SOX2-Dependent Transcription in Clock Neurons Promotes the Robustness of the Central Circadian Pacemaker

Highlights

- SOX2 is expressed in SCN clock neurons and activates Period2 gene transcription
- Ablation of SOX2 in SCN neurons severely disrupts circadian behavioral rhythms
- SOX2 promotes the robust expression of neuropeptides and their receptors in the SCN
- Ablation of SOX2 alters the transcriptional landscape of the SCN

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In Brief

SOX2 is a stem-cell-associated pluripotency transcription factor whose role in neuronal populations is undefined. Cheng et al. show that ablating SOX2 expression in SCN clock neurons severely disrupts circadian behavioral rhythms. SOX2 is a transcriptional activator of the Period2 gene, and its absence reduces neuropeptide signaling in the SCN.

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SOX2-Dependent Transcription in Clock Neurons Promotes the Robustness of the Central Circadian Pacemaker

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SUMMARY

Clock neurons within the mammalian suprachiasmatic nuclei (SCN) encode circadian time using interlocked transcription-translation feedback loops (TTFLs) that drive rhythmic gene expression. However, the contributions of other transcription factors outside of the circadian TTFLs to the functionality of the SCN remain obscure. Here, we report that the stem and progenitor cell transcription factor, sex-determining region Y-box 2 (SOX2), is expressed in adult SCN neurons and positively regulates transcription of the core clock gene, Period2. Mice lacking SOX2 selectively in SCN neurons display imprecise, poorly consolidated behavioral rhythms that do not entrain efficiently to environmental light cycles and that are highly susceptible to constant light-induced arrhythmicity. RNA sequencing revealed that Sox2 deficiency alters the SCN transcriptome, reducing the expression of core clock genes and neuropeptide-receptor systems. By defining the transcriptional landscape within SCN neurons, SOX2 enables the generation of robust, entrainable circadian rhythms that accurately reflect environmental time.

INTRODUCTION

Circadian rhythms evolved in many organisms to enable anticipatory adaptation of behavior and physiology to cyclical changes in the environment, the most notable being the 24-h day-night cycle. In mammals, these rhythms are driven by tissue-specific oscillators whose activities are orchestrated by a central circadian pacemaker, the hypothalamic suprachiasmatic nuclei (SCN) (Stephan and Zucker, 1972). The SCN generates self-sustained, ~24-h oscillations, entrains its phase to photic inputs from the retina, and communicates time-of-day information to peripheral oscillators through output signals (Mendoza-Viveros et al., 2017).

Each neuron within the SCN network harbors the molecular clock machinery and is capable of independent cellular oscillations (Mendoza-Viveros et al., 2017). The molecular clock is based on a series of transcription-translation feedback loops (TTFLs) that generate rhythmic expression of core clock genes through negative feedback inhibition. In the primary TTFL, heterodimers of the transcription factors, CLOCK and BMAL1, mediate expression of Period (Per1 and Per2) and Cryptochrome (Cry1 and Cry2) genes at E-box elements within their promoters (Shearman et al., 1997; Kume et al., 1999; Mendoza-Viveros et al., 2017). PER:CRY complexes subsequently inhibit their own gene transcription by repressing CLOCK:BMAL1 thereby closing the feedback loop (Kume et al., 1999). As the rate-limiting component of the repressive complex, PER holds particular significance for circadian clock regulation by defining period, phase, and clock-resetting properties (Shearman et al., 1997). Secondary feedback loops, such as those involved in D-box- and ROR/REV-ERB-response element (RORE)-mediated transcription, further regulate the primary TTFL (Mendoza-Viveros et al., 2017). Together, these TTFLs drive oscillations at the level of single cells, but tissue-level oscillations require coupling and synchronization among SCN neurons (Aton and Herzog, 2005). Different SCN neuronal populations synthesize distinct neuropeptides, including vasoactive intestinal peptide (VIP), arginine vasopressin (AVP), and gastrin-releasing peptide (GRP), that mediate interneuronal coupling within the SCN (Aton et al., 2005; Maywood et al., 2011). Neuropeptides, such as prokineticin 2 (PK2 and Prok2), can serve as functional outputs of the SCN, communicating phase information to other brain regions (Cheng et al., 2002).

Recently, we and others found that the transcription factor, SRY (sex-determining region Y)-box 2 (SOX2), is robustly expressed in neurons of the adult murine SCN (Hoeffer and Carter, 2014). SOX2 is a member of the SOX, or SRY-related high-mobility group (HMG)-box, family of transcription factors that play important roles in stem and progenitor cell differentiation and maintenance (Avilion et al., 2003). SOX2 is highly expressed in neural stem and progenitor cells during development, but its expression rapidly declines upon cell differentiation (Graham et al., 2003). In the adult brain, SOX2 is largely restricted to neural progenitors and glial populations: the SCN is the only adult brain
region that exhibits high SOX2 expression in mature neurons (Hoefflin and Carter, 2014). Intriguingly, we found that virtually all PER2+ cells in the adult murine brain co-expressed SOX2. These observations raise the possibility that SOX2 is a transcriptional regulator of PER2 and may play a role as a higher-order regulator of circadian rhythms within the central pacemaker.

Here, we examine the role of SOX2 in circadian timekeeping mechanisms within the SCN. We find that SOX2 can bind in vitro and in vivo to the Per2 gene promoter and induce its activity. Abolishing SOX2 expression in SCN neurons severely disrupts rhythms of locomotor activity in mice: notably, rhythms are longer and more fragmented, are more prone to constant light-induced arrhythmicity, and do not entrain efficiently to environmental light cycles. Sox2 deficiency reduces the expression of Per2 as well as neuropeptides and their cognate receptors within the SCN. These results reveal that SOX2 is essential for establishing the transcriptional landscape of the SCN and for facilitating the generation of robust and entrainable circadian rhythms.

RESULTS

SOX2 Interacts Directly with the mPeriod2 Promoter In Vitro and within the Adult Murine SCN

To understand the relationship between SOX2 and Per2, we mapped their expression throughout the adult mouse brain. Mapping was carried out using mPer2::DsRED transgenic mice, in which DsRED expression is transcriptionally regulated by the mPer2 promoter (Cheng et al., 2009). Nearly all DsRED+ cells expressed high levels of SOX2 (Figure S1A).

To test our hypothesis that SOX2 may be regulating mPer2 transcription, we examined the effects of SOX2 overexpression in Neuro2a cells on mPer2 promoter-driven luciferase (LUC) activity (using serial truncation constructs) and DsRED expression (Figures 1A, 1B, and S1B). SOX2 overexpression substantially increased mPer2-DsRED expression (Figure S1B) as well as mPer2-LUC activity in all truncation constructs (Figures 1A and 1B), suggesting that DNA elements within the proximal promoter region from −105 to +110 are responsible for SOX2-dependent mPer2 transactivation. A mutation in the noncanonical E-box (E2) enhancer site, which binds CLOCK:BMAL1 and drives mPer2 oscillations, did not alter SOX2-mediated mPer2 transcription (Figure S1C; Yoo et al., 2004). SOX2 was even more potent than CLOCK:BMAL1 in its ability to transactivate mPer2 (Figures S1C and S1D). SOX2 had only a modest effect on mPer1 promoter activity (Figure S1E) but activated the mPer2 promoter to a similar extent as it did to a bona fide SOX2 target sequence, the 6× tandem repeat of the Oct4-Sox2 binding site from the Fgf4 enhancer (Figure S1F). Altogether, these data reveal a potential role of SOX2 in enhancing mPer2 promoter activity independent of CLOCK:BMAL1 and the E2 site.

