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CENTRAL AND PERIPHERAL REGULATION OF CIRCADIAN GASTROINTESTINAL RHYTHMS

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CENTRAL AND PERIPHERAL REGULATION OF CIRCADIAN
GASTROINTESTINAL RHYTHMS

A thesis submitted in partial fulfillment of the requirements for the
degree of Master of Science in the College of Arts and Sciences in the
Department of Biology at the University of Kentucky

Jaclyn Malloy

Director: Dr. Vincent Cassone, Professor and Department Chair
Department of Biology

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ABSTRACT OF THESIS

CENTRAL AND PERIPHERAL REGULATION OF CIRCADIAN GASTROINTESTINAL RHYTHMS

Circadian clocks are responsible for daily rhythms in gastrointestinal function which are vital for normal digestive rhythms and health. The present study examines the roles of the circadian pacemaker, the suprachiasmatic nuclei (SCN), and the sympathetic nervous system in regulation of circadian gastrointestinal rhythms in *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, but attenuates stool output rhythms. Again, restricted feeding reestablishes these rhythms. *Ex vivo*, intestinal tissue from *mPer2^LUC* knockin mice expresses circadian rhythms of luciferase bioluminescence. 6-hydroxydopamine has little effect upon these rhythms, but timed administration of β-adrenergic agonist isoproterenol causes a phase-dependent phase shift in PERIOD2 expression rhythms. Collectively, the data suggest 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 gastrointestinal system.

KEYWORDS: colon, sympathetic nervous system, 6-hydroxydopamine, suprachiasmatic nucleus, period2

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CENTRAL AND PERIPHERAL REGULATION OF
CIRCADIAN GASTROINTESTINAL RHYTHMS

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6-OHDA: 6-hydroxydopamine
ANOVA: Analysis of Variance
ANS: Autonomic Nervous System
AP: anterior-posterior
CT: Circadian Time
DAB: diaminobenzidine
DD: dark-dark; constant darkness
DV: dorsoventral
FAA: Food Anticipatory Activity
FEO: Food Entrainable Oscillator
FITC: Fluorescein isothiocyanate
GIT: Gastrointestinal tract
LD: light-dark
LUC: luciferase reporter
ML: mediolateral
NE: norepinephrine
PRC: Phase Response Curve
RF: restricted feeding
RHT: retinohypothalamic tract
SCN: suprachiasmatic nuclei
SCNX: suprachiasmatic nuclei lesion
SNS: sympathetic nervous system
VIP: vasoactive intestinal polypeptide
ZT: Zeitgeber Time
Biological Rhythms

A wide array of organisms, ranging from prokaryotic cyanobacteria to complex eukaryotes, exhibit endogenously generated biological rhythms (Aschoff, 1981). Temporal oscillations can be observed within populations, single organisms, organs, and even individual isolated cells. The oscillations include a large range of cycle frequencies, ranging from several cycles per second to one cycle per year (Wollnik, 1989). These rhythms are broadly categorized as infradian, circadian or ultradian (Aschoff, 1981).

Infradian rhythms have a periodicity greater than ~24 hours, resulting in a cycle that repeats less frequently than a circadian rhythm (Aschoff, 1981). Infradian rhythms include biannual migratory restlessness and molting in birds (Gwinner and Czeschlik, 1978), and circannual seasonal torpor in hibernating animals (Walker et al., 1980). Infradian rhythms are also involved in reproductive processes such as the oestrous cycle, and in many species, photoperiodic breeding. Duration of daily light exposure determined by the season is a vital cue for animals that are short-day (such as sheep) (Foster et al., 1988), or long-day (such as the golden and Djungarian hamster) breeders (Darrow et al., 1980).

Ultradian rhythms have a periodicity which is less than ~24 hours. These rhythms repeat more frequently than a circadian rhythm (Aschoff, 1981). Various hormones, such as LH in humans (Bergendahl et al., 1996) and GH in the rat (Tannenbaum and Martin, 1976) exhibit ultradian secretion. Ultradian rhythms, unlike circadian rhythms and most infradian rhythms, do not correspond to any known physical environmental cycle (Gerkena and Daan, 1985). Postulations concerning the cause behind the generation of these rhythms include an “ultradian oscillator,” separate from the known circadian pacemaker in the hypothalamus (Kleitman, 1982) and circadian oscillators that have become desynchronized (Pittendrigh, 1974).

A Brief History of Circadian Time

Circadian rhythms have a cycle of about twenty-four hours, resulting in their Latin name circa “about” diem “a day” rhythms (Turek, 1985). The Earth completes a rotation around its axis every 23 hours, 56 minutes and 4 seconds, making one sidereal day (Van Allen, 2004). Organisms have adapted to predictable cyclic environmental conditions by means of a circadian clock able to process temporal
information (Turek, 1985). An approximate 24 hour clock confers an advantage, since it provides an organism with the ability to align behavioral, biochemical and physiological processes to a certain time of day (Panda et al., 2002).

Jean Jacques d’Ortous de Mairan (1729) performed the first experiment to record the phenomenon of free running. de Mairan noticed that plants seemed to have daily leaf movements, and put these plants (presumably *Mimosa pudica*) in constant darkness. He found that the plants continued to display these movements, and determined that the leaf movements were not passive responses to cyclic sunlight, but rather were a process innate to the plant itself. Kleitman and Richardson (1938) showed circadian patterns could be observed from humans in temporal isolation by performing an experiment in which they resided in the constant darkness of Mammoth Cave for 32 days. Wever and Aschoff (1963) organized a similar experiment that used a substantially larger number of subjects which were individually monitored while living in an underground bunker. The subjects were allowed to turn lights on and off and eat meals freely, allowing the subject to free run according to their endogenous period. Wever and Aschoff recorded several physiological parameters such as core body temperature and the sleep/wake cycle. Daily free running rhythms were observed in many biological processes by hourly measurements of the number of dividing cells in tissue, reaction to a drug, urine volume, or ability to solve arithmetical questions. Circadian oscillations are clearly endogenous, and adhere to an approximately 24 hour period in most subjects, but this does not mean that free running activity strictly aligns with the light-dark cycle the subject experienced prior to isolation. Instead, activity onset drifts slightly each day from the time of activity onset the previous day (Wever, 1979).

It is unknown at what time in history circadian rhythms evolved or whether internal or external factors were primarily responsible, but there has been speculation on the matter. Phylogenetic analysis of cyanobacteria, one of the oldest organisms on earth, suggests that two circadian pacemakers (kaiA and kaiB) each originated in cyanobacteria and the kaiABC complex arose approximately 1,000 million years ago (Dvornyk et al., 2003). Growth ring patterns of Cambrian corals suggest that circannual (and possibly circadian) rhythms were present at least 600 million years ago. Daily growth increments within each annual growth ring can be used to determine the number of days in a year at the time which these corals were living (Wells, 1963). Two primary factors proposed behind the evolution of the clock are internal and external coordination.
External coordination occurs when a rhythm is timed relative to predictable environmental changes such as sunrise and sunset (Woelfle et al., 2004). The external adaptive value of being synchronized to a light-dark cycle is most likely due to some cellular processes being affected by UV light. Nikaido and Johnson showed that the alga Chlamydomonas reinhardtii is most sensitive to ultraviolet radiation during the beginning of the night and end of the day as well as during the time of nuclear division. Circadian variation in UV sensitivity suggests that biological processes would be most selected for which are timed in a manner such that light-induced damage is minimized (Nikaido and Johnson, 2000).

Internal coordination occurs when the clock regulates the timing of internal events relative to one another such that the timing of these events is useful even in constant conditions (Woelfle et al., 2004). Internal coordination provides the benefit of allowing incompatible physiological processes to occur at separate temporal or spatial points in order to produce optimal effects of a biological event. Various species of cyanobacteria, for example, produce cellular reactions in different locations or have different timing of peak rhythmicity for incompatible functions (Mitsui et al., 1986).

