MQP-BIO-DSA-6944

CIRCADIAN RESPONSES TO TEMPERATURE CYCLES IN CRYB MUTANT DROSOPHILA

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

by

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April 26, 2007

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ABSTRACT

In *Drosophila melanogaster* circadian rhythms can be entrained by light, temperature, and social cues. Three essential proteins for the *Drosophila* circadian clock are PER, TIM, and the photoreceptor CRY. In cry^b mutant flies that lack CRY, PER and TIM do not cycle under light/dark conditions. A hypothesis was made that PER and TIM would oscillate normally in a temperature cycle under constant darkness in cry^b flies. Through immunoblotting it was found that both PER and TIM oscillate in cry^b flies similarly to that of wt, supporting the idea that CRY is only required for light responses, and not for temperature synchronization of Per and TIM cycling. A behavioral test was also performed to see if adding a temperature cycle into a light/dark (LD) cycle would cause cry^b fly activity to shift. Overall cry^b flies showed a greater sensitivity to temperature cycles than wild-type flies under these conditions.

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ACKNOWLEDGEMENTS

I would like to thank my Major Advisor, Dr. Patrick Emery for allowing me to work in his lab at UMass Medical School, and for all of his help throughout the entire project, and for teaching me how to perform an immunoblot. I would also like to thank the students in Dr. Emery's lab, especially Ania Busza for helping me and showing me how to collect behavior data. I would also like to thank the Rosbash Lab at Brandeis for providing the polyclonal antibodies to TIM and PER I used for my immunoblots. Lastly, I am grateful for the guidance of my WPI Project Advisor, Dr. Adams. He helped me initiate the project and prepare my final MQP report.

BACKGROUND

Circadian Rhythms

Animals and plants experience circadian rhythms, endogenous rhythms with a period of approximately 24 hours that are connected to the environment. In the early 1700s it was discovered that plants have biological rhythms when Jean-Jacques d'Ortous de Mairan noticed the daily movement of leaves, and designed an experiment to determine whether sunlight was required for this behavior. By keeping plants in the dark he determined that they experienced circadian leaf movements which originated from an endogenous clock (HHMI, 2007). More studies were done in the 1940s by Franz Halberg from the University of Minnesota, who advanced the study of the biological clock and its importance within animals (Dunlap et al., 2004). Halberg held a fellowship at Harvard in endocrinology, and he noticed in his patients that the timing between disease onset and the application of antibiotic treatment seemed to matter, and he was interested in finding patterns. Continuing research in endocrinology Halberg recognized phase differences between groups of mice and found synchronization patterns; he eventually created the term "circadian" (Halberg et al., 2003). Mairan and Halberg laid the foundation for circadian rhythms, but it was Aschoff and Pittendrigh who can be accredited with advancing the study of circadian entrainment. Both men worked to understand entrainment and our endogenous oscillators using different model experiments, such as *Drosophila*, and made great contributions to this field of study (Daan, 2000).

Drosophila melanogaster is a common fruit fly used in many circadian experiments, and it has considerably advanced the molecular understanding of circadian rhythms. The tiny size of the fly, the simple genetic makeup, as well as the fast growth rate makes *Drosophila* a perfect test species. They can also be easily mutagenized to identify critical genes of the circadian clocks and the specific proteins that run it. Also, large numbers of individuals can be collected when running experiments, yielding more data in less time. Importantly, the mechanisms of circadian rhythms in *Drosophila* were found to be similar to those of mammals.

Characteristics of Circadian Clocks

A circadian clock is an endogenous oscillator that tells time and generates overt rhythms, such as circadian behavior. This oscillator regulates behavioral output and sets the pace and phase of the other oscillators within the circadian system. Figure 1 represents a simple circadian system, and it can be broken into three parts: the input pathway, the circadian oscillator, and the output pathway. A stimulus from the environment (i.e. light) enters through an input pathway. The input pathway transmits this cue to the circadian oscillator, which then activates the output pathway. The output pathway is responsible for controlling different physiological and behavioral processes, and it produces the overt rhythm (Yu and Hardin, 2006).

