NEUROBIOLOGY

Haploinsufficiency of hnRNP U Changes Activity Pattern and Metabolic Rhythms

Beibei Lai,^{*} Jianghuan Zou,^{*} Zhaoyu Lin,^{*} Zhipeng Qu,^{*} Anying Song,^{*} Ying Xu,[†] and Xiang Gao^{*}

From the State Key Laboratory of Pharmaceutical Biotechnology and MOE Key Laboratory of Model Animal for Disease Study,^{*} Model Animal Research Center, Collaborative Innovation Center of Genetics and Development, Nanjing University, Nanjing; and the Medical College of Soochou University,[†] Suzhou, China

Accepted for publication
September 21, 2017.

Address correspondence to
Xiang Gao, Ph.D., Model Animal Research Center, Nanjing University, 12 Xuefu Road, Pukou Area, 210061, Nanjing, China; or Ying Xu, Ph.D., Medical College of Soochou University, 199 Renai Road, 215123, Suzhou, China.
E-mail: gaoxiang@nju.edu.cn or xuying@nicemice.cn.

The neuropeptides arginine vasopressin (Avp) and vasoactive intestinal polypeptide (Vip) are critical for the communication and coupling of suprachiasmatic nucleus neurons, which organize daily rhythms of physiology and behavior in mammals. However, how these peptides are regulated remains uncharacterized. We found that heterogeneous nuclear ribonucleoprotein U (hnRNP U) is essential for the expression of Avp and Vip. Loss of one copy of the *Hnnpu* gene resulted in fragmented locomotor activities and disrupted metabolic rhythms. *Hnnpu*^{+/-} mice were more active than wild-type mice in the daytime but more inactive at night. These phenotypes were partially rescued by microinfusion of Avp and Vip into free-moving animals. In addition, hnRNP U modulated Avp and Vip via directly binding to their promoters together with brain and muscle Arnt-like protein-1/circadian locomotor output cycles kaput heterodimers. Our work identifies hnRNP U as a novel regulator of the circadian pacemaker and provides new insights into the mechanism of rhythm output. (*Am J Pathol* 2018, 188: 173–183; <https://doi.org/10.1016/j.ajpath.2017.09.017>)

Haploinsufficiency is often associated with severe neurologic abnormalities, such as sleep disorders and autism disease.^{1–4} For instance, patients with sleep disturbances have one copy of a chromosomal microdeletion in 1q44, where the *Hnnpu* gene is located.⁵ Heterogeneous nuclear ribonucleoprotein U (HnRNP U) is expressed in a circadian manner in the suprachiasmatic nucleus (SCN).⁶ HnRNP U regulates oscillatory transcription of brain and muscle Arnt-like protein-1 (Bmal1) in NIH 3T3 cells.⁷ However, how hnRNP U regulates circadian rhythms in mammals remains unclear.

A robust and stable circadian oscillator is critical for physiologic and behavioral processes, such as locomotor activity, sleep-wake cycle, energy expenditure, and endocrine secretion.^{8–11} The circadian rhythm is controlled by a cell-autonomous molecular oscillator, which is a sophisticated transcriptional and posttranscriptional feedback loop.¹² In the positive limb of the loop, Bmal1/circadian locomotor output cycles kaput (Clock) heterodimers translocate into the nucleus and transcribe clock genes.^{13,14} In the negative limb, heterodimers composed of periods (Per1, Per2, and Per3) and cryptochromes (Cry1 and Cry2) inhibit

their own transcription by blocking Bmal1/Clock activity.^{15,16} These cellular and molecular processes last approximately 24 hours. Microarray assays demonstrate that approximately 10% of the transcriptome is under the control of circadian rhythms.^{17–19} Bmal1/Clock heterodimers transcribe many clock-controlled genes by binding to E-boxes in their promoters.²⁰

The oscillator in the SCN functions as a critical pacemaker that synchronizes peripheral tissues and cells through the nervous system and hormone pathways.^{21–23} SCN is a heterogeneous structure that consists of multiple types of neurons,²⁴ which secrete >100 identified neurotransmitters, neuropeptides, cytokines, and growth factors.^{25,26} Their coherences are subsequently responsible for proper timing of activity, feeding behavior, and metabolic processes.^{27–29}

Supported by National Natural Science Foundation of China grant 31301217 and Ministry of Science and Technology of the People's Republic of China grants 2014BAI02B01 and 2015BAI08B02 (X.G.).

B.L. and J.Z. contributed equally to this work.

Disclosures: None declared.

Two SCN-enriched neuropeptides are critical for synchronization: arginine vasopressin (Avp) and vasoactive intestinal polypeptide (Vip).^{30–32} Microinjection of Vip into SCN of freely moving animals at circadian time (CT) 12 to CT14 and CT22 to CT24 can induce phase delay and advance, respectively. This process is regarded as part of the mechanism of light entrainment.^{32–34} Deficiencies in the Avp primary receptor V1a results in arrhythmic locomotor activity, and deficiencies in Vip cause arrhythmic SCN neuron-firing rates.^{35–37} Similar to other clock-controlled genes, *Avp* and *Vip* are transcribed by Bmal1/Clock heterodimers through E-boxes, so they are stably expressed and secreted in a circadian manner.^{38,39} However, it is still unclear how *Avp* and *Vip* are regulated.

HnRNP U was first described as an RNA-binding protein that participates in pre-mRNA processing and alternative splicing.^{40,41} HnRNP U is also known as scaffold attachment factor A, a matrix protein required for higher-order DNA structure. By looping specific regions of DNA, hnRNP U mediates long-range regulation of gene expression.⁴² Recently, hnRNP U was found to form complexes with c-Myc-Max and regulate the clock-controlled gene *Odc* by binding to its E-box.⁴³ It is interesting to examine whether hnRNP U regulates *Avp* and *Vip* via a similar mechanism.

We found that mice with a heterozygous loss-of-function mutation in *Hnrnpu* (*Hnrnpu*^{+/-}) have significantly attenuated locomotor activity during the dark phase and increased activity during daytime. Correspondingly, *Hnrnpu*^{+/-} mice reduced food intake during nighttime, which is a typical food intake period for nocturnal rodents. Moreover, hnRNP U regulates *Avp* and *Vip* gene expression together with Bmal1/Clock at their promoters. These studies suggested that hnRNP U haploinsufficiency changes activity and metabolic rhythms through the SCN-enriched neuropeptides Avp and Vip.

Materials and Methods

Animals

Animals were housed in a specific pathogen-free facility accredited by Association for Assessment and Accreditation of Laboratory Animal Care International. Animal protocols and care were approved by the Institutional Animal Care and Use Committee of Model Animal Research Center of Nanjing University. Mice were housed and bred under 12 hours of light and 12 hours of dark cycles at 25°C ± 1°C, with water and food available *ad libitum*. At 1 or 2 weeks before experiments, mice were transferred into cages in light-tight chambers equipped with independent daylight fluorescent lamps. *Hnrnpu* knockout mice (frame shifted by deleting exon 3 to exon 6) were generated at the Model Animal Research Center. Then, *Hnrnpu*^{+/-} mice were continuously backcrossed to C57BL/6J strain for at least 10 generations. Genotyping primers are presented in Table 1.

