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Localization and expression of putative circadian clock transcripts in the brain of the nudibranch *Melibe leonina*

Victoria E. Duback¹, M. Sabrina Pankey¹, Rachel I. Thomas², Taylor L. Huyck², Izhar M. Mbarani², Kyle R. Bernier², Geoffrey M. Cook², Colleen A. O'Dowd¹, James M. Newcomb², and Winsor H. Watson III¹

¹University of New Hampshire, Durham, New Hampshire

²New England College, Henniker, New Hampshire

Abstract

The nudibranch, *Melibe leonina*, expresses a circadian rhythm of locomotion, and we recently determined the sequences of multiple circadian clock transcripts that may play a role in controlling these daily patterns of behavior. In this study, we used these genomics data to help us: 1) identify putative clock neurons using fluorescent *in situ* hybridization (FISH); and 2) determine if there is a daily rhythm of expression of clock transcripts in the *M. leonina* brain, using quantitative PCR. FISH indicated the presence of the clock-related transcripts *clock*, *period*, and *photoreceptive* and *non-photoreceptive cryptochrome* (*pcry* and *npcry*, respectively) in two bilateral neurons in each cerebropleural ganglion and a group of < 10 neurons in the anterolateral region of each pedal ganglion. Double-label experiments confirmed colocalization of all four clock transcripts with each other. Quantitative PCR demonstrated that the genes *clock*, *period*, *pcry* and *npcry* exhibited significant differences in expression levels over 24 hrs. These data suggest that the putative circadian clock network in *M. leonina* consists of a small number of identifiable neurons that express circadian genes with a daily rhythm.

Keywords

Gastropod; circadian rhythm; cryptochrome; biological clock; nudibranch; clock genes

1. Introduction

Many animals exhibit a daily rhythm of activity and tend to be more active either during the day (diurnal), or during the night (nocturnal). In addition, some animals have a crepuscular rhythm, expressing their strongest bouts of activity around sunrise and sunset (Dunlap, 1999;

*corresponding author: Dr. Winsor H. Watson III, Department of Biology, University of New Hampshire, Durham, NH 03824, Tel: 603-862-1629, win@unh.edu.

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Declarations of interest

None

Panda *et al.* 2002). All of these rhythmic patterns are strongly correlated with, and typically synchronized to, environmental cues such as the 24 hr light-dark (LD) cycle (Bunney & Bunney, 2000). Moreover, in the absence of LD cues, most animals continue to show a daily (~ 24 hr) rhythm of activity, indicating the presence of an endogenous circadian clock (Aschoff, 1965). The molecular mechanisms that give rise to these ~ 24 hr clocks have been elucidated in many organisms ranging from cyanobacteria to humans (Takahashi, 1991, 1995; Dunlap, 1999; Young & Kay, 2001; Hastings *et al.* 2007; Allada & Chung, 2010). As a result of these studies, it is now clear that most organisms, from prokaryotes to mammals, share a transcription-translation feedback loop of clock genes and their protein products that are either identical or quite similar in principle.

While we now know a great deal about daily behavioral rhythms and the molecular basis of the clocks underlying these rhythms, less is known about the connection between the molecular clockwork pathways and the neural networks responsible for influencing the expression of specific physiological and behavioral rhythms. Gastropods, due to their large identifiable neurons and stereotyped behaviors, are often suitable model systems for these types of investigations. Therefore, one of our major long-term goals is to develop a nudibranch model system that will facilitate investigations of the interactions between clock neurons and the neural networks controlling specific behaviors, such as swimming in *M. leonina* (Thompson & Watson, 2005; Sakurai *et al.*, 2014).