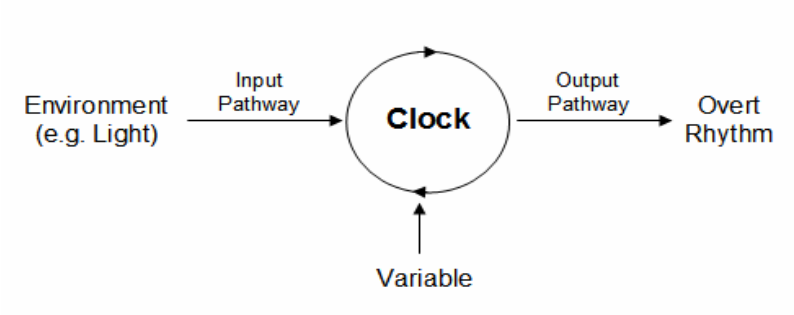

Figure 1: Diagram of a Simple Circadian System.
Redrawn from Williams and Sehgal, 2001.

The characteristics of circadian rhythms separate them from other types of bio-rhythms. They have a period of about 24 hours, and in order to maintain the periodicity of circadian behavior in a specific environment, the rate of oscillation is internally compensated when there are changes in temperature (Williams and Sehgal, 2001). The circadian clock is sensitive to changes in the environment, and the phase of a rhythm can be reset by a variation in that environment. For example, a strong pulse of light introduced into an input pathway can reset a rhythm's phase.

In fruit flies, circadian rhythms are well studied. They can be entrained by an environmental stimulus, called a zeitgeber, which determines the phase of circadian rhythms (Dunlap, 1999). Three inputs that can synchronize the *Drosophila* circadian clock are light, temperature, and social cues. The major stimulus known to synchronize the *Drosophila* circadian clock is light. To show successful entrainment, circadian rhythms must show persistence once a stimulus is no longer present, with the phase determined by the zeitgeber (Williams and Sehgal, 2001).

Physical Locations of the Clocks

Circadian clocks are present in different locations. There is a central clock, as well as peripheral clocks that make up the system. The main circadian pacemaker in mammals is known as the suprachiasmatic nucleus (SCN) (Mieda et al., 2006). Figure 2 shows the locations of the circadian pacemaker neurons of the fly head. The lateral neurons (LNs) (named according to where they are located in the brain) contain the central clock, and peripheral tissues, such as the photoreceptor cells of the fly eye, contain clocks as well (Chen et al., 1992).

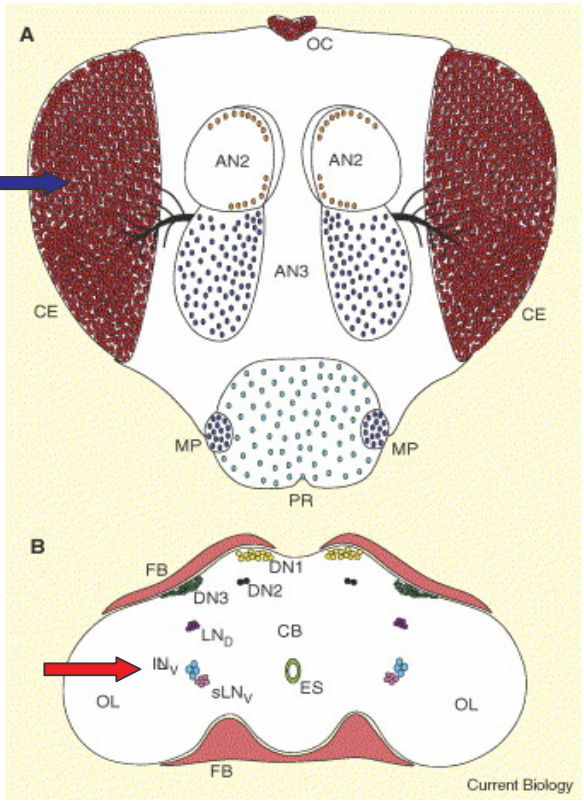

Figure 2: Fly Head Circadian Oscillator Locations.
Blue arrow: Peripheral Tissues (eye); Red arrow: Ventral lateral neurons (LN_Vs). (Hardin, 2005).

In *Drosophila* it has been shown that the lateral neurons express *per* and *tim* clock genes. The ventral lateral neurons contain the neuropeptide required for generating rhythmicity, PDF (or pigment-dispersing factor), and ultimately control locomotor activity and rest for *Drosophila* (Williams and Sehgal, 2001). Therefore flies that lack the lateral neurons are arrhythmic. Other neurons together with the ventral lateral (LN_vs) also control behavioral rhythms, and they are as follows: the dorsolateral (LN_ds) and 3 sets of dorsal neurons (DN(1-3)s) (Klarsfeld et al., 2004) (See Figure 2, above).