Next, we asked whether SOX2 can physically interact with the mPer2 promoter. Removing the HMG DNA-binding domain abrogated the ability of SOX2 to activate the mPer2−105→+110 promoter in Neuro2a cells (Figures 1C, 1D, S1G, and S1H). Deletion of the N- or C-terminal region, which are important for protein-protein interactions, reduced SOX2-dependent mPer2-LUC activity by ~50% (Figures 1C and 1D). Chromatin immuno-precipitation (ChiP) experiments using adult murine SCN tissues showed that endogenous SOX2 was significantly enriched at the proximal mPer2 promoter region in a time-of-day-independent manner (Figures 1E and 1F). SOX2 was not enriched at regions that were 2 kb up- or downstream of the mPer2 transcription start site (TSS) (Figure 1F) or at the proximal mPer1 promoter (Figure 1G). Luciferase assays using mPer2−105→+110-LUC constructs bearing specific deletions or mutations revealed potential SOX2-responsive elements within the region spanning −105 and −60 (Figures 1H and 1I). Electroencephaloretic mobility shift assays (EMSAs) demonstrated a direct interaction between SOX2 and the mPer2−105→+110 promoter DNA sequence (Figure 1J). Furthermore, DNase I footprinting uncovered two potential SOX2 binding sites (BSs), SOX2-BS1 and SOX2-BS2, within the mPer2 promoter (Figures 1K and 1L). Collectively, our data suggest that SOX2 positively regulates the transcription of mPer2 through physical associations with its promoter.

Ablation of SOX2 in SCN Neurons Reduces mPER2 and Neuropeptide Expression

To investigate the functions of SOX2 within the murine SCN, we ablated the Sox2 gene in GABAergic neurons by breeding mice that carry the loxP-flanked alleles of Sox2 (Sox2fl/fl) with those that express the cre recombinase under the control of the vesicular GABA transporter (Vgat) gene (Vgat-IRESCre; Shaham et al., 2009; Vong et al., 2011). Because nearly all SCN neurons are GABAergic, this results in highly efficient, neuron-restricted excision of the Sox2 coding exon within the SCN. Vgat-cre;Sox2fl/fl mice were born at the expected Mendelian ratio, were viable, and appeared grossly normal. Young (4 or 5 weeks old) Vgat-cre;Sox2fl/fl mice exhibited reduced body weights compared to sex- and age-matched controls (Figure S2A). Importantly, SOX2 expression was absent in all SCN neurons of Vgat-cre;Sox2fl/fl mice but was not affected in glial fibrillary acidic protein (GFAP)-positive cell populations in the SCN or in any other brain region examined (Figures 2A–2D and S2B).

To determine whether SOX2 is critical for the expression of Per2 within the adult SCN, we analyzed the circadian expression profile of PER2 in the SCN of Vgat-cre;Sox2fl/fl mice and two control groups, Sox2fl/+ littermates and heterozygous Vgatcre/+ animals. Vgatcre/+ and Sox2fl/+ mice (collectively referred to as control mice) were phenotypically indistinguishable from one another in terms of PER1 and PER2 expression (data not shown). The abundance of PER2 protein, and consequently the amplitude of PER2 rhythms, was significantly reduced in the SCN of Vgat-cre;Sox2fl/+ mice relative to controls (Figures 2E–2G). In contrast, Sox2 ablation had no significant effect on PER1 protein rhythms in the SCN (Figures 2H–2J). Cellular levels of PER2, but not PER1, were reduced in Vgat-cre;Sox2fl/+ SCN compared to controls (Figures S2C–S2F). The SCN of Vgat-cre;Sox2fl/+ mice showed no differences in CLOCK protein expression or the density of CLOCK+ nuclei (Figures S2G and S2H; data not shown).

To determine whether the effects of Sox2 disruption on PER2 expression are intrinsic to the fully developed SCN, we used adeno-associated virus (AAV)-mediated delivery of cre recombinase to ablate the Sox2 gene in neurons of SCN tissue explants prepared from Sox2fl/fl,Per2uc/+ pups at postnatal day (P) 12 (Figure 2K) and monitored PER2::LUCIFERASE rhythms in

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Figure 1. SOX2 Binds to and Promotes the Activation of the mPer2 Promoter In Vitro and within the Murine SCN

(A) Luciferase reporter constructs bearing truncations of the mPer2 promoter.
(B) mPer2-LUC assays in Neuro2a cells with or without overexpression of SOX2.
(C) mPer2-LUC assays in Neuro2a cells with or without overexpression of SOX2.
(D) mPer2-LUC assays in Neuro2a cells with or without overexpression of SOX2.
(E) Regions on the Period2 gene locus to which ChIP-qPCR primers anneal.
(F) Relative binding of SOX2 to the Per2 gene locus in SCN tissues. Binding to the −2,000 and +2,000 bp regions was assessed at CT 0 and to the −100 bp region at 4 CTs.
(G) Relative binding of SOX2 to the proximal Per1 promoter in SCN tissues at CT 0.
(H) mPer2-LUC constructs bearing one or more deletions (Δ) or mutations (Mut) at specific sites (gray boxes).
(I) mPer2-LUC assays in Neuro2a cells using the constructs shown in (H), with (gray or red bars) or without (white bar) overexpression of SOX2. mPer2-LUC represents the wild-type mPer2-LUC constructs.
(J) EMSA assay showing the migration of a FAM-labeled probe corresponding to the mPer2 promoter region (FAM-Period2) in the presence of different amounts of recombinant SOX2. In lane 5, unlabeled Period2 probe was added in 100-fold excess of the FAM-Period2 probe.
(K) The sequence of the mPer2 promoter region. The E2 enhancer site (E-BOX2) is indicated. SOX2 binding sites 1 (yellow) and 2 (green), identified by DNase I footprinting, are highlighted.
(L) DNase I footprint analysis of the mPeriod2 promoter sequence in the presence or absence of recombinant SOX2. Peaks corresponding to SOX2 binding sites 1 (yellow) and 2 (green) are boxed. Values represent mean ± SEM. For luciferase and ChIP assays, n = 3–6 per group.

*p < 0.05 versus empty vector (B, D, and I) or versus immunoglobulin G (IgG)-immunoprecipitation (IP) (F and G). #p < 0.05 versus (wild-type) SOX2.

See also Figure S1.
culture. Because most developmental milestones of the murine SCN have been reached by ~P10, our approach circumvents any potential effect of Sox2 ablation on SCN development. Compared to SCN explants transduced with control AAV5-human synapsin (hSyn)-TurboRFP, the amplitude of PER2::LUC rhythms was significantly reduced in cultures that had been transduced with AAV5-hSyn-Cre (Figures 2L and 2M). Collectively, our data suggest that Sox2 is important for the generation of high-amplitude PER2 rhythms within the SCN.

To determine whether other aspects of SCN physiology may be affected by Sox2 ablation, we assessed neuropeptide expression in the SCN of Vgat-cre;Sox2fl/fl mice. Sox2 protein was expressed in all AVP- and VIP-producing neurons of the SCN (Figure 2N). Abundance of AVP and VIP was significantly reduced in the SCN of Vgat-cre;Sox2fl/fl mice; however, the compartmentalization of the SCN into ventrolateral (core) and dorsomedial (shell) subregions was indistinguishable compared to control mice (Figures 2O–2Q). Expression of neuropeptide Y (NPY), which is synthesized by intergeniculate leaflet neurons, was expressed in all AVP- and VIP-producing neurons of the SCN (Figure 2N). Abundance of AVP and VIP was significantly reduced in the SCN of Vgat-cre;Sox2fl/fl mice (Figures 2O and 2R). Interestingly, there was an ~28% decrease in SCN volume and SCN neuron numbers in Vgat-cre;Sox2fl/fl mice compared to age-matched controls but no change in SCN neuronal density (Figures S2T–S2L). We conclude that the structural organization of the SCN is largely intact in Vgat-cre;Sox2fl/fl mice, but the absence of Sox2 severely blunts the expression of PER2, AVP, and VIP in the SCN.