Daily rhythms of locomotion have been demonstrated in a number of gastropods, including *Aplysia californica* (Kupfermann, 1968; Jacklet, 1972), *Bulla gouldiana* (Block & Davenport, 1982), *Bursatella leachi plei* (Block & Roberts 1981), *Helisoma trivolvis* (Kavaliers, 1981), *Limax maximus* (Sokolove *et al.*, 1977), *Melanoides tuberculata* (Beeston & Morgan, 1979), and *M. leonina* (Newcomb *et al.*, 2004; 2014). Furthermore, the isolated eyes and retinal neurons of some of these species exhibit circadian oscillations of neural activity (Jacklet, 1969; Block & Roberts, 1981; Block & Wallace, 1982; Michel *et al.*, 1993), suggesting that the eyes are the location of at least one clock system. Localization of putative circadian clocks has only been accomplished in two gastropods – *A. californica* and *B. gouldiana*, using immunohistochemistry with antibodies developed to a conserved region of the *Drosophila melanogaster* PERIOD protein (Siwicki *et al.*, 1989). PERIOD-immunoreactive neurons were located in the eyes of each species, as well as in approximately 10 neurons in the cerebral ganglion of *A. californica* (Siwicki *et al.*, 1989). Localization of period transcripts in *B. gouldiana* eyes has since been confirmed with *in situ* hybridization (Constance *et al.*, 2002). In both of these species, *period* oscillates in certain tissues, based on western blots (Siwicki *et al.*, 1989) and *in situ* hybridization (Constance *et al.*, 2002). However, of all of these gastropods, the one with the best-studied central pattern generator (CPG) underlying a behavior that is expressed with a circadian rhythm is *M. leonina* (Thompson & Watson, 2005; Newcomb *et al.*, 2014; Sakurai *et al.*, 2014). Thus, it may be the most promising model system for investigating the link between clock neurons and a specific behavior.

M. leonina displays two modes of locomotion – crawling and swimming (Agersborg, 1923; Lawrence & Watson, 2002) and the CPG underlying swimming consists of four pairs of bilateral neurons (Thompson & Watson, 2005; Sakurai *et al.*, 2014). *M. leonina* moves more

at night (Newcomb *et al.*, 2004) and this pattern of activity is controlled by an endogenous circadian clock (Newcomb *et al.*, 2014). Furthermore, transcripts of clock genes in *M. leonina* have been recently sequenced (Cook *et al.*, in press), making it possible to carry out experiments that were previously not possible. The goal of this project was to use these transcript sequences to accomplish two objectives: 1) determine the location of putative clock neurons that express these clock transcripts using RNA probes with fluorescent *in situ* hybridization (FISH); and 2) investigate the temporal expression profile of some of these clock genes.

2. Materials and Methods

2.1 Collection and housing of animals used for FISH

Adult *M. leonina* were collected from eelgrass beds in the Puget Sound near the University of Washington's Friday Harbor Laboratories, from kelp forests near Monterey Bay, CA (Monterey Abalone Company), and from floating kelp masses around Catalina Island, CA (Marinus Scientific). Animals were shipped overnight to New England College and maintained in aquaria containing artificial seawater (Instant Ocean, 32–35 ppt salinity). The tanks also contained eelgrass or kelp shipped from the collection sites. All aquaria were kept at 10–12°C, with a 6 am:6 pm, light/dark cycle. Animals were given at least 3 days to acclimate to the LD schedule prior to dissection.

2.2 FISH

Sequence-specific RNA probes were developed by Biosearch Technologies for *clock*, *period*, and *photoreceptive* and *non-photoreceptive cryptochrome* (*pcry* and *npcry*, respectively), based on sequences derived from RNA transcriptomes (Cook *et al.*, in press). In brief, 48 non-overlapping, 20-nucleotide RNA probes were designed for each transcript, with each probe being conjugated to a fluorophore. Amplification of the fluorescent signal results from multiple probes hybridizing to a single transcript. If less than five probes attach to a transcript, it will tend to blend into the background. FISH experiments were done in two stages. First, RNA probes for individual clock transcripts were used in individual brains to determine if each transcript could be identified ($n = 35$). These were accomplished with RNA probes conjugated to CAL Fluor 610 (peak excitation = 590 nm, peak emission = 610 nm). Dissections were done initially at 6 am, 12 pm, 6 pm, and 12 am, to determine if there were any differences in labeling over time. Dissection time had no apparent effect, so subsequent dissections were typically done around noon. Second, once each transcript was successfully identified, double-label experiments with two RNA probes conjugated to different fluorophores were used in individual brains ($n = 21$). In these experiments, one fluorophore was CAL Fluor 610 and the other was Quasar 670 (peak excitation = 647 nm, peak emission = 670 nm). Control experiments for each set of experiments involved omitting the RNA probes ($n = 3$).