Peripheral tissues have also been shown to express clock genes. These tissues contain light and temperature sensitive oscillators. The mechanisms between the central and peripheral oscillators are different, and this has been determined by experiments with mutant flies. For example, in cry^b flies circadian oscillation is normal in the ventral lateral neurons, but in most peripheral tissues the flies are no longer rhythmic (Stanewsky et al., 1998; Krishnan et al., 2001).

Clock Mutants

The study of mutant flies lead to the discovery of the clock genes. Flies that lack PER, called per^0, are arrhythmic and their locomotor activity is completely random when they are under constant darkness (DD). However, in a light:dark cycle (LD) their activity is much greater than *wt* under light and more suppressed under darkness (Collins et al., 2005). This affect is known as masking, or skewed activity due to light. Mutants have also been discovered that under constant conditions displayed longer or shorter periods than a wild type fly, called respectively per^{long} and per^{short}. per^{long} flies experience a period of around 28 hours, while per^{short} flies have a period of 19 hours (Konopka and Benzer,

1971). Although these are just a few examples, studying mutants helped discover the mechanisms behind the circadian clock.

Molecular Mechanisms of the Circadian Clock

A circadian clock runs as a molecular negative feedback loop, and the proteins that cycle within the clock regulate their own mRNA synthesis. The clock tells time by regulating protein levels so that they oscillate with a rhythm of approximately 24 hours (Williams and Sehgal, 2001). The main mechanism behind the *Drosophila* clock oscillation in the pacemaker neurons is the PER/TIM feedback loop (Figure 3). Period (PER) and Timeless (TIM) are the major proteins that repress transcription and their levels begin to rise at early night (Zeng et al., 1996), while Clock (CLK) and Cycle (CYC) are the two important transcription activating proteins.

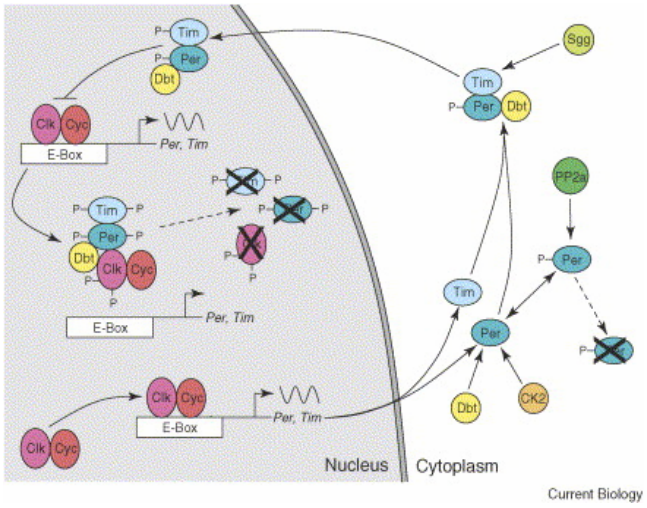

Figure 3: PER/TIM Feed back loop.
(Hardin, 2005).

The PER/TIM feedback loop begins to operate when CLK and CYC form a heterodimer and bind to an E-box upstream of *per* and *tim* genes, activating their transcription. PER then becomes phosphorylated by two kinases, Dbt (binds directly to

PER) and CK2. Once PER is phosphorylated TIM binds to the PER-Dbt complex, stabilizing it. TIM is phosphorylated by Sgg. The PER/TIM/DBT complex is transported into the nucleus. Here the complex binds to CLK-CYC, causing their dissociation from the E-box, and overall inhibiting transcription of PER and TIM. Dbt continues to phosphorylate PER as well as CLK, to promote their degradation. While this happens TIM is also degraded (Hardin, 2005).