SOX2 Is Essential for the Period, Precision, and Consolidation of Circadian Behavioral Rhythms

To determine whether loss of SOX2 expression affects behavioral outputs of the SCN, we examined wheel-running activity of mice housed under a 12-h light:12-h dark (12:12 LD) schedule, a skeleton photoperiod (1:10:1:12 LDLD), or constant dark (DD) conditions (Figures 3A, 3B, 3A, and 3B). The circadian behavior of Vgat-cre/+ and Sox2fl/fl control mice were phenotypically indistinguishable from one another under all light conditions (data not shown). In contrast, under LD conditions, Vgat-cre;Sox2fl/fl mice demonstrated weaker, poorly consoli-
Figure 3. Sox2 Ablation in GABAergic Neurons Leads to Lengthening and Poor Consolidation of Circadian Activity Rhythms

(A and B) Representative actograms of wheel-running activity of (A) control and (B) Vgat-cre;Sox2fl/fl mice. Periods of light are shaded in yellow.

(C) Onset error or instability of activity onsets (SD of onsets).

(D) Amplitude of activity rhythms.

(E) Duration of the active phase (alpha).

(F) Period length under DD.

(G) Percent activity per 1-h bin under 12:12 LD (gray), skeleton photoperiod (purple), and DD (blue).

(H) Representative photomicrographs of p-ERK1/2 expression in the SCN of control and Vgat-cre;Sox2fl/fl mice that received a light pulse (LP) at CT15 or CT22 or that served as dark controls (DD).

(I and J) Quantification of p-ERK1/2 expression in the shell or core SCN at (I) CT15 or (J) CT22.

(K) Representative photomicrographs of p-CREB (Ser133) expression in the SCN following a LP at CT 15. DD controls are also shown.

(L) Quantification of p-CREB intensity in the shell or core SCN.

Scale bars, 100 μm. Values represent mean ± SEM; n = 4 or 5 per group. *p < 0.05 versus control (C–F) or versus DD (I and J). #p < 0.05 versus control (I and J).

See also Figure S3.
Loss of SOX2 in SCN Neurons Promotes the Disruptive Effects of Constant Light and Impairs Re-entrainment to Shifted Environmental Light Cycles

The unstable activity onsets and variable phase angles exhibited by Vgat-cre;Sox2fl/fl mice under 12:12 LD and skeleton photoperiod suggest a potential effect of Sox2 ablation on light-induced entrainment. To test this, we subjected mice to experimental jetlag by abruptly delaying or advancing the LD schedule by 7 h. Control mice required 4.8 ± 0.5 and 7.7 ± 0.5 days to stably entrain to the delayed and advanced LD schedule, respectively (Figure 4A). Most of the Vgat-cre;Sox2fl/fl mice exhibited such erratic behavior that it was not possible to calculate the mean number of days to entrain (Figure 4B). Notably, a large proportion of Vgat-cre;Sox2fl/fl mice were either weakly or not entrained or were arrhythmic in the final 5 days of the delayed or advanced LD schedule (Figure 4C). During the first 3 days following transfer to a new light schedule, a considerable percentage of Vgat-cre;Sox2fl/fl mice were behaviorally arrhythmic, whereas all control mice maintained robust rhythms (Figure 4D). The erratic behavior of Vgat-cre;Sox2fl/fl mice in reaction to shifts or changes in the LD schedule was further demonstrated by the highly variable, and unpredictable, activity onsets (Figure 4E; Table S1). These results show that re-entrainment to shifted light cycles is severely impaired in mice lacking SOX2 in SCN neurons.

These entrainment phenotypes could be due to altered responsiveness of the SCN to light or to weak coupling within the SCN leading to sloppy rhythms. Given that Sox2 ablation suppressed the expression of AVP and VIP, we tested the hypothesis that coupling within the SCN is compromised in Vgat-cre;Sox2fl/fl mice using a constant light (LL)-induced desynchronization paradigm. Control mice exhibited increasingly longer behavioral rhythms when subjected to long-term LL conditions of escalating light intensities (10, 40, and 100 lux; Figures 4F and 4H; Table S1). Whereas 6% of control mice became arrhythmic at 100 lux, >70% of Vgat-cre;Sox2fl/fl mice became arrhythmic at 40 and 100 lux (Figures 4G and 4I; Table S1). The amplitude of activity rhythms under LL was greatly diminished in Vgat-cre;Sox2fl/fl mice, but period was largely unaffected (Figures 4H, 4J, and 4K; Table S1). Collectively, our results suggest that Sox2 is required for stable entrainment to environmental light and for buffering against the desynchronizing effects of constant light.

Sox2 Ablation Impairs Clock Gene Expression and Neuropeptide Signaling in the SCN

To understand the origins for the profound circadian behavioral phenotypes arising from Sox2 ablation within the SCN, we analyzed the SCN transcriptomes of Vgat-cre;Sox2fl/fl mice and Sox2fl/fl controls at 4 circadian times (CT 0, 6, 12, and 18) using RNA sequencing (RNA-seq) (see Data S1 for details). We first focused on the transcript abundance of clock genes. Consistent with our protein data, Per2 mRNA levels were significantly reduced, at CT 6, in Vgat-cre;Sox2fl/fl SCN compared to controls (Figure S4A). The expression of several other clock genes was also affected in these animals, including Cry1, Arntl, Rora, Hif, and Nr1d1 (REV-ERBα; Figures S4D, S4E, S4G, S4J, and S4L). The expression of Per1, Cry2, Clock, Rorγt, Nr1d2 (REV-ERβ), and Dbp was not perturbed (Figures S4B, S4C, S4F, S4H, S4I, and S4K). These data suggest that SOX2 in SCN neurons is required for appropriate expression of Per2 as well as other components of the clock machinery.

Using DESeq2 analysis and a BH.q value cutoff of 0.05, we identified 233 differentially expressed genes (DEGs) in the SCN of Vgat-cre;Sox2fl/fl mice (Data S2). Of those DEGs, 57 (24.5%) were upregulated in the Vgat-cre;Sox2fl/fl SCN and 176 (75.5%) were downregulated (Figure S5A). We subjected the 233 DEGs to Gene Ontology (GO) enrichment analysis, comparing them to a background list of 16,950 genes that were expressed in the control SCN. As expected, the terms “rhythmic process” (no. 5), “circadian rhythm” (no. 16), and “regulation of circadian rhythm” (no. 30) were featured in the list of top 30 most enriched biological process (BP) GO terms (Figure 5B; Data S3). Interestingly, the top 20 GO-BP terms also included “cell communication” (no. 1), “G-protein-coupled receptor signaling pathway” (no. 3), and “neuropeptide signaling pathway” (no. 14), suggesting that intercellular and/or peptidergic signaling may be affected in Vgat-cre;Sox2fl/fl SCN (Figure 5B). Along these lines, the list of top 10 most enriched molecular function (MF) GO terms