Brains and attached buccal ganglia were removed, pinned in a dish, and fixed overnight in a 3.7% formaldehyde fixation buffer (in phosphate buffered saline) at 4°C. RNase-sterile techniques were used throughout this entire procedure to minimize degradation of RNA and nuclease-free water was used when making all solutions. The following day, brains were

rinsed and then left overnight in 70% ethanol at 4°C. The brain was then incubated in 10% deionized formamide in Wash Buffer A (Biosearch Technologies) for 3 min on a shaker at room temperature, followed by incubation in the relevant FISH RNA probe (made against *clock*, *period*, *pcry*, or *npcry*). Initially probes for *clock* were diluted 1:100, 1:50 and 1:25 to determine the optimal dilution. Subsequently, all probes were diluted either 1:50 or 1:25 in 10% deionized formamide, made in Hybridization Buffer (Biosearch Technologies). Incubation in the FISH probe was done overnight on a shaker at 37°C. Double-label experiments included two FISH probes, with different fluorophores, each diluted to a 1:25 concentration in the final deionized formamide/Hybridization Buffer solution. Precautions were taken for the remainder of the protocol to keep light exposure to a minimum. Following overnight incubation in the FISH probe(s), brains were washed again in a 10% deionized formamide solution, made in Wash Buffer A, on a shaker for 30 min at 37°C, followed by a 3-min rinse in Wash Buffer B (Biosearch Technologies) on a shaker at room temperature. Brains were then dehydrated in increasing concentrations of ethanol (50%, 70%, 90%, 95%, 100%, and 100%), for 10 min each, at room temperature. Dehydrated brains were then cleared using methyl salicylate, and mounted in Cytoseal (Electron Microscopy).

2.3 Imaging of FISH preparations

Single-label FISH images were obtained on a Zeiss LSM 880 confocal microscope, using an excitation wavelength of 594 nm. Multiple images of optical sections of a brain were obtained at a high resolution and stitched together with Zen software. Double-label FISH preparations were imaged on a Zeiss Axio Scope.A1 epifluorescence microscope, using emission filter sets that prevented bleed through between CAL Fluor 610 and Quasar 670 fluorophores. Images were obtained with a Zeiss Axiocam digital camera and viewed/captured with Zen 2012. Pseudocolors of red and green were used to facilitate confirmation of double-labeled neurons. For each preparation, the location and numbers of labeled neurons were noted on a brain map.

2.4 Collection and dissections of animals for qPCR experiments

All the animals for the qPCR experiments were collected in Parks Bay off of Shaw Island, WA, (GPS: 48.565239, -122.980976), between March 2 and 11, 2017, and held in a flow-through seawater tank that was exposed to the ambient L:D cycle (which was roughly 12:12, due to the temporal proximity to the spring equinox). Animals were allowed at least 24 h after collection to adjust to their new conditions before the dissections began.

Prior to performing the dissections, all of the lab benches, dissection tools, and microscopes were cleaned with ethanol and RNase AWAY (Thermofisher Scientific). Over the course of three days, on two occasions, five animals were dissected every 3 hrs, for a total of 80 samples (n=10 for each time point). The dissections occurred at 6 am, 9 am, 12 pm, 3 pm, 6 pm, 9 pm, 12 am, and 3 am. During the night dissections (6 pm – 3 am), red light was used, as evidence suggests that *M. leonina* are not sensitive to this wavelength of light. Each dissection lasted ~ 10–30 min. The esophagus, to which the brain is firmly attached, was removed from each animal and placed in a tube containing 5 mL of RNAlater. The RNAlater samples were then covered with parafilm and stored at 4°C.

All 80 samples were shipped in a cooler, on ice, to the University of New Hampshire (UNH) overnight. At UNH the brain (paired cerebropleural and pedal ganglia) was removed from the esophagus and placed in PCR-clean Eppendorf tubes. The Eppendorf tubes were immediately flash frozen in a container holding a combination of 95% ethanol and dry ice, and then stored at -80°C until RNA extraction. All samples were processed within a month of arriving at UNH.

