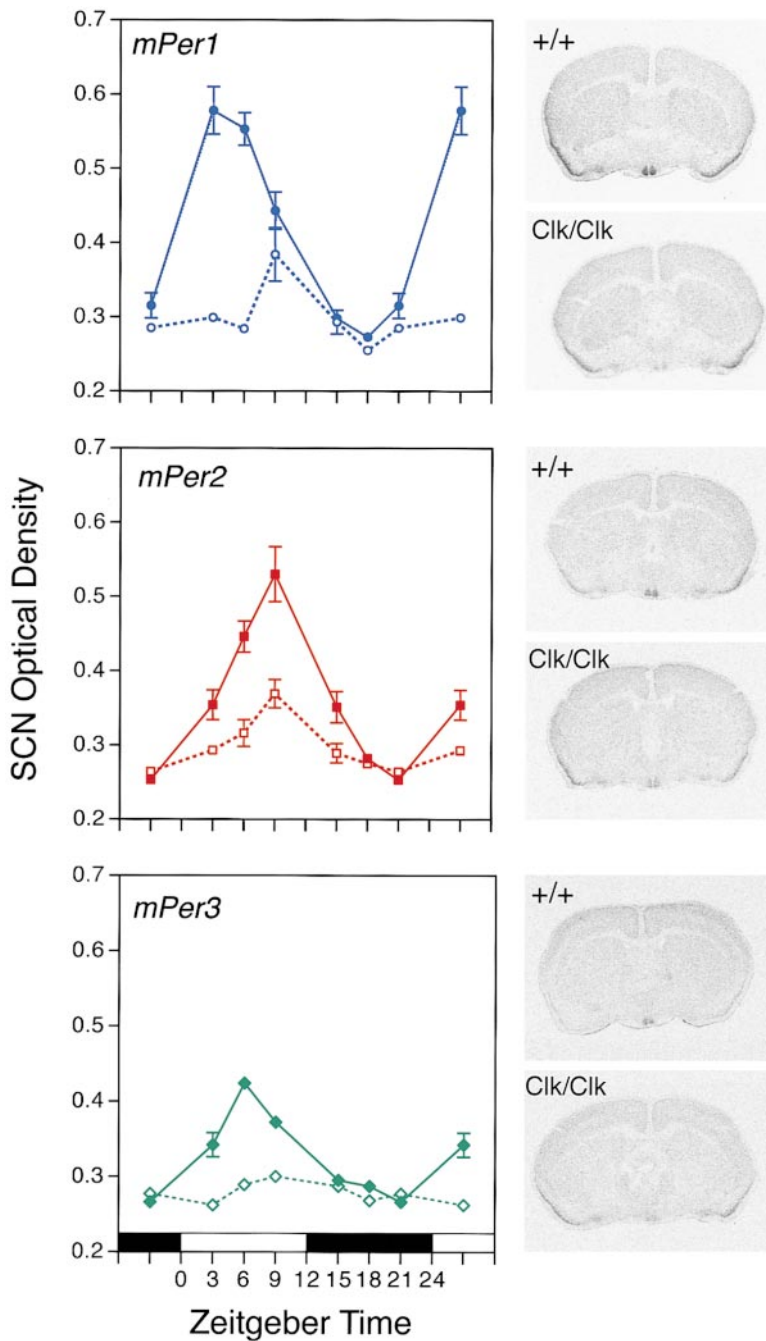


Figure 1. Rhythms of *mPer1*, *mPer2*, and *mPer3* RNA Levels Are Blunted in *Clock/Clock* Mice

Left panel depicts the temporal profiles of *mPer1* (blue), *mPer2* (red), and *mPer3* (green) RNA levels in the SCN of wild-type mice (solid lines) and *Clock/Clock* mice (dashed lines). Animals were studied in 12L:12D with lights on from Zeitgeber times 0–12. Each value for each genotype is the mean \pm SEM of 6–8 animals. Data at ZT 21 and ZT 3 are double plotted. Right panel depicts representative autoradiographs from wild-type and *Clock/Clock* (*Clk/Clk*) mice of coronal brain sections (15 μ m) at the level of the SCN at ZT 6. The brain sections were examined by in situ hybridization using cRNA probes. Magnification, 3.2 \times .

1997; McNeil et al., 1998). With the elucidation of a putative molecular mechanism for the SCN clock, a direct molecular connection between the core feedback loop and the regulation of CCGs can now be evaluated in mammals.

The most well-studied CCG in the mammalian SCN is the gene for the neuropeptide arginine vasopressin. Vasopressin is well known for its peripheral effects on salt and water balance (Schrier, 1985). Blood-borne peptide is largely derived from vasopressinergic neurons in the paraventricular nuclei (PVN) and supraoptic nuclei (SON) of the hypothalamus and released from the posterior pituitary. In addition to its effects on water balance, vasopressin has a number of distinct actions within the

central nervous system (CNS) (Ferris et al., 1984; Mihai et al., 1994a, 1994b; Ingram et al., 1996; Kalsbeek et al., 1996a, 1996b).

Vasopressin synthesized by SCN neurons is both functionally and anatomically compartmentalized for specific actions within the CNS (reviewed in Reppert et al., 1987). Vasopressin is synthesized and released in a circadian manner from neurons in the SCN. The SCN-derived peptide rhythm is driven by rhythmic transcription of the vasopressin gene (Carter and Murphy, 1992). Peptide released from axons within the SCN acts locally through vasopressin- V_1 receptors to modulate the amplitude of the SCN neuronal firing rate rhythm (Mihai et al., 1994a, 1994b; Ingram et al., 1996). Vasopressinergic

effluent projections from the SCN also transmit circadian output signals to distant hypothalamic and extrahypothalamic regions (Kalsbeek et al., 1996a, 1996b). An interesting aspect of the SCN vasopressin rhythm is that it is not dependent on negative feedback by the peptide. Indeed, homozygous Brattleboro rats, which have a deletion mutation in a structural portion of the vasopressin gene, still exhibit a vasopressin RNA rhythm in the SCN (Uhl and Reppert, 1986).

One way in which CCGs could be rhythmically regulated is by using the same transcriptional machinery that controls the clock feedback loop. In this report, we test the hypothesis that vasopressin gene transcription is positively regulated by CLOCK-BMAL1 heterodimers acting through an E box enhancer. Our data show that CLOCK-BMAL1 indeed serve a dual function within the SCN clockwork: they regulate both a core circadian feedback loop and a clock-controlled output rhythm. Moreover, the CLOCK-BMAL1 activation of vasopressin gene transcription can be inhibited by rhythmic components of the core transcriptional loop. The defined molecular link between the central feedback loop and vasopressin gene expression provides a mechanism that may be applicable to the regulation of CCGs within a variety of circadian clocks.