Cryptochrome

Cryptochrome (CRY) is an essential circadian photoreceptor of the clock. Similar to other clock genes its mRNA has been shown to undergo circadian oscillations, but CRY protein levels are mostly controlled by light (Emery et al., 1998). Studies of mutant flies without functional CRY(called cry^b), showed that CRY plays a role in the light-mediated entrainment and clock resetting, but it is not the sole input factor (Stanewsky et al., 1998). Cry^b mutants experience multiple problems; PER and TIM cannot cycle correctly in the photoreceptor cells under LD conditions, and the flies are unable to reset their circadian clocks when subjected to light pulses (light strongly resets a *wt* fly clock). Also, in constant light conditions, cry^b mutants are rhythmic (Emery et al., 2000), while wild-type flies are completely arrhythmic. Interestingly, cry^b flies can be entrained to LD cycles in other areas besides the photoreceptor cells, showing that circadian oscillations occur in multiple pathways: for example from eye input into the LN_vs (Stanewsky et al., 1998).

Cryptochrome Function

When CRY is in the presence of light, the PER/TIM feedback loop operates (as mentioned previously), but now the photoreceptive protein CRY is activated, causing it to bind to TIM (Busza et al., 2004). CRY triggers TIM degradation. This results in the subsequent degradation of PER, which is no longer protected by TIM. These mechanisms explain how light synchronize the circadian clock.

Studying Fly Circadian Rhythms

Wild type flies (*wt*) lay the foundation for studying rhythmicity due to zeitgebers. Entrainment to a light: dark cycle (LD) takes three full days. An LD cycle of 12 hours light and 12 hours dark (12:12 LD) is a general entrainment protocol used because it mimics normal day and night, and it represents half of the normal 24 hour period. *Wt* flies are entrained to LD cycles generated by incubators, and monitored with infrared light. The flies are placed singly in a glass tube that is capped at one end and filled with food in the other. The glass tubes are then snapped into place in a monitor that attaches inside an incubator in a dark room. The incubator takes many readings throughout the LD cycle and from these readings actograms are generated. Actograms show the measured fly activity. Figure 4 (below) gives an example of an actogram representing a 12:12 LD entrainment of *yw wt Drosophila;* the shaded area (with crosshatches) represents dark, and the white represents light. In a basic 12:12 LD cycle, *wt* flies show anticipation of both light on and light off transition, depicted on an actogram by increasing peaks of activity (the highest peaks). The actogram also shows persistence during the last 24hrs, which means the entrainment worked.

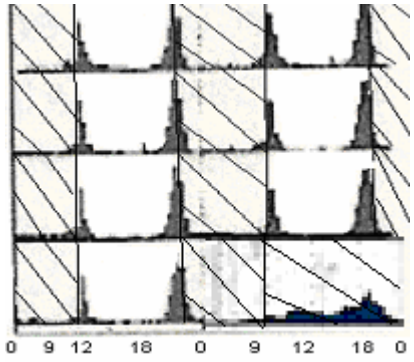

Figure 4: Actogram of LD cycle of *yw Drosophila*.
Y axis = activity per day; X axis = time (ZT). DD is represented by the crosshatching.
Redrawn from Kula et al., 2006.

The peaks represent when the flies are most active. Fly mutant actograms can be compared to *wt* to show when the flies are more active, and to correlate activity levels with specific protein levels. Also as a control, how flies act under constant darkness (DD cycle) helps to study how the flies respond solely to their internal 24 hour period.

The measurement of time is also different when working with circadian rhythms. The progress of time is measured in zeitgeber time (ZT). A ZT of 0 is said to be dawn, while a ZT of 12 is representative of dusk. In figure 4, time is represented in ZT because it relates to an entrainment where the zeitgeber is light, strongly influencing the activity of the flies (Daan et al., 2002).

This MQP project was performed in the laboratory of Dr. Patrick Emery at the University of Massachusetts Medical School. His lab has advanced our knowledge of circadian photoreception, in particular by elucidating mechanisms of the CRY input pathway.

PROJECT PURPOSE

The main purpose of my project was to gain a better understanding of the role of CRY in PER and TIM cycles after entrainment to temperature, and its importance in synchronizing to DD/temperature cycles. To accomplish this I focused mainly on comparing *wt* with cry^b mutant flies. I was shown general fly genetics, and how to screen for desired progeny. The *Drosophila* strains used were wild types *yw*, and mutant flies lacking CRY, *yw;* cry^b . I conducted temperature entrainments in constant darkness to gather data in order to perform immunoblots. I also learned how to run behavioral entrainments and create actograms based on the data collected from the fly activity. All of these techniques I used to test various hypotheses about PER and TIM cycling, and how CRY mutant flies react to entrainments. A hypothesis was made that PER and TIM would oscillate normally in the peripheral tissues in a temperature cycle under constant darkness in cry^b flies. A behavioral test was also performed to see whether adding a temperature cycle into an LD cycle would cause cry^b fly activity to shift, favoring temperature.