Examples and Properties of Daily Oscillations

Daily oscillations can be easily observed in many organisms. Locomotor activity in many animals, for example, contains an active and quiescent phase which repeats every 24 hours (Stephan and Zucker, 1972). Heliotropic plants such as members of the Fabaceae and Malvaceae families, open their leaves toward the sunlight during the daytime to maximize photosynthetic activity, then reorient away during the night (Ehleringer and Forseth, 1980). Similarly, flowers such as morning glories will often flower during the daytime and close at night (Lumsden and Furuya, 1986). Johnson et al. (1985) showed that subcellular scintillons of Gonyaulax polyedra dinoflagellates exhibit daily rhythmicity of bioluminescence. In mammals, various physiological processes show cyclic fluctuations including body temperature, blood pressure, immune function and metabolism (Keller et al., 2009; Sukumaran et al., 2010; Warren et al., 1993). Additionally, circadian rhythms are present in cellular processes such as mitosis and expression levels of many enzymes and hormones (Kang et al., 2008; Foulkes et al., 1996; Saito et al., 1975).

There are four properties that are observed in a circadian rhythm:

1) The rhythm has a periodicity of approximately twenty four hours
2) It is endogenously generated
3) It may be entrained by environmental cues within a periodicity close to twenty four hours (~23 to 25 hours)

4) It is temperature compensated, such that varying ambient temperature does not affect the period of the rhythm

(Pittendrigh and Daan, 1976)

Endogenous Rhythms and Entrainment

An endogenous rhythm in the absence of a repeated environmental cue exhibits a self-sustained periodicity (τ) of approximately 24 hours. Circadian Time (CT) is used to refer to a given time during free running where CT0 corresponds to activity onset in diurnal organisms, and CT12 in nocturnal organisms (Pittendrigh and Daan, 1976). Entrainment occurs when a repeated cue (called a Zeitgeber, German for “time giver”) provides temporal information that is sufficient to cause an organism to shift its phase and subsequent cycles with respect to exposure to the Zeitgeber. Light serves as a Zeitgeber by providing an environmental cue for the organism to be able to tell the time of day. Standard laboratory lighting conditions consist of twelve hours of light followed by twelve hours of dark (12:12 LD). In these conditions, ZT0 (Zeitgeber Time 0) serves as the indicator of the beginning of the light phase (Roenneberg et al., 2003). If an organism is diurnal, the beginning this light phase of an LD cycle (photophase) will initiate activity onset, whereas the dark phase of an LD cycle (scotophase) will mark activity onset for a nocturnal organism. Such parametric entrainment causes phase-shifting primarily due to the duration or intensity of a Zeitgeber, but the organism does not require exposure to light for an entire twelve hours in order to phase shift. Non-parametric entrainment can cause a phase-shift that occurs primarily due to the timing of the environmental cue. For example, a brief pulse of light at a certain time in DD (for nocturnal organisms, during subjective night) can also result in phase shifting (Figure 1.1 A-C). These timed light pulses which simulate dawn or dusk are known as a skeleton photoperiod. Phase advances (activity onset begins slightly earlier) and phase delays (activity onset begins slightly later) that occur due to a certain stimulus may be plotted over the course of a day to create a Phase Response Curve (PRC). Photic PRCs show the greatest effect of a light pulse at the beginning or end of subjective night (Figure 1.1 D) (Daan and Pittendrigh, 1976).

The Mammalian Basis of Circadian Rhythms

The currently accepted model of the physiological basis of circadian rhythms involves relaying environmental information, an input (light, for example), and this input is received by a pacemaker. The
pacemaker will then register this information and influence other oscillators accordingly, including peripheral oscillators to result in an observable rhythm, or “output”. In mammals, the suprachiasmatic nuclei (SCN) are responsible for maintaining overt rhythmic behavior (Albrecht and Eichele, 2003). The SCN are located bilateral to the third ventricle in the hypothalamus, dorsal to the chiasm of the optic nerves (Cassone et al., 1988). The SCN receive photic input as light meets the intrinsically photoreceptive ganglion cells of the retina and travels along the retinohypothalamic tract (RHT) to the SCN within the brain (Gooley et al., 2001). The SCN act as a pacemaker for peripheral oscillators that reside in every cell of an organism, though the mechanisms by which it relays temporal information to the periphery currently remain poorly understood (Guo et al., 2006).

**Properties of The SCN**

Approximately 20,000 putative cellular clocks compose the SCN and these individual neurons constituting the suprachiasmatic nuclei exhibit independent rhythms of firing rate and gene expression. Though slight interspecies variation exists among mammals, the SCN are described as being comprised of a dorsomedial shell and the ventrolateral core, each of which is marked by distinct cell types. The shell contains arginine vasopressin (AVP)-expressing cells. There are four groups of cells that exist within the core. Core cells express either vasoactive intestinal polypeptide (VIP), gastrin-releasing peptide (GRP), or calbindin (CalB) or are referred to as cap cells (Antle and Silver, 2005; Moore et al., 2002). Webb et al. (2009) showed that while individual SCN cells may express different neuropeptides, VIP cells in the ventral SCN are no more intrinsically rhythmic than the AVP neurons in the dorsomedial SCN. When SCN neurons are kept isolated in dispersed culture, most single neurons will not express PER2 rhythmically. Neurons that do remain rhythmic are no more likely to express AVP or VIP than those that are arrhythmic. Therefore the SCN, though heterogenous in arrangement and function of individual cells that compose the structure, do not have a specialized group of self-sustained pacemaker cells. Rather, while it is possible that self-sustained individual pacemaker cells do exist, such cells are unable to be categorized by peptide expression or location. It is imperative for individual neurons within the SCN network to remain coupled, because when SCN neurons are isolated, cellular desynchrony occurs (Webb et al., 2009). From this it is clear that intercellular communication is necessary to keep individual neurons in an SCN network stable and synchronous, though the responsible factors remain unknown.

Several possibilities have been proposed in an attempt to determine what gives the pacemaker its pacemaker qualities; one of the strongest candidates is vasoactive intestinal polypeptide. Mice with
a deficiency of VIP or its receptor (VPAC2) display disrupted locomotor rhythms in constant conditions. Half of all individual SCN neurons from VIP or VPAC2 null mice are arrhythmic in dispersed culture (Aton et al., 2005). While VIP is important in maintaining SCN synchrony, other factors are likely involved in the coupling process as well; the individual neurons in the SCN must oscillate as part of a neural network in order to produce coherent rhythmic output (Colwell, 2005).

**Lesions and Rescue**

Electrolytic ablation of the SCN results in abolished overt rhythmicity (Stephan and Zucker, 1972). The dimensions of the SCN in the house mouse, *Mus musculus* are $241 \pm 7 \mu m$ in mediolateral diameter, $261 \pm 7 \mu m$ in dorsoventral diameter, and $780 \pm 28 \mu m$ in rostrocaudal length (Cassone et al., 1988). Moore and Lenn (1972) discovered the RHT by means of tract-tracing in rodents while researching visual activity and regulation of pineal monoamine metabolism. This trace showing the novel connection between the eye and the hypothalamus prompted Moore et al. as well as Stephan and Zucker to show the SCN are a site in the pathway which relays photic information to the circadian rhythm pacemaker. Moore and Eichler (1972) as well as Stephan and Zucker (1972) performed electrolytic lesions of the SCN in rats. The animals became arrhythmic, showing that the SCN presumably were the circadian pacemaker. Lehman et al. (1987) in part established the role of the SCN by rescuing the arrhythmic phenotype. Adult hamsters that received an SCN lesion surgery displayed restored locomotor rhythmicity following brain graph implantations of fetal SCN brain tissue.

