Circadian rhythms of gastrointestinal function are regulated by both central and peripheral oscillators

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Malloy JN, Paulose JK, Li Y, Cassone VM. Circadian rhythms of gastrointestinal function are regulated by both central and peripheral oscillators. Am J Physiol Gastrointest Liver Physiol 303: G461–G473, 2012. First published June 21, 2012; doi:10.1152/ajpgi.00369.2011.—Circadian clocks are responsible for daily rhythms in a wide array of processes, including gastrointestinal (GI) function. These are vital for normal digestive rhythms and overall health. Previous studies demonstrated circadian clocks within the cells of GI tissue. The present study examines the roles played by the suprachiasmatic nuclei (SCN), master circadian pacemaker for overt circadian rhythms, and the sympathetic nervous system in regulation of circadian GI rhythms in the mouse Mus musculus. Surgical ablation of the SCN abolishes circadian locomotor, feeding, and stool output rhythms when animals are presented with food ad libitum, while restricted feeding reestablishes these rhythms temporarily. In intact mice, chemical sympathectomy with 6-hydroxydopamine has no effect on feeding and locomotor rhythmicity in light-dark cycles or constant darkness but attenuates stool weight and stool number rhythms. Again, however, restricted feeding reestablishes rhythms in locomotor activity, feeding, and stool output rhythms. Ex vivo, intestinal tissue from PER2::LUC transgenic mice expresses circadian rhythms of luciferase bioluminescence. Chemical sympathectomy has little effect on these rhythms, but timed administration of the β-adrenergic agonist isoproterenol causes a phase-dependent shift in PERIOD2 expression rhythms. Collectively, the data suggest that the SCN are required to maintain feeding, locomotor, and stool output rhythms during ad libitum conditions, acting at least in part through daily activation of sympathetic activity. Even so, this input is not necessary for entrainment to timed feeding, which may be the province of oscillators within the intestines themselves or other components of the GI system.

BIOLOGICAL RHYTHMS and the clocks that regulate them are important features of gastrointestinal (GI) and metabolic physiology that impact human health at several levels (5, 11, 12). Studies in healthy human volunteers have shown circadian changes in many aspects of GI function (12). For example, ambulatory colonic pressure recordings from healthy individuals exhibit maximal colonic activity during the day, especially following awakening and following a meal, and minimal activity during the night. GI disease states also emerge when circadian clocks are disrupted (19, 32, 26). GI symptoms, such as abdominal pain, constipation, and diarrhea, are more common in shift workers and time zone travelers than in the general population, and diagnoses of irritable bowel syndromes (IBS) have been associated with sleep disturbances, occurring in 26–55% of patients. It is unclear whether sleep disturbances are the cause or the effect of IBS, although the severity of bowel symptoms varies with the quality of the preceding night’s sleep in IBS patients. Furthermore, IBS, peptic ulcers, and GI cancers are more common in nurses who work rotating shifts than in those who work day-only or night-only shifts (23).

Circadian biological rhythms are fundamental properties of most organisms, ranging from Cyanobacteria to humans, and pervade all aspects of biological organization, from transcriptional regulation to behavior (1, 27). In animals, circadian rhythms are properties of a complex gene network of highly conserved “clock genes” and their outputs, which autonomously feed back to generate an oscillation of transcriptional activity with an ~24-h period. These genes are expressed rhythmically in many cell types and many tissues throughout the body (1) and are believed to mediate rhythmic processes locally in concert with the circadian system as a whole (1, 27). Briefly, positive elements clock and bmal1 are transcribed and translated and then form dimers that reenter the nucleus. There, the CLOCK/BMAL1 dimers stimulate transcription of genes containing an e-box or e-boxes on their promoters. Among these are the negative elements period (per) 1, 2, and 3 and cryptochrome (cry) 1 and 2. These are transcribed and translated and, in turn, form oligomers that enter the nucleus to interfere with CLOCK/BMAL1 activity (1, 20). Other components of this transcriptional, translational feedback loop modulate and stabilize the core of this loop. These are reviewed extensively elsewhere (17, 28, 37).