2.5 RNA extraction and qPCR

RNA was extracted and purified from each central nervous system (CNS) using TRIzol (Ambion, Fisher Scientific) and a Purelink RNA mini kit (Invitrogen, Thermofisher Scientific). Contaminate genomic DNA was removed with the on-column PureLink DNase treatment kit (Ambion, Thermofisher Scientific). Approximately 20 ng/ μl total RNA was obtained for each sample based on measurements obtained with both a Nanodrop (NanoDrop Spectrophotometer ND-1000, University of New Hampshire, Durham, NH) and Qubit 2.0 (Fluorometer, Invitrogen University of New Hampshire, Durham, NH).

DNA primers were designed using Primer3 (Table 1; Koressaar and Remm, 2007; Untergasser et al., 2012) so that amplicon sizes fell within an optimal range for qPCR assays (between 75 and 150 base pairs) for the four clock transcripts of interest (*clock*, *period*, *pcry*, and *npcry*) and the reference housekeeping gene *alpha tubulin* (*atub*). After an initial denaturation step (95°C for 10 min), cDNA synthesis was performed using a qScript cDNA supermix (Quantabio) following manufacturer protocols. A total of 3 μl of diluted cDNA was used for a 10 μl PCR reaction, and samples were amplified in triplicate for each primer. Complimentary DNA was amplified using an Agilent MX3000P qPCR system (Agilent) for 40 cycles (95°C for 30 s, 55°C for 60 s, 72°C for 60 s), using an Applied Biosystems SYBR select master mix (Applied Biosystems). These data were first normalized to the housekeeping gene *alpha tubulin*. The fold change over time was then graphed, which represents the expression levels of the genes per time point in comparison to the average expression level for each gene.

2.6 Statistical analyses

All qPCR statistical tests were performed with JMP13 (SAS, Cary, NC). Pairwise differences in expression level (ddCt values) between any two time points were determined with a students t-test.

3. Theory

Previous studies have indicated that *M. leonina* express a circadian rhythm of locomotion and also possess many of the core genes that control circadian rhythms in other organisms. Therefore, our working hypothesis was that we would see differences in the expression of these circadian genes in the brains of animals that were dissected in the night vs the day. Furthermore, in order to lay the foundation for future studies we sought to identify specific clock neurons using *in situ* hybridization. This effort was successful and makes it possible to carry out further intracellular investigations of clock neurons in *M. leonina*.

4. Results

4.1 Single-label FISH

Seven of 13 brains labeled with anti-clock FISH probes exhibited two *clock*-positive neurons in each cerebropleural ganglion (Fig. 1A). This variability was likely due to the fact that we initially used three different probe concentrations (1:100, 1:50, and 1:25); this pair of cells were clear in all four brains labeled with the 1:25 probe concentration. There were also *clock*-positive neurons in the anterolateral region of both pedal ganglia in 10 of 13 preparations (Fig. 1A). The number of pedal neurons in these ten preparations was quite variable: with 8.3 ± 8.2 and 8.8 ± 6.2 (mean $\pm$ standard deviation) neurons in the left and right pedal ganglia, respectively. These *clock*-positive pedal neurons were present in all four brains labelled with the 1:25 probe concentration, although the number of neurons was similar in preparations labelled with weaker probe concentrations. After these *clock* FISH trials, we concluded that a dilution of 1:100 was not strong enough to provide consistent results. Therefore, all subsequent FISH experiments were done with probe concentrations that were diluted 1:50 or 1:25, and these yielded indistinguishable results.

Period labeling was similar to *clock*, with 7 of 9 preparations exhibiting two *period*-positive neurons in each cerebropleural ganglion (Fig. 1B). In the other two brains, one preparation had two *period*-positive neurons in the left cerebropleural ganglion, but only one in the right ganglion, and in the other brain, there was one *period*-positive neuron in the left cerebropleural ganglion and no labeling in the right ganglion. In 6 of 9 preparations, there were also *period*-positive neurons present in the anterolateral region of both pedal ganglia (Fig. 1B). The number of *period*-positive neurons was 3.9 ± 2.7 in the left pedal ganglion and 4.7 ± 2.2 in the right pedal ganglion.