Figure 4. Sox2 Ablation in SCN Neurons Impairs Re-entrainment during Experimental Jetlag and Promotes Constant Light-Induced Arrhythmicity

(A and B) Representative actograms of (A) control and (B) Vgat-cre;Sox2fl/fl mice under jetlag conditions. Periods of light are shaded in yellow. (C) Percentage of mice that are stably entrained, weakly or not entrained, or arrhythmic in the last 5 days of the initial, delayed, or advanced LD schedule. (D) Percentage of mice showing rhythmic or arrhythmic behavior in the first 3 days after transitioning to the delayed LD (LD−delay), advanced LD (delay−Adv), or DD (Adv−DD) schedule. (E) Time of activity onsets of control (gray) and Vgat-cre;Sox2fl/fl mice (red), plotted for each day of the experiment. Time 12 (x axis) represents ZT 12 of the initial LD schedule. Day 43 onward represents DD. (F and G) Representative actograms of (F) control and (G) Vgat-cre;Sox2fl/fl mice under LL conditions of increasing light intensities. (H–K) Quantification of the (H) period length, (J) percentage of rhythmic mice, (J) amplitude determined from the χ2 periodogram, and (K) fast Fourier transformation (FFT) amplitude under LL conditions. Values represent mean ± SEM; n = 8–10 per group. *p < 0.05 versus control.
neuropeptides and their cognate G-protein-coupled receptors that are known to mediate intercellular signaling, either within the SCN or from the SCN to its effenter targets (Figures 5C–5L). Generally speaking, Sox2 ablation resulted in a significant reduction in the expression of one or both members of a neuropeptide-receptor pair in at least one time point. Vip, Prok2, and their respective receptors, Vipr2 and Prokr2, were drastically downregulated in the SCN of Vgat-cre;Sox2fl/fl mice (Figures 5F, 5G, 5K, and 5L). The expression of Avpr1a (encoding for an AVP receptor), neurexin S (Nms), Grp, and Grpr were also significantly diminished in the Sox2-deficient SCN (Figures 5D, 5E, 5H, and 5I). However, neither Avpr nor Nmur2 (a receptor for Nms) was markedly affected at the transcript level by Sox2 ablation (Figures 5C and 5J). Other genes that had previously been implicated in the function or regulation of the SCN (e.g., Fbxl21, Drd1, Rgs16, and Rasd1) were also downregulated in the mutant SCN (Figures 5M–5P). The results of the DEG analysis suggest that neuropeptide signaling may be impaired in the SCN of Vgat-cre;Sox2fl/fl mice.

To identify potential direct targets of SOX2, we compared our DEG dataset with a previously reported SOX2 ChIP-seq dataset from murine embryonic stem cells (ESCs) and ESC-derived neural progenitor cells (NPCs) (Lodato et al., 2013). The Lodato study identified SOX2 binding sites within 1 kb of a TSS and in distal enhancers. A total of 47 and 85 DEGs were identified in the ESC and NPC SOX2 ChIP-seq datasets, respectively (Figures S5A and S5B; Data S4). Most of the SOX2 binding sites in these DEGs were situated in distal enhancer regions (Figures S5A and S5B). Out of 7 DEGs that were selected for ChIP-qPCR validation, all but one (Hif1, Tctf12, Rora, Rgs16, Plk2, and Prok2) showed significant enrichment of SOX2 near the predicted TSS or distal enhancer region in SCN tissues extracted at one or more CTs (Figures S5C–S5I). Based on the high rate of ChIP-qPCR validation, the comparison with the Lodato dataset appears to yield a subset of DEGs that are probable, direct targets of SOX2 within the SCN.

Collectively, our RNA-seq data show that Sox2 deficiency alters the transcriptome of the SCN, reducing the expression of Per2, other clock genes, neuropeptides, and neuropeptide receptors. The ChIP-qPCR results further suggest that there are direct SOX2 target genes (including Per2) within the adult SCN.

**DISCUSSION**

Here, we have characterized the roles of SOX2 in SCN circadian timekeeping. We show that SOX2 is expressed in mature neurons of the SCN, where it can physically associate with the Per2 gene promoter. Ectopic expression of SOX2 in cultured cells induces the activity of the Per2 promoter. Using a conditional knockout mouse model where SOX2 expression is specifically ablated in SCN neurons, we show that SOX2 is critical for high-amplitude expression of PER2 in the SCN. Sox2 deficiency in SCN neurons has severe repercussions on behavioral rhythms: in addition to a lengthened period, rhythms are more unstable and fragmented, are more prone to the disruptive effects of constant light, and respond aberrantly to the entraining effects of environmental light cycles. RNA-seq analysis of the SCN reveals that Sox2 deficiency perturbs the transcriptome, reducing the expression of Per2, several other clock genes, and genes encoding for neuropeptides (Vip, Prok2, Grp, and Nms) and neuropeptide receptors (Vipr2, Prokr2, Grpr, and Avpr1a). Finally, we demonstrate that SOX2 binds to the promoters or enhancers of several genes in the adult SCN. These collective observations suggest that SOX2 functions in the adult SCN to control the transcriptional landscape, ensuring that the SCN generates appropriate rhythms and outputs.

We provide multiple lines of evidence to show that SOX2 is a transcriptional regulator of the Per2 gene. In vitro, SOX2 can directly interact with the proximal promoter region of mPer2 at two possible sites. SOX2 overexpression in Neuro2a cells activates the mPer2 promoter: this effect is dependent on the presence of SOX2’s HMG DNA-binding domain, partially dependent on the more distal putative SOX2 binding site, and independent of the E2 enhancer. Within the SCN of Vgat-cre;Sox2fl/fl mice, PER2 protein accumulation across the circadian cycle, examined at the level of single cells and of the whole SCN, was reduced in magnitude and amplitude. Reduced PER2 accumulation may delay the closing of the negative feedback loop and contribute to the lengthened behavioral period of Vgat-cre;Sox2fl/fl mice. Interestingly, we observed constitutive association of SOX2 at the Per2 promoter in SCN tissues throughout the circadian cycle but a selective effect of Sox2 ablation near the circadian peak (but not the nadir) of Per2 expression. These results suggest that SOX2 alone is not sufficient to enhance Per2 transcription in the SCN and that it may require a binding partner and/or work cooperatively with other transcription factors to activate the Per2 gene in a time-of-day-specific fashion.

However, SOX2-mediated Per2 transcription cannot solely explain the profound changes in behavioral rhythms of Vgat-cre;Sox2fl/fl mice. The expression of other clock genes, notably Rora, Amtl, and Cry1, is also perturbed in these animals,
although it is not clear whether these effects on the molecular clock machinery necessarily impacts oscillator strength and thus behavioral outputs. Perhaps more consequential is the pervasive reduction in the expression of neuropeptides and their co-regulated receptors within the SCN of Vgat-cre;Sox2fl/fl mice. Neuropeptides are important for intra-SCN synchrony (e.g., VIP, AVP, and NMS) and transmission of output signals to efferent targets of the SCN (e.g., PK2). Deficits in neuropeptide signaling are likely to affect coupling interactions within the SCN neuronal network, ultimately impacting its ability to maintain synchrony, to desynchronize, or to resynchronize depending on environmental parameters. Reduced coupling may explain the poor consolidation of behavioral rhythms of Vgat-cre;Sox2fl/fl mice. However, it cannot explain their erratic behavior under experimental jetlag conditions. Other behavioral phenotypes, such as reduced wheel-running activity, may stem from decreased SCN output signals to efferent targets, because the expression of PK2, an important output molecule, is strongly depressed in these animals.