Table 1 Genotyping Primers

Primer name	Sequence
Wild-type allele forward	5'-GAGGCATTACATAGTTGCTGAG-3'
Wild-type allele reverse	5'-GCCTAACATGGAACCTTGGACA-3'
Mutant allele forward	5'-GAGGCATTACATAGTTGCTGAG-3'
Mutant allele reverse	5'-GGGTTCAAGCTGGCTTAGTGAC-3'

The PCR products were 330 and 394 bp for the wild-type and mutant alleles, respectively.

Wheel-Running Activity Assay

A locomotor activity assay was performed with 3- to 4-month-old males and females. Adult mice of both genotypes were individually maintained in wheel running cages placed in light-tight cabinets with independent illumination. Mice had free access to food and water. Daily locomotor activity was measured by connecting the wheel and detector to a computer running ClockLab software version 7.0 (Actimetrics, Evanston, IL). Activity in 6-minute bins was recorded after approximately 1 week of habituation and entrainment. The initial photoschedule was white light between 8:00 and 20:00 and darkness between 20:00 and 8:00 (LD). Approximately 1 week later, mice were transferred to constant darkness (DD) conditions. Locomotor activity periods and amplitudes were assessed by counting wheel revolutions. The time of onset, the distribution of activity, and the χ^2 periodograms were analyzed using Clocklab software.

Open-Field Test

The open-field test was performed in 3- to 4-month-old male mice according to standard protocols at zeitgeber times (ZTs) 3 to 4 (11:00 to 12:00) and 15 to 16 (23:00 to 24:00). The open field consisted of a square with a length of 45 cm and a height of 35 cm (AES-LOC1; Anilab Software & Instruments Co. Ltd., Ningbo, China). Mice were placed in the field and then allowed to freely explore the field for 5 minutes. The total distance moved, the amount of time spent moving, and the mean speed were measured using video captured on infrared light video.

Metabolic Rhythms Measurement

Metabolic rhythms were continuously assessed and analyzed using the Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH). After 2 days of acclimation in the instrument room, mice were placed in individual chambers and had free access to water and food. Another group of mice were intraperitoneally implanted with capsule sensors to detect real-time body core temperature (RPC-1; DSI, St. Paul, MN). Mice were detected for 24 hours in 10-second time bins. The data were

then smoothed by averaging and transformed to ZT and double-plotted.

Real-Time Quantitative PCR

After entrainment, mice were sacrificed to collect tissues in 4-hour increments on the first day of DD. Livers and anterior ventral hypothalamus were collected, snap frozen in liquid nitrogen, and stored at -80°C . Total RNA was prepared using TRIzol Reagent (catalog number 10296028, Invitrogen, Carlsbad, CA) and reverse transcribed using a PrimeScript TR reagent kit (catalog number RR047A, TaKaRa, Dalian, China) according to the manufacturer's instructions. Real-time quantitative PCR (qPCR) was performed using the StepOnePlus Real-time PCR system (Applied Biosystems, Carlsbad, CA) with SYBR Premix Ex Taq reagents (catalog number RR820A, TaKaRa). Data were analyzed by StepOnePlus software version 2.1 (Applied Biosystems) using comparative Ct methods, and gene expression levels were calculated by normalizing to β -actin. Primers for detecting specific isoforms were designed by identification of the predicted alternative exon, and primers for detecting total RNA levels were designed via identification of the shared exon. The specific primers are provided in Table 2.

Immunostaining

Animals were anesthetized and perfused with 4% paraformaldehyde (PFA) at indicated time points. Brains were dissected carefully and fixed with 4% PFA for 4 hours at 4°C . After dehydrating in 30% sucrose, brains were embedded in optimal cutting temperature compound freeze medium at -80°C . Frozen brains were sliced into 10- μm -thick sections using a Leica CM1950 Cryostat (Leica Biosystems, Nussloch, German) and subjected to immunofluorescence staining. Briefly, the slides were blocked in 5% bovine serum albumin, incubated at 4°C in 1:200 hnRNP U antibody (ab180952; Abcam, Shanghai, China), washed with phosphate-buffered saline (PBS), and incubated in 1:200 goat anti-rabbit fluorescein isothiocyanate secondary antibody (F1262; Sigma, Shanghai, China). Nuclei were counterstained with 0.1% Hoechst 33,342 (Cell Signaling Technology, Shanghai, China). The images were obtained using a confocal microscope (Leica DM6000; Leica Biosystems) and analyzed using ImageJ software version 1.46 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>).

In Situ Hybridization

Animals were anesthetized and perfused with 4% PFA at indicated time points. Coronal serial sections (100- μm thick) through the SCN were cut on a Leica VT1000 S Vibrating blade microtome (Leica Biosystems). Given that the SCN region was not visible, 10 to 15 adjacent slides per mouse were prepared to ensure that sections that included

Table 2 Real-Time Quantitative PCR Primers

Primer name	Primer sequence
Neuropeptides	
<i>Avp</i> -F1	5'-ACTACGCTCTCCGCTTGTT-3'
<i>Avp</i> -R1	5'-GCAGATGCTTGGTCCGAAG-3'
<i>Vip</i> -F1	5'-CTTGGACAGCAGAGCACTAGC-3'
<i>Vip</i> -R1	5'-GAAGAGTATCAGGAATGCCAGGAA-3'
Central clock genes	
<i>Clock</i> QF	5'-GGTTTGATCACAGCCCAACT-3'
<i>Clock</i> QR	5'-CCTCCGCTGTGTCTATCTTTT-3'
<i>Cry1</i> QF	5'-TCCCCTCCCCTTTCTCTTTA-3'
<i>Cry1</i> QR	5'-TGAGTCATGATGGCGTCAAT-3'
<i>Cry2</i> QF	5'-AAGCTGAATTCGCGTCTGTT-3'
<i>Cry2</i> QR	5'-GTGGTTTCTGCCCATTCAGT-3'
<i>Bmal1</i> QF	5'-TGGAGGACTCCAGACATTC-3'
<i>Bmal1</i> QR	5'-TGGGACTACTGGATCCTTGG-3'
<i>Per2</i> QF	5'-CGCTTCATAGCCCGAGTGTA-3'
<i>Per2</i> QR	5'-TTCTGCCGTGTCACTGTTGG-3'
<i>Rev-erbα</i> QF	5'-GCTGCCATTGGAGTTGTCAC-3'
<i>Rev-erbα</i> QR	5'-GACATGACGACCTGGACTC-3'
<i>Rev-erbβ</i> QF	5'-GAAGAGTGACCGACAGATTG-3'
<i>Rev-erbβ</i> QR	5'-TCCGAAAGAAACCCTTACAGC-3'
<i>Rora</i> QF	5'-CCCTACTGTTCTTCACCA-3'
<i>Rora</i> QR	5'-AGCTGCCACATCACCTCTCT-3'
<i>Dbp</i> QF	5'-AAGAAGGCAAGGAAAGTCCAG-3'
<i>Dbp</i> QR	5'-ACCTCTTGGCTGCTTCATTG-3'
<i>Actβ</i> QF	5'-GTCCACCTTCCAGCAGATGT-3'
<i>Actβ</i> QR	5'-GAAAGGGTGTAACCGCAGC-3'
Alternative splicing	
<i>Clock</i> -EQF	5'-AGCATGGTCCAGATTCCATC-3'
<i>Clock</i> -EQR	5'-CAGGAGCAGTCACTAATTTGGTC-3'
<i>Clock</i> -GQF	5'-CTTCTGGTAACGCGAGAAAG-3'
<i>Clock</i> -GQR	5'-TCGAATCTCACTAGCATCTGACT-3'
ChIP	
<i>Avp</i> -CHIPM-F	5'-GGTGCTTCCTCTCATCTTATAC-3'
<i>Avp</i> -CHIPM-R	5'-GATTGGTGCTGTGCGATCA-3'
<i>Vip</i> -CHIPM-F	5'-CATCTTCTAGTTTCATCACAGCTAAG-3'
<i>Vip</i> -CHIPM-R	5'-TTATAGGGCTTGTCCTCGG-3'
<i>GapdhM</i> -F	5'-CTTGTGGCAAGAGGCTAGGG-3'
<i>GapdhM</i> -R	5'-CAGGGACGTGCTGACTGGC-3'
<i>Avp</i> -CHIPR-F	5'-GATGCTTCCTCTTCTCTACAC-3'
<i>Avp</i> -CHIPR-R	5'-GATTGGTGCTGTGCAATCA-3'
<i>Vip</i> -CHIPR-F	5'-CGTCTTCCAGTTTCATCACAACTAAG-3'
<i>Vip</i> -CHIPR-R	5'-TTATAGGGCTTGTCCTTAGG-3'
<i>GapdhR</i> -F	5'-GGCAGCCAAGGAAAGAGAGTC-3'
<i>GapdhR</i> -R	5'-TGAGTTGTACCGCCAGGAT-3'