Results

Rhythms of *mPer1*, *mPer2*, and *mPer3* RNA Levels in the SCN Are Regulated by CLOCK

Both in vitro and in vivo studies indicate that *mPer1* transcription is regulated by CLOCK-BMAL1 heterodimers binding to E box enhancers (Gekakis et al., 1998). Furthermore, the mutant CLOCK protein (CLOCK-Δ19) produces a dominant-negative phenotype. In vitro studies show that CLOCK-Δ19-BMAL1 heterodimers, while still capable of binding DNA, have defective transcriptional activity. *mPer2* and *mPer3* are probably under similar transcriptional regulation because the phase of their RNA rhythms in the SCN is similar to that for *mPer1*. We thus used in situ hybridization to compare the RNA rhythms for each of the *mPer* genes in the SCN of wild-type and *Clock/Clock* mice. RNA rhythms were assessed by examining six time points over a 24 hr period in a 12 hr light:12 hr dark (12L:12D) lighting cycle. Diurnal lighting was used because of the marked difference in free-running circadian periods between wild-type animals (23.5 hr) and *Clock/Clock* animals (28 hr) in constant darkness (DD) (Vitaterna et al., 1994). This large period difference in DD could hinder an accurate comparison of *mPer* rhythms between the two genotypes. In LD, on the other hand, locomotor activity rhythms of wild-type and *Clock/Clock* animals are entrained, and the phase relationship of the behavioral rhythms to LD is similar between the two genotypes (Vitaterna et al., 1994).

Wild-type animals exhibited a robust daily rhythm in RNA levels for each of the *mPer* genes (Figure 1). The *mPer1* and *mPer3* RNA oscillations preceded the *mPer2* RNA oscillation by ca. 3 hr, similar to what has been previously described (Tei et al., 1997; Zylka et al., 1998a). In contrast to wild-type animals, *Clock/Clock* animals manifested an RNA rhythm for each *mPer* gene that,

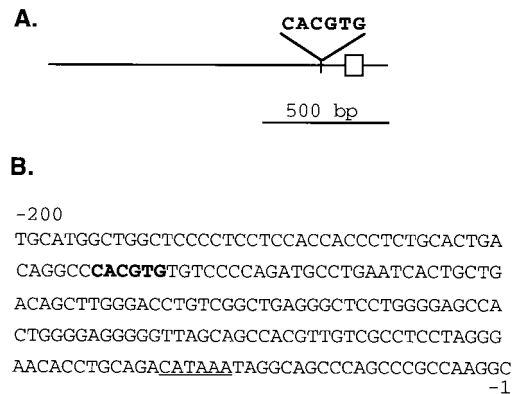


Figure 2. A CACGTG E Box Enhancer Resides in the 5' Flanking Region of the Mouse Vasopressin Gene

(A) Location of the CACGTG E box in the 5' flanking region of the vasopressin gene. The box represents the first exon.

(B) Sequence of 200 bp of the 5' flanking region. The numbers indicate the distance in nucleotides from the transcription start site (Hara et al., 1990). The sequence depicted was obtained from sequencing PCR products of genomic DNA. The underlined region indicates the modified TATA box. The bold print denotes the CACGTG E box.

while still apparent (ANOVA, $p < 0.01$), was severely blunted (Figure 1). A similar decrease in the amplitude of each of the three *mPer* rhythms was subsequently found in *Clock/Clock* animals studied at the same six time points during the first day in constant darkness (DD) (data not shown). These in vivo data are consistent with the in vitro transcriptional studies for *mPer1* (Gekakis et al., 1998), as well as a preliminary report of decreased *mPer1* expression in *Clock/Clock* mice (see Gekakis et al., 1998), and further suggest that CLOCK regulates the amplitude of the rhythms in *mPer2* and *mPer3* transcription.

Since three CACGTG E box enhancers reside within 2.0 kb of the 5' flanking region of the *mPer1* gene (Gekakis et al., 1998), we analyzed the *mPer2* and *mPer3* genes for CACGTG E boxes. Sequence analysis of 1.6 kb of the 5' flanking region of the *mPer2* gene and 2.0 kb of the 5' flanking regions of the *mPer3* gene revealed no CACGTG motifs. We next used restriction endonuclease mapping with the enzyme PmlI, which specifically recognizes the CACGTG sequence to determine where this site resides in the *mPer2* and *mPer3* genes. For the *mPer2* gene, no PmlI sites were found in 12.5 kb of the 5' flanking region. One PmlI site was found in the first intron, ca 2 kb downstream of the *mPer2* transcription start site. For the *mPer3* gene, no PmlI sites were found in 12 kb of the 5' flanking region. One PmlI site was found in the 4th intron, ca 3.5 kb downstream from the *mPer3* transcription start site.

The Clock-Controlled Rhythm of Vasopressin RNA Levels Is Selectively Abolished in the SCN of *Clock/Clock* Mice

Previous studies have shown that vasopressin RNA levels exhibit a prominent circadian rhythm in the SCN with high levels during the day and low levels at night (Uhl and Reppert, 1986). This suggested that this CCG may be under similar transcriptional control as the *mPer*