In 5 of 6 brains labeled with probes for *npcry*, there were multiple bilaterally symmetric neurons in each cerebropleural ganglion (Fig. 1C). Three of these brains exhibited two labeled neurons in each cerebropleural ganglion, similar to *clock* and *period*, while a fourth preparation had three *npcry*-positive neurons in the left cerebropleural ganglion, and the fifth brain had three labeled neurons in both cerebropleural ganglia. The same five brains that exhibited *npcry*-positive neurons in the cerebropleural ganglia also had labeling for *npcry* in the anterolateral region of each pedal ganglion (Fig. 1C). There was an average of 4.8 ± 2.5 *npcry*-positive neurons in the left pedal ganglion, and 5.2 ± 2.3 neurons in the right pedal ganglion.

The fourth RNA probe for *pcry* labeled two neurons in each cerebropleural ganglion in all seven preparations (Fig. 1D). All seven brains also exhibited *pcry*-positive neurons in the anterolateral region of both pedal ganglia, with 9.3 ± 4.1 and 9.3 ± 4.9 neurons in the left and right pedal ganglia, respectively (Fig. 1D).

All of the FISH labeling was punctate at higher magnification (Fig. 2), illustrating labeling of individual transcripts by the accumulation of upwards of 40 custom FISH probes per transcript. This labeling was also relegated to the cytoplasm, and did not appear in the nucleus (Fig. 2).

4.2 Double-label FISH

As seen in Fig. 1, all four RNA probes labeled similar numbers of neurons in similar areas of the brain. To determine if multiple probes were labeling the same neurons, additional experiments were done with RNA probes that had different fluorophores, to enable double-labeled preparations. The following combinations of probes were used: *clock* and *period* ($n = 6$), *period* and *npcry* ($n = 9$), and *npcry* and *pcry* ($n = 6$). By association, these combinations enabled us to determine potential co-localization of all four RNA probes. Based on the single-label experiments above, RNA probes were used at a concentration of 1:25. In all preparations, there was 100% colocalization between probes (Fig. 3). The pattern of labeling (two bilaterally symmetric cerebropleural neurons and two small groups of bilaterally symmetric pedal neurons) and the numbers of labeled neurons in each area were similar to single-label FISH experiments (see Fig. 1). Control preparations ($n = 3$), that lacked RNA FISH probes, did not exhibit any labeling (not shown).

4.3 qPCR

The brain (cerebropleural and pedal ganglia) exhibited oscillating expression of circadian clock genes over a period of 24 hr (Fig. 4). Note that all of the expression levels are expressed in comparison to the average expression for each gene, meaning that positive expression is higher than the average and negative expression is lower. There were significant variances in expression levels overall in a 24 hr period for *pcry* and *period* (Fig. 4C, D; ANOVA; $p=0.0001$ and $p=0.0084$, respectively), and fluctuations trending towards significance for *clock* (Fig. 4A; ANOVA; $p=0.0893$). No significant differences were seen overall for *npcry* (Fig. 4B; ANOVA; $p=0.1253$). *Clock* tended to have high gene expression at night, between 15 h (circadian time, middle of the night) and 2 h (right after sunrise), which gradually declined until 9 h (sunset) (Fig. 4A). The circadian clock gene *npcry* showed weak expression between 6 h (middle of the day) and 12 h (sunset), and then it subsequently increased from 18 h (middle of night) to 21 h (end of night) (Fig. 4B). *Pcry* had several time points with significant differences over the 24 h period (Fig. 4C), which was similar to the pattern for *period* (Fig. 4D). There were two troughs, at 3 h (day) and 21 h (night), and the expression slowly increased until 18 h (midnight) for both. There also was a peak in expression at 24 h for *pcry* and *period*.

In general, when comparing all circadian clock genes, there was a tendency for peak expression around 18 h (night). However, the expression patterns for *clock* and *npcry* indicate relatively moderate changes throughout the night whereas *pcry* and *period* show greater fluctuations in expression. For all of the genes, expression was fairly low during the day.