In mammals, circadian function is also hierarchically organized, in that the hypothalamic suprachiasmatic nuclei (SCN) serve as the conduits for light-dark (LD) cycles and as central pacemakers for the entire system (22). Entrainment or synchronization of circadian rhythms to LD cycles is mediated through a specific retinohypothalamic tract from the inner retina to the SCN, and interruption of the retinohypothalamic tract results in animals “free-running,” or expressing an endogenous period of approximately, but not exactly, 24 h (18). Surgical destruction or isolation of the SCN results in arrhythmicity in all behavioral and physiological outputs in every mammal studied, and transplantation of SCN tissue or cells into the brains of SCN-lesioned (SCNX) rodents restores behavioral rhythms (21). These studies strongly indicate that the SCN are indeed the master pacemakers for all biological clock function in mammals, except for two details. 1) Although the SCN express all the “clock genes” rhythmically, as one might expect for the master pacemaker, clock genes are expressed in many parts of the brain and peripheral tissues in a rhythmic fashion in vivo and ex vivo. 2) Although SCN lesion abolishes free-running rhythmicity, Comperatore and Stephan (7) showed that rhythmic presentation of food to SCNX rodents can entrain antici-

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regulation of sympathetic tone is responsible for central and circadian rhythms and to ask whether the known role of SCN-dependent activity are in synchrony (9). Therefore, the present presentation, such that SCN-dependent activity and FEO-de-
circumstances, the GIT clock acts independently of the SCN. Entrain to a timed meal, this does not mean that, under normal
in colonic activity in vivo and ex vivo (15). Thus the GIT
rhythmically (16). Clock genes are rhythmically expressed in
the entire GI tract (GIT) at the mRNA and protein levels, and
timed feeding shifts the phase of these rhythms in vivo and ex vivo (14, 25). This effect is also unaffected by vagotomy. Finally, knockout of per1 and per2 abolishes circadian rhythms
colonic activity in vivo and ex vivo (15). Thus the GIT
contains a circadian oscillator capable of entraining to timed
meals.

However, even though the GIT contains a clock that can
entrain to a timed meal, this does not mean that, under normal circumstances, the GIT clock acts independently of the SCN. Indeed, intact mice and rats will entrain to a timed food presentation, such that SCN-dependent activity and FEO-de-
pendent activity are in synchrony (9). Therefore, the present
study was conducted to determine the role of the SCN in GIT
circadian rhythms and to ask whether the known role of SCN
regulation of sympathetic tone is responsible for central and
peripheral coordination of GIT function.

METHODS

Animals

All in vivo experiments utilized male wild-type Sv129 mice
(Jackson Laboratory, Bar Harbor, ME). All colonic tissue cultured for
ex vivo experiments was excised from transgenic PER2::LUC C57/
BL6 mice (36) (gift from Dr. Shin Yamazaki, Vanderbilt University,
Nashville, TN). Animals were treated in accordance with the guide-
lines of the University of Kentucky Institutional Animal Care and Use
Committee, which approved our protocols.

Procedures

Male mice (n = 18) were placed in polycarbonate cages, and
locomotor activity was recorded continuously from running wheels
equipped with magnetic switches attached to the wire cage tops.
Activity was recorded, compiled, and analyzed using data acquisition
and control systems (VitalView version 4.1) from Mini Mitte (Sun
River, OR), and data were analyzed with ActiView software.

Mice were kept in a 12:12-h LD cycle (150-lux illumination) for
14–21 days before receiving experimental or control surgeries (see
below). The animals were allowed to recover in LD for 14 days; then
food and stool collection (see below) was conducted, and data were
recorded every 4 h for 3 days. Mice were allowed to recover in
constant darkness (DD) for 7 days. They remained in DD for 3
additional days, during which food and stool collections were con-
ducted every 4 h in reference to individual activity onset (circadian
time) (12). Mice remained in DD for another 7 days for recovery. All
mice were then subjected to a restricted feeding (RF) regimen while
they remained in DD for 10 days. Food and stool collections were
conducted during the final 3 days of RF. Then animals were allowed
to recover for 7 days in DD. Half of the animals subjected to an
experimental surgery and half of the mice subjected to control surgery
were injected intraperitoneally with 6-hydroxycamphine (6-OHDA)
(see below). The remaining mice were injected with ascorbate vehicle.
All injections were given prior to each change in light cycle and
feeding regimen (LD, DD, RF), which were repeated corresponding to
the order specified above. As described above, food and stool mea-
surements were performed for 3 days every 4 h, and animals were
allowed to recover for 1 wk in DD following each change in light
cycle or feeding regimen. After the second RF regimen, mice were
released into DD until they were euthanized for organ harvest. All
observations and manipulations of animals in DD were conducted
with the aid of Night Owl (Optics Planet) infrared binocular viewers.

