Is the circadian clock protein npCRYPTOCHROME2 in the eyestalks of the American lobster, *Homarus americanus*?

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Introduction:

Almost all living organisms exhibit circadian rhythms of movement, metabolic rates, and other behaviors. These rhythms are driven by endogenous clocks that have a period of approximately 24 hours and persist under constant conditions (Hill et al., 2004). The core molecular components of the circadian clock include PERIOD (PER), CLOCK (CLK), CYCLE (CYC), TIMELESS (TIM), and CRYPTOCROME2 (CRY2) (Konopka and Benzer, 1971; Bell-Pederson et al., 2005; Hardin, 2009). These proteins work together in a negative feedback loop to either up or down regulate gene expression over the course of 24 hours (Tomioka and Matsumoto, 2010; Hill et all., 2004). Cell expression of the circadian clock have been seen in different locations in variety of species.

The circadian clock has been located in the optic lobe of crickets and beetles and in the central brain of moths, flies and mosquitos (Tomioka and Matsumoto, 2010). In a recent study, it has been found that PERIOD cells are in higher abundancy at mid-dark hours in the eye stalks of lobster, *Homarus americanus* (Grabek and Chabot, 2012). Although PERIOD has been detected in the eyestalks of lobster, the exact location of the circadian clock is still unknown. Another clock protein, CRYPTOCHROME2 was located in the nervous system of crayfish, *Procambarus clarkii* (Kobayashi et al.). Recent transcriptomic evidence suggests that CRYPTOCHROME2 is also in the central nervous system of lobsters (Wilk, unpublished), and that a commercial antibody may be used to detect and quantify this protein.

The specific aim of this experiment was to use immunohistochemistry to localize npCRYPTOCHROME2 in the eyes and brains of lobster. Central nervous system tissue was dissected at subjective mid-light and mid-dark periods. By removing the tissue at mid-light and mid-dark it can be determined if the expression of npCRYPTOCHROME2 proteins is differs between times of day.

Material and Methods:

Species and environmental conditions:

Twelve American lobsters (Homarus americanus) were bought from a local supply and transferred to a 113.6 L tank located in laboratory Plymouth State University. Temperature was controlled by an Arctica ® commercial series titanium chiller. The initial temperature was 7°C and increased a degree each day until reaching 17°C. At approximately this temperature lobsters have the most robust circadian rhythms (Jury et al., 2008). The lobsters were exposed to a 12:12 LD cycle for 10 days to allow for entrainment.

Dissection and tissue sample collection:

Eye stalk and brain tissue were dissected at mid light at 7am (n= 4) and mid dark at 7pm (n=4). The tissue samples collected were incubated in a perfusion solution for Homarus americanus with a pH equal to 7.4.

Washes and Dehydration/Rehydration

The tissues were fixed at 4 degrees Celsius in 4% paraformaldehyde (pH = 7.2-7.4) buffered with 0.1 M Sorenson's buffer for 90 minutes.

Dehydration and rehydration followed with 30% ethanol, 50% EtOH, 70% EtOH, 80% EtOH, 90% EtOH, 95% EtOH and then in the reverse order 70% EtOH, 50% EtOH, 30% EtOH for 10 minutes each. Lastly, a final wash with distilled water was completed for 10 minutes. The tissue was then transferred into a small beaker filled with 30% sucrose in Sorenson's Buffer approximately 12 hours.

Embedding and Sectioning:

Each tissue was mounted to the orientation assembly and embedded with Tissue-Tek O.C.T. Frozen slices were cut and placed on slides. Each slice was cut into about 10-50 µm sections and placed from left to right on the correctly labeled slides. The slides were then stored in the refrigerator at -20 degree Celsius for up to 24 hours.

Wash, Blocking and Antibodies:

A square about 1.5- 2 cm was drawn on the slides around each tissue with a hydrophobic pen. The sections created were then washed by applying PTA with a pipette of 1-20 µL for ten minutes. The tissues were blocked with 6% solution of goat serum in PTA (100mLs of Phosphate buffer, 0.1g Sodium azide, 0.3 mL Triton X-100) for an hour at room temperature. Primary antibody were diluted in 6% goat serum PTA to reach a desired concentration of 1: 499. Primary antibodies used were human anti-rabbit polyclonal CRY 1, 5mL aliquots. Tissues were incubated for two hours. After the primary antibody incubation, three 10 minute washes of Sorenson's buffer were done. A diluted secondary fluorescent antibody in 6% goat serum PTA was created using Alexafluor 488 Goat Anti-Rabbit, 10 µL aliquot. Desired concentration was 1:500. Three 5 minute washes of Sorenson's buffer was applied to the tissue. Fluid was removed from slides with two methods, tapping off of the fluid and/or manually pipetting it off into a waste beaker.