**Food As A Zeitgeber**

Light is the primary, but not the only Zeitgeber, for an organism. Food is able to serve as a non-photonic Zeitgeber during restricted food availability. During a restricted feeding (RF) regimen, the organism is allowed food for only a few hours at the same time each day, with ZT0 designating the time of food presentation. Locomotor activity in most laboratory rodents will phase shift after a few days such that Food Anticipatory Activity (FAA) begins several hours just prior to food presentation. FAA persists for several days when food is withheld following RF, demonstrating that an endogenous mechanism drives this anticipatory behavior (Figure 1.2). The proposed endogenous means by which this occurs is referred to as the Food Entrainable Oscillator (FEO) (Stephan, 2002). The anatomical location of this oscillator has not been identified, but many experiments have better defined the properties of this food entrainment.
Surprisingly, the highly predatory kowari responds to light and food zeitgebers in a similar hierarchy as standard laboratory rodents. *Dasyuroides byrnei*, more commonly known as the kowari, is a predatory marsupial. Being predatory, the kowari was proposed to have a periodism similar to its mammalian, avian or insect prey, and may therefore respond more strongly to a timed feeding regimen than standard laboratory rodents. Kennedy *et al.* (1991) showed that this was not the case in these predatory marsupials. Kowaris did not demonstrate entrainment of free running locomotor rhythms in DD during restricted feeding, nor did the RF schedule take precedence to circadian activity rhythms in 12:12LD. Some, but not all, of the animals showed FAA. This ability of RF to uncouple the SCN from the FEO has been observed in parameters aside from locomotor activity. SCN firing rate and expression of circadian genes within the SCN remain entrained to a light-dark cycle, whereas the circadian genes in peripheral organs phase shift relative to food availability (Damiola *et al.*, 2000). Only in rare circumstances where rhythmicity is perturbed such as in constant light (Novakova *et al.*, 2011), or hypocaloric conditions (Mendoza *et al.*, 2005), has it been reported that circadian genes within the SCN phase shift relative to a feeding regimen. Though both light and food are able to serve as Zeitgebers, it is clear that the SCN and the FEO are spatially distinct oscillators. Comperatore and Stephan (1990) showed that mice that received an SCN ablation continued to show food anticipatory rhythms (Figure 1.2).

Searching for the FEO in other central and peripheral locations aside from the SCN has also been inconclusive. FAA was also not abolished by several other hypothalamic lesions, including lesions of the ventromedial, paraventricular, or lateral hypothalamic nuclei (Mistlberger and Rusak, 1988). Ablation of extrahypothalamic locations including hippocampus, nucleus accumbens, amygdala, neocortex and several brainstem regions such as the nucleus of the solitary tract also fail to abolish food anticipatory rhythms (Mistlberger, 2011; Mistlberger and Mumby, 1992). In addition, subdiaphragmatic vagotomy (Comperatore *et al.*, 1990) and capsaicin-induced visceral deafferentation had little effect on anticipatory wheel running during RF (Davidson and Comperatore, 1998). While this showed that neither ablation of the SCN nor peripheral neural signals are essential for FAA during RF, other studies noted a correlation between the digestive system and anticipation of food presentation during timed feeding.

Comperatore and Stephan (1987) showed that duodenal activity will entrain to an RF regimen as seen by irregular contractions (ICs) that occur 3-5 hours preceding food access. During food deprivation,
distribution of ICs showed circadian variation, suggesting a circadian mechanism drove these ICs which are present in the absence of a Zeitgeber. Davidson and Stephan (1999) showed that, though the concentrations of plasma glucose and insulin remained nearly constant before and during FAA, the concentration of glucagon decreases during FAA as compared to just prior to FAA. Challet et al., (1998) also focused upon the role of glucose in food-related activity rhythms. Mice with lesions of glucose-sensitive neurons created by gold-thioglucose injection that were fed a hypocaloric diet, did not phase-shift by one hour in DD as compared to control animals fed a hypocaloric diet. It is possible that glucose-sensitive neurons are involved in regulating circadian rhythmicity during calorie restriction, providing another link of metabolism and the digestive system to behavioral rhythmicity. Stephan and Davidson, (1998) showed rats’ activity phase shifted with respect to glucose presented at the same time daily, but did not phase shift when presented with the artificial sweetener saccharin, nor to certain lipids such as mineral or vegetable oils. A phase shift due to glucose but not saccharin, showed that nutritive value of the food was a crucial factor in determining whether the animal would phase-shift. Indeed, mice fed a palatable non-nutritive mash do not show FAA, in contrast to those mice which were fed a palatable nutrient-rich mash (Mistlberger and Rusak, 1987). Nutritive content and not gastric distension, appears responsible for this effect, demonstrated in a study (Stephan, 1997) in which mice were fed the same quantity of nutritive chow mixed with varying amounts of non-nutritive cellulose bulk. The cellulose bulk had little to no effect on the phase resetting of the FEO, indicating that caloric content of the meal served as the Zeitgeber.

This is not to say that the satiating nature of the food does not play a role in food entrainment, however. Mendoza et al. (2010) showed the behavioral rhythms of mice entrained to a palatable snack, suggesting that feedback to the SCN occurs as a result of the hedonic qualities of palatable food. This relay of signals to the SCN may be due to dopaminergic neurons involved in reward and motivation-related behaviors, such as those cells composing the mesolimbic pathway from the ventral tegmental area to the nucleus accumbens (Kelley and Berridge, 2002).

**Clock Genes and the GIT**

Within each cell composing central and peripheral tissue, there is a molecular means by which keeping time occurs. The mammalian mechanism is a molecular feedback loop composed of both positive and negative elements. Positive elements such as CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain and Muscle Arnt-Like Protein 1) heterodimerize and enter the nucleus to
promote transcription of several genes including the period genes (per1, 2 and 3) and the cryptochrome genes (cry 1 and 2) (Bell-Pederson et al., 2005). This occurs when proteins containing a basic helix-loop-helix structural motif bind to E-boxes within a promotor containing a canonical sequence (CACGTG) and promotes transcription (Albrecht and Eichele, 2003). Once translated into proteins, PER and CRY form a heterodimer that is able to inhibit the transcription-promoting properties of BMAL1-CLOCK. Other players exist in this molecular clockwork mechanism, including two nuclear receptors Rev-erba and Retinoic Acid Orphan Receptor-alpha (ROR-α), which are also transcriptional targets of the BMAL1-CLOCK complex, and in turn, are able to inhibit and promote bmal1 transcription respectively (Bell-Pederson et al., 2005). Post-translational modifications also occur within this loop to maintain optimal rhythmic function, such as phosphorylation of PER by caseine kinase 1 epsilon, which promotes degradation of the protein (Albrecht and Eichele, 2003).

Clock genes are rhythmically expressed in the gastrointestinal tract (GIT) and rhythmicity of these genes is fundamental to maintaining circadian GI function. Bmal1, per1, per2, per3, cry1 and cry2 each show a rhythmic daily profile of mRNA transcription in murine colonic tissue. Expression levels of PER2 and BMAL1 are rhythmic over 24 hours in the myenteric plexus and colonic epithelium (Hoogerwerf et al., 2007). Clock genes are also expressed in human colonic crypts (Pardini et al., 2005), and epithelial cells and within the myenteric plexus (Hoogerwerf, 2010), showing that similar studies in rodents are relevant to human health and physiology. Wildtype mice exhibit circadian variation in intracolonic pressure, which is regulated by clock genes (Hoogerwerf et al., 2010) and though intestinal motility in mice is not necessarily the same as in any given mammal, clock genes are believed to be involved in both cases. Kumar et al. (1986) showed sheep circadian intestinal motility decreases during the night and increases during the day. Feeding disrupts this peristalsis, referred to as the migratory motor complex (MMC), in many mammals, but not in ruminants. Ruminants exhibit circadian MMC function even when fed during the evening, suggesting these rhythms are not due to intraluminal content alone but may be due in part to clock gene oscillation (Bron and Furness, 2009). Exposing mice to a RF regimen shifts clock gene expression in GI tissue relative to food availability (Hoogerwerf et al., 2007). (RF does not cause a clock gene expression shift within the central clock, however, consistent with the concept that the SCN and oscillators involved in food entrainment are distinct oscillators).