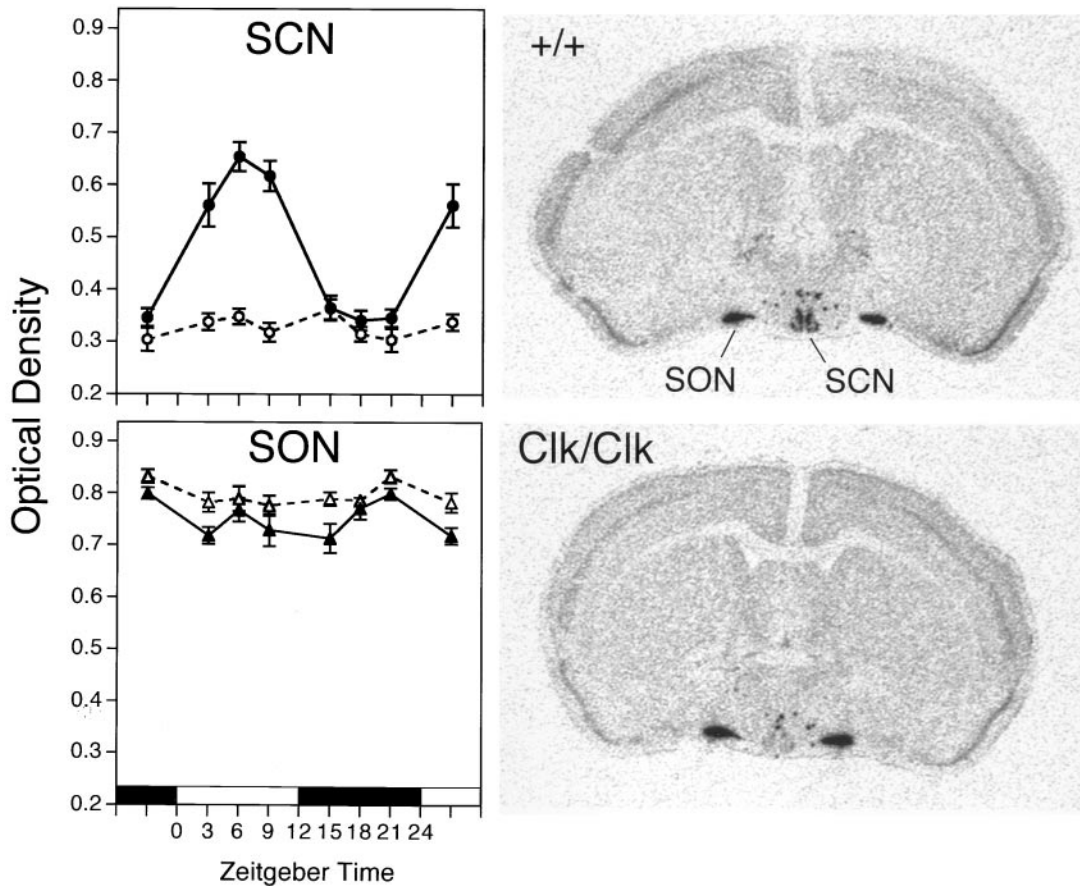


Figure 3. Vasopressin RNA Levels Are Reduced in the SCN, but Not SON, of *Clock/Clock* Mice
 Left panel depicts the temporal profiles of vasopressin RNA levels in the SCN (upper) and SON (lower) of wild-type mice (solid lines) and *Clock/Clock* mice (dashed lines). Animals were studied in 12L:12D with lights on from Zeitgeber times 0–12. Each value for each genotype is the mean \pm SEM of 6–8 animals. Data at ZT 21 and ZT 3 are double plotted. Right panel depicts representative autoradiographs from wild-type and *Clock/Clock* (*Clk/Clk*) mice of coronal brain sections (15 μ m) at the level of the SCN and SON at ZT 6. The brain sections were examined by in situ hybridization using cRNA probes. Magnification, 7.8 \times .

genes in the SCN. We thus examined published sequence of the 5' flanking region of the vasopressin gene to determine whether it contains CACGTG E boxes (Hara et al., 1990). At positions -154 to -149, a CACGTG E box does indeed exist (Figure 2). This is found not only in the 5' flanking regions of the mouse gene, but it is also conserved at the same location in the vasopressin gene of all mammals examined, which includes rats, cattle, and humans (Hara et al., 1990). There were no other CACGTG E boxes within the 1.5 kb 5' flanking region reported (Hara et al., 1990).

Since the phase of the vasopressin RNA rhythm is similar to that for the *mPer* RNA rhythms, and CLOCK is known to enhance *mPer1* gene expression through E boxes in the 5' flanking region, we predicted that the *Clock* mutation would similarly blunt the vasopressin RNA rhythm. We thus used in situ hybridization to examine the vasopressin RNA rhythms in the SCN of wild-type and *Clock/Clock* mice.

In wild-type mice, the vasopressin RNA rhythm was prominent, and the phase of the rhythm in LD appeared synchronous with that for the *mPer1* RNA rhythm (Figure 3). In striking contrast, no vasopressin RNA rhythm was

apparent in *Clock/Clock* mice; RNA levels remained at low nighttime levels throughout the period of study (Figure 3). Furthermore, the vasopressin RNA rhythm was severely blunted in *Clock/Clock* animals studied during the first day in constant darkness (data not shown). These data suggest that the rhythmic expression of the vasopressin and *mPer* genes is under similar transcriptional control and that this control is dependent on a functional CLOCK protein.

It is known that the vasopressin gene is differentially regulated in different brain regions (reviewed in Reppert et al., 1987). This is most readily apparent when the regulation of the gene in the SCN and SON is compared in the same brain section. For example, vasopressin gene expression in the SCN is under circadian control, whereas vasopressin gene expression in the SON is under osmotic but not circadian control. We therefore determined whether vasopressin RNA levels were lowered in the SON of *Clock/Clock* animals.

Vasopressin RNA levels in the SON of wild-type mice did not exhibit a daily rhythm (Figure 3). In addition, RNA levels were not decreased in *Clock/Clock* animals compared to wild-type mice (Figure 3). This result shows

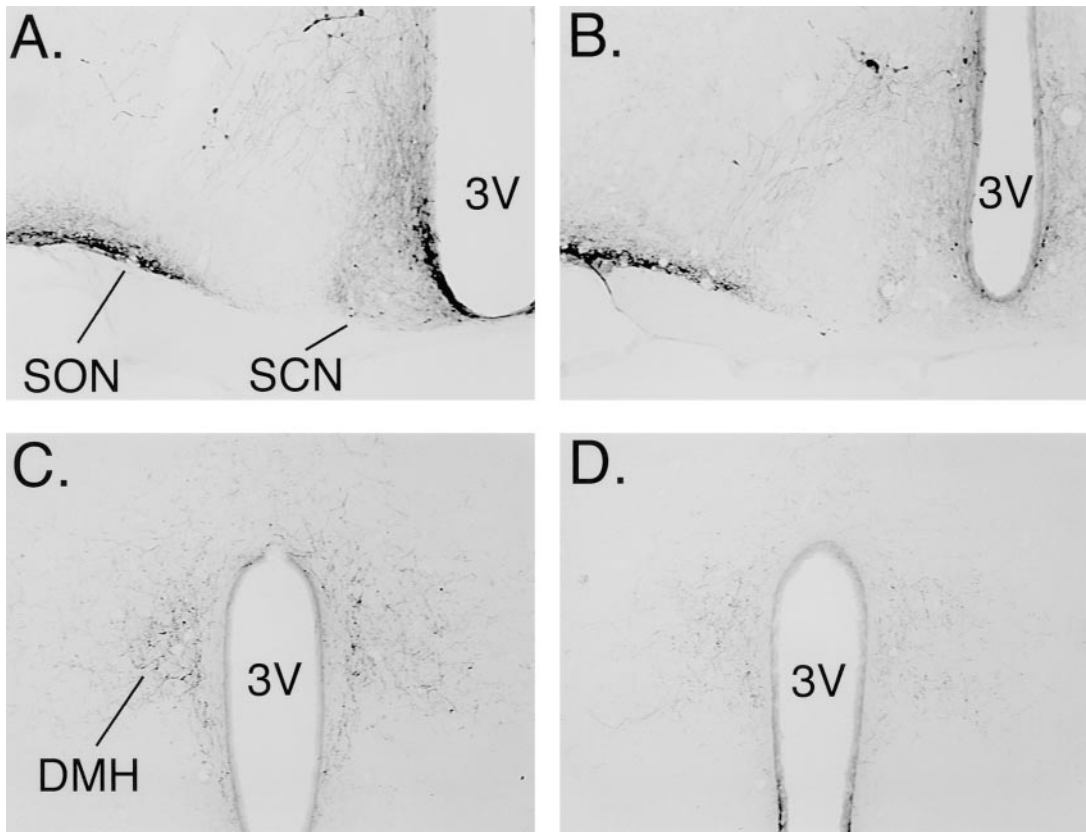


Figure 4. Vasopressin Immunoreactivity Is Selectively Decreased in the SCN and Its Efferents of *Clock/Clock* Mice