Surgery

Mice were anesthetized with ketamine and xylazine (80 and 20
mg/kg body wt, respectively). A stereotaxic instrument (Stoelting,
Wood Dale, IL) was used to position a stainless steel electrode (100
μm) within the SCN according to stereotaxic coordinates of the mouse
brain (9a). Electrolytic lesions of the SCN (anteroposterior 5.40 ± 9 cm,
medialateral 2.60 ± 3 cm bilaterally, dorsoventral 3.00 ± 4 mm) were
produced using the custom-made electrode connected to a stimulator
(SD9K square-pulse model, Astro-Med) set to 50 V. Current was
applied for 20 s, followed by a 10-s pause and another 10 s of
stimulation. Mice that received current to regions of the brain other
than the SCN were considered animals that received a sham operation.
All sham lesions were located in tissue surrounding the SCN or were
incomplete SCN lesions. Sham animals were verified upon recovery
from surgery by rhythmic locomotor activity records. The surgical site
was held closed using a 9-mm wound-closing kit (Stoelting). The
lesion site was later verified by immunohistochemical staining on
histological sections.

Food Consumption and Stool Measurement

During periods of GI data collection, the cage bottoms were lined
with one layer of Versi-Dry lab soaker material (VWR, Philadelphia,
PA). Food (2018 Teklad Global 18% Protein Rodent Diet, Teklad
Diets, Madison, WI) weight was measured under infrared light before
and after every 4-h time point during the 3-day collections, with the
typical of the timed feeding experiment, during which food con-
sumption was measured during the 4-h time point daily over which
food was available. At the end of every 4-h time point, stools were
collected, counted, and weighed. Stool weight and stool number for
each mouse were recorded. Cages were changed and new material
was placed in the cage bottom following each time point. Water was
available ad libitum throughout all experiments.

Restricted Feeding

The RF protocol was similar to that employed by Gooley et al. (10).
Mice were given access to food from zeitgeber time (ZT) 0 (12 PM or
time of previous lights on) to ZT4 (4 PM) for a total of 10 days. The
initial 7 days served to ensure entrainment to the timed feeding by
development of anticipatory activity associated with food availability
(FAA); during the following 3 days, food consumption and stool output
were measured.

6-OHDA Protocol

6-OHDA (50 mg/kg; Sigma, St. Louis, MO) in 0.2% ascorbate is a
neurotoxin that causes the degeneration of sympathetic terminals in
the peripheral sympathetic nervous system (24). It is an accepted way
to determine whether sympathetic activity mediates downstream pro-
cesses. In this case, 6-OHDA was injected intraperitoneally 1–2 days
prior to the start of each of the final three in vivo experiments (3
injections per mouse in total). Controls received ascorbate only. Half
of the animals from the SCNX and sham groups received 6-OHDA.
treatment. Experiments were performed during LD, DD, and RF, as described above.

Immunohistochemistry

To ascertain the completeness of SCN lesion, we visualized immunoreactivity for VIP in the brain, because VIP marks the presence of SCN tissue. We also visualized tyrosine hydroxylase (TH) immunoreactivity in the brain and intestines. TH immunoreactivity in the brain should not be affected by 6-OHDA, since 6-OHDA typically does not pass the blood-brain barrier (BBB) but should decrease TH immunoreactivity in the intestines. After data collection and recovery in DD, all mice were euthanized by transcardial perfusion with PBS and 4% paraformaldehyde. A separate set of mice (n = 4) were treated with 6-OHDA or ascorbate (n = 2 each), allowed to survive for 3 days, and then euthanized as described above. These mice were treated to gain an estimate of the effect of 6-OHDA at the time at which the actual behavioral and physiological measurements were obtained. Brain tissue was harvested and cryoprotected in a sucrose solution gradient (10%, 20%, and 30%). The brain tissue was kept at −80°C until sectioned. A cryostat (Leica CM 1850) was used to slice the tissue into 40-μm coronal sections, which were placed into PBS. Sections were washed thoroughly with PBS and incubated for 15 min in H2O2 solution [1.2 ml of 30% H2O2 in 100 ml of double-distilled H2O (ddH2O)]; then they were washed again. Sections were left to incubate in PBS-goat serum-Triton X (PBSGT) for 30 min at room temperature and washed again. Finally, sections were incubated in diaminobenzidine-nickel-H2O2 solution until immunoreactivity became discernible. The sections were washed and transferred to slides to dry overnight. On the following day, the slides were dehydrated in ethanol and cleared in xylene before coverslips were applied.

