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5/7/21

Final Written Lab Report

Introduction

Circadian rhythms are one of the most unifying and conserved traits across life. Internal clocks control the hormone release, molecular rhythmicity, and sleep-wake cycle from cyanobacteria to humans.[1] While the existence of the clocks themselves are apparent in almost all forms of life, their actual mechanism remained unexplored for centuries. Research has revealed that there isn't only one singular 'clock' in an organism, rather every cell has an independent 'clock' and these cell-autonomous clocks are coordinated within an organism.[2] In the 1970s, the suprachiasmatic nucleus was discovered in mammals as the central neural driving force behind the internal clock; however, little is known about what drives this clock in other animals, as well as if that is the only coordinating coupler. Without this coordinating entrainer the cells would oscillate on independent free-running cycles and eventually would run out of sync. Similarly, questions remain about the feedback-loop that entrains the circadian rhythm in non-mammals, specifically if photoreceptors are the only mechanism of entrainment and if the neural ganglia house the only organismal entraining mechanism. Answering these questions in easier to study model organisms could give insight into the extent of the chronobiological system in mammals. *Lumbriculus variegatus*, an aquatic annelid worm, is a model organism for chronobiological study in order to discover the fundamental basis of circadian rhythms due to its hardiness, photoreceptive function, and ability to survive the removal of the head and tail body segments.[3]

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In most animals, the circadian clock is entrained by changes in light. At the beginning of the photoperiod day, the RNA levels of Timeless (TIM) and Period (PER) start to increase. The regulation of the transcription and location of these two proteins are crucial to the photoperiod response. PER protein, following phosphorylation by CK1, feeds back into the nucleus to suppress clock-controlled gene expression. PER is not able to enter the nucleus without TIM via the formation of a heterodimer, and only takes place during the photoperiod night. PER and TIM are degraded when the photoperiod night ends, and are then transcribed throughout the day. When it is close to night, there is enough mRNA of PER and TIM to create their proteins. In the middle of the night, there are sufficient amounts of proteins to slow the accumulation of the mRNA. The dimer is then destroyed at daybreak. Thus, this dimer is responsible for the coupling of light to intracellular circadian cycles. The presence, expression, and location of this protein in lumbriculid freshwater worms is unknown and will be examined in these experiments. This dimer is a transcriptional repressing component of the internal biological clock, whose feedback suppression of gene expression allows animals to anticipate regular daily environmental changes and to maintain an approximate 24-hour rhythm of behavior, even in constant darkness. PER is gradually phosphorylated over the course of the night and early morning, in some species, and is then degraded. Phosphorylation of this protein regulates its stability and cellular localization, but the physiologically relevant sites have been difficult to identify despite knowing the relevant kinase.[4]

Performance of immunohistological staining will allow the expression and location of the TIM protein to be determined in *Lumbriculus*. By conducting antibody-labelling experiments across multiple fixed time points, we will also reveal if this core clock protein is rhythmic by

examining the intensity of staining. This analysis will also demonstrate the cellular and tissue-specific nature of the TIM expression. In so doing, we will test the hypothesis that the Timeless protein is expressed in *Lumbriculus* body tissues, specificially brain and ventral nerve cord neurons, and is product of a rhythmically expressed core clock gene. Fluorescence images will be collected and staining as a proxy for the PER protein will be quantified for intensity, rhythmicity and cellular localization.

Materials and Methods

Firstly, the worms will be entrained into a light-dark cycle. They will then be collected at multiple time points. There will be twelve 10 mL tubes labeled by the time points, each being four hours apart. Every tube will contain 12 mL of spring water and four medium to large-sized worms. Six of the tubes contain animals that were treated with the CK1 inhibitor, while the rest of the tubes contain untreated worms. Each tube will be collected at its allocated time point. 0.25 micrometers of nicotine will be added to the tube at each time point for ten to fifteen minutes to immobilize the worms. After pouring out the nicotine, 4mL of 4 % paraformaldehyde, the fixative, will be added. Lumbriculus heads containing the brain-suboesophageal ganglion complexes were amputated following nicotine anestesia (0.25 micromoles) in spring water for 10 minutes and then fixed in 4% paraformaldehyde in PBS (phosphate buffered saline) at 4°C overnight. After thorough washing with PBS, the heads were dissected to remove all body cavity organs, leaving only the nervous system and underlying body wall of a fileted preparation. The dissected heads were then washed in PBS supplemented with 0.2% Tween 20 (PBS-T) and then