Photomicrographs show representative vasopressin staining in the SCN and SON in wild-type mice (A) and *Clock/Clock* animals (B). Also depicted are representative images of vasopressin staining in SCN-vasopressinergic projections in the dorsomedial nucleus of the hypothalamus (DMH) in wild-type (C) and *Clock* mutant (D) mice. Vasopressin staining was significantly reduced in the SCN of *Clock/Clock* mice (gray level values: +/+, 38.47 ± 1.26 , $n = 5$; *Clock/Clock*, 30.89 ± 1.38 , $n = 6$; $p < 0.005$, t test), but not in SON (gray level values: +/+, 38.80 ± 2.75 , $n = 5$; *Clock/Clock*, 39.58 ± 4.20 , $n = 6$; $p < 0.88$). Vasopressin staining was significantly reduced in the DMN of *Clock/Clock* mice (gray-level thresholds: +/+, 48.81 ± 6.04 , $n = 5$; *Clock/Clock*, 23.50 ± 5.37 , $n = 6$; $p < 0.05$) and anteroventral part of the periventricular nucleus of the hypothalamus (gray-level thresholds: +/+, 9.77 ± 2.71 , $n = 5$; *Clock/Clock*, 3.49 ± 0.85 , $n = 6$; $p < 0.05$; data not shown). Magnification, $68\times$.

that the decrease in vasopressin gene expression in *Clock/Clock* mice is specific for circadian enhancement. Interestingly, we found a strong hybridization signal for *Clock* by in situ hybridization in both the SCN and SON of wild-type mice (data not shown). Although the hybridization signal for *Bmal1* RNA was strong in the SCN of wild-type mice, it was barely detectable in the SON (data not shown). Thus, BMAL1 may be a limiting factor preventing rhythmic vasopressin expression in SON.

Vasopressin Peptide Levels Are Selectively Reduced in the SCN of *Clock/Clock* Animals

Since vasopressin RNA levels are reduced to constant low nighttime values in *Clock/Clock* mice, peptide levels should also be severely reduced in mutant animals. This hypothesis was tested by examining the brains of wild-type and *Clock/Clock* mice for vasopressin immunoreactivity by immunocytochemistry. Animals were examined at midday when vasopressin peptide levels are the highest (Tominaga et al., 1992).

In wild-type animals, vasopressin immunoreactivity

was prominently expressed in the SCN (Figure 4). Intense immunoreactivity was also found in the magnocellular neurons of the SON. In *Clock/Clock* mice, vasopressin immunoreactivity was unaltered in SON ($p > 0.5$) but was selectively decreased in the SCN ($p < 0.005$) (Figure 4). This decreased staining appeared to reflect a decrease in staining intensity rather than a decrease in the number of SCN neurons expressing a detectable level of vasopressin. Vasopressinergic fibers from the SCN that terminate in the dorsomedial nucleus of the hypothalamus and anteroventral part of the periventricular nucleus of the hypothalamus were analyzed and also had reduced staining in *Clock/Clock* animals ($p < 0.05$) (Figure 4). These data indicate that vasopressinergic output from the SCN is markedly reduced in *Clock/Clock* mice.

SCN Gene Expression Is Not Globally Reduced in *Clock/Clock* Animals

Since the rhythmically expressed genes in the SCN so far examined are all downregulated in *Clock/Clock* animals, we examined RNA levels for other SCN-expressed

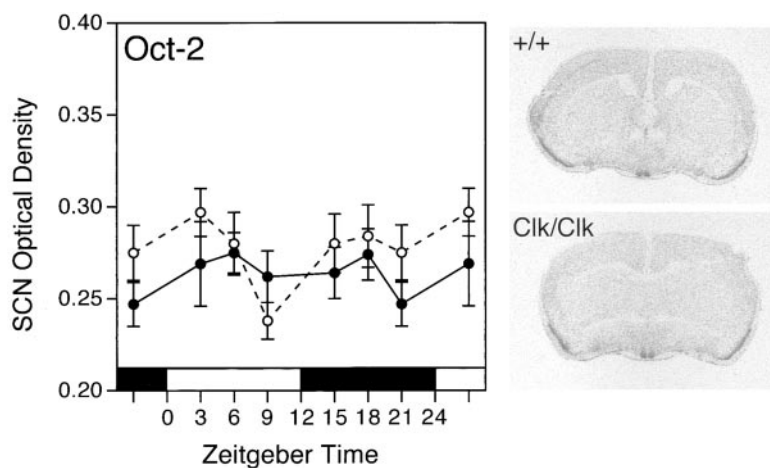


Figure 5. *Oct-2* RNA Levels Are Not Reduced in *Clock/Clock* Mice

Temporal profiles of *Oct-2* RNA levels in the SCN of wild-type mice (solid lines) and *Clock/Clock* mice (dashed lines) are depicted. Animals were studied in 12L:12D with lights on from Zeitgeber times 0–12. Each value for each genotype is the mean \pm SEM of 6–8 animals. Data at ZT 21 and ZT 3 are double plotted. Right panel depicts representative autoradiographs from wild-type and *Clock/Clock* (*Clk/Clk*) mice of coronal brain sections (15 μ m) at the level of the SCN and SCN at ZT 18. The brain sections were examined by in situ hybridization using cRNA probes. Magnification, 3.4 \times .

genes that do not exhibit a circadian rhythm in steady-state RNA levels. This was examined to determine whether the *Clock* mutation has broader effects on SCN function than those that can be explained by action solely on CACGTG E boxes to enhance expression of rhythmically expressed genes. For example, bHLH-PAS transcription factors can have several different partners and thus elicit a broad range of effects on development and nuclear signaling (Hogenesch et al., 1998; Takahata et al., 1998).

In a preliminary study, expression of genes for the NMDAR1 glutamate receptor, 5-HT_{2c} receptor, dopamine D1 receptor, and *Oct-2* was examined at midlight and middark in 12L:12D. In each instance, gene expression was not altered in *Clock/Clock* animals, compared to wild-type mice (data not shown). We next compared the daily pattern of *Oct-2* gene expression between the two genotypes. This was evaluated by examining six time points over a 24 hr period in 12L:12D. The *Oct-2* gene was chosen for this phase of study because its expression is restricted in adult rodents to only a few brain regions, including the SCN; the *Oct-2* gene is expressed throughout the SCN; and *Oct-2* RNA levels do not oscillate in the SCN (Rivkees et al., 1992). *Oct-2* is a member of the POU family of DNA-binding proteins and regulates the expression of specific genes in peripheral tissues (Clerc et al., 1988). The role of *Oct-2* in SCN function is unknown (Ninkina et al., 1995).