Western Blot

Samples (2 × 3 mm) from proximal colonic tissue were homogenized directly in 0.5 ml of Laemmli buffer and boiled for 30 min. Protein content from each sample was determined by Nanodrop, and 50 μg of individual samples were loaded accordingly. Samples and protein ladder (Precision Plus Protein Dual Color Standards, catalog no. 161-0374, Bio-Rad) were separated on a 10% SDS-polyacrylamide gel by SDS-PAGE using 1× running buffer (30.28 g of Tris base, 144.0 g of glycine, and 10 g of SDS in 10 liters of ddH2O). The gel was then used for protein transfer to a nitrocellulose membrane (Whatman, Dassel, Germany) by electroblotting in transfer buffer (3.03 g of Tris, 14.4 g of glycine, 200 ml of MeOH, and 0.05% SDS in 1 liter of ddH2O). The membrane was rinsed in PBS for 5 min and then blocked in 5% nonfat dry milk in PBS with 0.1% Tween 20 (PBT) for 30 min. Rabbit polyclonal IgG anti-TH antibody (EMD Millipore) was added at a 1:1,000 dilution and left overnight on a rotator at 4°C. The membrane was blocked for 7 min in 5% nonfat dry milk in PBT twice and washed in 1× casein (rabbit IgG Vectastain ABC-Amp Kit, Vector Laboratories) for 7 min. The membrane was incubated in biotinylated anti-rabbit IgG (rabbit IgG Vectastain ABC-Amp Kit) for 40 min. After two 7-min washes in PBT with 5% milk and one 7-min wash in 1× casein solution, the membrane was incubated for 30 min in ABC-Amp solution (rabbit IgG Vectastain ABC-Amp Kit) and then washed three times in 1× casein for 7 min. For equilibration, the membrane was incubated in 0.1 M Tris buffer (pH 9.5) for 10 min. Immunoreactive bands were detected using an alkaline phosphatase substrate staining solution [Alkaline Phosphatase Substrate Kit IV 5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT), Vector Laboratories]. Blots were scanned and imported into National Institutes of Health ImageJ, where individual lanes were quantified as density relative to internal standard. Internal standards of separate blots were normalized to each other for averaging purposes.

Ex Vivo Tissue Excision and Preparation

Unless otherwise noted, all reagents were purchased from Sigma. Five-week-old transgenic mice were anesthetized with ketamine and xylazine (80 and 20 mg/kg body wt, respectively). Colonic tissues were excised from the cecum to ~2 mm proximal to the rectum and placed into ice-cold Krebs solution (in mM: 117 NaCl, 5 KCl, 1.2 MgSO4, 25 NaHCO3, 1.2 NaH2PO4, 10 glucose, and 2.5 CaCl2, pH 7.6). The distal colon was rinsed in Krebs solution, bisected longitudinally, and cut into two to three 1- to 3-mm2 sections. Each piece was placed lumen-side-up onto sterile mesh in recording medium consisting of HEPES-buffered DMEM (33) with 0.1 mM luciferin ( Biosynth, Staad, Switzerland) in a 35-mm petri dish and sealed with a 40-μm cover glass ( Fisher Scientific, Pittsburg, PA). The dishes were placed into a LumiCycle photoluminometer (Actimetrics), which measured mPER2::LUCIFERASE bioluminescence every 10 min. The plates were removed when the oscillations appeared fully damped.

6-OHDA Ex Vivo

At 48 h prior to tissue excision and culture, mice (n = 4 for each treatment) were injected intraperitoneally with 6-OHDA (50 mg/kg) or 0.2% ascorbate vehicle and euthanized as described above. Distal colon pieces were cultured as described above, and bioluminescence data were collected until the tissues showed no discernible rhythmicity.

Isoproterenol Ex Vivo

Isoproterenol is a β-adrenergic agonist that can mimic the effects of endogenous norepinephrine (NE) in a wide array of peripheral tissues. We therefore asked whether isoproterenol affected the circadian rhythm of PER2 expression in explanted intestinal tissue. Tissues (n = 6–8 from 3 mice for each experiment) were excised, cut into sections, and placed into a LumiCycle, as described above. Bioluminescence was recorded for 2–3 days to determine the peak phase of PER2 expression. On the 3rd day in culture, the medium was replaced with fresh medium containing 100 μM isoproterenol or 0.2% ascorbate vehicle at 4, 9, 12, 16, or 21 h following the previous peak expression. As described above, recording continued until tissue rhythms damped to arrhythmicity.

Statistics

In vivo. Significance between peaks and troughs of rhythmic food consumption and stool production during ad libitum feeding and RF was determined by one- or two-way repeated-measures ANOVA, which was followed by a Student-Newman-Keuls post hoc test using SigmaStat 3.5 software. Rhythmicity (P < 0.05) was determined using CircWave v1.4 (R. A. Hut). Amplitude between sham groups was assessed using raw data and a Student’s t-test in SigmaStat 3.5. All tests were performed at a 95% confidence level (α = 0.05).