One outstanding issue that our study has not addressed is the impact of Sox2 ablation on SCN development and its potential contribution to the circadian phenotype of adult Vgat-cre;Sox2fl/fl mice. Although we have presented clear evidence to support our conclusion that SOX2 acts as a transcriptional regulator in the adult SCN, it may have additional functions during SCN development, especially in light of the ~28% reduction in SCN size and neuron number in our mutant mice. Whether this change in SCN neuron number affects the function of the adult SCN remains unknown. Furthermore, some of the gene expression changes that we observed in the SCN of adult Vgat-cre;Sox2fl/fl mice may be a secondary consequence of altered SCN development rather than a direct (or indirect) transcriptional effect of SOX2 on these genes in the adult SCN. Future investigations should examine the role of SOX2 in SCN development and parse out the relative contributions of its developmental and adult functions to the phenotypes of Vgat-cre;Sox2fl/fl mice.

In conclusion, our findings reveal SOX2 to be an important regulator of SCN clock function. SOX2 in SCN neurons is critical for high-magnitude expression of several clock genes, including Per2, its direct transcriptional target. Sox2 ablation also strongly attenuates the expression of neuropeptides and receptors that serve as coupling agents or clock output effectors. Most importantly, the absence of SOX2 in SCN neurons leads to severe and widespread changes in behavioral rhythms. Overall, our study shows that neuronal SOX2 expression is vital for defining the transcriptional landscape of the SCN and promoting its function as the central circadian pacemaker.

**STAR★METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **METHOD DETAILS**
  - Behavioral Analyses
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
  - Image Acquisition and Quantification
  - Statistical Analysis
  - Computational Analysis
- **DATA AND SOFTWARE AVAILABILITY**

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found with this article online at https://doi.org/10.1016/j.celrep.2019.02.068.

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**AUTHOR CONTRIBUTIONS**

A.H.C. conducted experiments, analyzed data, performed bioinformatics and statistical analyses, prepared figures, and contributed to manuscript writing. P.B.-C. conducted experiments and analyzed data. S.H., C.L., and S.W.F. assisted in experiments and statistical analyses. R.W.N. mapped the raw sequencing data. C.-K.C. assisted in bioinformatics analyses. H.-Y.M.C. conceived and designed the study, conducted experiments, analyzed data, and wrote the manuscript with input from all authors.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**REFERENCES**


## STAR★METHODS

### KEY RESOURCES TABLE

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Experimental Models: Cell Lines

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Animals
All animal handling and experimental procedures were performed at the University of Toronto Mississauga (UTM) Animal Facility and were approved by the UTM Animal Care Committee, complying with guidelines established by the University of Toronto Animal Care Committee and the Canadian Council on Animal Care. The following mouse strains were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred in-house to generate the appropriate genotypes for this study: homozygous Sox2\textsuperscript{fl/fl} mice in which the coding exon of Sox2 is flanked by loxP sequences (Sox2\textsuperscript{tm1.1Lan}); homozygous Vgat\textsuperscript{-IRES-cre} (Vgat\textsuperscript{cre/cre}) knockin mice in which the IRES-cre recombinase cassette is inserted downstream of the stop codon of the endogenous vesicular GABA transporter (vgat) gene (Slc32a1\textsuperscript{tm2(cre)Lowl}); PER2::LUC mice in which the firefly luciferase (luc) gene is inserted in-frame into exon 23 of the wild-type sequence (Per2\textsuperscript{tm1Jt}/J); and C57BL/6J mice. The \textit{mPeriod2::DsRED} mouse strain (Cheng et al., 2009) was also used. Vgat\textsuperscript{cre/cre} mice were bred to Sox2\textsuperscript{fl/fl} mice, and a breeding colony was maintained by mating Sox2\textsuperscript{fl/fl} mice with Vgat\textsuperscript{cre/cre}/Sox2\textsuperscript{fl/fl} mice. \textit{mPeriod2::DsRED} and Vgat\textsuperscript{cre/cre} mice were bred to C57BL/6J mice to generate hemizygous or heterozygous mice, respectively, for experiments. Littermate controls were used wherever possible for experiments. For behavioral and immunostaining experiments,
data from Vgat<sup>cre/+/</sup> and Sox2<sup>fl/fl</sup> controls were pooled as they were phenotypically indistinguishable from one another. For RNA-seq experiments, only Sox2<sup>fl/fl</sup> mice were used as controls. Unless otherwise specified, mice were bred and maintained on a fixed 12-hr light:12-hr dark (12:12 LD) schedule in which lights on and lights off corresponded to 8 am and 8 pm Eastern Standard Time, respectively.

**METHOD DETAILS**

**Behavioral Analyses**

**Circadian behavioral paradigms and analyses**

Starting at 5 to 8 weeks of age, male and female mice were singly housed in running-wheel cages within ventilated, light-tight cabinets under computer-controlled lighting schedules (Phenome Technologies). Illumination within the cabinets was provided by white light-emitting diodes (LEDs), with intensity set at 40 Lux (0.50 μE) at cage level unless otherwise indicated. Wheel revolutions were recorded and analyzed using ClockLab software (Actimetrics). In all experiments, mice were initially entrained to a 12:12 LD schedule for at least 2 weeks prior to indicated changes in the light schedules. To assess entrainment under a skeleton photoperiod, mice were maintained for 2 weeks under a 1:10:1:12 LDLD schedule, where the L portions (40 Lux, 0.50 μE) corresponded to Zeitgeber time (ZT) 0-1 and ZT 11-12 of the previous 12:12 LD schedule. Free-running rhythms were examined by releasing mice from 12:12 LD or 1:10:1:12 LDLD into constant darkness (DD) for 2 weeks. To determine the effects of constant light (LL), mice were exposed to LL in which the light intensity increased stepwise (10 Lux [0.13 μE], 40 Lux [0.50 μE], 100 Lux [1.25 μE]) every 2 weeks. For the jetlag paradigm, mice were subjected to an abrupt 7-hr delay of the 12:12 LD schedule for 2 weeks, followed by a 7-hr advance of the LD schedule for an additional 2 weeks. Times of activity onset and offset were generated by ClockLab and corrected when necessary following visual inspection of the actograms. The onset was considered as the first 5-min bin where 20% of maximum activity during that cycle was displayed, and the offset was the last 5-min bin with 5% of peak activity. Mean onset/offset times were calculated for each animal. Onset error was defined as the standard deviation of daily onsets over 7 consecutive days. Total (mean) daily wheel revolutions were calculated by taking the average of daily wheel revolutions for 7 consecutive days. Period length was measured by fitting a regression line through daily activity onsets or by using the χ<sup>2</sup> periodogram. Amplitude was measured by the χ<sup>2</sup> periodogram and Fast Fourier Transformation (FFT). Arrhythmicity was determined by χ<sup>2</sup> periodogram analysis.

**Weights test**

The apparatus consists of a series of 8 weights (12.95, 18.65, 24.35, 30.05, 35.75, 41.45, 47.15, and 52.85 g) constructed from a piece of plastic bath sponge and 1 to 8 steel chain links. Five-week-old male and female mice were tested for their ability to hold onto each weight for a minimum of 3 s. Mice were held by the tail, allowed to grasp the sponge portion of the first weight (12.95 g) from a bench-top with their forepaws, and raised until the weight was cleared entirely from the bench. Each mouse was given 3 chances to hold onto the weight for at least 3 s. Only mice that had one successful attempt were tested on the next weight in the series. For each mouse, the fail weight was the weight at which all 3 attempts were unsuccessful. Percent success represented the percentage of mice that had one successful attempt for each weight in the series.