Oct-2 RNA levels in the SCN of wild-type mice did not exhibit a daily rhythm (Figure 5), consistent with previous findings in rats (Rivkees et al., 1992). In addition, RNA levels were not altered in *Clock/Clock* animals compared to wild-type mice. Taken together, these data suggest that the *Clock* mutation does not result in a global downregulation of SCN gene expression.

An E Box Element in the Vasopressin Gene Is Necessary for CLOCK-BMAL1-Mediated Transcriptional Activation

To directly examine the ability of CLOCK-BMAL1 heterodimers to drive vasopressin transcription through an E box enhancer, a 200 bp fragment of the 5' flanking region of the mouse vasopressin gene containing the endogenous promoter and the CACGTG E box was subcloned

into a promoterless luciferase reporter vector (pGL3-Basic). Using a luciferase reporter gene assay (Gekakis et al., 1998), we found that CLOCK and BMAL1 together induced a large increase in transcriptional activity through the vasopressin promoter (47.5-fold; Figure 6); no increase in transcriptional activity was detected when either CLOCK or BMAL1 was examined alone. This CLOCK-BMAL1 activation was dependent on the E box element, because mutation of the CACGTG sequence to GGACCT within the 200 bp fragment reduced the transcriptional activation by CLOCK and BMAL1 from 47.5-fold to 2.6-fold.

We also examined the transcriptional activity of a 54 bp construct in which three copies of an 18 bp sequence centered on the vasopressin E box were linked in tandem and subcloned into a luciferase reporter vector containing an SV40 promoter (pGL3-Promoter; Figure 6). CLOCK along with BMAL1 (but neither alone) caused a substantial increase in transcriptional activity through the 54 bp tandem repeat (18.0-fold). This CLOCK-BMAL1-dependent activation was reduced from 18.0-fold to 2.6-fold when the three E boxes were mutated. These results indicate that CLOCK and BMAL1 together enhance transcription of the vasopressin gene through the CACGTG E box element.

Since our in vivo studies showed that the vasopressin RNA rhythm is abolished in *Clock/Clock* mice, we reasoned that the mutant protein, CLOCK- Δ 19, together with BMAL1 would not be able to markedly activate transcription through the CACGTG E box in vitro. Indeed, when CLOCK- Δ 19 was substituted for CLOCK in our transcriptional assays, CLOCK- Δ 19 in combination with BMAL1 resulted in only a 3.6-fold activation from the 200 bp 5' flanking region of the vasopressin gene (Figure 6). This small increase in transcriptional activity is consistent with the notion based on analysis of behavioral rhythms in *Clock/Clock* mice that CLOCK- Δ 19 is defective, but not completely deficient in transcriptional activity (Vitaterna et al., 1994). CLOCK- Δ 19 together with BMAL1 caused a small increase in transcription through the 54 bp tandem E box repeat above basal levels (1.3-fold), similar to what has been previously shown for a tandem construct of *mPer1* E boxes (Gekakis et al., 1998).

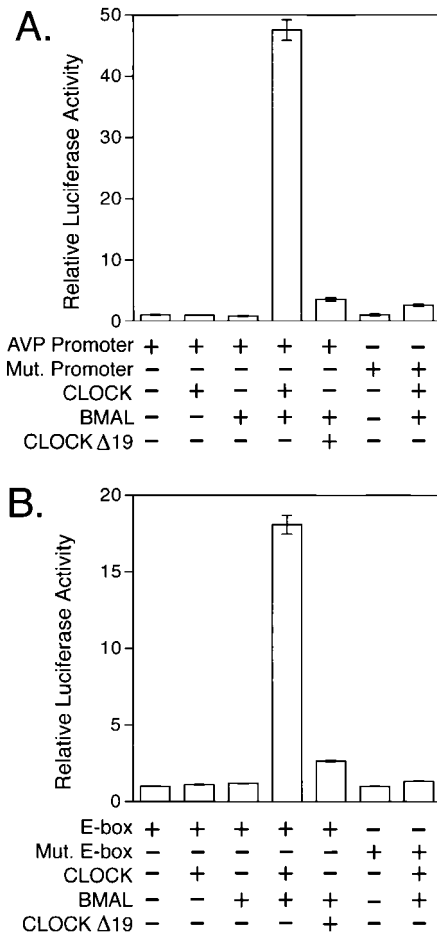


Figure 6. CLOCK and BMAL1 Together Act through a CACGTG E Box to Activate Vasopressin Gene Transcription
(A) Transcriptional activation of luciferase reporter (pGL3-Basic) containing a 200 bp fragment of the 5' flanking region of the mouse vasopressin gene that includes the endogenous promoter and either the CACGTG E box or a mutated E box (GGACCT). Presence (+) or absence (-) of reporter and expression plasmids is denoted. Each value is the mean \pm SEM of three replicates from a single assay. The results shown are representative of three independent experiments.
(B) Transcriptional activation of an SV40-driven luciferase reporter (pGL3-Promoter) containing a 54 bp construct in which three copies of either the vasopressin E box (CACGTG) or the mutated E box (GGACCT) and flanking sequences were linked in tandem. Presence (+) or absence (-) of reporter (10 ng) and expression plasmids (0.50 μ g of each plasmid) is denoted. Each value is the mean \pm SEM of three replicates for a single assay. The results shown are representative of three independent experiments.

Negative Regulation of CLOCK-BMAL1-Mediated Vasopressin Transcription

We next examined the negative elements of the SCN clock feedback loop, using the 200 bp vasopressin promoter fragment. For this phase of study, the amount of *mClock* and hamster (*hBmal1*) transfected was reduced by 50% to 0.25 μ g each so that *mPer* and *mTim* constructs could be cotransfected, while still keeping the total amount of DNA transfected constant (1 μ g). This decrease in *mClock* and *hBmal1* resulted in an easily quantifiable 12.5-fold increase in transcription that was on the linear part of the dose-response curve (Figure 7A).