ChIP, chromatin immunoprecipitation; CHIPM, CHIP-mouse; CHIPR, CHIP-rat; E, exon; F, forward; G, global; *GapdhM*, *Gapdh*-mouse; *GapdhR*, *Gapdh*-rat; Q, quantitative; R, reverse.

the region were obtained. Digoxin-labeled *Avp* probe and *Vip* probe were synthesized with Promega reagents according to the manufacturer's instructions. Briefly, sections were subject to bleaching (6% H_2O_2 , 40 minutes, room temperature), cells permeabilization (10 $\mu\text{g}/\text{mL}$ of protease K, 10 minutes, room temperature), protease K inactivation (2 mg/mL of glycine, 5 minutes, room temperature), fixation (0.2% glutaraldehyde and 4% PFA, 20 minutes, room

temperature), prehybridization (1 hour, 70°C), hybridization (specific probes, 12 hours, 70°C), RNase A treatment (10 µg/mL of RNase A, 2 hours, 37°C), blocking (1% heat inactivated sheep serum, 30 minutes, 4°C), hybridization (1:2000 alkaline phosphatase–linked DIG antibody, 11093274910; Roche, Shanghai, China, 12 hours, 4°C), and staining (nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolylphosphate, room temperature, in the dark). After staining in the dark, SCN regions were visible. Then, slices were selected, mounted, and photographed.

Stereotaxic Microinjection

Mice were entrained to LD cycles and then placed in DD conditions to detect circadian phases and periods. Unilateral cannulas (outer diameter = 0.21 mm, inner diameter = 0.11 mm) for infusion of peptides were implanted into the brain of anesthetized mice. Stereotaxic techniques were used to position the cannulas (bregma: anteroposterior, −0.46 mm; mediolateral, 0.12 mm; dorsoventral, −5.40 mm). The microsyringe was controlled at a speed of 250 nL/minute by a micromanipulator (RWD Life Science Co. Ltd, Shenzhen, China). Activity counts 1 week before and after injection were analyzed. Vip was purchased from Sigma-Aldrich (St. Louis, MO). Avp and a scrambled polypeptide were synthesized from GenScript (Nanjing, China).

Cell Culture

Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (26104-079; Gibco, Carlsbad, CA) and 60 µg/mL of penicillin and 50 µg/mL of streptomycin at 37°C in the presence of 5% carbon dioxide. Transient transfection of plasmid DNA using Lipofectamine 2000 (Invitrogen) was performed according to the manufacturer's instructions. The siRNAs were synthesized at Genepharma (Shanghai, China) and are presented in Table 3. Avp (−600 to +50 bp) and Vip (−550 to +30 bp) promoters were inserted into PGL3-Basic plasmid (pGL3-Avp and pGL3-Vip). pGL3 and siRNA and pRL-TK vector were cotransfected into HEK 293T cells. Luciferase activity was detected using a dual-reporter assay system

Table 3 siRNA Sequence

siRNA	Sequence
hsiU1 (for HEK 293T cells)	5'-GAUGAACACUUCGAUGACAdTdT-3'
hsiU2 (for HEK 293T cells)	5'-AGACCACGAGAAGAUAUdTdT-3'
rsiU1 (for SCN2.2 cell)	5'-GACGAACACUUCGAUGACAdTdT-3'
rsiU2 (for SCN2.2 cell)	5'-AGACCCCGGAAGAUAUdTdT-3'
siSCR (as negative control)	5'-UUCUCCGAACGUGUCACGUAdTdT-3'

hsiU, human hnRNP U–specific siRNAs; rsiU, rat hnRNP U–specific siRNAs; siSCR, scrambled siRNA.

following the manufacturer's manual (E1910; Promega, Beijing, China).

Chromatin Immunoprecipitation

HnRNP U (ab20666), Clock (ab3517), and Bmal1 (NB100-2288) antibodies were purchased from Abcam and NOVUS Biologicals (Littleton, CO). Protein G sepharose beads (GE Healthcare, Chicago, IL) were prepared by washing with protease inhibitor cocktail (Roche) and phenylmethylsulfonyl fluoride added PBS (PBS++). Then, the beads were incubated with 1-mg antibodies at 4°C overnight.

SCN2.2 cells and anterior ventral hypothalamus were crosslinked with 1% formaldehyde immediately. Then, 1.375 mol/L glycine was used to stop the linkage reaction, and the cells were washed in PBS++ twice. Cells were scraped and treated with ice-cold cell lysis buffer to collect nuclei. Then, the nuclei were treated with nuclear lysis buffer. Sonication was performed at a high-level grade (Bioruptor UCD-200; Diagenode, Liege, Belgium) for 15 minutes (5 minutes each three times). Samples were gently incubated with beads at 4°C overnight and then washed with high-salt washing buffer, low-salt washing buffer, LiCl washing buffer, and immunoprecipitation buffer twice for each. DNA was extracted after decrosslinking at 65°C and then subjected to qPCR using the primers listed in Table 2. Relative binding efficiency for a specific protein was normalized to normal IgG (nIgG).

Statistical Analysis

Data are presented as means ± SD. Analyses were performed using GraphPad Prism software version 5 (GraphPad Software, La Jolla, CA). Statistical significance between two groups was determined using an unpaired two-tailed *t*-test. Multigroup analysis was conducted using two-way analysis of variance. If significant differences were detected, the Bonferroni post hoc test was applied. *P* < 0.05 was considered statistically significant.