Notably, particular clock genes are responsible for circadian colonic motility as noted by studies on period double-knockout mice. Per1 per2 double-knockout mice do not display day-night differences in stool output, intracolonic pressure or response to acetylcholine (Hoogerwerf et al., 2010). Pan and
Hussian (2009) showed that Clock plays several important roles in intestinal metabolism. Clock mutant (Clk$^{mt/mt}$) mice show peak expression of clock genes in intestinal tissue that more closely resembles the postprandial peak observed in the liver rather than intestine, suggesting that Clock is involved in the circadian clock gene expression rhythms of intestinal tissue. Clk$^{mt/mt}$ mice also do not show circadian expression of nutrient transport proteins such as certain glucose transporters (GLUT2, GLUT5) compared to wildtype controls. This indicates the presence of Clock is of higher value to regulate these proteins than is the presence of food within the gastrointestinal lumen. Rats, even when rendered behaviorally arrhythmic by constant light, displayed impaired, but not abolished, clock gene ($per1$, $per2$, $bmal1$ and $rev-erba$) rhythmicity in the liver, duodenum and colon. This was particularly noticeable in the duodenum, where rhythmicity was preserved in all clock genes studied (Polidarova $et$ $al$. , 2011).

It is important to recognize that while clock genes are expressed rhythmically in intestinal tissue and are necessary for rhythmic activity, they are not the only genes that are involved in circadian processes in peripheral tissue. Eight to ten percent of genes in peripheral organs are under clock control. Within the distal colon, out of the 34,000 colonic transcripts analyzed by microarray, 1,248 (3.7%) were rhythmic. When corrected for gene redundancy or unclassified transcripts, 906 unique and classified genes were identified. Of these, approximately 7% (62/906) are associated with development of colorectal cancer and 1.8% (18/906) are involved in gastrointestinal functions such as motility or secretion (VIP, CFTR). Though it remains to be determined, it is possible that these rhythmic genes are under regulation of the canonical clock genes (Hoogerwerf $et$ $al$. , 2008).

**GIT Rhythms Ex Vivo**

Gastrointestinal circadian rhythms can be observed ex vivo in tissue, cellular and molecular function. Hoogerwerf $et$ $al$. (2010) showed that distal colonic rings in culture from wildtype mice exhibit a significant day-night difference in response to acetylcholine. Cells within the alimentary canal undergo circadian proliferation. Esophageal, gastric, duodenal and jejunal tissue display rhythms that vary in amplitude, but each display the lowest daily value of DNA synthesis near transition from light to darkness (Scheving, 2000). Many tissue types in the GIT, such as colonic, gastric and hepatic tissue excised from $Per1$-luciferase transgenic rats all exhibit circadian $per1$ expression in constant darkness (Davidson $et$ $al$. , 2003). These studies show that extra-SCN oscillators residing in colonic tissue are capable of time-keeping mechanisms, and are able to function semi-independently of the central pacemaker.
**Experimental Aims**

Though research has previously established the expression and roles of several clock genes in the GI tract, multiple questions concerning circadian influence in GI rhythms still exist. The research above has made it clear that the GIT contains oscillators within the cells of its tissue that have endogenous rhythms and can entrain to certain environmental cues. Our research aims to discern the extent of influence that the central pacemaker has upon the GI clock. Additionally, if communication does exist between the SCN and tissue composing the GIT, it is of interest by which means this interaction occurs. Colonic motility, being an autonomic process, raises the obvious potential of being regulated, at least in part, by the autonomic nervous system. Disrupting parasympathetic signaling by subdiaphragmatic vagotomy has no effect on clock gene expression (Hoogerwerf et al., 2007), making the sympathetic nervous system (SNS) an apparent option to consider. With regard to this, an aim of the project is to determine what involvement the SNS has in maintaining circadian colonic motility.
Figure 1.1
Entrainment and Phase Shifts

A: Illustrated representation of an actogram demonstrating rhythmic locomotor activity of a nocturnal organism. White bars indicate subjective day and dark bars indicate subjective night during a 12:12 LD cycle. DD (grey bar) causes the organism to free run. Exposure to a light pulse during previous subjective day has no effect on the phase of free running rhythms. B: DD causes the organism to free run. A light pulse shortly following activity onset results in a phase delay of activity. C: Exposure to a light pulse toward activity offset during DD results in a phase advance. D: A Phase Response Curve indicating phase shifts caused by the time of exposure to the light pulses in panels A-C.

Modified from *Physiology of Circadian Entrainment* Golombek and Rosenstein, 2010.
Figure 1.2
Food Anticipatory Activity

A: Activity of an individual rhythmic rat during 12:12 LD. FAA begins during RF several hours prior to food presentation. This activity persists during food deprivation (FD). B: Activity of an individual rat with an SCN-lesion is arrhythmic when food is available *ad libitum*. During restricted feeding, the rodent demonstrates FAA which persists during FD.

From *Neurobiology of Food Anticipatory Circadian Rhythms* Mistlberger, 2011.
Introduction for Chapter II

Several studies have made it clear that the gastrointestinal tract itself contains a clock. GIT tissues exhibit rhythmic transcript (Hoogerwerf et al., 2008) and protein rhythms, and clock gene expression within the colon will entrain to food availability (Hoogerwerf et al., 2007). While the GIT harbors a clock within its tissues, it is the SCN that are responsible for overt rhythmicity in mammals since surgical ablation of this structure results in behavioral arrhythmicity (Stephan and Zucker, 1972). Though the SCN clearly play into the generation and maintenance of physiological rhythms in this way, it was previously unknown to what extent the central pacemaker regulates circadian gastrointestinal motility rhythms. Other studies, such as that of Polidarova et al. (2011), studied the effect of the SCN on daily GIT clock gene expression by perturbation of the pacemaker by exposure to constant light. Our study examined the role of the SCN in GIT function using a more direct method of SCN disruption. We ablated the SCN by electrolytic lesion surgery and recorded stool weight, number, food consumption and locomotor activity from each animal over three days in LD, DD and during a timed feeding regimen.

A. Methods

1. Animals

All in vivo experiments utilized male wild-type Sv/129 mice (Jackson Laboratory, Bar Harbor ME). Animals were treated in accordance with the University of Kentucky and IACUC guidelines.

2. Procedure

Male mice (n=18) were kept in LD (150 lux illuminance) for 14-21 days before receiving experimental or control surgeries (see below). The animals were then allowed to recover in LD for 14 days after which food and stool collection (see below) was conducted, and data were recorded every four hours for 3 days. Mice were allotted a recovery period in DD for 7 days, after which the animals remained in DD for 3 additional days. During these three days, food and stool collections were conducted every four hours in reference to individual activity onset (Circadian Time (CT) 12). Mice remained in DD for another 7 days for recovery. All mice were then subjected to a restricted feeding (RF) regimen while still in DD for 10 days. Food and stool collections were conducted during the final 3 days of RF. Animals were given another 7-day DD recovery period. All
observations and manipulations of animals in DD were conducted with the aid of Night Owl (Optics Planet) infrared binocular viewers.

3. Locomotor Activity

Mice 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 from Minimitter (Sun River, OR), VitalView software V 4.1 and analyzed with Actiview software (Mini Mitter Company Inc. Sun River, OR).

4. Surgery

Mice were anesthetized with 80 mg ketamine and 20 mg xylazine/kg. 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 (Franklin and Paxinos, 3rd Ed.) Electrolytic lesions of the SCN (AP 5.40 + 9 cm, ML 2.60 + 3 cm bilaterally, DV 3.00 + 4 mm) were produced using the custom made electrode connected to a stimulator (SD9K Square Pulse Model, Astro-Med Inc.) set to 50V. Current was applied for 20 seconds, followed by a 10 second pause and 10 additional seconds of stimulation. Mice that received current to regions of the brain other than the SCN were considered animals that received a sham operation. Sham animals were verified upon recovery from surgery by rhythmic locomotor activity records. The surgical site was held closed using a 9mm wound closing kit (Stoelting, Wood Dale IL). The lesion site was later verified by immunohistochemical staining on histological sections.