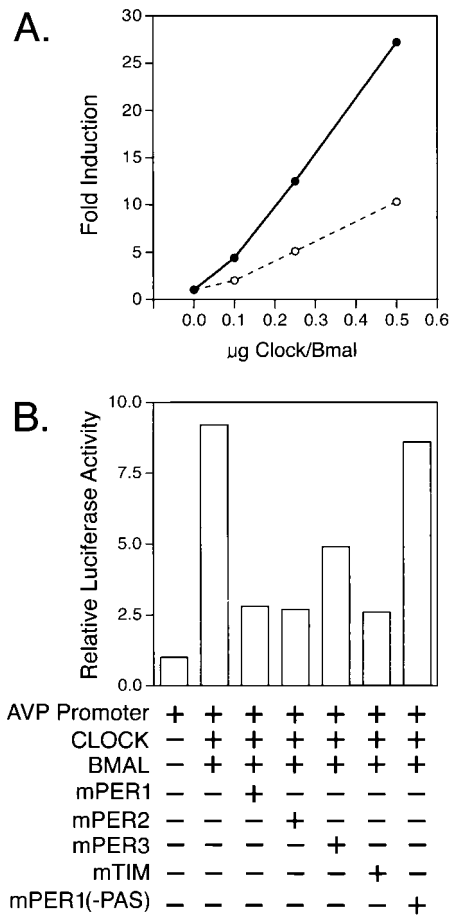


Figure 7. Negative Regulation of CLOCK-BMAL1-Mediated Transcription of the Vasopressin Gene
(A) Dose-response curve of CLOCK-BMAL1-mediated transcriptional activation of a luciferase reporter (PGL3-Basic) containing a 200 bp fragment of the 5' flanking region of the mouse vasopressin gene. Increasing doses of *Clock* and *Bmal1* were transfected using either 1 μ g of total DNA per well (solid line) or 2 μ g of total DNA (dashed line). Each value is the mean of duplicate determinations from the same assay; duplicate values did not vary by more than 10% from the mean.
(B) Inhibition of CLOCK-BMAL1-mediated vasopressin transcription by the mPER and mTIM proteins. Presence (+) or absence (-) of reporter (10 ng) and expression plasmids (0.25 μ g of each plasmid) are denoted. Each value is the mean of duplicate determinations from a single assay; duplicate values did not vary by more than 15% from the mean. A similar pattern of inhibition was found in two other experiments.

Using this modified assay, mPER1, mPER2, mPER3, and TIM each substantially inhibited CLOCK-BMAL1-mediated transcription of the vasopressin gene (Figure 7B). Deletion of the protein dimerization PAS domain and cytoplasmic localization domain from mPER1 (amino acids 179–495 of GenBank accession no. AF022992 deleted) eliminated the inhibitory effect of mPER1 on CLOCK-BMAL1-mediated transcription (Figure 7B). This finding suggests that mPER1-mediated inhibition is dependent on protein-protein interactions. The marked inhibition of vasopressin transcription when each of the mPER and mTIM expression plasmids was transfected alone,

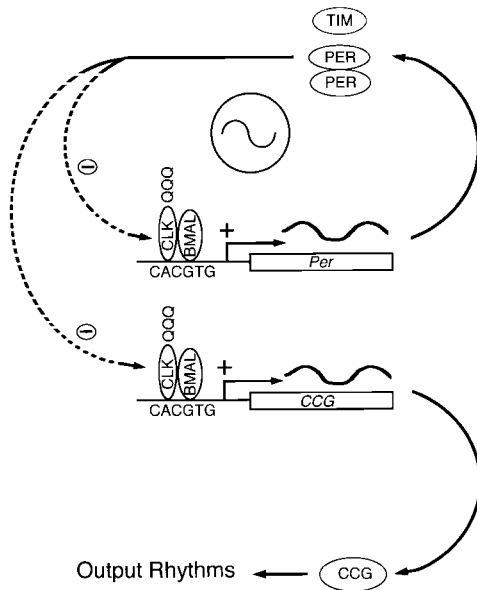


Figure 8. Model of the Dual Regulation of a Core Feedback Loop and CCGs
Both the positive and negative transcriptional elements of the core feedback loop can also drive transcriptional rhythms in clock-controlled genes (CCGs).

however, along with the endogenous expression of *mPer1,2,3* and *mTim* in NIH 3T3 cells (data not shown), made analysis of specific mPER-mPER and mPER-mTIM interactions on inhibition difficult to assess.

mPER1 also caused a significant 12%–33% decrease in CLOCK-BMAL1-mediated transcription through *mPer1* E boxes (one sample t test, $p < 0.0005$ vs 0% reduction, $n = 7$ experiments). The negative effect of mPER1 on its own transcription was quite variable because of the modest and variable CLOCK-BMAL1-mediated increase in transcription (4.1- to 7.8-fold increase) with this reporter.

The data clearly show that the rhythmic components of the SCN clockwork (the three mPER proteins) can inhibit CLOCK-BMAL1-mediated vasopressin transcription. Furthermore, the data are consistent with our hypothesis that PER-PER interactions are important regulators of the negative limb of the mammalian feedback loop (Zylka et al., 1998b).

Discussion

We have discovered a molecular mechanism that links the central circadian feedback loop in the SCN to the regulation of CCGs. This mechanism involves using the same positive and negative elements that drive the core feedback loop (Figure 8). Our data specifically suggest that CLOCK-BMAL1 heterodimers act through an E box enhancer to drive the rhythmic transcription of the vasopressin gene in the SCN.

Although the negative limb of the mammalian feedback loop has not yet been precisely defined, our transcriptional studies suggest that negative feedback of vasopressin transcription involves the mPER proteins

(Figure 7), consistent with our previous yeast two-hybrid results (Zylka et al., 1998b). mTIM also strongly inhibits CLOCK-BMAL1-mediated transcription of the vasopressin promoter, similar to what has been recently reported for the *mPer1* promoter (Sangoram et al., 1998). It is worth noting, however, that in marked contrast to the fly, where *tim* RNA and protein levels exhibit robust rhythms, *mTim* RNA and protein levels are expressed at low, nonrhythmic levels in the SCN (Zylka et al., 1998b; Hastings et al., unpublished data). mTIM could play a modulatory role in negative feedback, with the predominant action coming from the mPER proteins. Importantly, the vasopressin peptide is not involved in negative feedback of the vasopressin rhythm (Uhl and Reppert, 1986). Also, since *mClock* RNA levels are not rhythmic (Tei et al., 1997; Shearman et al., 1999), it is unlikely that a CLOCK rhythm regulates both the positive and negative aspects of the rhythm in vasopressin transcription. These findings, along with our transcriptional studies, strongly suggest that the same feedback element(s) which shut down *mPer1* transcription also negatively regulate vasopressin transcription.

The marked reduction in amplitude of the *mPer* RNA rhythms in *Clock/Clock* mice provides compelling evidence that CLOCK participates in the rhythmic expression of all three *mPer* genes. In fact, a CACGTG E box element resides in an intron of both the *mPer2* and *mPer3* genes through which CLOCK-BMAL1 heterodimers could potentially enhance transcription. In vitro studies indicate that CLOCK-BMAL1 heterodimers are highly specific in their preference for CACGTG E boxes (Darlington et al., 1998). It is also possible that CLOCK heterodimerizes with other partners and/or activates transcription through as yet unidentified DNA binding elements in the *mPer* genes. The delayed phase of the *mPer1* and *mPer3* rhythms, relative to the phase of the *mPer1* and *mPer3* rhythms, further suggests the existence of a different transcriptional activation cascade. On the other hand, these phase differences may merely reflect differences in the kinetics of gene transcription and RNA turnover among the three *mPer* genes, even though transcription of each may be driven by CLOCK-BMAL1 heterodimers acting through CACGTG E boxes.