METHODS

Fly Handling

Care and Handling

All *Drosophila* fly types used in this study were obtained from Dr. Emery's fly stocks. The stocks were grown in plastic bottles containing a medium of cornmeal, molasses, yeast, agar, and alcohol. To optimize the growth period of larvae, the bottles were placed in an incubation room at 25°C.

When collecting flies for an entrainment, the flies were anesthetized using carbon dioxide sent into the bottles by a thin tube with a connected needle point at the end. After about 30 seconds the flies would fall asleep, and could be placed on a CO_2 pad set under a microscope. The desired number of flies would then be counted and placed in separate tubes containing food, and labeled accordingly.

Flies had to be continuously transferred approximately once a week to keep the stocks from developing bacteria or from going old. Newly grown flies were transferred into new bottles to lay their eggs, and after about a week of transferring that stock, they were dumped into soap and water to kill them. This ensured that entrainments were done using young flies.

Sex Determination and Virgin/non-virgin

Young males were chosen for behavioral entrainments because they are more active. About an even mix of male and female were collected for head extracts. Male *Drosophila* have a distinct sex organ and a rounder shape to the abdomen. Another way to

spot a male is the sex comb on the front legs, and the shorter, smaller, and darker colored abdomen. Females have more of a V shape to their abdomen, with a much whiter belly and no sex comb on their upper front legs. The females are larger in size, with darker backside/shoulders than the male. Virgin females are very easily recognized. They have a large, white belly and a V shaped abdomen. Virgins are also much lighter in color than the non-virgins, and they look almost swollen.

Behavioral Fly Entrainment

Behavioral entrainments were done with four different fly phenotypes, wild type *yw*; and mutant cry^b, $glass60^j$, and $glass60^j cry^b$. $Glass60^j$ mutants are visually blind to light but still have CRY, and the double mutant $glass60^j cry^b$ flies are totally blind to light because they lack both the circadian photoreceptor CRY and they are visually blind. These double mutant flies should exhibit no entrainment to light. Both these mutants were compared to *wt* and cry^b flies.

Flies were collected separately by phenotype and placed singly into glass tubes containing food. The tubes fit into two monitors that held 64 tubes total, and 16 flies of each type were collected for entrainment (2 monitors total). For the advanced temperature cycle, one incubator was set with an LD cycle that was lights on at 8AM and off at 8PM, with the temperature initially set at constant 25°C. This ran for 3 days to entrain the flies to the LD cycle; a temperature cycle was then added in an advanced phase of 6 hrs from the start of the LD cycle. At 2 PM the temperature would drop to 20°C, and at 2 AM the temperature would increase to 29°C. This ran for 6 days. Lastly, to show persistence of the entrainment, the incubator was set to a constant 20°C in DD for 3 days.

Another entrainment was set up similarly to the advanced temperature cycle, but instead the temperature had a delayed onset of 6 hrs. At 2 PM the temperature rose to 29°C, and dropped to 20°C at 2 AM. This was done to see if there was a correlation between the time when it got warmer/colder, and if it made a difference in fly activity. Lastly, an entrainment with temperature ramping was set up, so that as the lights went on at 8AM, the temperature would increase gradually (in even periods) from 20ºC to 29ºC, and back down when lights went off at 8PM. All activity data was collected on a computer and observed using MatLab.

Preparation of Fly Extracts for Immunoblots

Fly Entrainment for Immunoblot Testing

The fly types collected for entrainment prior to immunoblotting were the wild type *yw,* and cry^b mutant *yw;*cry^b (one immunoblot was done with the fly head extracts of the TIM mutant, tim^0). Six tubes of *yw* and 6 tubes of cry^b flies were collected, containing 20 flies each (12 tubes total). Half of the tubes (3 *yw* and 3 cry^b) were placed in one incubator set at DD with a temperature cycle of 29°C at 9AM, and 20°C at 9 PM. These flies were collected to represent times ZT 2, 10, and 18. A second incubator contained the other half of the tubes, and represented times ZT 6, 14, and 22; its temperature cycle set as 20°C at 9AM, and 29°C at 9 PM. After three days of entrainment, the first two time points were collected for each fly type at ZT 2 and 14. The next two time points collected were ZT 6 and 18, and finally the last set of time points were collected, ZT 10 and 22. Flies were always collected in 15mL tubes on dry ice, labeled with the correct ZT, and placed in the

-80°C freezer between collection times. This procedure did not vary significantly between different immunoblots.