5. Food Consumption and Stool Measurement

During periods of gastrointestinal data collection, the cage bottoms were lined with one layer of Versidry lab soaker material (VWR International, Philadelphia PA). Food (2018 Teklad Global 18% Protein Rodent Diet, Teklad Diets, Madison WI) weight was measured before and after every four hour time-point during the three day collections, with the exception of the timed feeding experiment during which food consumption was measured during the four hour time-point daily over which food was available. At the end of every four-hour time-point, stools were collected, counted and weighed. Stool weight and stool number for each mouse was 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.
6. Restricted Feeding

The RF protocol was similar to that employed by Gooley et al. (2006). Mice were given access to food from Zeitgeber Time (ZT) 0 (12:00 pm or time of previous lights on) to ZT4 (4:00 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), and during the following three days, food consumption and stool output was measured.

7. Immunohistochemical Staining

To ascertain the completeness of SCN lesion, we visualized immunoreactivity for vasoactive intestinal polypeptide (VIP) in the brain, because VIP marks the presence of SCN tissue. 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. Using a cryostat (Leica CM 1850), the tissue was sliced into 40μm coronal sections which were placed into PBS. Sections were washed thoroughly with PBS and incubated for 15 minutes in H₂O₂ treatment (1.2 ml 30% hydrogen peroxide in 100 ml ddH₂O) before washing again. Sections were left to incubate in PBSGT (PBS, Goat serum, Triton X) for 30 minutes at room temperature, then incubated at 4°C for 48 hours in a 1:1,000 dilution of primary antiserum (Rabbit α-VIP; Bachem, Santa Cruz) in PBSGT. Sections were thoroughly washed with PBS and incubated in a 1:500 dilution of secondary antisera (Goat α-Rabbit; Vector Labs Inc) in PBSGT at room temperature for 2 hours. After washing, sections were exposed to avidin/biotin (Vectastain ABC Kit, Vector Labs Inc) in PBSGT for 90 minutes at room temperature and washed again. Sections were lastly incubated in diaminobenzidine (DAB)/Nickel/H₂O₂ solution until immunohistochemical staining became clearly visible (about 3 minutes). This solution was composed of 100mg/50ml NiCl in Tris buffer added to 100mg/50ml DAB in Tris Buffer and 50μl 30% H₂O₂. The sections were washed and transferred to slides to dry overnight. The following day, the slides were dehydrated in ethanol and cleared in xylenes prior to cover-slipping.

8. Statistical Analysis

In vivo. Significance between peaks and troughs of rhythmic food consumption and stool production during ad libitum food and RF was determined by use of one- or two-way repeated measures analysis of variance (RM ANOVA) which was followed by a Student-Newman-Keuls post hoc test using SigmaStat 3.5 software. Rhythmicity (p<.05) was determined using CircWave V1.4 (R. A. Hut). Amplitude between sham groups was assessed using a Student’s t-test in SigmaStat 3.5.
All tests were performed at a 95% confidence level (α=.05).

B. Results

1. Locomotor Activity

The locomotor activity of representative mice throughout the various light cycles and feeding regimens can be seen in the actograms in Figure 2.1. As expected, mice in LD that received a sham operation (Figure 2.1A) retained circadian locomotor rhythms (Figure 2.1C black arrow), whereas ablation of the SCN (Figure 2.1B) resulted in locomotor arrhythmicity (Figure 2.1D black arrow). In DD, shams exhibited free-running rhythms of wheel-running activity in which they expressed endogenous periods of 23.43 + 0.27 hrs (Figure 2.1C grey arrow), and lesioned animals continued to be arrhythmic (Figure 2.1D grey arrow). All animals exhibited food anticipatory activity (FAA) preceding food presentation during RF (Figure 2.1C black boxes, Figure 2.1D black boxes).

2. Surgery

All SCN lesions resulted in complete ablation of the SCN. All sham lesions were located in tissue surrounding the SCN or were incomplete SCN lesions (Figure 2.2).

3. Food Consumption and Stool Measurement

Figure 2.3 shows feeding and defecation measurements of both SCNX and sham mice. In shams in LD, significant differences exist between the peaks and troughs, though the lesioned animals were arrhythmic (Figure 2.3A, 2.3B and 2.3C). In constant darkness, sham mice expressed rhythmic locomotor activity, feeding and stool production. SCNX animals expressed arrhythmic feeding, stool weight and number in both LD and DD (Figure 2.3D, 2.3E and 2.3F).

4. Restricted Feeding

During RF in DD, two-way ANOVAs reported no significant differences between the food consumption of SCNX and sham groups on any day during RF (Figure 2.4A). Both SCNX and sham animals showed significant rhythms in amplitude of colonic motility rhythms, (Figure 2.4B, Figure 2.4C) which peak just following the window of food availability.

5. Immunohistochemical Staining

Vasoactive intestinal polypeptide is a protein that is expressed prominently in the SCN, making
the bilateral nuclei clearly visible following immunohistochemical staining with VIP antisera. Complete SCN lesions showed no VIP immunoreactivity in the SCN region of the hypothalamic tissues (Figure 2.1A and 2.1B).

C. Discussion

There is little doubt that the mammalian gastrointestinal system contains a circadian clock capable of entraining to and anticipation of the availability of food, and this clock may bear direct relevance to gastrointestinal health and disease (Hoogerwerf, 2009). First, between 0.6 to 3.7% of the colonic transcriptome is expressed rhythmically on a circadian basis. Rhythmic transcripts include known signals involved in intestinal motility, inflammation, cell signaling and cell-cycle, including several linked to colorectal cancer (Hoogerwerf et al., 2008). Secondly, the myenteric plexus and many cell types of the endothelium express circadian patterns of mRNA and protein levels corresponding to known “canonical" clock genes both 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 (Hoogerwerf et al., 2007; Sladek et al., 2007). Further, 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 responses to exogenous acetylcholine (Hoogerwerf et al., 2010).

The circadian molecular clockworks are necessary for the expression of circadian rhythms of intestinal function. Knockout of either per1 or per2 alters the period and decreases the amplitude of many measures of colonic motility, but double knockout 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 (Hoogerwerf et al., 2010).

However, the overwhelming evidence for a gastrointestinal 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 all abolished by SCNX in both LD and DD. Similarly, Polidorova et al. (2011) have 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. Secondly, timed feeding reinstates circadian patterns of feeding and defecation. These measures of GIT activity also anticipate the presence of a meal. Similarly, Polidorova et al. (2011) also show that rhythms of GIT clock gene expression in rats are restored with timed feeding. Finally, colonic tissue retains rhythmic PER2 expression *ex vivo* (Chapter IV).

How can we reconcile the fact that SCNX 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 approximately 10 days. It is likely that restoration of rhythmicity by the GIT requires either stimulation from a meal itself and/or circadian signals from the SCN.
Figure 2.1
Light Cycles and Feeding Regimens of Operated Animals

(A) Intact bilateral suprachiasmatic nuclei immunostained for VIP in a sham operated animal. (B) A SCNX mouse in which the SCN has been electrolytically lesioned. (C) Actogram showing the rhythmic circadian locomotor activity of a sham operated animal. (D) Actogram showing arrhythmic locomotor behavior of a SCNX animal. Arrows on actograms indicate the beginning of a new light cycle (black arrows, LD; grey arrows, DD). Boxes on actograms in panels C and D indicate the four hours of mid-day food availability during RF.
Figure 2.2
Locations of Individual Sham and SCNX Lesions

Top: Locations of sham lesions of individual mice (n=8) are denoted by grey ovals on Figure 37 of the Mouse Brain In Stereotaxic Coordinates (Franklin and Paxinos, 3rd Edition). Bottom: Size and locations of SCNX lesions in individual mice (n=10). The location of the suprachiasmatic nuclei (abbreviated as SCh) is visible on the lower center of each atlas figure.
Figure 2.3
Food consumption and stool output are rhythmic in sham (n=8), and arrhythmic in SCN-ablated animals (n=10) during ad lib food availability.