The decreased amplitude of the *mPer* RNA rhythms in *Clock/Clock* mice likely leads to decreased mPER synthesis. Based on a recent model of the *Drosophila* circadian clock, decreased PER synthesis should lead to a lengthening of circadian period (Leloup and Goldbeter, 1998). Thus, blunting of the *mPer* RNA rhythms may be the molecular cause of lengthening of circadian period in *Clock/Clock* mice and is consistent with the proposal that the *mPer* genes are essential rhythmic elements of a core feedback loop.

The differential regulation of vasopressin gene expression by CLOCK in the SCN and SON is striking. Previous studies have shown that the vasopressin RNA levels are differentially regulated in these nuclei by various stimuli (reviewed in Reppert et al., 1987). The data presented here provide a direct mechanism for the positive regulation of rhythmic vasopressin gene transcription in the SCN by CLOCK. Previous in vitro studies have shown that the positive and negative regulation of vasopressin gene expression is complex and involves

several *cis-* and *trans-*acting factors (Iwasaki et al., 1997). Thus, there are probably a number of tissue-specific elements that participate in the differential regulation of vasopressin transcription seen in different brain regions. Since *Clock* is avidly expressed in SON and vasopressin RNA levels are not altered in the SON of *Clock/Clock* mice, transcription factors other than CLOCK are of primary importance for regulating vasopressin gene expression in that nucleus.

Vasopressin output from the SCN is seriously impaired in *Clock* mutant animals. The low, nonrhythmic vasopressin RNA levels in the SCN of *Clock/Clock* mice are also reflected in markedly diminished peptide levels in both the SCN itself and in vasopressinergic efferent projections from the SCN. This altered circadian output may contribute to some of the circadian abnormalities manifested by *Clock/Clock* mice, such as the decreased amplitude of the locomotor activity rhythm (Vitaterna et al., 1994; see below).

Based on studies in rats, vasopressin is an important output of the SCN clockwork. Vasopressin release within the SCN modulates the amplitude of the firing rate rhythm. The firing rate rhythm is arguably the most important output of the SCN clock, involved in rhythmically regulating efferent projections to various brain regions (Schwartz et al., 1987). Evidence supporting a role for vasopressin in modulating the SCN activity rhythm is substantial. Vasopressin acting through vasopressin- V_1 receptors excites SCN neurons in a dose-dependent, pharmacologically specific manner (Mihai et al., 1994a). This SCN excitation shows daily sensitivity, with increased vasopressin sensitivity during the subjective day; this correlates nicely with the peak in the SCN firing rate rhythm. Moreover, application of a specific vasopressin antagonist suppresses spontaneous SCN neuronal firing during the subjective day (Mihai et al., 1994b). Importantly, vasopressin-deficient Brattleboro rats have a dampened amplitude of the firing rate rhythm (Ingram et al., 1996). Although homozygous Brattleboro rats express circadian rhythms, several of their expressed rhythms have decreased amplitude (Groblewski et al., 1981; Brown and Nunez, 1989). Diminished rhythm amplitude in Brattleboro rats could be explained by vasopressin's normal augmentation of the firing rate rhythm.

Vasopressin of SCN origin also appears to have a specific output function in driving rhythmic hypothalamo-pituitary-adrenal axis (HPA) activity (Kalsbeek et al., 1996a, 1996b). Vasopressin released from SCN terminals in the dorsomedial hypothalamus and subparaventricular zone has a pronounced inhibitory effect on the release of the adrenal hormone corticosterone (Kalsbeek et al., 1996b). Vasopressin release might affect descending projections from brain stem and spinal cord, thereby decreasing splanchnic neural activity and corticosterone release. The vasopressin-mediated inhibition of corticosterone release is one aspect of a two-part regulatory mechanism controlling the corticosterone rhythm. The second mechanism involves a vasopressin-independent stimulatory input from the SCN to the HPA (Kalsbeek et al., 1996b).

In conclusion, our data provide a precedent for the molecular control of a CCG in the SCN. We predict that

other CCGs in vertebrates and in *Drosophila* could use this dual transcriptional control mechanism. Activation of CCGs through such a mechanism provides the first level of circadian regulation outside a central clock leading to output. The continued unraveling of successive mechanistic layers should ultimately provide a full understanding of the events leading from clock genes to the temporal regulation of behavior.

Experimental Procedures

Animals and Tissue Collection

A breeding colony of mice carrying the *Clock* mutation was established using *Clock* mutant mice on a BALB/c background generously provided by Martha H. Vitaterna and Joseph S. Takahashi, Northwestern University. For studies, both male and female mice 5–15 weeks of age were used. Mice were housed within a temperature-controlled facility (23°C–24°C). The lighting cycle consisted of 12L:12D, except as noted. The light was provided by cool white fluorescent bulbs. Animals were killed by decapitation to avoid acute changes in gene expression.

Genotyping

Genotypes were determined using a PCR mutagenesis method that introduces a restriction site, allowing direct detection of the *Clock* mutation. The mutant allele has an A to T transversion in the third nucleotide of the intron splice donor site (from CAGgtaac to CAGgttac, where lower case represents the intronic sequence), resulting in skipping of exon 19. The 5' primer (63 nt, 5'-GCAAGAAGAACTAAGGAAATTC AAGAGCAACTTCAGATGGTCCATGGTCAAGGGCTA CAGTT-3') is based on the sequence of exon 18 and includes the first two nucleotides of the intron but terminates with CAGtt (rather than CAGgt). The 3' primer (5'-TAGTGCCTAGATGGCCCTGTTGG-3') is the reverse complement of intronic sequence terminating 398 bp 3' of the mutation (sequence information generously provided by David P. King, Northwestern University). PCR amplification results in introduction of a HincII site (Gttaac) in the wild-type allele, while the corresponding section of the mutant allele (Gtttac) is not cut by HincII.

Mouse genomic DNA was extracted from tail biopsies by proteinase K digestion and isopropanol precipitation. Genomic DNA was subjected to polymerase chain reaction (PCR) amplification using a hot-start protocol and Taq DNA polymerase. Cycling parameters were 94°C for 3 min., 30 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min, followed by a final extension at 72°C for 10 min. Amplification products were digested with HincII (New England Biolabs) without extraction and were separated by 1.5% agarose gel electrophoresis. The *Clock* allele produces an amplified product of 460 bp that is unaffected by HincII. The wild-type allele is sensitive to HincII; cleavage of the 5' primer results in a 398 bp band.

In preliminary experiments, the PCR product was subcloned and sequenced to verify the identity of the amplified band and the fidelity of the primer-based mutagenesis method.