Preparation of Fly Head Extracts

The fly heads were collected (on dry ice) and transferred from the 15mL tubes into separate 0.5 ml Eppendorf tubes and labeled according to fly type and ZT. A volume of 1 ml of extraction buffer (20 mM Hepes, pH 7.5; 100 mM KCl; 5% glycerol; 10 mM EDTA; 0.1% Triton X-100; 1 mM DDT; 1X Complete Protease Inhibitors) was mixed with 1µl of 1M DDT, along with 40 µl of 25X Complete Protease Inhibitors. 30 µl of this mix was added to each tube (1 tube at a time). Each sample was then homogenized with a motorized pestle and centrifuged in a microcentrifuge three times total to clarify the lysates before adding 9 µl of gel loading buffer. Samples were spun one last time, then placed on dry ice while another sample was being homogenized. The samples were denatured prior to loading into the gel by placing the samples into boiling water for 10 minutes, and then centrifuged for 5 minutes. 12 µl of Benchmark Protein Ladder (Invitrogen) was loaded as a marker, and then between 8–9 µl of sample were added per lane.

Immunoblots

Gel Composition

The resolving gel for PER and TIM was a 6% polyacrylamide gel. Two resolving gels were prepared by adding 2 mL of 29.6% acrylamide 0.4% Bisacrylamide solution in a 15mL sterile tube. To that I added 5.2 mL H_2O, 2.7 mL 1.5M Tris-HCl (pH 8.8), 100 µl 10% SDS, 20 µl of 25% APS, and 10 µl TEMED. The gels were poured into the plates

preset in a holder. The resolving gels were left to polymerize for half an hour. The stacking gel was then prepared, and it was premixed (H_2O; 29.6% acrylamide/ 0.4% Bis; 1M Tris-HCl, pH 6.8; 10% SDS). I added 2 mL stacking gel into a 15mL tube, and then added 4 µl of 25% APS and 2 µl of TEMED. I added the stacking gel on top of the resolving gel, placing the comb on top for the wells, then allowed the gel to polymerize for another 30 minutes. The running buffer was prepared accordingly: 1 L of dH_2O, 3 gm of Tris, 14.4 gm of Glycine, and 10 mL of 10% SDS were mixed, then poured around the gels once they polymerized.

Electrophoresis and Blotting

The boiled extract samples were added to the lanes, and electrophoresis was first performed at 80 volts until the proteins reached the resolving gel then increased to 150 volts for about 2 hours. The gels were placed on the blotter for 1 hour at a constant 240 mA, and transferred to a nitrocellulose (NC) membrane. To normalize the protein content the NC was soaked in Ponceau S stain (Sigma) for 1 minute to verify that the lanes loaded correctly, and then cleansed with deionized water (2X) until all Ponceau was removed.

Antibody Incubations

The NC membrane was first blocked in 5% milk/TBST [2 grams of powdered milk (Dry milk, blotting grade, Bio-Rad) to 40 mL TBST (TBS 1X + 0.05% Tween-20)] two times, 15 minutes each. The TIM antibody (Polyclonal antiserum to TIM and PER were kindly donated from the Rosbash lab at Brandeis University) was then poured undiluted over the membrane, and incubated overnight at 4°C.

After washing the membrane 6 times (5 minutes each) with TBST, the secondary antibody was prepared: In a tube 0.15 g milk and 15 mL TBST were mixed, and then 1.5 µl of anti-rat-IgG-HRP was added. The mixture was poured over the membrane and incubated at room temperature for 2 hours. The membrane was again washed 6 times (5 min each) with TBST before detection.

Detection

The HRP signal on the membrane was detected with enhanced chemiluminescence (ECL) for approximately 1 minute then exposed on film (the exact exposure time varied).