(A) Measurements of food consumption in SCNX and shams during a three day time series in LD. (B) Stool weight of operated animals during LD. (C) Stool number of operated animals during LD. (D) Food consumption of SCNX and shams during three days of constant darkness. (E) Stool weight of SCNX and shams in DD. (F) Stool number measurements of SCNX and sham animals during DD. For determination of circadian rhythmicity of a data set, peak values were compared to the corresponding following and/or preceding trough by use of one-way ANOVA. Significance in shams is denoted by *P<.05 in panels A-F. No significance was reported in SCNX animals. Results are expressed as the mean ± SEM.
Figure 2.4
Colonic motility in SCNX and sham animals directly entrains to food availability.

Food was available for four hours daily (12:00pm-4:00pm) during ZT0-ZT4, as indicated by the vertical grey bars in panels B and C. Bars along the x-axis in panels B and C represent the previous LD cycle. (A) Measurements of food consumption during each day of RF in SCNX and sham mice. (B) Stool weight during RF. (C) Stool number during RF. Significance is denoted by *P<.05 for shams or **P<.05 for SCNX data in panels B and C. Two-Way ANOVA showed no significant difference between groups in panel A. Results are expressed as the mean ± SEM.
CHAPTER III: *In Vivo* Experiments- Role of the SNS in GIT Rhythms

Introduction for Chapter III

The results from Chapter II indicate the necessity of the SCN to maintain rhythmic feeding, locomotor and colonic activity during *ad libitum* conditions. This shows that the SCN communicates by some means with the oscillators in the GIT, but like the relay mechanisms of most central-to-peripheral temporal information, the exact means by which it occurs was unknown. GIT peristalsis, being an autonomic function, may rely on ANS regulation by parasympathetic or sympathetic signaling. A previous study by Hoogerwerf *et al.* (2007) demonstrated that gastric clock gene expression was not affected by vagotomy, suggesting that this clock gene expression is not mediated through the vagal nerve. This was particularly relevant following a study conducted by Hoogerwerf *et al.* (2010) which showed *per1/per2*KO mice exhibit arrhythmic colonic motility, indicating circadian colonic motility is regulated by the clock genes *period1* and *period2*. For this reason our study focused on examining the role of day-night differences in sympathetic tone in the regulation of GIT rhythmicity. We ablated the SNS using three total 50mg/kg doses of the neurotoxin 6-hydroxydopamine in half of the SCNX animals and half of the sham animals from Chapter II. We then measured food consumption, stool weight and number and locomotor activity for 3 days in LD, DD and during RF, as described in Chapter II.

A. Methods

1. Animals

   All *in vivo* experiments utilized male wild-type Sv/129 mice (Jackson Laboratory, Bar Harbor ME). Animals were treated in accordance with the University of Kentucky and IACUC guidelines.

2. Procedure

   Half of the animals that received an experimental surgery (see Chapter II) and half of the mice that received a control surgery, were injected intraperitoneally with 6-hydroxydopamine (see below). 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 in Chapter II. As described above, food and stool measurements were performed for 3 days every four hours and one week recovery in DD was allotted following each change in light cycles or feeding.
regimens. Following the second RF regimen, mice were released into DD until being sacrificed for organ harvest. All observations and manipulations of animals in DD were conducted with the aid of Night Owl (Optics Planet) infrared binocular viewers.

3. Locomotor Activity
Male mice (n=18) were placed in polycarbonate cages, and locomotor activity was recorded continuously as specified in Chapter II.

4. Intraperitoneal Drug Administration
Fifty mg/kg 6-hydroxydopamine (6-OHDA; Sigma, St. Louis MO) in 0.2% ascorbate is a neurotoxin that causes the degeneration of sympathetic terminals in the peripheral sympathetic nervous system (Pawlik et al., 2011). It is an accepted way to determine whether sympathetic activity mediates downstream processes. 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 the animals from SCNX and sham groups received 6-OHDA treatment. Experiments were performed during LD, DD and RF, as described previously.

5. Food Consumption and Stool Measurement
Data collection was completed as described in Chapter II.

6. Restricted Feeding
The RF protocol was repeated as described in Chapter II.

7. Immunohistochemical Staining
Tyrosine hydroxylase (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 blood brain barrier (BBB) and enter the brain. TH-immunoreactivity in the brain should not be affected by 6-OHDA, since 6-OHDA typically does not pass the blood brain barrier but should decrease TH-immunoreactivity in the intestines. Following data collection and recovery in DD, all mice were sacrificed by transcardial perfusion with phosphate-buffered saline and 4% paraformaldehyde. A separate set of mice (N=4) were administered either 6-
OHDA or ascorbate (N=2 each), allowed to survive 3 days and then sacrificed as 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 prepared similarly to IHC Methods in Chapter II. Brain tissue was incubated at 4°C for 48 hours in a 1:1,000 dilution of primary antisera (Rabbit α-TH; Santa Cruz) in PBSGT. For TH immunofluorescence, tissues were incubated in a 1:500 dilution of Rabbit α-TH in PBSGT (EMD Millipore, Billerica MA). Sections were thoroughly washed with PBS and incubated in a 1:500 dilution of secondary antisera (Goat α-Rabbit; Vector Labs Inc) in PBSGT at room temperature for 2 hours. For immunofluorescent detection, tissues were incubated in a 1:100 dilution of FITC-Goat α-Rabbit (Vector Laboratories Inc, Burlingame CA).

8. Western Blot

Samples (2mm x 3mm pieces) from proximal colonic tissue were homogenized directly in 0.5mL of Laemmli buffer and boiled for 30 minutes. Protein content from each sample was determined by Nanodrop, and 50μg of individual samples were loaded accordingly. Samples and protein ladder (Precision Plus Protein Dual Color Standards #161-0374, BioRad) were separated on a 10% SDS polyacrylamide gel by SDS-PAGE using 1x Running Buffer (30.28g Tris Base, 144.0g glycine, 10g SDS in 10 L ddH₂O). The gel was then used for protein transfer to a nitrocellulose membrane (Whatman, Dassel Germany) by electroblotting in Transfer buffer (3.03g Tris, 14.4g glycine, 200ml MeOH, 0.05% SDS in 1L ddH₂O). The membrane was then rinsed in PBS for 5 minutes followed by blocking in 5% non-fat dry milk in PBT (PBS with 0.1% Tween-20) for 30 minutes. Rabbit polyclonal IgG α-TH antibody (EMD Millipore Corporation, Billerica MA) was added at a 1:1000 dilution, and left overnight on a rotator at 4°C. The membrane was blocked for 7 minutes in 5% non-fat dry milk in PBT twice, and washed in 1x caseine (Rabbit IgG Vectastain ABC-Amp Kit, Vector Labs Inc) for 7 minutes. The membrane was then incubated in biotinylated α-rabbit IgG (Rabbit IgG Vectastain ABC-Amp Kit, Vector Labs Inc) for 40 minutes. Two 7-minute washes in PBT with 5% milk, and one 7-minute wash in 1x caseine solution were performed. The membrane was incubated for 30 minutes in ABC-Amp solution (Rabbit IgG Vectastain ABC-Amp Kit, Vector Labs Inc) then washed 3 times in 1x caseine for 7 minutes. Equilibration was performed by incubating the membrane in 0.1M Tris buffer (pH 9.5) for 10 minutes. Immunoreactive bands were detected using an alkaline phosphatase substrate staining solution (Alkaline Phosphatase Substrate Kit IV BCIP/NBT, Vector Labs Inc). Blots were scanned and imported into ImageJ (NIH) where individual lanes were quantified as density relative to internal standard. Internal standards of separate blots were normalized to each other for averaging purposes.
9. Statistical Analysis
All tests were performed as in Chapter II.

B. Results

1. Locomotor Activity
Sham mice that received 6-OHDA expressed free-running periods of 23.8 + 0.08 hrs, while those receiving 6-OHDA expressed periods of 23.58 + 0.21 hrs. No statistical differences among these periods were detected. Again, SCNX mice continued to be arrhythmic. All animals exhibited food anticipatory activity (FAA) preceding food presentation during RF (Figure 3.1A black boxes, Figure 3.1B black boxes).