blocked with 10% normal goat serum in PBS-T (at room temperature). The preparations were then incubated with the DpTIM-GP47 primary antibody (diluted 1:500 in PBS-T with goat serum) overnight at 4°C. Following rinsing with PBS-T (three times for 10 min at room temperature), samples were incubated with secondary antibody (goat anti-guinea pig AlexFluor388) diluted 1:200 in PBS-T with goat serum) for 1 hr at room temperature.

Polyclonal antibodies against purified monarch butterfly Timeless (TIM) protein were generated in guinea pig. The immunogen contained amino acids (aa) 251-450 of the TIM protein. Much this carboxy terminal region of the TIM protein is highly conserved in its amino acid sequence between the polychaete worm, Platynereis, the fruit fly (Drosophila melanogaster, Dm) and the monarch (Danaus plexippus, Dp). The first 110 amino acids of the immunogen's 200 aa have high consensus in their sequence. Antisera (DpTIM-GP47) immunoreactivity in the monarch was previously confirmed in Western blot, immunoprecipitation, and immunocytochemistry experiments in the lab of Steven Reppert (Zhu et al., 2008). The antibodies were kindly provided by Christine Merlin.

By staining the antibody, we can determine where the protein is located. We can also determine the rhythmic period immunoreactivity by comparing the intensity of the stains at the various time points.

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Results

We prepared a mixture of glycerol, PBS, and DMSO and placed the dissected heads on a slide with this mixture. The animals from the time points were not viewed under the microscope, only a tube of preparation with the TIM and SER antibodies were viewed.

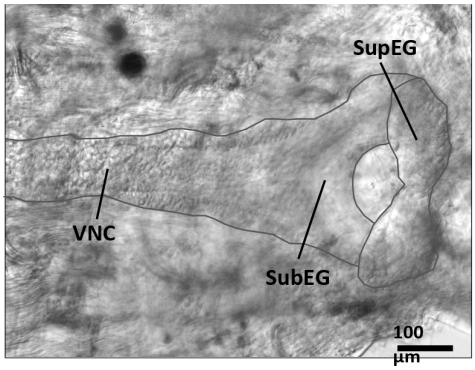

Figure 1 Transmitted light (differential interference contrast; DIC) micrograph of nervous system in the first 3-4 head segments of Lumbriculus. The brain is composed of subesophageal ganglion (SubEG) and a pair of supraesophageal ganglia (SupEG). Gray line indicates the boundaries of the central nervous system.

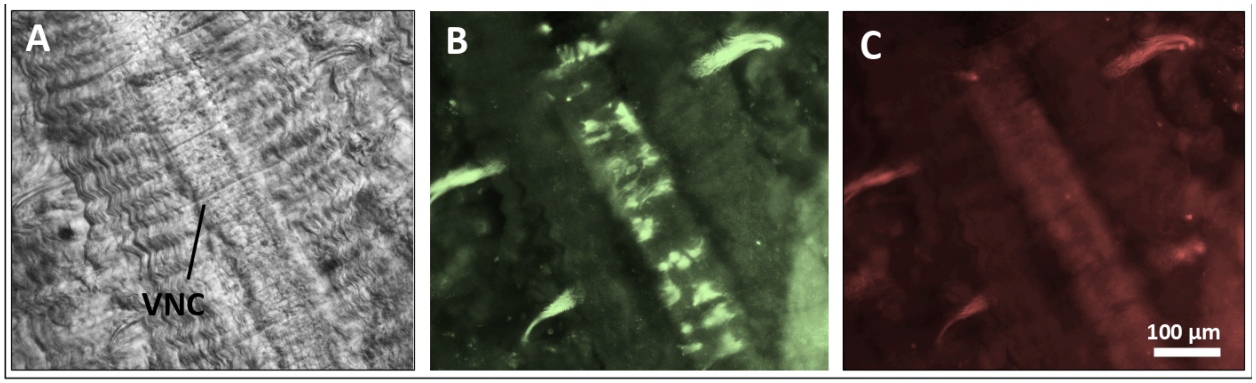

Figure 2 Timeless protein antibody immunoreactivity in head ventral nerve cord of Lumbriculus. (A) Transmitted light (differential interference contrast; DIC) micrograph of nervous system in anterior segments 8-10 in Lumbriculus. The ventral nerve cord (VNC) is indicated in the center