Mounting, Storing and Visualization:

Finally the tissues were mounted using just a drop of mounting medium, a cover slip and then a sealing agent around the coverslip to prevent the tissues from drying out. The tissue sections were visualized and imaged using an Olympus BX53 compound fluorescent microscope. Images were scaled and analyzed through ImageJ.

npCRY2 cells in mid-dark vs no cells in mid-light

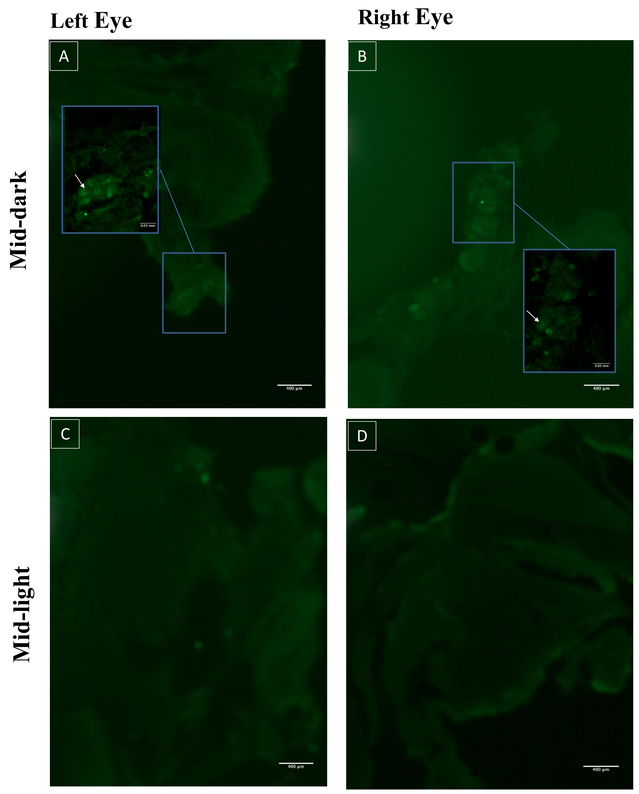

Figure 1: Images of the eye tissues of *Homarus americanus*. (A) npCRY2 immunoreactive cells (white arrows) in the left eye tissue at mid-dark. (B) npCRY2 immunoreactive cells (white arrows) in the right eye tissue at mid-dark. . (C) No npCRY2 fluorescing cells present in left eye. (D) No npCRY2 fluorescing cells present in right eye.

- Bilateral staining suggests clock may be in the eyes
- Only found in 1 of 3 mid-dark animals
- Zero of four animals in mid-light had specific binding present
- Suggests that npCRY2 is not up-regulated during mid-light

No clear npCRY2 in the brain

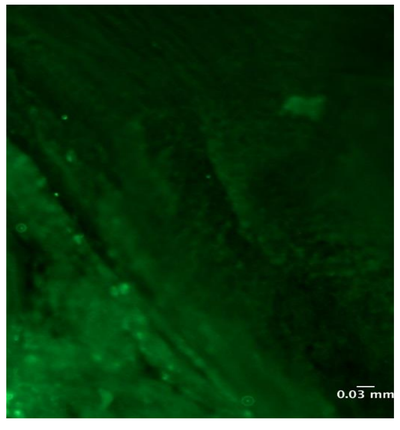

Figure 3: Image of the brain from *Homarus americanus.* No npCRY2-immunoreactive cells present in the brain.

- No npCRY2 in brain tissue is expected (Grabek and Chabot 2012; Wilk, unpublished).

npCRY2 cells in the VNC ganglia

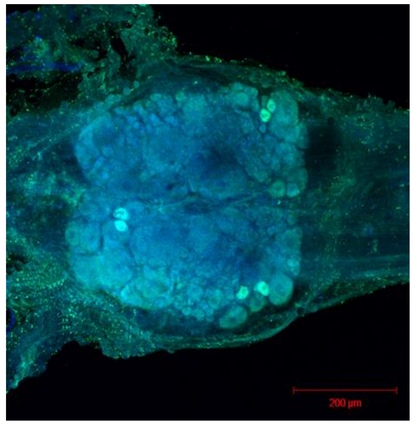

Figure 4: *Homarus americanus* npCRYPTOCROME2 immunofluorescently stained cells in Ventral Nerve Cord (VNC) ganglia tissue (Watson, unpublished)

- npCRY2 fluorescing cells found in the VNC ganglia
- Suggest clock may be in VNC

Discussion/Conclusions:

- There was npCRY2 immunoreactive cells found in eye tissues in one mid-dark animal suggesting that npCRY2 is present at night. Similar findings of npCRY2 have been found in mosquitos (Gentile at el., 2009).
- The cells found in our study were located bilaterally in the eyes as previous studies had suggested clock presence to be (Strauss and Dirksen 2010). PER like protein was found in the eye stalks of lobsters (Grabek and Chabot 2012), furthermore npCRY2 and other core clock proteins were also found in the eyestalks of crayfish (Fanjul-Moles et al., 2004).
  - All three findings Suggest that the clock is located in the eyes of this species
- NpCRY2 was also found to be present at night in the ventral nerve cord (VNC) of the lobster (Figure 3) suggesting that there is another clock located in the VNC and additionally supporting that npCRY2 is upregulated at night.
- It was expected that the four mid-dark animals would exhibit immunoreactivity with npCRY2 antibodies at subjective night (Gentile at el., 2009). However, even though the epitope of our CRY2 antibody shows a high degree of sequence homology with lobster npCRY2 (Wilk unpublished, 2015), three of the four mid-dark animals did not show expected results.
- Future directions
  - An increased "n"
  - Blocking for a longer time period and performing a higher number of washes should decrease the amount of nonspecific binding.
  - Careful maintenance of tissue structure integrity by using auto pipettes to remove fluids from slides instead of tapping it off
  - Dissections at numerous different time points

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