**Kondziela’s inverted screen test (hanging wire test)**

The inverted screen consisted of a 43-cm square wire mesh screen surrounded by a 4-cm deep wooden frame. Five-week-old male and female mice were tested individually by placing them at the center of the screen and slowly inverting the screen. The screen was held approximately 50 cm above a large rat cage filled with crinkle paper for up to 1 min. Animals that fell before 1 min had elapsed were scored as having failed the test. All control and Vgat-cre;Sox2<sup>fl/fl</sup> mice passed this test.

**Grip meter**

A grip meter (Columbus Instruments) was used to assess forelimb grip strength of five-week-old male and female mice. The mouse was held by the tail and positioned so that its forepaws gripped the horizontal bar of the apparatus. The mouse was gently pulled by the tail until it lost grip of the bar. For each animal, the greatest kilogram force (kgF) recorded in three attempts was assigned as the peak force. The peak force was also expressed relative to the animal’s body weight. For this and the two aforementioned tests, performance was assessed between ZT 0 and ZT 4 under ambient conditions.

**Tissue Harvest**

Mice (both sexes, 5 to 8 weeks of age) were maintained on a fixed 12:12 LD schedule and released in DD for two consecutive cycles prior to further treatment and tissue harvest. To examine light-induced pERK1/2 and pCREB expression in the SCN, mice received a 15-min light pulse (LP) of 50 Lux intensity (0.63 μE) at circadian time (CT) 15 or 22. Tissues were harvested immediately after the end of the LP. For all other experiments, tissues were harvested at prescribed CTs on the third day of DD. Mice were killed by a 15-min light pulse (LP) of 50 Lux intensity (0.63 μE) at cage level unless otherwise indicated. Wheel revolutions were recorded and analyzed using ClockLab software (Actimetrics). In all experiments, mice were initially entrained to a 12:12 LD schedule for at least 2 weeks prior to indicated changes in the light schedules. To assess entrainment under a skeleton photoperiod, mice were maintained for 2 weeks under a 1:10:1:12 LDLD schedule, where the L portions (40 Lux, 0.50 μE) corresponded to Zeitgeber time (ZT) 0-1 and ZT 11-12 of the previous 12:12 LD schedule. Free-running rhythms were examined by releasing mice from 12:12 LD or 1:10:1:12 LDLD into constant darkness (DD) for 2 weeks. To determine the effects of constant light (LL), mice were exposed to LL in which the light intensity increased stepwise (10 Lux [0.13 μE], 40 Lux [0.50 μE], 100 Lux [1.25 μE]) every 2 weeks. For the jetlag paradigm, mice were subjected to an abrupt 7-hr delay of the 12:12 LD schedule for 2 weeks, followed by a 7-hr advance of the LD schedule for an additional 2 weeks. Times of activity onset and offset were generated by ClockLab and corrected when necessary following visual inspection of the actograms. The onset was considered as the first 5-min bin where 20% of maximum activity during that cycle was displayed, and the offset was the last 5-min bin with 5% of peak activity. Mean onset/offset times were calculated for each animal. Onset error was defined as the standard deviation of daily onsets over 7 consecutive days. Total (mean) daily wheel revolutions were calculated by taking the average of daily wheel revolutions for 7 consecutive days. Period length was measured by fitting a regression line through daily activity onsets or by using the χ<sup>2</sup> periodogram. Amplitude was measured by the χ<sup>2</sup> periodogram and Fast Fourier Transformation (FFT). Arrhythmicity was determined by χ<sup>2</sup> periodogram analysis.

**Weights test**

The apparatus consists of a series of 8 weights (12.95, 18.65, 24.35, 30.05, 35.75, 41.45, 47.15, and 52.85 g) constructed from a piece of plastic bath sponge and 1 to 8 steel chain links. Five-week-old male and female mice were tested for their ability to hold onto each weight for a minimum of 3 s. Mice were held by the tail, allowed to grasp the sponge portion of the first weight (12.95 g) from a bench-top with their forepaws, and raised until the weight was cleared entirely from the bench. Each mouse was given 3 chances to hold onto the weight for at least 3 s. Only mice that had one successful attempt were tested on the next weight in the series. For each mouse, the fail weight was the weight at which all 3 attempts were unsuccessful. Percent success represented the percentage of mice that had one successful attempt for each weight in the series.

**Kondziela’s inverted screen test (hanging wire test)**

The inverted screen consisted of a 43-cm square wire mesh screen surrounded by a 4-cm deep wooden frame. Five-week-old male and female mice were tested individually by placing them at the center of the screen and slowly inverting the screen. The screen was held approximately 50 cm above a large rat cage filled with crinkle paper for up to 1 min. Animals that fell before 1 min had elapsed were scored as having failed the test. All control and Vgat-cre;Sox2<sup>fl/fl</sup> mice passed this test.

**Grip meter**

A grip meter (Columbus Instruments) was used to assess forelimb grip strength of five-week-old male and female mice. The mouse was held by the tail and positioned so that its forepaws gripped the horizontal bar of the apparatus. The mouse was gently pulled by the tail until it lost grip of the bar. For each animal, the greatest kilogram force (kgF) recorded in three attempts was assigned as the peak force. The peak force was also expressed relative to the animal’s body weight. For this and the two aforementioned tests, performance was assessed between ZT 0 and ZT 4 under ambient conditions.

**Tissue Harvest**

Mice (both sexes, 5 to 8 weeks of age) were maintained on a fixed 12:12 LD schedule and released in DD for two consecutive cycles prior to further treatment and tissue harvest. To examine light-induced pERK1/2 and pCREB expression in the SCN, mice received a 15-min light pulse (LP) of 50 Lux intensity (0.63 μE) at circadian time (CT) 15 or 22. Tissues were harvested immediately after the end of the LP. For all other experiments, tissues were harvested at prescribed CTs on the third day of DD. Mice were killed by cervical dislocation and their brains were rapidly dissected under dim red light. Brains were sectioned in ice-cold oxygenated media or diethyl pyrocarbonate (DEPC)-treated phosphate buffered saline (PBS), pH 7.4 (for RNA-sequencing experiments) with an oscillating tissue slicer (Electron Microscopy Sciences) to obtain an 800-μm thick coronal slice containing the SCN or other brain regions. For immunostaining experiments, tissue slices were fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 6 hr at room temperature, cryoprotected in 30% sucrose in PBS at 4°C overnight, cut into 30-μm or 40-μm thin sections using a freezing microtome (Leica Microsystems), and stored in 30% sucrose at 4°C until further use. For chromatin immunoprecipitation (ChIP) and RNA-seq experiments, the 800-μm coronal slice containing the SCN was placed on a dry ice-chilled glass slide and the SCN
was microdissected using a sharp scalpel blade. SCN tissues were frozen immediately on dry ice and stored at −80°C until further processing. For RNA-seq and ChIP experiments, each sample represented a pool of SCN tissues from 3 or 4 mice, respectively.

**Immunofluorescence (IF), Immunohistochemistry (IHC), and Immunocytochemistry (ICC)**

For IF, tissues were washed 5 × 5 min in PBS (pH 7.4) with 0.1% Triton X-100 (PBST), incubated for 1 hr at room temperature (RT) in blocking solution (10% horse serum in PBST), and incubated overnight at 4°C in fresh blocking solution containing the primary antibody. The next day, tissues were washed 5 × 5 min with PBST and incubated for 2 hr at RT, protected from light, with the appropriate secondary AlexaFlour antibody (1:1000) diluted in blocking solution. Sections were washed 5 × 5 min with PBST, incubated for 10 min in DAPI (1:10,000) diluted in PBS, washed twice in PBST, and mounted onto glass microscope slides. Slides were coverslipped with Fluorescence Mounting Medium and sealed with nail polish.