of the image. (B) AlexaFuor488 fluorescence reveals the TIM protein staining in numerous cells entirely restricted to the ventral nerve cord. Autofluorescent setae on either side of the cord roughly indicated the separation of adjacent body segments. (C) Excitation outside the blue wavelength shows no emission in the red spectrum, demonstrating that the TIM immunoreactivity is not a product of autofluorescence.

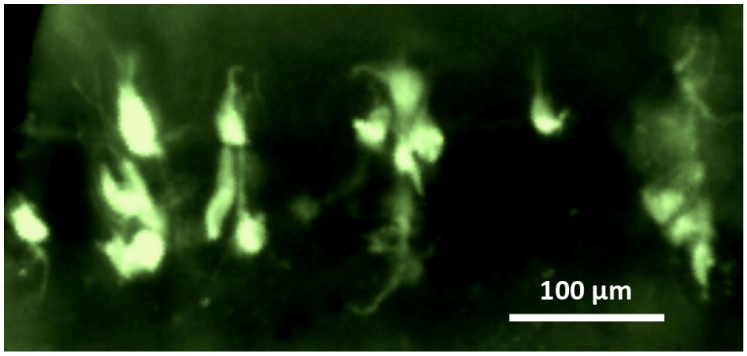

Figure 3 Timeless protein antibody immunoreactivity in the ventral nerve cord of Lumbriculus. AlexaFuor488 fluorescence TIM protein staining in 15-20 neurons within a signal body segment of the ventral nervous system. Similar patterns of neuronal TIM staining exists along the length of cord, along posterior body segments have not yet been examined.

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Conclusion

Cell bodies were observed below the head, after the first eight segments, and were very bright. This leads to multiple possible explanations. One being that the animal may contain a similar amino sequence to TIM that the antibody binds to and may not be exclusively binding to TIM. Another possibility is that TIM may have diverged and evolved to perform a function that is not involved in the clock, such as being involved in development. Since the *Lumbriculus variegatus* reproduced asexually and needs development, it may be that the TIM protein is regulated in regeneration. This can be studied in the future by performing an experiment involving asexual reproduction and presence of TIM in the ventral nerve cord. This may explain why some animals showed staining while others did not. Another explanation may be that the animals may contain genetic variation due to being closely related species or subspecies.

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References

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Roenneberg, T., & Merrow, M. (2005). Circadian clocks — the fall and rise of physiology. *Nature Reviews Molecular Cell Biology*, *6*(12), 965–971. doi: 10.1038/nrm1766

2

Helm, B., Visser, M. E., Schwartz, W., Kronfeld-Schor, N., Gerkema, M., Piersma, T., & Bloch, G. (2017). Two sides of a coin: ecological and chronobiological perspectives of timing in the wild. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *372*(1734), 20160246. doi: 10.1098/rstb.2016.0246

3

Zoran, M. J., & Martinez, V. G. (n.d.). Lumbriculus variegatus and the Need for Speed: A Model System for Rapid Escape, Regeneration and Asexual Reproduction. *Annelids in Modern Biology*, 185–202. doi: 10.1002/9780470455203.ch10

4

Blau, Justin. “PERspective on PER Phosphorylation.” *Genes & Development*, Cold Spring Harbor Lab, 1 Jan. 1970

Lybrand, Zane R., et al. “Coupled Sensory Interneurons Mediate Escape Neural Circuit Processing in an Aquatic Annelid Worm, Lumbriculus Variegatus.” *Wiley Online Library*, John Wiley & Sons, Ltd, 4 Oct. 2019, onlinelibrary.wiley.com/doi/full/10.1002/cne.24769.