2. Food Consumption and Stool Measurement
Following IP injection of 6-OHDA or ascorbate, food consumption and stool output rhythms of the SCNX animals were still abolished in both LD (Figure 3.2A, Figure 3.2B, Figure 3.2C) and DD (Figure 3.2D, Figure 3.2E Figure 3.2F). Importantly, the sham control animals maintained clear rhythmicity in locomotor activity, feeding (Figure 3.3A) and stool output in LD, while the sham 6-OHDA animals only retained rhythmicity in locomotor activity, feeding activity (Figure 3.3A), but showed attenuated stool output rhythms (Figure 3.3B, Figure 3.3C). This persisted in DD (Figure 3.3D, Figure 3.3E, Figure 3.3F), as determined by a one-way ANOVA, and there was a significant difference (P = .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 shams in Figure 3.3. All sham control rhythms passed CircWave analysis for all three days (Figure 3.3A, Figure 3.3B, Figure 3.3C, Figure 3.3D, Figure 3.3E, Figure 3.3F). Notably, all three days of food consumption in the sham 6-OHDA-treated animals were considered rhythmic by CircWave in LD, but stool weight and stool number rhythms were not (Figure 3.3A, Figure 3.3B, Figure 3.3C). From this, it may be concluded that 6-OHDA-induced sympathectomy specifically attenuates, but does not abolish, circadian colonic rhythmicity.

3. Restricted Feeding
During the second exposure to a RF regimen, there were no significant differences in food consumption between any of the 4 groups according to two-way ANOVA (Figure 3.4A). Similar to the entrainment seen during RF in Figure 2.4, all animals show significant rhythmicity in all stool output
parameters (Figure 3.4B, Figure 3.4C, Figure 3.4D, Figure 3.4E).

4. Immunohistochemical Staining

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 (Figure 3.5A-D). Both western blot analysis (see below) and immunofluorescent visualization of TH-immunoreactive fibers (Figure 3.5E-F) show that the 6-OHDA treatment decreased or abolished intestinal TH-immunoreactivity.

5. Western Blot

Tyrosine Hydroxylase immunoreactive bands were detected on the nitrocellulose membrane at 60kDa (Figure 3.5G). Bands from 6-OHDA-treated tissues showed a significant decrease in relative density compared to vehicle-treated tissues as analyzed using t-test (p<0.001, n=4 tissues per treatment, Figure 3.5H).

C. Discussion

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, pineal and adrenal glands such that maximal turnover is during the subjective night, when locomotor activity is highest (Brusco et al., 1998), and these rhythms are abolished by SCNX (Warren et al., 1994). 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, SCNX abolishes circadian patterns of heart rate and heart rate variability (Warren et al., 1994). This effect is simulated by chemical sympathectomy through previous treatment with guanethidine. Similarly, SCNX and surgical denervation of sympathetic afferents to the liver abolishes circadian patterns of hepatic gluconeogenesis and several hepatic enzymes and glucocorticoid rhythms (Cialotto et al., 2005, Cialotto et al., 2008, Shibata 2004).

In the present study, 6-OHDA treatment, 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 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 both 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.
Figure 3.1
Locomotor Activity of Shams and SCNX animals which received 6-OHDA injection.

(A) Locomotor activity of a representative sham mouse which received 50 mg/kg 6-OHDA. After injection, the locomotor activity remains rhythmic in LD and DD, and FAA is visible. (B) Locomotor activity of a SCN lesioned animal which received 50 mg/kg 6-OHDA. Following injection, the locomotor activity remains arrhythmic in LD and DD, and FAA develops during restricted food availability. The locomotor activity of operated animals which received ascorbate injections in place of 6-hydroxydopamine can be seen in Figure 2.1C and 2.1D. Arrows on the actograms indicate the start of a new light cycle (black arrows, 12:12LD; grey arrows DD) and boxes indicate four hours of mid-day food availability during RF.
Figure 3.2
SCN-ablation yields arrhythmic food consumption and stool output during *ad lib* food availability in 6-OHDA (n=6) and vehicle-treated (n=4) mice.

(A) SCNX food consumption in LD. (B) SCNX stool weight in LD. (C) SCNX stool number in LD. (D) SCNX food consumption in DD. (E) SCNX stool weight in DD. (F) SCNX stool number in DD. Results are expressed as the mean ± SEM.
Figure 3.3
6-OHDA-treated sham animals show differences in stool output rhythms in contrast to vehicle-treated shams during ad lib conditions.

(A) Food consumption of 6-OHDA-treated shams (n=3) and vehicle-treated shams (n=5). (B) Stool weight of shams in LD. (C) Stool number of shams in LD. (D) Food Consumption of shams during DD. (E) Stool weight of shams during DD. (F) Stool number of shams during DD. Significance is denoted by *P<.05 for sham controls or **P<.05 for sham 6-OHDA-treatment data in panels A-F. Results are expressed as the mean ± SEM.
Figure 3.4
Colonic motility in all treated animals directly entrains to food availability.

Food was available for four hours daily (12:00pm-4:00pm) during ZT0-ZT4, as indicated by the vertical grey bars in panels B-E. (A) Food consumption of each surgery/treatment group. (B) Stool weight of lesioned animals during RF. (C) Stool number of lesioned animals during RF. (D) Stool weight of shams during RF. (E) Stool number of shams during RF. Significance is denoted by *P<.05 for sham controls or ♦P<.05 for sham 6-OHDA-treatment data in panels B-E. Two-way ANOVA showed no significant difference of feeding activity between groups in panel A. Results are expressed as the mean ± SEM.
SCN ablation and 6-OHDA treatment do not decrease the number of TH-expressing neurons within the arcuate nucleus, but 6-OHDA treatment does decrease TH immunoreactivity in the colon.

Representative histological section showing TH immunohistochemical staining of the arcuate nucleus in the brain of (A) a sham ascorbate-treated animal, (B) a sham 6-OHDA-treated animal, (C) an SCNX ascorbate-treated animal, and (D) an SCNX 6-OHDA-treated animal. Representative distal colonic sections showing immunofluorescent detection of TH expressing neurons in (E) ascorbate-treated and (F) 6-OHDA-treated animals. Bars on the bottom right of panels E and F indicate 100μm. (G) Representative western blot showing immunoreactive TH. From left to right, 6-hydroxydopamine-treated tissue samples can be seen in lanes 1 and 2, and ascorbate-treated tissues can be seen in lanes 3 and 4. (H) An average composite of the relative density of experimental and control immunoreactive TH bands of four independent western blots.
CHAPTER IV: Ex Vivo Experiments- PERIOD2 Expression in Colonic Tissue

Introduction for Chapter IV

As discussed in Chapter III, the sympathetic nervous system relays temporal information from the SCN to oscillators within the GIT. This process in part regulates sympathetic tone, resulting in circadian colonic motility patterns. We investigated the specific response of gastrointestinal tissue to pharmacological compounds which affect SNS neurons (6-OHDA and isoproterenol). Tissues excised from mPer2\textsuperscript{LUC} mice express the clock gene period2 and a luciferase reporter as a fusion protein. A photoluminometer records luminescence and these traces and values can then be used to analyze the properties of the rhythm, such as period, amplitude (value from peak to trough) and oscillatory phase. We administered 6-hydroxydopamine by intraperitoneal injection in order to study the result of sympathectomy upon intestinal tissue PER2 rhythms. We administered the beta-adrenergic receptor agonist isoproterenol to intestinal tissues as a “pulse” directly into the culture media since isoproterenol has a brief half-life and could not be administered chronically. This beta-adrenergic agonist was used to study the effects of SNS intestinal plexus stimulation on the GIT PER2 rhythms.

A. Methods

1. Animals

All colonic tissue cultured for ex vivo experiments were excised from mPer2\textsuperscript{LUC} knockin C57/BL6 mice (Yoo et al., 2004) (Gift from Dr. Shin Yamazaki, Vanderbilt University, Nashville TN). Animals were treated in accordance with the University of Kentucky and IACUC guidelines.

