Supplemental Materials and Methods:

**Generation of the Six3-Cre;Lhx1^{lox/lox} Line**

The Six3Cre allele was originally introduced into fertilized C57BL6 X DBA2J eggs to generate the founder line, which was subsequently backcrossed to C57BL6 (Furuta et al, 2000). The Lhx1 floxed allele was originally introduced into Sv129 stem cells to generate the founder line, which was crossed to a C57BL6 CMV-Cre line to remove neomycin (Kwan and Behringer, 2002). To our knowledge, the Lhx1 floxed line remained mixed C57BL6/Sv129 when we received it. We anticipate that the Six3-Cre;Lhx1^{lox/lox} mutants and Lhx1^{lox/lox} littermate controls used in our studies are thus primarily of C57BL6 background with no more than ~25% Sv129 background, perhaps with a very small residual contribution from DBA2J. The Sv129 contribution is likely approximately equally distributed in experimental and control littermates, since both have two floxed Lhx1 alleles.

**Real-time Per2::luciferase measurement.**

SCN slice cultures were created as described previously (Abe et al, 2002). Briefly, brains were removed from CO2-anesthetized and decapitated mice and placed in cold Hank’s Buffered Salt Solution (HBSS). Coronal sections of the brain were sliced (300 μm thickness) with a vibratome and transferred to cold HBSS. SCN was identified under a dissecting microscope and isolated from surrounding tissue as a 1.5x1.5mm square by scalpels. SCN explants were placed on Millicell membranes (catalog #PICM030-50; pore size, 0.45 μm; Millipore, Bedford, MA) with 1 ml of DMEM (catalog #13000–021; Life Technologies, Grand Island, NY) supplemented with 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, B-27 serum-free supplement (catalog # 17504-044; Gibco/Life Technologies, Grand Island, NY) and 0.1 mM beetle luciferin (catalog #E1601; Promega, Madison, WI). Cultures were sealed in a 35 mm Petri dish with vacuum grease. Cultures were maintained at 36°C in darkness, and their bioluminescence was continuously monitored with a photomultiplier tube (catalog #HC135-11MOD; Hamamatsu, Shizouka, Japan) for >4 d as described previously (Geusz et al., 1997).

Seventeen additional SCN explants were placed on a single Millicell membrane using the culture conditions described above. The culture was sealed in a 35 mm Petri dish with vacuum grease and maintained in darkness at 34°C in a light-tight incubator (Onyx Box, Stanford Photonics, Inc, Palo Alto, CA). Per2::luc bioluminescence was monitored with 15 second exposures every 10 minutes for 5 days with a charge-coupled device camera (Stanford Photonics, Inc, Palo Alto, CA).

**Luminescence Analysis**

Period and amplitude of Per2::luc PMT traces were determined using modified published methods (Herzog et al, 2004). Data was detrended by subtracting 24 h running average from raw data, then analyzed by Fast-Fourier Nonlinear Least Squares, fitting for phase of the first peak, dominant period between 18-32 h, and relative amplitude. CCD images were stacked into 60-minute summed frames using Fiji software (Schindelin et al, 2012). Analysis was performed for 101 2 x 2 pixel regions of interest (ROIs) distributed in a standard array over each SCN slice. We excluded slices (1 Lhx1^{lox/lox} and 3 Six3Cre;Lhx1^{lox/lox}) if bioluminescence was <12 counts per hour between 48-72 h of
recording. We measured period and mean bioluminescence for each ROI with Chronostar V2.0 (a generous gift from A. Kramer, Charite, Berlin, Germany). All ROI periods ranged from 18 to 32 h. Mean period and bioluminescence were calculated from 101 ROIs per SCN slice.

Cannula Injections and Analysis

Prior to injection, all mice were entrained to 12:12 LD and released into constant darkness. Wheel-running behavior of the mice was monitored, and once the mutants’ activity patterns had begun to fragment, estimated CT14 was calculated for each mouse and injections were carried out. Animals were allowed to free-run for 5-7 days after each injection, long enough to allow post-injection phase angle and period to be reliably calculated. Once most mutants’ activity had become too fragmented for CT to reliably be calculated, the animals were re-entrained to 12:12 LD and re-released into DD. In practice, quantitative data could only be collected from behavior before and after the first injection in each round of entrainment and release into DD; rhythms were often too degraded to quantify period and, especially, phase in mutant animals after a second injection on the same round of entrainment. Over time, the mutants’ behavioral phenotype worsened, and some mutants’ rhythms broke down too quickly for CT to be reliably estimated. In these cases, arrhythmic mice were injected at the same solar time as the rhythmic mouse that had most closely tracked its CT in prior rounds of entrainment and release into DD.