Results

Attenuated Locomotor Activity in *Hnnpu*^{+/-} Mice

In a systematic phenotyping project, we monitored the wheel-running activity of *Hnnpu*^{+/-} mice on the C57BL/6J background because *Hnnpu*^{-/-} mice were embryonically lethal.⁴⁴ Mice were placed in LD conditions (light from 8:00 to 20:00) for at least 1 week and then released into DD conditions according to standard protocols as reported in our previous studies.^{45,46} The representative double-plotted wheel-running actograms demonstrates that *Hnnpu*^{+/-} mice had robust rest-activity rhythms, whereas 8 of 11 *Hnnpu*^{+/-} mice exhibited remarkably decreased activity during nighttime with increased activity during daytime. Moreover, 3/11 *Hnnpu*^{+/-} mice were completely

Figure 1 The locomotor activity patterns change in *Hnmpu*^{+/-} mice. **A:** Representative double-plotted actograms of wheel-running show disorganized activity rhythms in *Hnmpu*^{+/-} mice. *Hnmpu*^{+/+} mice become active immediately after dark, but *Hnmpu*^{+/-} mice do not exhibit a discernible onset of activity. The onset line (the orange line) is ambiguous in eight *Hnmpu*^{+/-} mice (middle) and undetectable in the remaining three mice (right). **B:** Periods of locomotor activity in constant darkness (DD) are normal in *Hnmpu*^{+/+} and *Hnmpu*^{+/-} mice. **C** and **D:** Distribution of locomotor activity shows that *Hnmpu*^{+/-} mice are relatively more active in the daytime but more inactive at night compared with wild-type mice. **E** and **F:** Batch analysis in white light between 8:00 and 20:00 and darkness between 20:00 and 8:00 (LD) and DD conditions were performed using χ^2 periodograms. The circadian rhythm amplitudes of *Hnmpu*^{+/-} mice are reduced. The diagonal lines indicate the 99% CI. Data are presented as means $\pm$ SD (**B–F**). $n = 7$ *Hnmpu*^{+/+} mice; $n = 11$ *Hnmpu*^{+/-} mice. ** $P < 0.01$, *** $P < 0.001$, unpaired two-tailed t -test. D, day; N, night; SD, subjective day; SN, subjective night.

arrhythmic in DD conditions (Figure 1A). Then, circadian period was estimated by χ^2 periodograms. The endogenous dominant period lengths were not altered in *Hnmpu*^{+/-} mice compared with *Hnmpu*^{+/+} mice during 2 weeks of DD conditions (Figure 1B).

Quantification of wheel-running activity revealed that *Hnmpu*^{+/-} mice had considerably reduced locomotor activity compared with *Hnmpu*^{+/+} mice at night. In contrast,

the relative activity of *Hnmpu*^{+/-} mice in the daytime was considerably increased compared with wild-type controls. Therefore, *Hnmpu*^{+/-} mice tended to move in the daytime in LD or subjective day in DD (Figure 1, C and D).

Rhythmic power in LD and DD conditions was measured by batch analysis using χ^2 periodograms. The peaks in the corresponding χ^2 periodograms indicated the dominant periods during days 1 to 7 (LD stage) and days 8 to 24 (DD

Figure 2 *Hnmpu*^{+/-} mice are more active in the daytime compared with wild-type mice. **A–C:** *Hnmpu*^{+/-} mice explore more and move faster in the daytime compared with wild-type mice. **D–F:** *Hnmpu*^{+/-} mice explore less and move slower at night compared with wild-type mice. $n = 6$ *Hnmpu*^{+/+} mice; $n = 11$ *Hnmpu*^{+/-} mice. Data are expressed as means $\pm$ SD. * $P < 0.05$, unpaired two-tailed t -test.

Figure 3 Metabolic rhythm changes in *Hnrnpu*^{+/-} mice. **A, B, D, and E:** Consecutive data were recorded and analyzed. Oxygen consumption and carbon dioxide production decrease significantly at night in *Hnrnpu*^{+/-} mice. **C, F, G, and J:** Respiratory exchange ratio (RER) and food intake values decrease in *Hnrnpu*^{+/-} mice at night. **H and K:** Thermogenesis decreases in *Hnrnpu*^{+/-} mice at night. **I and L:** Core body temperature of *Hnrnpu*^{+/-} mice is increased compared with wild-type controls in the daytime but reduces at night. Data are expressed as means $\pm$ SD. $n = 7$ *Hnrnpu*^{+/+} mice; $n = 8$ *Hnrnpu*^{+/-} mice. Dashes over the curves indicate time points with significant differences. * $P < 0.05$, ** $P < 0.01$, unpaired two-tailed t -test.

stage). The decreased peaks demonstrated that *Hnrnpu*^{+/-} mice exhibited significantly attenuated circadian rhythms and considerably reduced amplitudes compared with wild-type mice (Figure 1, E and F).

To confirm this phenotype of abnormal activity, mice were subjected to an open-field test at ZT3 to ZT4 and ZT15 to ZT16, separately. *Hnrnpu*^{+/-} mice were more active than *Hnrnpu*^{+/+} mice during daytime. The total moving distance, total moving duration, and mean speed were increased in *Hnrnpu*^{+/-} mice compared with wild-type mice (Figure 2, A–C). In contrast, *Hnrnpu*^{+/-} mice exhibited reduced moving distance, moving duration, and speed at ZT15 to ZT16, indicating that *Hnrnpu*^{+/-} mice were more inactive at night (Figure 2, D–F). In summary, the above data indicate that hnRNP U haploinsufficiency affects mouse activity pattern.

Aberrant Metabolic Rhythms in *Hnrnpu*^{+/-} Mice

Because the *Hnrnpu*^{+/-} mice displayed rest-activity abnormalities, it was assessed whether hnRNP U functioned specifically in animal behavior patterns or also physiologies or even the SCN pacemaker. To address this issue, we monitored mice using the Comprehensive Lab Animal Monitoring System. In LD conditions, *Hnrnpu*^{+/+} mice displayed overt circadian rhythms in oxygen consumption and carbon dioxide production. Consistent with the wheel-

running test, *Hnrnpu*^{+/-} mice displayed reduced oxygen and carbon dioxide peak values (Figure 3, A, B, D, and E). Similarly, the peaks of food intake and the respiratory exchange ratio were largely decreased in *Hnrnpu*^{+/-} mice at night (Figure 3, C, G, F, and J). The reduced activity, respiration, and ingestion at night resulted in a reduced peak of heat production (Figure 3, H and K). Furthermore, telemetric devices were implanted into the peritoneal cavity, and real-time core body temperature was measured. The body temperature of *Hnrnpu*^{+/-} mice was increased compared with that of wild-type controls in the daytime but was lower at night (Figure 3, I and L). Altogether these observations suggest that hnRNP U haploinsufficiency specifically affects not only animal activity-rest patterns but also physiologic rhythms.