Membrane Re-Testing

In some cases, the membrane was incubated in stripping buffer (62.5 mM Tris-HCl, pH 6.7; 2% SDS; 100 mM β-mercaptoethanol) for 30 minutes, then washed two times (10 minutes each) with TBST. The membrane was then incubated with PER antibody overnight, as described above. The secondary antibody for PER detection was anti-Rabbit HRP, exactly as for TIM.

RESULTS

The main purpose of my project was to gain a better understanding of the role of CRY in PER and TIM cycles after entrainment to temperature, and its importance in synchronizing to DD/temperature cycles. I learned basic *Drosophila* genetics in order to study the cry^b mutant and developed experiment techniques relevant to the study of circadian rhythms, specifically an immunoblot.

PER and TIM Oscillations in cry^b Mutants under a Temperature Cycle

The first hypothesis, to see if the PER and TIM proteins cycled normally in cry^b *Drosophila* under a DD/temperature cycle, was tested by incubator entrainments, and data was obtained through immunoblotting. Figures 5 – 9 represent the TIM and PER specific immunoblots performed that supported the hypothesis that cry^b mutant flies would in fact cycle the proteins normally in DD/temperature cycles.

Figure 5 is a photograph of the Ponceau S staining that was performed prior to membrane detection with ECL (same as Figure 7). This stain was a deep red color that was helpful because it showed that the lanes were roughly even on the membrane (except for lane 6).

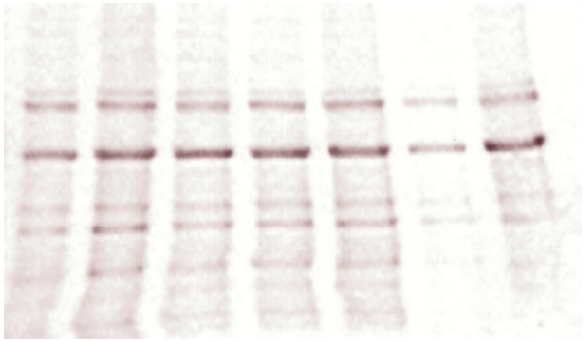

Figure 5: Ponceau S Stain of NC Membrane post immunoblot.

Figure 6 is a membrane probed with TIM (30 seconds exposure). Wild type *yw* and mutant *cry*^{b} *Drosophila* were entrained in DD with an alternating temperature cycle of 20°C and 29°C for three days (see Methods section for Entrainment details). Their heads were collected and extracted for immunoblotting and then detected for TIM protein cycling.

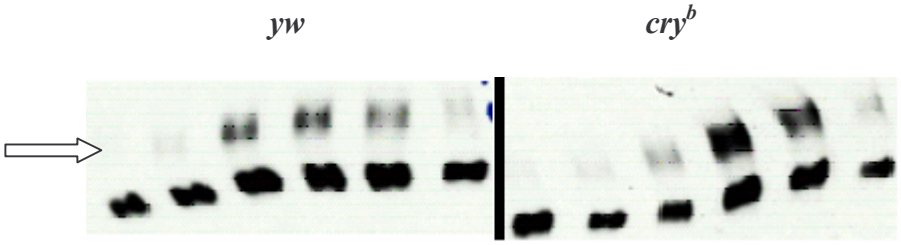

Figure 6: Immunoblot - Membrane Detected with TIM antibody (30 second exposure). X-axis from left to right (for both *yw* and *cry*^{b}) represents times ZT 2, 6, 10, 14, 18, 22. The arrow shows the location of TIM. (Note that the gel transferred a bit skewed and the lanes are uneven, but show good protein cycling).

Figure 7 represents another immunoblot detected with TIM antibody, with a slightly different entrainment. A mutant type *tim*^{0} was collected at ZT 10 and 22 to ensure that the band detected in *wt* or *cry*^{b} flies is TIM. The entrainment was performed similarly to that of Figure 5. The exposure time was 1 minute. The data was a bit dense but the cycling was noticeable. The figure was set up a bit differently, integrating the fly types according to ZT time.