Ex vivo. Bioluminescence rhythms were detrended by application of a low-order polynomial to the raw data. The resulting baseline was then subtracted from the data set (LumiCycle Analysis Software, Actimetrics). The period and amplitude were determined from these baseline-subtracted data using LumiCycle Analysis Software. For the
6-OHDA-treated samples, the damping coefficient of each rhythm was calculated using a previously published equation (35): \[ \beta_j = \frac{2(t - t_0)}{\ln(A_j/A_0)} \] where the damping coefficient (\( \beta_j \)) is expressed as the ratio of amplitude from the 1st day of observation (\( A_0 \)) to the average amplitude from \( t \) until the oscillation becomes desynchronized (\( t_0 \)). Average period and damping coefficients between groups were compared by one-way ANOVA. For the timed isoproterenol pulse, peak phase of bioluminescence on the day before and on the 2nd and 3rd days after drug administration was determined for each tissue in CircWave by recording each center-of-gravity value. Each individual trace was analyzed to determine if the pulse resulted in a peak phase earlier (advance) or later (delay) than the prepulse phase, and the resulting change in phase was assigned a positive or negative value, respectively. Average change in phase (phase-response curve) was then subjected to one-way ANOVA to determine significant differences pre- and postpulse between treatment groups. Changes in phase, positive for phase advances and negative for phase delays, were plotted against the time of the pulse relative to peak PER2 expression. All tests were performed at a 95% confidence level (\( \alpha = 0.05 \)).

RESULTS

Locomotor Activity

The locomotor activity of representative mice throughout the various light cycles and feeding regimens can be seen in the actograms in Fig. 1. As expected, sham-operated mice in LD (Fig. 1A) retained circadian locomotor rhythms (Fig. 1C, black arrows), whereas ablation of the SCN (Fig. 1B) resulted in locomotor arrhythmicity (Fig. 1D, black arrows). In DD, sham animals exhibited free-running rhythms of wheel-running activity in which they expressed endogenous periods of 23.43 ± 0.27 h (Fig. 1C, gray arrows), and lesioned animals continued to be arrhythmic (Fig. 1D, gray arrows). Sham mice that received 6-OHDA expressed free-running periods of 23.8 ± 0.08 h, while mice receiving 6-OHDA expressed periods of 23.58 ± 0.21 h. No statistical differences among these periods were detected. Again, SCNX mice continued to be arrhythmic. All animals exhibited FAA preceding food presentation during RF (black boxes in Fig. 1, C and D).

Food Consumption and Stool Measurement

Figure 2 shows feeding and defecation measurements of SCNX and sham mice prior to drug or vehicle treatment. In sham animals in LD, significant differences exist between the peaks and troughs, although the SCNX animals were arrhythmic (Fig. 2, A–C). In DD, sham mice expressed rhythmic locomotor activity, feeding, and stool production. SCNX animals expressed arrhythmic feeding, stool weight, and stool number in LD and DD (Fig. 2, D–F). During RF in DD, two-way ANOVAs reported no significant differences between the food consumption of SCNX and sham groups on any day (Fig. 3A). SCNX and sham animals showed significant rhythms in amplitude of colonic motility (Fig. 3, B and C), which peak just following the window of food availability.

After intraperitoneal injection of 6-OHDA or ascorbate, food consumption and stool output rhythms of the SCNX animals were still abolished in LD (Fig. 4, A–C) and DD (Fig. 4, D–F). Importantly, the sham control animals maintained clear rhythmicity in locomotor activity, feeding (Fig. 5A), and stool output in LD, while the sham 6-OHDA-treated animals only retained rhythmicity in locomotor activity and feeding activity (Fig. 5A) but showed attenuated stool output rhythms.
This persisted in DD (Fig. 5, D–F), as determined by a one-way ANOVA, and there was a significant difference ($P < 0.032$) in rhythm amplitude of stool number output between the two groups as determined by a $t$-test. CircWave analysis was also employed for further quantitative information about the rhythms of the 6-OHDA-treated sham animals in Fig. 5. All sham control rhythms passed CircWave analysis for all 3 days (Fig. 5, A–C). Thus it may be concluded that 6-OHDA-induced sympathectomy specifically attenuates, but does not abolish, circadian colonic rhythmicity.