Intact Central Molecular Clock in *Hnrnpu*^{+/-} Mice

The aberrant patterns of locomotor activity and metabolic rhythms prompted us to measure the integrity of the molecular oscillator and SCN output pathway. The mRNA profiles of core clock genes were examined in the SCN and liver as described previously.⁴⁵

Mice were entrained to LD cycles 1 week before the experiments. Tissues were collected at 4-hour intervals during the first day in DD for 24 hours. Relative gene levels were detected with qPCR and normalized to β -actin. No

Figure 4 Central clock genes are intact in the suprachiasmatic nucleus (SCN) of *Hnmpu*^{+/-} mice. **A–E:** Representative mRNA profiles of central clock genes were analyzed in the SCN. Tissues were collected at 4-hour intervals during the first day in constant darkness (DD). Relative gene levels were detected with real-time quantitative PCR and normalized to β -actin. No significant difference is detected in brain and muscle *Arnt*-like protein-1 (*Bmal1*), circadian locomotor output cycles kaput (*Clock*), period 2 (*Per2*), cryptochrome-1 (*Cry1*), or cryptochrome-2 (*Cry2*) between *Hnmpu*^{+/+} and *Hnmpu*^{+/-} mice. Data are expressed as means $\pm$ SD (**B–D, G, and H**). Two-way analysis of variance followed by Bonferroni post hoc test, $n = 21$. CT, circadian time.

significant difference was detected in *Bmal1*, *Clock*, *Per2*, *Cry1*, or *Cry2* between *Hnmpu*^{+/+} and *Hnmpu*^{+/-} SCN at any time points (**Figure 4**).

Similarly, no differences in *Bmal1*, *Clock*, *Per1*, *Per2*, *Cry1*, *Cry2*, *Dbp*, *Rev-erb α* , *Rev-erb β* , and *Ror α* mRNA levels were noted in the liver (**Supplemental Figure S1, A–J**). hnRNP U has been reported as a regulator of alternative splicing, so different splicing isoforms of the *Clock* gene in the liver were examined.^{41,47} A pair of specific qPCR primers were designed to evaluate the expression of some specific isoforms, and another pair were used to detect total mRNA levels. The relative ratio could be obtained by dividing the specific isoform by total levels. No difference was detected between *Hnmpu*^{+/+} and *Hnmpu*^{+/-} mice (**Supplemental Figure S1, K and L**). These data excluded the possibility that hnRNP U affects the expression profiles of core *Clock* genes to a great extent.

Reduced Levels of Avp and Vip in *Hnmpu*^{+/-} Mice

Then, we hypothesized that hnRNP U regulates the SCN pacemaker output pathway. The expression of hnRNP U was determined in the SCN via immunostaining and found that the hnRNP U level was decreased significantly in *Hnmpu*^{+/-} mice. The optical density was measured with ImageJ (**Figure 5, A and B**).

Avp and Vip are critical components of the circadian pacemaker. Mice defective in Avp and Vip signaling exhibit phenotypes similar to *Hnmpu*^{+/-} mice.^{34–37,48–50} Thus, the Avp and Vip mRNA levels were examined in the anterior ventral hypothalamus using qPCR. Both Avp and Vip were expressed in a circadian manner.^{35,49,51,52} Distinct decreases in Avp and Vip were noted especially at the peak levels (**Figure 5, C and D**). To verify the above results, we performed *in situ* hybridization with Avp and Vip probes. Avp and Vip levels in *Hnmpu*^{+/-} mice were prominently reduced compared with wild-type controls (**Figure 5, E and F**). The SCN structures in *Hnmpu*^{+/-} mice were grossly normal (**Figure 5, A, E, and F**), and SCN sizes were normal in both genotypes (**Figure 5G**).

To further examine whether hnRNP U directly regulated the transcription of these two neuropeptides, luciferase was used under the control of the Avp promoter and Vip promoter to evaluate the influence of hnRNP U as described in the **Materials and Methods**. Luciferase vectors were cotransfected with hnRNP U–specific siRNAs (sihnRNP U1 and sihnRNP U2) or scrambled siRNA (siSCR) into HEK 293T cells.^{40,41} The RNA interference (RNAi) efficiency was approximately 40% to 80% as detected by qPCR 24, 48, and 72 hours after transfection (**Supplemental Figure S2**). The transcriptional activity of both promoters was significantly inhibited by hnRNP U knockdown (**Figure 5H**), demonstrating that hnRNP U mediates Avp and Vip expression at the transcription level.

Abnormal Activity Is Partially Restored by Avp and Vip

To explore the causal link between these neuropeptides and abnormal activity in *Hnmpu*^{+/-} mice, suprachiasmatic microinjection was performed with synthetic Avp and Vip neuropeptides for a consecutive 7 days. Before injection, mice were entrained to LD cycles and then transferred to DD. After stable rhythms were observed, 0.5 μ L of Avp (150 nmol), Vip (150 nmol), or scrambled peptide (150 nmol) was infused into the SCN region at CT4 to CT6 every day.^{27,30,32,33} The activity distribution was assessed before and after injection. The daytime activity was significantly decreased in *Hnmpu*^{+/-} mice after Avp or Vip injection, indicating that the activity pattern was partially rescued by these two neuropeptides (**Figure 6**). These data suggest that Avp and Vip are associated with hnRNP U–mediated circadian outputs.

HnRNP U Bound to the Avp and Vip Promoters

Because hnRNP U RNAi inhibited Avp-promoter and Vip-promoter activity in the luciferase assay, we hypothesized that hnRNP U regulated Avp and Vip expression by binding to their promoters directly. Chromatin immunoprecipitation was performed using anti-hnRNP U antibody and nIgG. The binding efficiency was detected in mouse anterior ventral hypothalamus. The efficiency was reduced in *Hnmpu*^{+/-} mice compared with littermate controls (**Figure 7A**). To

Figure 5 Arginine vasopressin (Avp) and vasoactive intestinal polypeptide (Vip) transcription is suppressed in the *Hnmpu*^{+/-} suprachiasmatic nucleus (SCN). **A** and **B**: Immunofluorescence reveals that the expression levels are decreased in *Hnmpu*^{+/-} mice. **C** and **D**: Avp and Vip mRNA expression levels in the SCN are reduced in *Hnmpu*^{+/-} mice. **E** and **F**: *In situ* hybridization in the SCN exhibits less Avp and Vip in *Hnmpu*^{+/-} mice. **G**: Histogram revealing similar lengths from the rostral end to the caudal end of the SCN in both groups. **H**: The pGL3-Avp and pGL3-Vip luciferase activities are suppressed by heterogeneous nuclear ribonucleoprotein U (hnRNP U) RNA interference, whereas pGL3-Basic is not affected. Data are expressed as means ± SD (**B–D**, **G**, and **H**). *n* = 3 *Hnmpu*^{+/-} mice (**A** and **B**); *n* = 21 *Hnmpu*^{+/-} mice (**C** and **D**); *n* = 4 mice with Avp and 3 mice with Vip (**E** and **F**); *n* = 5 (**G**); *n* = 6 (**H**). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, unpaired two-tailed *t*-test (**B** and **G**) and two-way analysis of variance followed by Bonferroni post hoc test (**C**, **D**, and **H**). Scale bars: 200 μm. CT, circadian time; SiSCR, scrambled siRNA.

further explore the molecular mechanism, their interaction was examined in the SCN2.2 cell line.⁵³ hnRNP U bound to Avp and Vip promoters in SCN2.2 cells (Figure 7B).