In Situ Hybridization

Antisense and sense cRNA probes were generated from each plasmid by *in vitro* transcription in the presence of ^{35}S -UTP (1200 Ci/mmol), as previously described (Weaver, 1993). The following probes were used: rat vasopressin (cDNA courtesy of Tom Sherman, U of Pittsburgh), nt 19–254 (Exon C); rat NMDAR1 receptor, nt 1318–1936 isolated by RT-PCR corresponding to GenBank accession number x63255; rat 5-HT $_2$ c receptor (cDNA provided by D. Julius, University of California, San Francisco), full-length cDNA, GenBank accession number M21410; mouse D1 dopamine receptor (cDNA fragment generated by RT-PCR), corresponding to nt 483–1183 of the rat D1 dopamine receptor, GenBank accession number m35077; *mOct-2* (cDNA provided by Lynn M. Corcoran, Melbourne, Australia), entire cDNA, GenBank accession number x53654; *mPer1*, nt 340–761, GenBank accession number AF022992; *mPer2*, nt 9–489, GenBank accession number AF035830; *mPer3*, nt 1637–2223, GenBank accession number AF050182; *mClock* (cDNA fragment generated by

RT-PCR), nt 1343–1831, GenBank accession number AF000998; and *mBMAL1* (cDNA fragment generated by RT-PCR), corresponding to nt 784–1282, GenBank accession number AF015953. Probe quality and size were confirmed by determining ^{35}S incorporation into TCA-precipitable material, and by gel electrophoresis and subsequent autoradiography of the gel.

Prehybridization, hybridization, and wash procedures have been previously described in detail (Weaver, 1993). Probe (50 μl at 10^7 cpm/ml) was applied to each slide. Coverslipped slides were then incubated in humidified chambers overnight at 55°C . Following completion of the wash steps, slides were air dried and apposed to Kodak BioMax MR film for 1–11 days.

Densitometric analysis of hybridization intensity was accomplished using NIH Image software on a Macintosh computer; data are expressed as absolute optical density values as determined by calibration with Kodak photographic step tablet #3. ^{14}C standards (American Radiolabeled Chemicals) included in each cassette were used to verify that the optical density values measured were within the linear response range of the film.

Immunocytochemistry

The mice were overdosed with pentobarbital and intracardially perfused with 0.9% saline followed by 5% acrolein in 0.1 M sodium phosphate buffer (pH 7.6). Fixed brains were placed in a solution of 30% sucrose in 0.1 M sodium phosphate buffer (pH 7.6) and stored at 4°C . Brains were sectioned into 40 μm transverse sections. Vasopressin immunocytochemistry and specificity tests were done as described in Zhou et al. (1994).

An observer blind to the genetic background analyzed the density of vasopressin immunoreactivity in the SCN and SON using NIH Image program (W. Raskind, NIH). Microscopic images were captured under bright-field illumination using a CCD72 camera (Dage, MT) attached to a Quick Capture frame grabber board (Data Translation) in a Macintosh IIfx computer. The density of the vasopressin immunoreactivity in the SCN was measured in a sampling area of 0.3×0.5 mm, placed with its ventral border flush with the ventral border of the SCN and centered around the third ventricle in every second section throughout the SCN. The density of vasopressin immunoreactivity in the SON was measured bilaterally in a sampling area of 0.3×0.5 mm, placed with its lateral and ventral border flush with the lateral and ventral border of the SON, respectively, in the section that contained the caudal pole of the SCN. The light intensity and camera setting were kept constant across the sections in order to standardize measurements. The density of vasopressin immunoreactivity was calculated as the average gray level in the sampling area minus the gray level of the background.

The density of vasopressin immunoreactive fibers in the anteroventral periventricular nucleus of the preoptic area, and the dorsomedial hypothalamic nucleus was examined in the two consecutive sections that contained the highest fiber density in these areas. Fiber density was analyzed by computerized gray-level thresholding using the NIH IMAGE program (Shipley et al., 1989). The light intensity and camera setting were kept constant across the sections in order to standardize measurements. For the two areas, the density was expressed as the proportion of the area covered by vasopressin immunoreactive fibers in a 0.15×0.15 mm sampling area immediately bordering the ventricular wall.

Transcriptional Assay

Mouse NIH 3T3 cells were grown in Dulbecco's Modified Eagle Medium (GIBCO-BRL) supplemented with 10% calf serum. Cells were plated at ca. 3×10^5 cells per well in six-well plates 1 day before transfection. Cells were transfected with Lipofectamine-Plus (GIBCO-BRL). Unless otherwise specified, cells in each well were transfected with 1 μg (total) of expression plasmids with the indicated inserts in pcDNA3.1 vector (Invitrogen), 10 ng of firefly luciferase reporter plasmid (either pGL3-Promoter or pGL3-Basic vectors; Promega), and 25 ng of CMV- β -gal internal control plasmid (Clontech). The total amount of DNA per well was adjusted by the adding pcDNA3.1 vector as carrier. Forty-eight hours after transfection, cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline and lysed with 200 μl per well reporter lysis buffer (Promega). Portions of the cell extract were assayed for β -gal activity

(Tropix) and firefly luciferase activity (Promega) by luminometry. For each sample, luciferase activity was normalized by determining luciferase: β -gal activity ratios and averaging the values from triplicate wells. All experiments were repeated at least three times.

Reporter constructs were made as follows: a 200 bp piece of the 5' flanking region of the mouse vasopressin gene (-1 to -200; Figure 3) was amplified from genomic DNA by PCR and subcloned into the pGL3-Basic vector. PCR by overlap extension was used with an oligonucleotide containing the mutated sequence to construct the same 200 bp fragment of the vasopressin gene with the E box mutated to GGACCT; it was also subcloned into the pGL3-Basic vector. A 54 bp construct in which three copies of the vasopressin E box and flanking sequences were linked in tandem was constructed by annealing the oligodeoxynucleotides 5'-GATCTCAGGC CCACGTGTGTCCCAGGCCACGTGTGTCCCAGGCCACGTG TGTTCCCA-3' and 5-GATCTGGGACACACGTGGGCCTGGGGAC ACACGTGGGCCTGGGGACACACGTGGGCCTGA-3. The annealed oligos were phosphorylated with T4 polynucleotide kinase and ligated into the pGL3-Promoter vector. A comparable 54 bp construct in which the three E boxes were mutated was constructed with the oligonucleotides 5-GATCTCAGGCCGGACCTTGTCCCAGGCCGG ACCTTGTCCCAGGCCGGACCTTGTCCCA-3 and 5-GATCTGGGA CAAGG TCCGGCCTGGGGACAAGGTCGGGCCTGGGGACAAGGT CCGGCCTGA-3. The annealed oligos were phosphorylated and ligated into the pGL3-Promoter vector. All constructs were sequenced and shown to be correct.

Expression constructs for mouse CLOCK, CLOCK- $\Delta 19$, and hamster BMAL1 were each subcloned into pcDNA3.1 (see Gekakis et al., 1998) and generously provided by Charles J. Weitz, Harvard Medical School. The full-length coding regions of mPER1, mPER1 missing PAS, and cytoplasmic localization domains, mPER2, mPER3, and mTIM were each subcloned into pcDNA3.1.

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