Fig. 2. Food consumption and stool output are rhythmic in sham ($n = 8$) and arrhythmic in SCNX ($n = 10$) animals during ad libitum food availability. A–C: food consumption, stool weight, and stool number in SCNX and sham animals during 3 days in LD. ZT, zeitgeber time. D–F: food consumption, stool weight, and stool number in SCNX and sham animals during 3 days in DD. CT, circadian time. For determination of circadian rhythmicity of a data set, peak values were compared with the corresponding following and/or preceding trough by 1-way ANOVA. *Significance in sham animals ($P < 0.05$). No significance was reported in SCNX animals. Values are means ± SE.
Similar to the entrainment during RF in Fig. 3, all animals show significant rhythmicity in all stool output parameters (Fig. 6, B–E).

**Immunohistochemistry**

VIP is a protein that is expressed prominently in the SCN, making the bilateral nuclei clearly visible following immunohistochemical staining with VIP antiserum. Complete SCN lesions showed no VIP immunoreactivity in the SCN region of the hypothalamic tissues. TH, involved in dopamine synthesis, is present in dopaminergic neurons. Neurons that have undergone a sympathectomy (such as by 6-OHDA) will no longer show TH expression. When injected peripherally, 6-OHDA cannot cross the BBB and enter the brain. In sham and SCNX animals, there was no significant decrease in TH-expressing neurons in the brain, indicating that SCN ablation surgery did not compromise the structural or functional integrity of the BBB (Fig. 7, A–D). Western blot analysis (see below) and immunofluorescent visualization of TH-immunoreactive fibers (Fig. 7, E and F) show that 6-OHDA decreased or abolished intestinal TH immunoreactivity.

**Western Blot**

TH-immunoreactive bands were detected on the nitrocellulose membrane at 60 kDa (Fig. 7G). Bands from 6-OHDA-treated tissues showed a significant decrease in relative density compared with vehicle-treated tissues as analyzed by t-test (P < 0.001, n = 4 tissues per treatment; Fig. 7H).

**6-OHDA Ex Vivo**

To determine whether 6-OHDA-induced peripheral sympathectomy affected the endogenous clock within the GIT, mPER2 bioluminescence was measured ex vivo from mice that had been subjected to this treatment. Figure 8A summarizes the data from this experiment. Control tissues had an average period of 24.00 ± 0.02 h and damping coefficient of 0.56 ± 0.08, while 6-OHDA-treated tissues oscillated with an average period of 24.14 ± 0.064 h with a damping coefficient of 0.47 ± 0.05. Student’s t-test analysis showed significant difference in period (t = 2.052, P < 0.05) but no difference in damping coefficient. Representative traces of PER2 expression rhythms from 6-OHDA-treated and control tissues in Fig. 8B highlight the difference in period length and the similar rate of damping.

**Isoproterenol Ex Vivo**

To determine if isoproterenol was able to affect PER2 expression rhythms in GI tissues, medium of colonic tissue in culture for several days was replaced with medium containing isoproterenol or ascorbate (control) can be seen in Fig. 8C, with the gap between day 4 and day 5 denoting media exchange. Isoproterenol administration resulted in a phase shift that was dependent on the time of drug administration. Figure 8D shows the composite data from five differently timed isoproterenol pulses at different phases relative to the previous peak of PER2::LUC bioluminescence. At 4 h after peak expression, as PER2 levels were declining, isoproterenol administration caused a phase delay of 2 h.

Fig. 3. Colonic motility in SCNX and sham animals directly entrains to food availability. Food was available for 4 h daily (from 12 PM to 4 PM) during ZT0–ZT4 (vertical gray bars in B and C). Bars along the x-axis in B and C represent the previous LD cycle. A: food consumption during each day of RF in SCNX and sham mice. B: stool weight during RF. C: stool number during RF. *P < 0.05. No significant difference was shown by 2-way ANOVA between groups in A. Values are means ± SE.
After 5 h, as PER2 approached nadir, the pulse resulted in a 2-h advance in phase. The most dramatic phase shift of 12-h delay (or advance) was produced when isoproterenol was administered at the trough of bioluminescence. The phase shifts became less dramatic when the drug was administered during the rising phases, with a 3-h delay 16 h after peak expression and a 2-h advance at 19 h after peak expression. Vehicle-treated samples showed no phase shift at any time point (Fig. 8D).

DISCUSSION

There is little doubt that the mammalian GI system contains a circadian clock capable of entraining to and anticipating the availability of food, and this clock may bear direct relevance to GI health and disease (13). First, 0.6–3.7% of the colonic transcriptome is expressed rhythmically on a circadian basis. Rhythmic transcripts include known signals involved in intest-
tinal motility, inflammation, cell signaling, and cell cycle, including several linked to colorectal cancer (16). Second, the myenteric plexus and many cell types of the endothelium express circadian patterns of mRNA and protein levels corresponding to known “canonical” clock genes in vivo and ex vivo. In vivo, intestinal clock gene rhythms phase shift in response to a shift in a timed food presentation, even though rhythms of clock genes within the SCN do not respond to this cue (14, 30). Furthermore, these rhythms correspond to congruent rhythms of intestinal motility in vivo, in terms of rhythmic defecation and intracolonic pressure, and ex vivo, in terms of rhythms of spontaneous contractility and in response to exogenous acetylcholine (15).