hnRNP U affected some transcription factor binding to specific promoters.^{54,55} Avp and Vip are two well-

established clock-controlled genes, and Bmal1 and Clock transcribed these genes periodically by binding to their promoters.^{38,39} Thus, we hypothesized that hnRNP U might affect the binding between Bmal1/Clock heterodimers and Avp and Vip promoters in a similar manner. The binding

Figure 6 Suprachiasmatic microinjection partially rescues the activity patterns. **A**: Mice were entrained to white light between 8:00 and 20:00 and darkness between 20:00 and 8:00 (LD) cycles and then released into constant darkness (DD). Arginine vasopressin (Avp) (†), vasoactive intestinal polypeptide (*), and a scrambled peptide (†) were injected into the suprachiasmatic nucleus. The locomotor activity patterns 1 week before and after injection are presented in the red and yellow rhombi. **B**: *Hnmpu*^{+/-} mice activity in the daytime is not reduced after control injection. **C**: *Hnmpu*^{+/-} mice activity in the daytime significantly reduces after Avp injection. **D**: *Hnmpu*^{+/-} mice activity in the daytime reduces after Vip injection. Data are expressed as means ± SD (**B–D**). *n* = 3. **P* < 0.05, unpaired two-tailed *t*-test. Ctl, control.

Figure 7 Heterogeneous nuclear ribonucleoprotein U (hnRNP U) bound to arginine vasopressin (Avp) and vasoactive intestinal polypeptide (Vip) promoters. **A:** hnRNP U bound to Avp and Vip promoters in the *Hnrnpu*^{+/+} hypothalamus but not *Hnrnpu*^{+/-} hypothalamus. **B:** hnRNP U bound to Avp and Vip promoters in the SCN2.2 cells. **C:** hnRNP U knockdown disrupts brain and muscle Arnt-like protein-1 (Bmal1) binding to Avp and Vip promoters in suprachiasmatic nucleus (SCN) 2.2 cells. **D:** hnRNP U knockdown disrupts circadian locomotor output cycles kaput (Clock) binding to Avp and Vip promoters in SCN2.2 cells. Data are normalized to normal IgG (nIgG) and presented as means ± SD. *n* = 5 *Hnrnpu*^{+/+} mice; *n* = 3 *Hnrnpu*^{+/-} mice; and *n* = 8 nIgG mice (**A**); *n* = 3 (**B–D**). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, two-way analysis of variance followed by Bonferroni post hoc test (**A**, **C** and **D**) and unpaired two-tailed *t*-test (**B**). αBmal1, anti-Bmal1; αClock, anti-clock; αhnRNP U, anti-hnRNP U; αnIgG, anti-normal IgG; ChIP, chromatin immunoprecipitation.

efficiency was separately examined in SCN2.2 cells transfected with siSCR or small interfering hnRNP U1. hnRNP U RNAi strongly suppressed Bmal1 and Clock binding to the Avp and Vip promoters (Figure 7, C and D). These results demonstrate that hnRNP U is indispensable for Bmal1 and Clock binding to Avp and Vip promoters.

Discussion

Circadian expression of hnRNP U in rodents has been known for years. Patients with one copy deletion of *Hnrnpu* experience sleep disorders.^{5,47} Up to half of elderly individuals have chronic sleep disturbances, and one-quarter to a half have several kinds of nocturnal restlessness at some stage of diseases.⁵⁶ In this report, we found that *Hnrnpu* haploinsufficiency in mice led to abnormal rest-activity cycles with obviously increased activity in the daytime and reduced activity at nighttime. Defective expression of Avp and Vip participate in mediating these irregular activity patterns and abnormal metabolic rhythms.

Avp and Vip are crucial for the pacemaker to synchronize with the clock in peripheral tissues. The phenotypes of *Hnrnpu*^{+/-} mice were similar to *Vip*^{-/-} mice or *Vla*^{-/-} mice with defects in central and peripheral clock coupling.^{35–37} All these mice expressed fragmented locomotor activities and ambiguous activity onset lines. *Hnrnpu*^{+/-} mice were similar to LIM homeodomain transcription factor 1 (*Lhx1*) knockout mice given that *Lhx1* is a regulator of SCN-enriched neuropeptides. Without *Lhx1*, mice were deficient in Avp and Vip.^{57,58} Thus, it is reasonable to speculate that HnRNP U regulates circadian rhythm outputs through Avp and Vip.

Several studies have indicated that hnRNP U bound directly to DNA and regulated gene expression.^{7,43,54,55} In

most cases, hnRNP U bound to DNA regions named matrix associated regions in a nonspecific manner, whereas data in this paper and other papers have indicated that hnRNP U could bind to specific DNA regions with some other specific transcription factors and regulators. Our previous work suggested that hnRNP U participated in the long-distance regulation of *Shh* by binding to a *cis*-element 1 Mb away from the transcription starting point.⁴² In embryonic stem cells, hnRNP U bound to *oct4* proximal promoter and recruited RNA polymerase II.⁵⁴ Moreover, hnRNP U bound to E-box regions of the clock-controlled gene *Odc* and activated its transcription together with the c-Myc-Max complex.⁴³ Both Avp and Vip are well-established clock-controlled genes that contain E-boxes in their promoters.^{38,39} hnRNP U interacted with Avp and Vip promoters together with Bmal1 and Clock. In addition, hnRNP U might not bind to all E-boxes because the expression of *Per2*, *Cry1*, and *Dbp* was not altered in *Hnrnpu*^{+/-} mice. Other potential *cis*-elements and *trans*-acting factors that regulate the expression of these genes might exist.

Interestingly, changes in the Bmal1 transcript were not observed in mutant mice, although it has been reported that hnRNP U regulates Bmal1 expression in NIH 3T3 cells.⁷ We hypothesized that the residual hnRNP U in heterozygous mice might be sufficient to sustain typical Bmal1 expression, whereas Avp and Vip promoters are more sensitive to hnRNP U dosage.

Significantly, mammals maintain circadian rhythms not only in an appropriate period and phase but also in adequate robustness and amplitude. For diurnal animals, like humans, most activity should occur in the daytime, whereas for nocturnal animals, like mice, most activity should be at night. Logically, there are two main possible factors related to circadian expression of activity: the stability of the oscillator per se and the extent of coupling between the

pacemaker and peripheral oscillators.⁵⁹ The data presented here underline the significance of the latter factor.

In summary, our work identifies distinct transcriptional networks of *Avp*, *Vip*, *Bmal1*, *Clock*, and *hnRNP U*. *hnRNP U* mediates *Bmal1/Clock*, transcribing these neuropeptides periodically. These results not only provide new insights into the highly complicated regulation of SCN but also aid understanding of the pathogenesis of human *1q44* deletion syndrome.⁵

Acknowledgments

All authors contributed intellectually; X.G., Y.X., and B.L. conceptualized and designed the research; B.L. and J.Z. performed most experiments and analyzed data; B.L. drafted the manuscript; Z.L. revised the manuscript; X.G. approved the final version of the manuscript; Z.Q. aided in the wheel running test; and A.S. assisted with molecular experiments.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2017.09.017>.