When comparing individual time points to one another, for each circadian gene of interest, some significant differences were apparent between specific pairs of time points (Fig. 5). For example, gene expression for *clock* quickly up-regulated from hour 12 (sunset) to hour 18 (midnight) (Fig. 4A, Fig. 5A) and *clock* gene expression at hour 18 (midnight) and 21 (early morning) was very different than the expression at hour 6 (midday) and 9 (afternoon). These trends signify differences in expression from the day hours to the night hours for *clock*. *Non-photoreceptive cryptochrome* also showed gene up regulation from right after midnight to

early morning with limited to no expression seen throughout the day (Fig. 5B). Trends toward expression differences were seen between hour 21 (early morning) and the daytime hours (Fig. 5B) as the expression was slowly increasing over time until its peak at hour 21. The expression of *Pcry* at hour 18 (midnight) and hour 24 (sunrise) was significantly higher than every other hour, including all of the day hours (Figs. 4B, 5C). However, there was no difference between the expression at midnight and sunrise, suggesting that the expression began to down regulate from the early morning until sunrise (Fig. 5C). Like *pcry*, the significant differences in expression levels over time for *period* were between high levels at hour 18 (midnight) and 24 (sunrise) in comparison to all the other hours (Fig. 5D). Both *pcry* and *period* had troughs in expression at hour 3 (10 am; real time), and then leveled off until after sunset, before rapidly up regulating during the night. Every gene of interest showed significant differences between the expression during at least one daytime hour and one nighttime hour, suggesting differential expression over a 24-hour period and potentially daily rhythmic expression over time.

5. Discussion

5.1 Clock transcript localization

FISH labeling revealed a small group of neurons in the brain of *M. leonina* that consistently exhibited the presence of four putative clock gene transcripts (Figs. 1–3). These included two bilaterally symmetric neurons near the center of each cerebropleural ganglion, and a group of < 10 neurons in the anterolateral region of each pedal ganglion. Thus, the putative circadian clock in *M. leonina* likely consists of a network of ~20 neurons in two bilaterally symmetric regions of the brain.

A putative circadian circuit of ~20 neurons would rank *M. leonina*'s clock as one of the smaller clocks in bilaterians, with the exception of several Lepidopteran insects that appear to have a central clock of only ~8 neurons (Sauman & Reppert, 1996; Wise *et al.*, 2002; Sehadová *et al.*, 2004; Sauman *et al.*, 2005; Zhu *et al.*, 2008). The central circadian clock is more than 2–3 times larger in crickets (~50 neurons; Shao *et al.*, 2006, 2008), cockroaches (~50 neurons; Wen & Lee, 2008), and the housefly, *Musca domestica* (~50 neurons; Codd *et al.*, 2007), more than seven times larger in *D. melanogaster* (~150 neurons; Allada & Chung, 2010) and three orders of magnitude larger in the suprachiasmatic nucleus of mammals (~20,000 neurons; Stephan & Zucker, 1972; Inouye & Kawamura, 1979; Ralph *et al.*, 1990). Immunohistochemistry data from several decades ago suggest that ~10 neurons in the cerebral ganglion of *A. californica* contain PERIOD (Siwicki *et al.*, 1989). However, western blots indicated that the antibodies bound to proteins that were 48 and 66 kDa in size, which is significantly smaller than most PERIOD proteins. There are two predicted PERIOD-like proteins for *A. californica* now available on NCBI (Accession numbers XP_005093378.1 and XP_012944985.1), and the predicted size of these proteins is 127 and 176 kDa, respectively (as determined by the ExPASy Compute pI/Mw tool [https://web.expasy.org/compute_pi/]). Furthermore, the only other known PERIOD protein in gastropods is for *B. gouldiana* (101 kDa; Constance *et al.*, 2002). Therefore, it is possible that the antibody was not labeling PERIOD in the study by Siwicki *et al.*, (1989), and that the number of neurons in a putative central clock in the brain of *A. californica* still remains to be determined.