For IHC, tissues were washed 5 × 5 min in PBST, treated with 0.3% H2O2 in PBS for 20 min at RT, washed 5 × 5 min in PBST, and incubated for 1 hr at RT in blocking solution. Tissues were incubated overnight at 4°C in primary antibodies diluted in fresh blocking solution. The next day, sections were washed 5 × 5 min in PBST, incubated for 2 hr at RT with the appropriate biotinylated secondary antibody diluted in blocking solution, washed 5 × 5 min in PBST, and incubated for 45 min with Avidin-Biotinylated horse radish peroxidase (HRP) Complexes (ABC) from the VECTASTAIN ABC-HRP kit. After washing 5 × 5 min in PBST, sections were developed with the 3,3′-diaminobenzidine (DAB) HRP substrate according to the manufacturer’s instructions. For each experiment, all sections were processed at the same time and with the same DAB development time. Sections were mounted onto gelatin-coated glass microscope slides, dehydrated, and coverslipped with Permount Mounting Media.

For ICC, Neuro2a cells were plated onto glass coverslips in 24-well plates. Cells were washed with PBS (pH 7.4), fixed for 10 min in 4% PFA in PBS, rinsed with ice-cold PBS, and washed 5 × 5 min in PBST. Immunolabeling was performed as described above for IF. Coverslips were mounted onto glass microscope slides with Fluorescence Mounting Medium and sealed with nail polish.

For SCN explants, membrane-attached SCN tissues were fixed for 1 hr in 4% PFA, washed 2 × 10 min in PBS, permeabilized in PBS with 0.3% Triton X-100 for 1 hr, and incubated in blocking buffer (10% horse serum, 1% BSA, 0.3% Triton X-100 in PBS, pH 7.4) for 3 hr at RT. Primary antibodies were added in blocking solution and incubated O/N at 4°C. The next day, tissues were washed 4 × 10 min in PBS with 0.3% Triton X-100, incubated with the appropriate secondary AlexaFlour antibody at RT for 3 hr, and washed 4 × 10 min in PBS with 0.3% Triton X-100. Tissues were then incubated for 30 min in DAPI (1:10,000) diluted in PBS, washed 2 × 10 min in PBS, and mounted onto glass microscope slides with glycerol for confocal imaging.

**Bioluminescence Recordings and Viral Transduction of SCN Explants**

Twelve-day-old Sox2fl/fl;Per2Luc/+/Sox18-LUC, and the pGL3-DmPeriod2-105 to +110-LUC construct was used to generate pGL3-DmPeriod2-105 to +110-POU-LUC. To generate the pCMV-FLAG-SOX2 construct, the murine Sox2 cDNA sequence was amplified by PCR using the pCAG-HA-SOX2-IP plasmid (Plasmid #13459, Addgene) as template and the following primers (fwd 5′-AAAGAAACCGCCTTTGTTAACAATGATGGAGCAGGAGCTG-3′; rev 5′-GAAAGATTCCTACATGTCGAGAGGGC-3′), and subsequently cloned into the NotI and BglII sites of the pCMV10-3xFLAG vector. SOX2 deletion mutants were generated with the O5 Site-Directed Mutagenesis Kit and pCMV-FLAG-SOX2 as the template. Briefly, 5 ng of plasmid DNA was amplified using the following PCR conditions (98°C for 30 s; 25 cycles of 98°C for 10 s, 60°C for 20 s, 72°C for 4 min; 72°C for 2 min) and primers flanking the deleted region. Following a 15-min incubation at RT in Kinase-Ligase-DpnI (KLD) mix, 1 μL of the PCR reaction was transformed into TOP10 cells and plated onto LB agar plates containing ampicillin. The following constructs were generated: pCMV10-3xFLAG-SOX2 Δsat1-39
(SOX2ΔN-Terminal), pCMV10-3xFLAG-SOX2Δaa446-110 (SOX2ΔNLS1-ΔHMG-ΔNLS2); pCMV10-3xFlag-SOX2Δaa67-108 (SOX2ΔHMG); pCMV10-3xFLAG-SOX2Δaa206-251 (SOX2ΔSRD); pCMV10-3xFLAG-SOX2Δaa121-319 (SOX2ΔC-Terminal). All constructs were confirmed by sequencing at The Center for Applied Genomics (TCAG, Toronto, Canada). The pTolo-EX5-Sox2 vector was generated by Tolo Biotechnology (Shanghai, China). Briefly, the coding sequence of the murine Sox2 gene was first synthesized and codon-optimized for expression in Escherichia coli cells, and then cloned into the Ncol and ScaI sites of the pTolo-Ex5 bacterial expression plasmid.

**Cell Culture, Plasmid Transfections and Luciferase Assays**

Neuro2a (N2a) cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin at 37°C/5% CO2. N2a cells were transfected with plasmid constructs using Lipofectamine 3000 in DMEM and returned to complete growth media 5 hr later. For luciferase experiments, 30 hr post-transfection, cells were washed twice with ice-cold PBS and processed using the Dual-Glo Luciferase Assay System according to manufacturer’s instructions. To normalize measurements of firefly luciferase activity, cells were co-transfected with a construct expressing Renilla luciferase under the control of the thymidine kinase promoter.

**Western Blotting**

Transfected N2a cells were washed twice with ice-cold PBS and lysed in RIPA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, Protease Inhibitor Cocktail). Lysates were centrifuged at 14,000 x g for 20 min, and protein concentration of the supernatant was determined using the Bradford method. Protein samples were run on a 10% Tris-glycine SDS-PAGE gel and transferred onto PVDF membrane. Membranes were incubated O/N at 4°C with primary antibodies diluted in blocking solution. The next day, membranes were washed 5 x 5 min with TBST, incubated for 2 hr at RT in HRP-conjugated secondary antibodies, and washed again 5 x 5 min in TBST. Signals were detected by chemiluminescence using the SuperSignal West Femto Maximum Sensitivity Substrate and developed on X-ray film.

**Protein Purification, Electrophoretic Mobility Shift Assay (EMSA) and Dnase I Footprinting**

Protein purification, EMSA and DNase I footprinting experiments were performed by Tolo Biotechnology.

**SOX2 protein purification**

Single colonies of BL21(DE3) cells transformed with the pTolo-ES5-Sox2 plasmid were grown in 5 mL of LB broth at 37°C until the OD600 reached 0.6. The starter culture was diluted 1:100 in 200 mL LB of broth and grown at 37°C/5% CO2. N2a cells were transfected with plasmid constructs using Lipofectamine 3000 in DMEM and returned to complete growth media 5 hr later. For luciferase experiments, 30 hr post-transfection, cells were washed twice with ice-cold PBS and processed using the Dual-Glo Luciferase Assay System according to manufacturer’s instructions. To normalize measurements of firefly luciferase activity, cells were co-transfected with a construct expressing Renilla luciferase under the control of the thymidine kinase promoter.