The circadian molecular clockworks are necessary for the expression of circadian rhythms of intestinal function. Knockout of per1 or per2 alters the period and decreases the ampli-
Fig. 6. Colonic motility in all treated animals directly entrains to food availability. Food was available for 4 h daily (from 12 PM to 4 PM) during ZT0–ZT4 (vertical gray bars in B–E). A: food consumption of each surgery/treatment group. B and C: stool weight and stool number of SCNX animals during RF. D and E: stool weight and stool number of sham animals during RF. *P < 0.05 for sham control. *P < 0.05 for sham 6-OHDA. No significant difference (by 2-way ANOVA) in food consumption was shown between groups in A. Values are means ± SE.
tude of many measures of colonic motility, but double knock-out of per1 and per2 abolishes these rhythms. Circadian rhythms of stool number and weight in LD and DD and intracolonic pressure changes in vivo are absent in per1/per2 double-knockout mice. Ex vivo, while colonic circular muscle contractility is expressed on a circadian basis in wild-type mice, the contractility is arrhythmic on a circadian basis in per1/per2 mice as well. Thus, at least the per1/per2 arms of the molecular feedback loop are required for circadian patterns of intestinal function, and it is likely that the entire gene network is involved (15).

However, the overwhelming evidence for a GI clock does not necessarily mean that circadian patterns of intestinal function are independent of central nervous pacemakers in the SCN. In the present study, we have shown that the SCN are critical for self-sustaining rhythms in the GIT. Circadian rhythms of locomotor activity, food consumption, stool number, and stool weight are abolished by SCN lesion in LD and DD. Similarly, Polidarová et al. (25) recently found that rats made arrhythmic with constant bright light (LL) also expressed disrupted or arrhythmic patterns of clock gene mRNA in the colon, duodenum, and liver. These authors inferred that the SCN was made arrhythmic with exposure to LL, whereas the present study explicitly addresses it. Even so, the present study also corroborates the view that some circadian functions are retained in SCNX mice. First, SCNX mice anticipate the presence of a timed meal by anticipatory wheel-running behavior. Second, timed feeding reinstates circadian patterns of feeding and defecation. These measures of GIT activity also anticipate the presence of a meal. Similarly, Polidarová et al. (25) also showed that rhythms of GIT clock gene expression in rats are restored with timed feeding. Finally, colonic tissue retains rhythmic PER2 expression ex vivo.

How can we reconcile the fact that SCN lesion completely abolishes behavioral and intestinal circadian rhythms and the fact that feeding and intestinal rhythms can still anticipate a meal? While it is true that the GIT retains circadian patterns of clock gene expression and motility ex vivo, the amplitude of these rhythms is reduced, and the rhythms damp over ~10 days. It is likely that restoration of rhythmicity by the GIT requires stimulation from a meal itself and/or circadian signals from the SCN.

One pathway by which the SCN is known to influence downstream processes is through the regulation of sympathetic tone. In rats, at least, sympathetic tone is rhythmic in sympathetic ganglia, the heart, and pineal and adrenal glands, such that maximal turnover occurs during the subjective night, when locomotor activity is highest (2), and these rhythms are abolished by SCN lesion (34). Sympathetic activity is required for the expression of several peripheral circadian rhythms, most notably circadian patterns of heart rate and liver function. In the case of circadian regulation of heart rate in rats, SCN lesion abolishes circadian patterns of heart rate and heart rate variability (34). This effect is simulated by chemical sympathectomy through previous treatment with guanethidine. Similarly, SCN lesion and surgical denervation of sympathetic afferents to the liver abolish circadian patterns of hepatic gluconeogenesis and several hepatic enzymes and glucocorticoid rhythms (3, 4, 29).