2. Tissue Excision And Preparation

Unless otherwise noted, all reagents were purchased from Sigma (St. Louis MO). 5-week old knockin mice were anesthetized with 80mg ketamine and 20mg xylazine / kg body weight. Colonic tissues were excised from the caecum to ~2mm proximal to rectum and placed into ice-cold Kreb’s solution (117mM NaCl, 5mM KCl, 1.2mM MgSO\textsubscript{4}, 25mM NaHCO\textsubscript{3}, 1.2mM NaH\textsubscript{2}PO\textsubscript{4}, 10mM glucose and 2.5mM CaCl\textsubscript{2}; pH 7.6). The distal colon was rinsed in Kreb’s, bisected longitudinally, and cut into two to three, 1 – 3mm\textsuperscript{2} sections. Each piece was placed lumen side up onto sterile mesh in recording media consisting of HEPES buffered DMEM (Vujovic et al., 2008) with 0.1mM luciferin (Biosynth AG, Staad Switzerland) in a 35 mm petri dish and sealed with 40 mm coverglass (Fisher Scientific, Pittsburg PA).
The dishes were placed into a Lumicycle photoluminometer (Actimetrics, Il.) which measured mPER2::LUCIFERASE bioluminescence every 10 minutes. The plates were removed when the oscillations appeared fully damped.

3. 6-Hydroxydopamine

Forty-eight hours prior to tissue excision and culture, mice (n=4 for each treatment) were injected intraperitoneally with either 50mg/kg 6-OHDA or 0.2% ascorbate vehicle. Animals were sacrificed as above, and distal colon pieces were cultured as above and bioluminescence data collected until the tissues showed no discernible rhythmicity.

4. Isoproterenol

Isoproterenol is a β-adrenergic agonist that can mimic the effects of endogenous norepinephrine effects 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 in order to determine the peak phase of PER2 expression. On the third day in culture, the media was replaced with fresh media containing 100 μM isoproterenol or 0.2% ascorbate vehicle at 4, 9, 12, 16 or 21 hours following the previous peak expression. As above, recording continued until tissue rhythms damped to arrhythmicity.

5. Statistical Analysis

Ex vivo. Bioluminescence rhythms were de-trended by applying a low order polynomial to the raw data. The resulting baseline was then subtracted from the dataset (Lumicycle Analysis Software, Actimetrics, Il.). 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 (Wu et al., 2000): \[ \beta_J = \frac{2}{(t-t_0)} \ln \frac{A_{jt}}{A_j}. \] In this equation, the damping coefficient (\( \beta_J \)) is expressed as a ratio of amplitude from the first day of observation (\( A_{jt} \)) over the average amplitude from t until the oscillation becomes desynchronized (t0). \( A_j \) represents the rhythm amplitude prior to decoupling. 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 second and third day 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 pre-pulse phase, and the resulting change in phase assigned a positive or negative value, respectively. Average change in phase (phase response curve; PRC) was then subjected to one-way ANOVA to determine significant differences pre- and post-pulse 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 (α=.05).

B. Results

1. 6-Hydroxydopamine

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 4.1A summarizes the data from this experiment. Control tissues (black bars) had an average period of 24.00 ± 0.02 hrs and damping coefficient of 0.56 ± 0.08 while 6-OHDA-treated tissues (grey bars) oscillated with an average period of 24.14 ± 0.064 with a damping coefficient of 0.47 ± 0.05. Student’s t-test analysis showed significant difference in period (=2.052, p-value<0.05), but no difference in damping coefficient. Representative traces of PER2 expression rhythms from 6-OHDA and control treated tissues in Figure 4.1B highlight the difference in period length and the similar rate of damping.

2. Isoproterenol

To determine if isoproterenol was able to affect PER2 expression rhythms in GI tissues, media of colonic tissue in culture for several days was replaced with media containing isoproterenol or vehicle at different times relative to peak PER2 bioluminescence. Representative traces of PER2 expression in tissues treated with isoproterenol (grey trace) or ascorbate (black trace) can be seen in Figure 4.1C, with the gap between day 4 and day 5 denoting media exchange (grey arrow). Isoproterenol administration resulted in a phase shift that was dependent upon the time of drug administration. Figure 4.1D shows the composite data from 5 differently timed isoproterenol pulses at different phases relative to the previous peak of PER2::LUC bioluminescence. At 4 hours post-peak expression, as PER2 levels were declining, isoproterenol administration caused a phase delay of 2 hours. Five hours later, as PER2 approached nadir, the pulse resulted in a 2-hour advance in phase. The most dramatic phase
shift of 12-hour 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-hour delay 16 hours after peak expression and 2-hour advance at 19 hours post-peak. Vehicle-treated samples showed no phase shift at any time point (Figure 4.1D, grey circles).

C. Discussion

The SCN interact with the 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 the GIT clock is only sensitive to sympathetic input at certain times of day. PER2 expression rhythms are phase-delayed some 12 hours when presented 12 circadian hours following the last PER2 peak, but have little effect or phase-advances the rhythm at other circadian phases. This is not due to effects on myenteric plexus catecholaminergic neurons, because pretreatment of mPer2LUC mice with 6-OHDA has relatively little effect on rhythms of luciferase bioluminescence (Figure 4.1). 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 tyrosine hydroxylase (TH) in cell bodies or known projection areas of catecholamine neurons. Further, there is no effect of 6-OHDA on free-running locomotor rhythms, punctuating the view that the effects of 6-OHDA are peripheral.
6-OHDA has little effect and isoproterenol causes a phase shift in PER2 expression ex vivo.

(A) Period and damping factor in tissues from vehicle treated (n=4) and 6-OHDA (n=4) treated animals. Results are expressed as the mean + SEM. Significance by Student’s t-test is denoted by *P<.05.

(B) Representative traces of baseline subtracted luciferase bioluminescence in counts per second from tissues of vehicle and 6-OHDA-treated animals.

(C) Representative traces of baseline subtracted luciferase bioluminescence in counts per second from tissues treated with a pulse of isoproterenol (grey trace) or vehicle (black trace) solutions. Phase resetting occurs after treatment (grey arrow) in isoproterenol-treated tissues, but not control-treated tissues.

(D) The phase response curve (PRC) generated using PER2 expression data from isoproterenol and vehicle-treated tissues (black and grey circles, respectively). Significance between groups was determined by means of two-way ANOVA, where *P<.005 and + P<.001. Phase shifting occurs depending upon time of isoproterenol treatment in relation to peak PER2 expression.
CHAPTER V: Final Remarks

The most likely scenario for the mechanisms behind gastrointestinal circadian rhythmicity is that circadian oscillators within the SCN, entrained to the LD cycle, influence a circadian change in sympathetic tone by increasing release of post-ganglionic 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 as motility, enzymatic activity, cell signaling and even inflammation (Figure 5.1). However, as is the case with any sympathetically regulated process, other regulators such as stress, changes in blood pressure and temperature fluctuations, sympathetic output can change outside the purview of the clock. Further, the GIT itself can detect the regular presence or absence of food even in the absence of SCN-sympathetic input (Figure 5.1). 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 (Hoogerwerf 2006, Hoogerwerf 2009). Previously, Davidson and colleagues investigated the role of peripheral tissues - including liver and GIT tissues – in FAA during restricted feeding and concluded that the FEO that drives FAA is not located in the periphery, suggesting that further inquiries should focus instead on the CNS (Davidson et al., 2003). The results presented here, while incapable of providing an anatomical 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 gastrointestinal 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.
A model for circadian GIT motility rhythms. The SCN pacemaker relays temporal information by means of signaling by the sympathetic nervous system. These signals will reach the periphery through SNS afferents, some of which innervate GI tissue. Neurons within the plexus of the GIT and/or peripheral oscillators within the cells of this tissue will then regulate GIT motility rhythms accordingly. The FEO and SCN remain coupled during ad libitum food availability (black dashed arrows), though the exact means by which this occurs and is maintained is not known, due largely to the unknown anatomical location of the FEO. Therefore, during ad lib food availability, both the SCN and the FEO influence GIT rhythms. During restricted feeding, however, even in the absence of SCN or SNS input, stool rhythms entrain to timed food availability (grey arrow).
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