References

- Han S, Tai C, Westenbroek RE, Yu FH, Cheah CS, Potter GB, Rubenstein JL, Scheuer T, de la Iglesia HO, Catterall WA: Autistic-like behaviour in *Scn1a*+/- mice and rescue by enhanced GABA-mediated neurotransmission. *Nature* 2012, 489:385–390
- Huang TN, Chuang HC, Chou WH, Chen CY, Wang HF, Chou SJ, Hsueh YP: *Tbr1* haploinsufficiency impairs amygdalar axonal projections and results in cognitive abnormality. *Nat Neurosci* 2014, 17: 240–247
- Carpinelli MR, Kruse EA, Arhatari BD, Debrincat MA, Ogier JM, Bories J, Kile BT, Burt RA: Mice haploinsufficient for *Ets1* and *Fli1* display middle ear abnormalities and model aspects of Jacobsen syndrome. *Am J Pathol* 2015, 185:1867–1876
- Rahrmann EP, Moriarity BS, Otto GM, Watson AL, Choi K, Collins MH, Wallace MR, Webber BR, Forster CL, Rizzardi AE: *Trp53* haploinsufficiency modifies EGFR-driven peripheral nerve sheath tumorigenesis. *Am J Pathol* 2014, 184:2082–2098
- Thierry G, Beneteau C, Pichon O, Flori E, Isidor B, Popelard F, Delrue MA, Duboscq-Bidot L, Thuresson AC, van Bon BW, Cailley D, Rooryck C, Paubel A, Metay C, Dusser A, Pasquier L, Beri M, Bonnet C, Jaillard S, Dubourg C, Tou B, Quere MP, Soussi-Zander C, Toutain A, Lacombe D, Arveiler B, de Vries BB, Jonveaux P, David A, Le Caignec C: Molecular characterization of *1q44* microdeletion in 11 patients reveals three candidate genes for intellectual disability and seizures. *Am J Med Genet A* 2012, 158A: 1633–1640
- Tamaru T, Isojima Y, Nagai K, Takamatsu K: Circadian expression of *hnRNP U*, a nuclear multi-potent regulatory protein, in the murine suprachiasmatic nucleus. *Neurosci Lett* 2003, 341:111–114
- Onishi Y, Hanai S, Ohno T, Hara Y, Ishida N: Rhythmic SAF-A binding underlies circadian transcription of the *Bmal1* gene. *Mol Cell Biol* 2008, 28:3477–3488
- Saper CB, Scammell TE, Lu J: Hypothalamic regulation of sleep and circadian rhythms. *Nature* 2005, 437:1257–1263
- De Leersnyder H: Inverted rhythm of melatonin secretion in Smith-Magenis syndrome: from symptoms to treatment. *Trends Endocrinol Metab* 2006, 17:291–298
- Orozco-Solis R, Aguilar-Arnal L, Murakami M, Peruquetti R, Ramadori G, Coppari R, Sassone-Corsi P: The circadian clock in the ventromedial hypothalamus controls cyclic energy expenditure. *Cell Metab* 2016, 23:467–478
- Chen W, Yeh J, Peng M, Shie S, Lin S, Yang C, Chen T, Hung K, Wang C, Hsieh I: Circadian CLOCK mediates activation of transforming growth factor- β signaling and renal fibrosis through cyclooxygenase 2. *Am J Pathol* 2015, 185:3152–3163
- Shearman LP, Sriram S, Weaver DR, Maywood ES, Chaves I, Zheng B, Kume K, Lee CC, van der Horst GT, Hastings MH, Reppert SM: Interacting molecular loops in the mammalian circadian clock. *Science* 2000, 288:1013–1019
- Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, Bradfield CA: *Mop3* is an essential component of the master circadian pacemaker in mammals. *Cell* 2000, 103:1009–1017
- Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL, McDonald JD, Dove WF, Pinto LH, Turek FW, Takahashi JS: Mutagenesis and mapping of a mouse gene, *Clock*, essential for circadian behavior. *Science* 1994, 264:719–725
- Tei H, Okamura H, Shigeyoshi Y, Fukuhara C, Ozawa R, Hirose M, Sakaki Y: Circadian oscillation of a mammalian homologue of the *Drosophila* period gene. *Nature* 1997, 389:512–516
- van der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S, Takao M, de Wit J, Verkerk A, Eker AP, van Leenen D, Buijs R, Bootsma D, Hoeijmakers JH, Yasui A: Mammalian *Cry1* and *Cry2* are essential for maintenance of circadian rhythms. *Nature* 1999, 398: 627–630
- Miller BH, McDearmon EL, Panda S, Hayes KR, Zhang J, Andrews JL, Antoch MP, Walker JR, Esser KA, Hogenesch JB, Takahashi JS: Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation. *Proc Natl Acad Sci U S A* 2007, 104:3342–3347
- Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS, Hogenesch JB: Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* 2002, 109:307–320
- Storch KF, Lipan O, Leykin I, Viswanathan N, Davis FC, Wong WH, Weitz CJ: Extensive and divergent circadian gene expression in liver and heart. *Nature* 2002, 417:78–83
- Schibler U: Circadian rhythms: new cogwheels in the clockworks. *Nature* 1998, 393:620–621
- Buijs RM, la Fleur SE, Wortel J, Van Heyningen C, Zuidam L, Mettenleiter TC, Kalsbeek A, Nagai K, Nijima A: The suprachiasmatic nucleus balances sympathetic and parasympathetic output to peripheral organs through separate preautonomic neurons. *J Comp Neurol* 2003, 464:36–48
- Stephan FK, Zucker I: Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proc Natl Acad Sci U S A* 1972, 69:1583–1586
- Vogelbaum MA, Galef J, Menaker M: Factors determining the restoration of circadian behavior by hypothalamic transplants. *J Neural Transplant Plast* 1993, 4:239–256
- Antle MC, Silver R: Orchestrating time: arrangements of the brain circadian clock. *Trends Neurosci* 2005, 28:145–151
- Lee JE, Atkins N Jr, Hatcher NG, Zamdborg L, Gillette MU, Sweedler JV, Kelleher NL: Endogenous peptide discovery of the rat circadian clock: a focused study of the suprachiasmatic nucleus by ultrahigh performance tandem mass spectrometry. *Mol Cell Proteomics* 2010, 9:285–297
- Abrahamson EE, Moore RY: Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections. *Brain Res* 2001, 916:172–191
- An S, Harang R, Meeker K, Granados-Fuentes D, Tsai CA, Mazuski C, Kim J, Doyle FJ 3rd, Petzold LR, Herzog ED: A