Most prior electrophysiological and histological evidence point to the eyes as the location of the circadian clock in gastropods (Jacklet, 1969; Block & Roberts, 1981; Block & Wallace, 1982; Siwicki *et al.*, 1989; Michel *et al.*, 1993; Constance *et al.*, 2002). In this study, our use of wholemount preparations meant that the FISH probes probably did not have access to retinal neurons inside the eye due to lack of permeabilization of the tough connective tissue surrounding the eye. Furthermore, *M. leonina* eyes exhibit strong autofluorescence over a wide range of excitation/emission wavelengths, including those of our fluorophores. Therefore, an additional clock in the eyes of *M. leonina* is still a distinct possibility, although *M. leonina* without eyes are still capable of maintaining a circadian rhythm of locomotion (Newcomb *et al.*, 2014). There is also prior evidence to suggest that gastropods may have multiple circadian clocks. For example, in *A. californica*, some animals can maintain circadian rhythms of locomotion in constant darkness after eye removal, suggesting that there must be additional clocks outside of the eyes (Lickey *et al.*, 1977). There is some evidence to suggest that the abdominal ganglia may contain a circadian clock in *A. californica* (Audesirk & Strumwasser, 1975; Beiswanger & Jacklet, 1975), but the ability of such a clock to maintain circadian activity in constant darkness has not been definitely demonstrated. Furthermore, lesion studies suggest that the circadian system in *A. californica* lies within the cerebral and buccal ganglia, and sensory structures associated with these regions of the brain (Roberts & Block, 1982).

Regardless of whether or not there is an additional clock in the eyes of *M. leonina*, the evidence presented here, as well as in a previous study (Newcomb *et al.*, 2014) suggests that there are putative clock neurons in both the cerebropleural and pedal ganglia that may be important in regulating circadian rhythms of locomotion, such as swimming. The neural circuit underlying swimming in this animal consists of four bilateral pairs of neurons, one of which is in the cerebropleural ganglia and the other three are located in the pedal ganglia (Thompson & Watson, 2005; Sakurai *et al.*, 2014). The two pairs of cerebropleural neurons identified in this study, which contain clock transcripts, are in the same region of the cerebropleural ganglia as Swim Interneuron 1 (Thompson & Watson, 2005). In contrast, the pedal clock neurons identified here are anterior and lateral to the pedal swim interneurons, although these pedal swim interneurons are known to have extensive arborizations in the pedal ganglia and neuropil (Thompson & Watson, 2005; Sakurai *et al.*, 2014). Future studies are planned to investigate whether or not the newly identified clock neurons connect to, or modulate, the swim interneurons in *M. leonina*.

5.2 Clock transcript expression

In general, the qPCR data obtained is consistent with the hypothesis that there are clock neurons in the *M. leonina* brain that utilize much of the same molecular machinery found in other animals. *Npcry* was up regulated at night compared to the day, and both *period* and *pcry* had peaks of expression around the same time, and slowly ramped up to this peak. Thus, in general, we see expression levels of clock genes that tend to increase in the late evening, and then decline late in the day and into the early evening.

The expression of *M. leonina bmal* was not measured in this study, but we hypothesize that *bmal* oscillates independently of *period* and *npcry* and, in turn, works with *clock* to shut off

the expression of *period*, similar to what happens in the mammalian clock (Shearman et al., 2000). Although all of the circadian clock genes show low levels of expression during sunset, *period* and *pcry* have slight peaks shortly thereafter, and *timeless* (data not shown) shows trends mimicking this pattern. This could be when the genes are translated into proteins in the cytoplasm before beginning the negative feedback, shutting off their own transcription and promoting the upregulation of the other genes (Dunlap, 1999; Shearman et al., 2000; Allada & Chung, 2010).

The molecular machinery driving circadian rhythms has now been elucidated in a number of organisms. Based on these studies, there are several different ways that gene expression can lead to circadian rhythms of activity (Bunney & Bunney, 2000; Young & Kay, 2001). The two most well-studied and common patterns are found in the fruit fly and mouse. Both the molecules and oscillation patterns we have documented in brain of *M. leonina* are more similar to the mouse model than the *D. melanogaster* model. For example, the *D. melanogaster* model contains *cycle*, a circadian gene that binds with *clock* at the E-box promotor and helps regulate the transcription of *period* and *timeless*. In the *Mus* genus *bmal*, a homolog of *cycle*, binds to *clock* and regulates the transcription of *period* and *npcri* (Shearman et al., 2000). Although not included in this study, Cook *et al.*, (in press) have demonstrated the presence of *bmal* in the *M. leonina* transcriptome. Therefore, the molecular mechanisms involved in the *M. leonina* circadian clock(s) contains the same genes present in the mammalian system and the connection/pathway of these genes is also similar. The times of up and down regulation for each gene vary from a mouse to *M. leonina*, but this is to be expected, because they live in different environments and express a different range and pattern of behaviors.