Human prokineticin-2 isoform 2 (H-7342, Bachem, Torrance, CA) and porcine gastrin releasing peptide (H-1635, Bachem, Torrance, CA) were used in this study. The peptide was re-suspended in 0.9% sterile saline, aliquoted, and stored at -80°C. Aliquots were thawed on ice no more than 1 h before the injection(s) for which they were used and stored on ice until immediately before use. 2 uL icv injections were given under dim red light using a syringe type microinjector (EW-07844-00, Gilmont, Libertyville, IL). The mice were then left in DD for at least 5 days post-injection, and their wheel running behavior was analyzed as described in our Methods. After Prok2 injection, heteroscedastic two-tailed t-tests were used to analyze the significance of absolute phase and period changes in Excel. Since the mutants’ rhythms were less robust than controls, their estimated CTs were less accurate. In cases where post-injection rhythms were robust enough to allow it, we calculated the true CT at the injection time post-hoc and computed its correlation with the injection effect by linear regression in Excel. After Grp injection, phase and period of mutant mice often became incalculable. Thus, instead of comparing period and phase shifts, animals of either genotype without calculable phase prior to injection were excluded, and the number of remaining mutant and control animals with calculable or incalculable phase post-injection were compared using a non-parametric 2x2 Fisher’s exact test in GraphPad.

Generally, investigators performing circadian cannulation studies sample a range of circadian times and neuropeptide dosages during the course of the study. In our case, this was infeasible. Only ~30% of mutants had robust enough rhythms to be cannulated in the first place, and because the mice had to regularly be re-entrained and re-released into DD over the course of the study, each injection took well over a month to perform. Furthermore, the rhythms of even Lhx1-deficient mice with relatively mild behavioral phenotypes degraded with age, making it progressively more difficult to reliably determine the mutants’ CTs as they aged. Due to these constraints, we chose to inject
Prok2 at CT14 using a 114 uM injected peptide concentration, based upon the injection time and dosage shown to induce masking in a previous Prok2 icv cannulation study in wild-type rats (Cheng et al, 2002). We chose to inject Grp at CT14 using a 114 uM injected peptide concentration based upon the injection time at which maximum phase shift occurs following Grp cannulation directly into hamster SCN (Piggins et al, 1995) and our calculation that this dosage would result in a CSF concentration of exogenous Grp well within the 100 nM to 10 uM concentration range of Grp shown to maximally shift the electrophysiological rhythms of rat SCN in culture (McArthur et al, 2000).

**Supplemental References:**
Cheng, M.Y., Bullock, C.M., Li, C., Lee, A.G., Bermak, J.C., Belluzzi, J., Weaver, D.R., Furuta, Y., Lagutin, O., Hogan, B.L., and Oliver, G.C. (2000). Retina- and ventral forebrain-specific Cre recombinase activity in transgenic mice. Genesis 26, 130-132.

Kwan, K.M., and Behringer, R.R. (2002). Conditional inactivation of Lim1 function. Genesis 32, 118-120.

Leslie, F.M., and Zhou, Q.Y. (2002). Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. Nature 417, 405-410.

McArthur, A.J., Coogan, A.N., Ajpru, S., Sugden, D., Biello, S.M., and Piggins, H.D. (2000). Gastrin-releasing peptide phase-shifts suprachiasmatic nuclei neuronal rhythms in vitro. J. Neurosci. 20, 5496-5502.

Piggins, H.D., Antle, M.C., and Rusak, B. (1995). Neuropeptides phase shift the mammalian circadian pacemaker. J. Neurosci. 15, 5612-5622.

**Supplemental Figure and Table Legends:**

**Figure S1:** All Circadian Phenotypes of Six3-Cre;Lhx1<sup>lox/lox</sup> Mice Are Due to Selective Lhx1 Deletion in Anterior Hypothalamus, Related to Figure 1.