- neuropeptide speeds circadian entrainment by reducing intercellular synchrony. *Proc Natl Acad Sci U S A* 2013, 110:E4355–E4361
28. Herzog ED: Neurons and networks in daily rhythms. *Nat Rev Neurosci* 2007, 8:790–802
 29. Maywood ES, Reddy AB, Wong GK, O'Neill JS, O'Brien JA, McMahon DG, Harmar AJ, Okamura H, Hastings MH: Synchronization and maintenance of timekeeping in suprachiasmatic circadian clock cells by neuropeptidergic signaling. *Curr Biol* 2006, 16:599–605
 30. Shinohara K, Honma S, Katsuno Y, Abe H, Honma K: Two distinct oscillators in the rat suprachiasmatic nucleus in vitro. *Proc Natl Acad Sci U S A* 1995, 92:7396–7400
 31. Tousson E, Meissl H: Suprachiasmatic nuclei grafts restore the circadian rhythm in the paraventricular nucleus of the hypothalamus. *J Neurosci* 2004, 24:2983–2988
 32. Piggins HD, Antle MC, Rusak B: Neuropeptides phase shift the mammalian circadian pacemaker. *J Neurosci* 1995, 15:5612–5622
 33. Albers HE, Gillespie CF, Babagbemi TO, Huhman KL: Analysis of the phase shifting effects of gastrin releasing peptide when microinjected into the suprachiasmatic region. *Neurosci Lett* 1995, 191:63–66
 34. Hughes AT, Piggins HD: Behavioral responses of *Vipr2*^{-/-} mice to light. *J Biol Rhythms* 2008, 23:211–219
 35. Li JD, Burton KJ, Zhang C, Hu SB, Zhou QY: Vasopressin receptor V1a regulates circadian rhythms of locomotor activity and expression of clock-controlled genes in the suprachiasmatic nuclei. *Am J Physiol Regul Integr Comp Physiol* 2009, 296:R824–R830
 36. Colwell CS, Michel S, Itri J, Rodriguez W, Tam J, Lelievre V, Hu Z, Liu X, Waschek JA: Disrupted circadian rhythms in VIP- and PHI-deficient mice. *Am J Physiol Regul Integr Comp Physiol* 2003, 285:R939–R949
 37. Aton SJ, Colwell CS, Harmar AJ, Waschek J, Herzog ED: Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons. *Nat Neurosci* 2005, 8:476–483
 38. Reppert SM, Weaver DR: Molecular analysis of mammalian circadian rhythms. *Annu Rev Physiol* 2001, 63:647–676
 39. Hurst WJ, Mitchell JW, Gillette MU: Synchronization and phase-resetting by glutamate of an immortalized SCN cell line. *Biochem Biophys Res Commun* 2002, 298:133–143
 40. Xiao R, Tang P, Yang B, Huang J, Zhou Y, Shao C, Li H, Sun H, Zhang Y, Fu XD: Nuclear matrix factor hnRNP U/SAF-A exerts a global control of alternative splicing by regulating U2 snRNP maturation. *Mol Cell* 2012, 45:656–668
 41. Ye J, Beetz N, O'Keefe S, Tapia JC, Macpherson L, Chen WV, Bassel-Duby R, Olson EN, Maniatis T: hnRNP U protein is required for normal pre-mRNA splicing and postnatal heart development and function. *Proc Natl Acad Sci U S A* 2015, 112:E3020–E3029
 42. Zhao J, Ding J, Li Y, Ren K, Sha J, Zhu M, Gao X: HnRNP U mediates the long-range regulation of *Shh* expression during limb development. *Hum Mol Genet* 2009, 18:3090–3097
 43. Matsuoka Y, Uehara N, Tsubura A: hnRNP U interacts with the c-Myc-Max complex on the E-box promoter region inducing the ornithine decarboxylase gene. *Oncol Rep* 2009, 22:249–255
 44. Roshon MJ, Ruley HE: Hypomorphic mutation in hnRNP U results in post-implantation lethality. *Transgenic Res* 2005, 14:179–192
 45. Wang X, Tang J, Xing L, Shi G, Ruan H, Gu X, Liu Z, Wu X, Gao X, Xu Y: Interaction of MAGED1 with nuclear receptors affects circadian clock function. *EMBO J* 2010, 29:1389–1400
 46. Qu Z, Zhang H, Huang M, Shi G, Liu Z, Xie P, Li H, Wang W, Xu G, Zhang Y, Yang L, Huang G, Takahashi JS, Zhang WJ, Xu Y: Loss of ZBTB20 impairs circadian output and leads to unimodal behavioral rhythms. *Elife* 2016, 5
 47. McGlincy NJ, Valomon A, Chesham JE, Maywood ES, Hastings MH, Ule J: Regulation of alternative splicing by the circadian clock and food related cues. *Genome Biol* 2012, 13:R54
 48. Cutler DJ, Haraura M, Reed HE, Shen S, Sheward WJ, Morrison CF, Marston HM, Harmar AJ, Piggins HD: The mouse VPAC2 receptor confers suprachiasmatic nuclei cellular rhythmicity and responsiveness to vasoactive intestinal polypeptide in vitro. *Eur J Neurosci* 2003, 17:197–204
 49. Harmar AJ, Marston HM, Shen S, Spratt C, West KM, Sheward WJ, Morrison CF, Dorin JR, Piggins HD, Reubi JC, Kelly JS, Maywood ES, Hastings MH: The VPAC(2) receptor is essential for circadian function in the mouse suprachiasmatic nuclei. *Cell* 2002, 109:497–508
 50. Hughes AT, Guilding C, Lennox L, Samuels RE, McMahon DG, Piggins HD: Live imaging of altered *period1* expression in the suprachiasmatic nuclei of *Vipr2*^{-/-} mice. *J Neurochem* 2008, 106:1646–1657
 51. Georg B, Hannibal J, Fahrenkrug J: Lack of the PAC1 receptor alters the circadian expression of VIP mRNA in the suprachiasmatic nucleus of mice. *Brain Res* 2007, 1135:52–57
 52. Dardente H, Menet JS, Challet E, Tournier BB, Pevet P, Masson-Pevet M: Daily and circadian expression of neuropeptides in the suprachiasmatic nuclei of nocturnal and diurnal rodents. *Brain Res Mol Brain Res* 2004, 124:143–151
 53. Earnest DJ, Liang FQ, Ratcliff M, Cassone VM: Immortal time: circadian clock properties of rat suprachiasmatic cell lines. *Science* 1999, 283:693–695
 54. Vizlin-Hodzic D, Johansson H, Ryme J, Simonsson T, Simonsson S: SAF-A has a role in transcriptional regulation of Oct4 in ES cells through promoter binding. *Cell Reprogram* 2011, 13:13–27
 55. Vizlin-Hodzic D, Runnberg R, Ryme J, Simonsson S, Simonsson T: SAF-A forms a complex with BRG1 and both components are required for RNA polymerase II mediated transcription. *PLoS One* 2011, 6:e28049
 56. van Someren V, Burmester M, Alusi G, Lane R: Are sleep studies worth doing? *Arch Dis Child* 2000, 83:76–81
 57. Bedont JL, LeGates TA, Slat EA, Byerly MS, Wang H, Hu J, Rupp AC, Qian J, Wong GW, Herzog ED, Hattar S, Blackshaw S: *Lhx1* controls terminal differentiation and circadian function of the suprachiasmatic nucleus. *Cell Rep* 2014, 7:609–622
 58. Hatori M, Gill S, Mure LS, Goulding M, O'Leary DD, Panda S: *Lhx1* maintains synchrony among circadian oscillator neurons of the SCN. *Elife* 2014, 3:e03357
 59. Satinoff E, Li H, Tchong TK, Liu C, McArthur AJ, Medanic M, Gillette MU: Do the suprachiasmatic nuclei oscillate in old rats as they do in young ones? *Am J Physiol* 1993, 265:R1216–R1222