6. Conclusions

1. In the gastropod, *M. leonina*, clock gene transcripts are expressed in a relatively small number of neurons in the brain. There are a pair of putative clock neurons in the cerebropleural ganglia as well as a cluster of 5–10 neurons in each pedal ganglia.
2. *M. leonina* transcripts from the circadian genes *cryptochrome* (both *photoreceptive* and *nonphotoreceptive*), *clock* and *period* are all expressed differentially over time.
3. These data indicate that *M. leonina* might be a good model system for investigating the influence of identifiable clock neurons on the neural networks responsible for generating stereotyped behaviors at specific times of the day or night.

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Figure 1.

RNA FISH labeling in *Melibe* brains for four different circadian clock transcripts: clock (**A**), period (**B**), *npcri* (**C**), and *pcry* (**D**). All four probes consistently labeled two cerebropleural neurons in the middle of each cerebropleural ganglion (arrowheads), and a small group of neurons in the anterolateral region of each pedal ganglion (arrows). The boxed region in (**B**) is shown at higher magnification in Figure 2. The fluorophore was CAL Fluor 610, but images were subsequently changed to a green color to improve contrast. The scale bar represents 200 μ m.

Figure 2.

Close up image of the two cerebropleural neurons (outlined in dotted lines) in the boxed region of Figure 1B. The labeling in each cell is punctate and illustrates individual RNA transcripts in the cytoplasm. The scale bar represents 5 μm .

Figure 3.

Double-label FISH experiments demonstrate consistent colocalization of clock and period, period and *npcry*, and *npcry* and *pcry*. Yellow cells in the merged images indicate overlap between the red and green fluorophores. In each instance, neurons in locations that were similar to those seen in single-label FISH experiments (see Fig. 1) were labeled – two cells in each cerebropleural ganglion and a small group of neurons in the anterolateral region of each pedal ganglion.

Figure 4.

Average fold change (log2) values over time for *M. leonina* brain circadian clock gene transcripts (A) *clock*, (B) *npcri*, (C) *pcry*, and (D) *period*; n=10 for each gene and time point. The yellow and black bars represent times of day (yellow) and night (black), which was approximately 12:12 when these experiments took place. Time points represent circadian time (0 is lights on, which was 7 AM in real time; 12 is lights off, which was 7 PM). Time 0 was double plotted as time 24. Error bars represent the standard error of the mean. In addition to the average expression fold change (log2), all data points are plotted to show actual variability.

Figure 5.

Heat maps showing time points where gene expression was significantly different for each clock gene of interest, *clock* (A), *npcry* (B), *pcry* (C), and *period* (D) (student's t-test). The scale is from dark blue to white. The middle of the color scale signifies 0.05 (light blue), anything less than that value (darker blue) is significantly different, whereas anything lighter (white) is not different.

Table 1

Forward and reverse qPCR primer sequences, for circadian clock and housekeeping (control) transcripts.

Gene	Forward primer	Reverse primer
<i>alphatubulin</i>	5'-GCCCTACAACCTCCATCCTGA-3'	5'-GTAGGTGGGACGCTCAATGT-3'
<i>period</i>	5'-CTGATGACGGTGTGTTGTCC-3'	5'-AAGACCAGAAACGGCTGCTA-3'
<i>photoreceptive cryptochrome</i>	5'-GGTTTGGAGACCTTTGACCA-3'	5'-GCGAGGGCACTGTAATACCT-3'
<i>non-photoreceptive cryptochrome</i>	5'-ACAAACGGTTCCAGTCCATC-3'	5'-TGACAGGTGTGACGCTCTTC-3'
<i>clock</i>	5'-ACCAGCACAGAGCTTGTAC-3'	5'-ATCCTGCAGCTTCTTGGACT-3'