**DNase I footprinting**

DNase I footprinting assays were performed as previously described (Wang et al., 2012) with minor modifications. For each assay, 400 ng of FAM-labeled probe was incubated with 0 or 6 μg of recombinant SOX2 protein in a total volume of 40 μL at 30°C. After the incubation, a 10 μL reaction volume containing 40 ng of FAM-labeled probe and 0 to 3 μg of recombinant SOX2 protein was added to a reaction to test for competitive binding. Samples were incubated for 30 min at 30°C and loaded onto a 1% agarose gel buffered with 0.5x TBE. Gels were scanned with ImageQuant LAS 4000 mini (GE Healthcare), with the exception that the GeneScan-LIZ500 size standard (Applied Biosystems) was used.

**Chromatin Immunoprecipitation (ChIP) and Quantitative PCR**

N2a cells transfected with either pcDNA3.1 or pCMV10-3X-FLAG-SOX2 were fixed with 0.75% PFA for 10 min at room temperature and quenched with 125 mM glycine. Cells were washed 3x with protease inhibitor cocktail-supplemented PBS (PBS-PI), scraped, and pelleted by centrifugation. Cell pellets were homogenized in SDS lysis buffer (50 mM Tris pH 8.0, 10 mM EDTA, 1% SDS, protease inhibitors) and incubated on ice for 10 min. For SCN samples, 1% PFA was added to frozen tissues, incubated at 37°C for 10 min, and quenched with 125 mM glycine. Tissues were washed 5x with PBS-PI, homogenized in SDS lysis buffer and incubated on ice for 10 min. Cell lysates were sonicated for 11 cycles, whereas tissue lysates were sonicated for 9 cycles at power 40% on ice (10 s ON,
Cell counts within the SCN. Staining was measured in a non-immunoreactive region adjacent to each SCN and subtracted from the immunoreactive intensity. For quantification of staining intensity, 10x bright-field images of the bilateral SCN were acquired. The area of each SCN was delineated using the “analyze particle” function with size set at \( > 30 \, \mu \text{m}^2 \). For total SCN cell counts and density measurements, 40x confocal images were acquired that covered the entire surface of the bilateral SCN from four 40-\( \mu \text{m} \)-thick coronal sections containing 2 central, 1 rostral, and 1 caudal SCN. The total number of MeCP2\(^+\) cells within the SCN was counted manually through all z-planes of these confocal images. MeCP2\(^+\) cell density measurements were calculated for each section. A mean density measurement was calculated for each animal, since the cell density was not significantly different across the rostral-caudal SCN axis. To estimate the total number of SCN neurons per animal, all SCN-containing coronal sections were stained with DAPI, and the area occupied by the SCN was measured for each section. The number of SCN neurons per section was calculated by multiplying the mean cell density by the SCN area. The sum of these values for all sections represented the estimated total number of SCN neurons per animal.

**Statistical Analysis**

Data were analyzed using one-way analysis of variance (ANOVA), two-way ANOVA, linear mixed effects modeling fit (LMM), and Fisher’s Exact Test with Minitab software version 17.1.0 and R version 3.5.0. Post hoc significance of pairwise comparisons was assessed using Tukey’s Honest Significant Difference test with \( \alpha \) set at 0.05. The significance of viral transduction on the relative amplitude of PER2::LUC rhythms over time was assessed with LMM by restricted maximum likelihood.

**Computational Analysis**

RNA-Seq reads in FASTQ format for each of 40 replicates were trimmed using Trimmomatic v.0.36. Trimmomatic was used to remove contaminating TruSeq3 paired-end adapters and low quality leading or trailing bases. Bases 3’ of the first 4bp window were removed.

**RNA-sequencing**

RNA extraction was performed using the RNeasy Micro Kit according to the manufacturer’s instructions. RNA samples were submitted to the Génome Québec Innovation Centre at McGill University (Montréal, Canada) for RNA-seq library preparation and sequencing. Total RNA was quantified using a Nanodrop Spectrophotometer ND-1000 (Nanodrop Technologies) and its integrity was assessed on a 2100 Bioanalyzer (Agilent Technologies). Libraries were generated from 250 ng of total RNA as described below. mRNA enrichment was performed using the NEBNext Poly(A) Magnetic Isolation Module (New England BioLabs). cDNA synthesis was achieved with the NEBNext RNA First Strand Synthesis and NEBNext Ultra Directional RNA Second Strand Synthesis Modules (New England BioLabs). The remaining steps of library preparation were done using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs). Adapters and PCR primers were purchased from New England BioLabs. Libraries were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies) and the Kapa Illumina GA with Revised Primers-SYBR Fast Universal Kit (Kapa Biosystems). Average size fragment was determined using a LabChip GX (PerkinElmer) instrument. RNA libraries were sequenced (100bp paired-end reads, 86.6 million reads on average per sample [range 40.9M-224.1M reads per replicate]) on an Illumina HiSeq4000 platform. A total of 40 SCN samples (n = 5 per genotype per CT) were sequenced.
with a mean PHRED score below 15 were trimmed. Reads with fewer than 36bp after quality trimming were discarded but their mates were retained if they passed quality trimming. After quality trimming, we retained 95.4% of reads with a mean of 1.44 bases trimmed from each read. Both paired and unpaired, trimmed reads for each replicate were aligned to the Mus musculus reference genome (GRCm38) using Bowtie2. Approximately 85.1% of reads aligned to the reference genome (range 84.0%–87.6%) with a mean mismatch rate of 0.7%. Each of the resulting SAM files was compressed into BAM format using samtools view. Details on alignment and quantification are included in Data S1.

The Mus musculus gene annotation from Ensembl (release 90) was loaded as gene model using makeTxDbFromGFF from the GenomicFeatures package. The function summarizeOverlaps from the GenomicAlignments package was used to generate a SummarizedExperiment object that contained the information about each library: genotype (control or Vgat-cre;Sox2fl/fl), circadian time (CT 0, 6, 12, or 18), the replicate (1 - 5 for each genotype-time combination), and number of reads per gene. On average, 624 reads aligned to each gene in the genome, and the mean number of reads per gene varied from 293 to 1557 among replicates. Genes with counts-per-million (cpm) below 0.1 in at least 35 of the 40 libraries were not considered to be expressed in the SCN and were thus discarded from further analysis. Expression data of the remaining 26,798 genes were used for downstream analysis.

Differential expression analysis was performed using the DESeq2 package with the assumption of negative binomial distribution for RNA-seq data. For each time point, genes were considered differentially expressed in Vgat-cre;Sox2fl/fl SCN compared to controls when the reported benjamini-hochberg adjusted p values (PADJ) is less than 0.05.

For constructing hierarchically clustered heatmaps of DEGs, variance stabilizing transformations (VST) (Anders and Huber, 2010) were first applied to the normalized counts to remove the dependence of the variance on the mean. The amount by which each gene deviates in a specific sample from the average VST transformed values across all samples was used to construct clustered heatmaps.

The 233 DEGs were submitted to STRING for Gene Ontology (GO) enrichment analysis. A total of 211 DEGs were mapped and compared against a control SCN background gene list of 16,950 genes. Significantly enrichment GO terms and KEGG pathways (FDR < 0.05) were summarized in Data S3.

ChIP-seq data of bound promoters, active enhancers, and poised enhancers for SOX2 in ESCs and NPCs were extracted from Table S2 of Lodato et al. (2013). Active enhancers and poised enhancers were grouped together for comparison. The list of SOX2 binding sites were crosschecked with our list of DEGs.

DATA AND SOFTWARE AVAILABILITY

The RNA-seq data reported in this paper have been deposited to the ArrayExpress database at EMBL-EBI (https://www.ebi.ac.uk/arrayexpress) under accession number ArrayExpress: E-MTAB-7496.