(A) α-YFP IHC (green) and DAPI stain (blue) in Six3-Cre;ROSA26::YFP brains showed robust Cre activity in SCN, vSPZ, and POA, as well as more modest activity throughout anterior hypothalamus, but little to none in more posterior regions. Coronal planes are arranged from anterior (left) to posterior (right). Scale bar = 500um. (B) Sagital schematic view of Six3-Cre activity and Lhx1 expression at E12.5. (C-H) Coronal high-magnification ISH of Lhx1 expression in Lhx1<sup>lox/lox</sup> control (C-H) and Six3-Cre;Lhx1<sup>lox/lox</sup> mutant (C’-H’) mice in P0 SCN (C,C’), and adult SCN (D,D’), vSPZ (E,E’), POA (F,F’), IGL (G,G’), and cerebellum (H,H’). Scale bar = 100um. (I) Coronal low-magnification ISH of Lhx1 expression in Lhx1<sup>lox/lox</sup> control (top) and Six3-Cre;Lhx1<sup>lox/lox</sup> mutant (bottom) mouse brain at the level of POA (left), SCN (middle), and SPZ (right). (J-M) ISH of SCN from adult Six3-Cre;Lhx1<sup>+/+</sup> mice for Vip (J), Grp (K), Avp (L), and Lhx1 (M), showing no evidence for developmental defects in Six3-Cre;Lhx1<sup>+/+</sup> mice. Scale
Figure S2: SCN regional differentiation occurs normally in Six3-Cre;Lhx1^{lox/lox} mice, and loss of neuropeptidergic markers is not due to selective apoptosis in neonatal SCN, Related to Figure 2.

(A-D) ISH of SCN from Lhx1^{lox/lox} control (A-L) and Six3-Cre;Lhx1^{lox/lox} mutant (A’-L’) mice for Id4 (A,A’), Arx (B,B’), Dlx2 (C,C’), Nr1d1 (D,D’), Rorb (E,E’), Sst (F,F’), Agt (G,G’), Nts (H,H’), Cck (I,I’), Gal (J,J’), Scg2 (K,K’), and Pcsk1n (L,L’). Adult SCN was used for all ISHs except A-C, which were P0. (M) Pan-neuronal α-HuC/D IHC (red) and DAPI stain (blue) of SCN from (left to right) P0, P4, P7, and adult Lhx1^{lox/lox} control (M) and Six3-Cre;Lhx1^{lox/lox} mutant (M’) mice. Scale bar = 100um. (N) TUNEL stain (red) and DAPI stain (blue) of SCN from (left to right) P0, P1, P2, and P3 Lhx1^{lox/lox} control (N) and Six3-Cre;Lhx1^{lox/lox} mutant (N’) mice. (O-R) ISH of SCN from P0 Lhx1^{lox/lox} control (O-R) and Six3-Cre;Lhx1^{lox/lox} mutant (O’-R’) mice for Vip (O,O’), Prok2 (P,P’), Grp (Q,Q’), and Avp (R,R’).

Figure S3: Clock Gene ISH Time-Course, Related to Figure 3.

(A-C) ISH of SCN from adult Lhx1^{lox/lox} control (A-C) and Six3-Cre;Lhx1^{lox/lox} mutant (A’-C’) mice for Per1 (A,A’), Per2 (B,B’), and Avp (C,C’) at six circadian time points ranging from CT4-CT24. Scale bar = 100um.

Figure S4: ipRGC Input and Behavioral Masking Are Intact in Six3-Cre;Lhx1^{lox/lox} Mice, Related to Figure 4.

(A) ipRGCs projections (shown in blue) to the SCN are intact in Lhx1^{lox/lox};Opn4^{tau-LacZ} and Six3-Cre;Lhx1^{lox/lox};Opn4^{tau-LacZ} mice. (B) ipRGC projections to IGL and OPN are intact in Lhx1^{lox/lox};Opn4^{tau-LacZ} and Six3-Cre;Lhx1^{lox/lox};Opn4^{tau-LacZ} mice. (C) Pupillary light reflex is intact in Six3-Cre;Lhx1^{lox/lox} mice and comparable to controls (n=10-11 per group, p<0.0001). Data represent Mean ± SEM. ***p<0.001. (D-E) Cholera toxin labeling shows intact retinal projections (shown in brown). (F) Six3-Cre; Lhx1^{lox/lox} mice show a small defect in visual acuity in the optomotor task (n=8,10 per group, p=0.03). Data represent Mean ± SEM. *p<0.05. (G) No difference was observed in masking in response to a 3-hour masking pulse (Lhx1^{lox/lox}: 0.08 ± 0.04, n=5; Six3-Cre;Lhx1^{lox/lox}: 0.39 ± 0.28, n=5) or (H) T7 light cycle (Lhx1^{lox/lox}: 0.76 ± 0.03, n=16; Six3-Cre;Lhx1^{lox/lox}: 0.71 ± 0.04, n=13). Mice in T7 confined their activity to the dark phase (One sample t-test μ=50%: p=0.0037, 0.0361). Horizontal line (G) and Bars (H) represent mean. Error bars represent SEM.