![Image](https://example.com/image.png)

**Fig. 7.** SCN lesion and 6-OHDA treatment do not decrease the number of tyrosine hydroxylase (TH)-expressing neurons within the arcuate nucleus, but 6-OHDA treatment does decrease TH immunoreactivity in the colon. A–D: representative histological sections showing TH immunohistochemical staining of the arcuate nucleus in the brain of an ascorbate-treated sham animal, a 6-OHDA-treated sham animal, an ascorbate-treated SCNX animal, and a 6-OHDA-treated SCNX animal. E and F: representative distal colonic sections showing immunofluorescent detection of TH-expressing neurons in ascorbate-treated (E) and 6-OHDA-treated (F) animals. Scale bars, 100 μm. G: representative Western blot showing immunoreactive TH. Left to right, 6-OHDA-treated tissues (lanes 1 and 2) and ascorbate-treated tissues (lanes 3 and 4). H: average composite of relative density of experimental and control immunoreactive TH bands of 4 independent Western blots.
In the present study, treatment with 6-OHDA, a neurotoxin that destroys sympathetic terminals, reduced the amplitude or abolished circadian patterns of stool number and weight but had little effect on the rhythms of food consumption of sham mice, suggesting that sympathetic activity is required for sustained circadian patterns of intestinal motility but has relatively little effect on feeding. In SCNX mice, 6-OHDA had no additional effect on the already arrhythmic pattern of food consumption, stool number, and stool weight. Nonetheless, timed feeding restored the rhythms of locomotor activity, stool number, stool weight, and anticipatory locomotor activity of sham and SCNX mice. The most likely scenario is that the GIT does indeed contain a circadian clock capable of responding to and anticipating the regular presence or absence of food. However, it is coordinated on a daily basis with the light-sensitive circadian clock in the SCN via the circadian changes in sympathetic tone under the control of SCN efferents. The SCN pacemaker interacts with GIT oscillators through entrainment of circadian clocks residing in the GIT. This is evident in the fact that the β-adrenergic agonist isoproterenol phase shifts circadian rhythms of PER2 expression in colonic tissues ex vivo in a phase-dependent fashion, indicating that the GIT clock is only sensitive to sympathetic input at certain times of day. PER2 expression rhythms are phase-delayed some 12 h when presented 12 circadian hours following the last PER2 peak but are either not affected or phase-advanced at other circadian phases. This is not due to effects on myenteric plexus catecholaminergic neurons, because pretreatment of PER2::LUC mice with 6-OHDA has relatively little effect on rhythms of luciferase bioluminescence. It is also not likely due to effects of 6-OHDA on the central nervous appetitive apparatus, since no effect of 6-OHDA can be observed in immunohistochemical detection of TH in cell bodies or known projection areas of catecholamine neurons. Furthermore, there is no effect of 6-OHDA on free-running locomotor rhythms, punctuating the view that the effects of 6-OHDA are peripheral. The most likely scenario is that circadian oscillators within the SCN, entrained to the LD cycle, influence a circadian change in sympathetic tone by increasing release of postganglionic NE primarily during the subjective night. Sympathetic NE entrains circadian oscillators within the myenteric plexus of the GIT, which in turn regulate downstream processes such
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as motility, enzymatic activity, cell signaling, and even inflammation. However, as is the case with any sympathetically regulated process and other regulators, such as stress, changes in blood pressure, and temperature fluctuations, sympathetic output can change outside the purview of the clock. Furthermore, the GIT itself can detect the regular presence or absence of food, even in the absence of SCN sympathetic input. The mechanisms by which the GIT might anticipate food are unknown but may involve GIT hormones and/or hepatic input, which is also known to contain a clock (12, 13). Davidson and colleagues (8) investigated the role of peripheral tissues, including liver and GIT tissues, in FAA during RF and concluded that the FEO that drives FAA is not located in the periphery, suggesting that further inquiries should focus instead on the central nervous system (CNS). The results presented here, while incapable of providing an anatomic location of the FEO, are in agreement that GIT tissues act independently of the CNS and implicate sympathetic signaling as a possible mechanism through which the CNS communicates time of day to the GIT. The presence of a circadian clock within the GIT serves as an adaptive mechanism by which the repeated, predictable presence of food can entrain the GI apparatus for efficient metabolism of foodstuffs. Among the questions that remain are, of course, the mechanisms by which the GIT anticipates food and the pathways by which peripheral detection of a meal influences behavior such as anticipatory wheel running.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.N.M. and V.M.C. are responsible for conception and design of the research; J.N.M., J.K.P., and Y.L. performed the experiments; J.N.M., J.K.P., Y.L., and V.M.C. analyzed the data; J.N.M., J.K.P., Y.L., and V.M.C. interpreted the results of the experiments; J.N.M. and J.K.P. prepared the figures; J.N.M., J.K.P., and V.M.C. drafted the manuscript; J.N.M., J.K.P., and V.M.C. edited and revised the manuscript; J.N.M., J.K.P., Y.L., and V.M.C. approved the final version of the manuscript.

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