Figure S5: Correlation of Behavior and Histology in Cannulated Mice, Related to Figure 5.

(A-B) Actograms of wheel running activity of Lhx1^{lox/lox} (letter) and Six3-Cre;Lhx1^{lox/lox} (letter’) mice before and after each individual icv injection (red asterisk) of Grp (A) Prok2 (B) in DD. (C) SCN ISH for Lhx1, Vip, Grp, and Avp in each Lhx1^{lox/lox} and Six3-Cre;Lhx1^{lox/lox} mouse whose brain we were able to collect. (D-E) Correlation of cell counts of individual ISH for Vip (left), Grp (middle), and Avp (right) in Six3-Cre;Lhx1^{lox/lox} SCN with phase shift (D) and period shift (E) of the corresponding mouse.
after i.c.v. Prok2 injection (n=6, linear regression, scatter plots depict neuropeptide
counts vs. behavior of individual cannulated mutants after each of two Prok2 injections).

**Table S1: Neuropeptide-expressing Cell Counts, Related to Figure 1.**
Mean +/- SEM labeled cells from neuropeptide ISH and Hu IHC of adult Lhx1\textsuperscript{lox/lox}
control and Six3-Cre;Lhx1\textsuperscript{lox/lox} mutant SCN hemispheres. Absolute numbers and
percentages of cells lost in mutants relative to controls are included.

**Table S2: Putative Lhx1-Binding Sites in SCN-Enriched Pool, Related to Figure 1.**
All genes selected for the SCN-enriched pool are listed. Genes with putative Lhx1
binding sites are highlighted in red. Down-regulated neuropeptides with putative Lhx1
binding sites are highlighted in blue.
Figure S2
Figure S3
|          | Lhx1\textsuperscript{lox/lox} | Six3-Cre;Lhx1\textsuperscript{lox/lox} | # Cells Lost | % Cells Lost |
|----------|-------------------------------|----------------------------------------|--------------|--------------|
| Vip      | 875 +/- 100                   | 12 +/- 7                               | 863          | 98.60        |
| Prok2    | 927 +/- 104                   | 25 +/- 20                              | 902          | 97.30        |
| Grp      | 467 +/- 39                    | 38 +/- 24                              | 429          | 91.86        |
| Avp      | 3142 +/- 265                  | 552 +/- 276                            | 2590         | 82.43        |
| Enk      | 497 +/- 70                    | 87 +/- 27                              | 410          | 82.49        |
| Nms      | 630 +/- 88                    | 30 +/- 23                              | 600          | 95.24        |
| Hu/DAPI  | 7038 +/- 96                   | 4945 +/- 285                           | 2093         | 29.74        |

Table S1
| Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol |
|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| 181009M01Rik | Cacna1g     | Ephb1      | Lhx1        | Pcdh11x     | Rasa4       | Setd6       | Trip10      | 2310079N02Rik | Calcr       | Fhl1        | Lhx8        | Pde5a       | Rasd1       | Slc2a13     | Trp53i11    | Adck4       | Ccdc109b    | Flrt3       | Magel2      | Pde7b       | Rasl11b     | Slc39a6     | Vgf         | Adcy3       | Cdh13       | Gpr56       | Myo10       | Per1        | Rftn1       | Slc6a11     | Vip         | Avp         | Creb3l1     | Grp         | Myt1        | Per2        | Rgs16       | Snx25       | Vipr2       | Avpr1a      | Ctf1        | Grpr        | Nms         | Per3        | Rgs4        | Spon1       | Ysk4        | B3gat2      | D130067I03Rik | Id4        | Nnat        | Pik3r3      | Rora        | Stk32a      | Zfp462       | B630019K06Rik | Dlk1       | Igfbp5      | Nov         | Plc14       | Rorb        | Syt10       | Zic1        | BC022623    | Drd1a       | Iqsec3      | Nr1d1       | Plekhg5     | Rps6ka2     | Tle4        | Zim1        | Bmal1       | Dusp4       | Itgb8       | Nr1d2       | Prok2       | Sat1        | Tnfrsf191   | Btg1        | Ednrb       | Kcnk13      | Nxnx        | Prokr2      | Sema6d      | Tnrc4       |            |