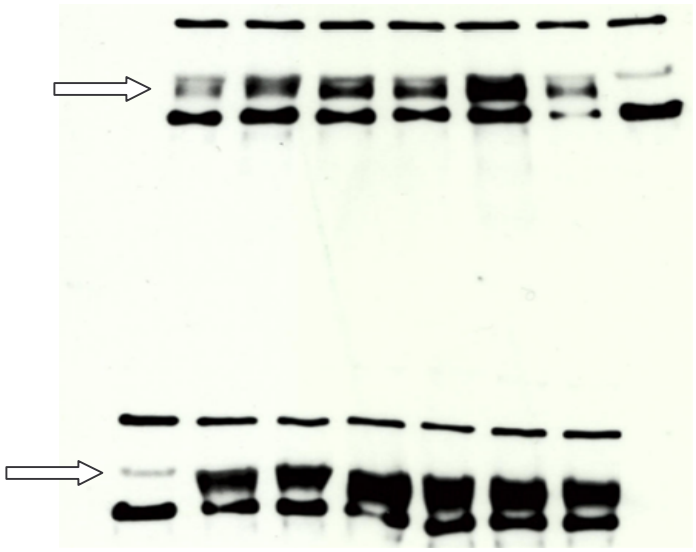

Figure 7: Immunoblot – Detected with TIM antibody (1 minute exposure). X-axis (top row, left to right): *yw* ZT2; *cry*^{b} 2; *yw* 6; *cry*^{b} 6; *yw*10; *cry*^{b} 10; *tim*^{0} 10. X-axis (bottom row, right to left): *yw* 14; *cry*^{b} 14; *yw* 18; *cry*^{b} 18; *yw* 22; *cry*^{b} 22; *tim*^{0} 22. The arrows designate TIM.

Figure 8 is another TIM detected immunoblot with a 1 minute exposure time. This was a repeated experiment with slightly different collection times: ZTs 1, 5, 9, 13, 17, and 21 (only up to ZT 17 for *cry*^{b}). This was done to show that TIM protein levels do rise highest near ZT 13, and fall back down again at ZT 21. The entrainment again was similar to that of Figure 5, except the fly tubes were collected 1 ZT earlier each collection point.

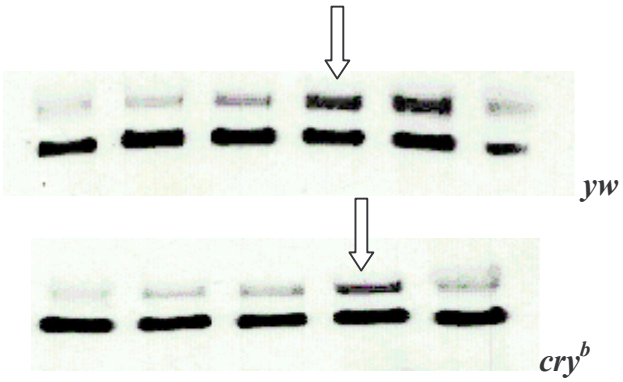

Figure 8: Immunoblot – Detected with TIM antibody (1 minute exposure). Top Panel: *yw*; x-axis from left to right: ZT 1, 5, 9, 13, 17, 21. Bottom Panel: *cry*^{b}; x-axis from left to right: ZT 1, 5, 9, 13, 17. The arrows show where TIM peaks.

Figure 9 is an immunoblot for the other major clock protein PER. The methods for collecting PER data were the same as TIM data, only the antibodies were for PER. The same membranes were used as they were for TIM, and they were stripped prior to PER detection. In figure 9 the PER detection is from the same membrane as Figure 6, and the exposure time was 5 minutes. The difference with PER from TIM, when looking for it on the immunoblot, it appears as almost two bands instead of 1.

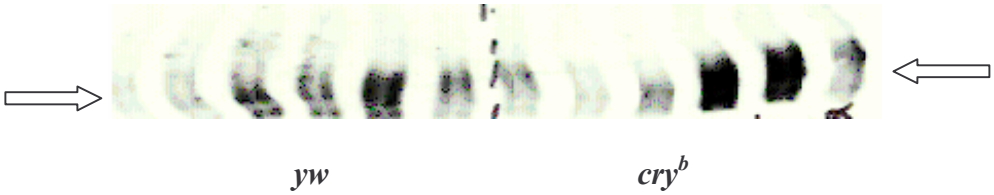

Figure 9: Immunoblot - Membrane Detected with PER antibody (5 minute exposure). X-axis from left to right (for both yw and cry^b) represents times ZT 2, 6, 10, 14, 18, 22. The arrow shows the location of PER.

Figure 10 is an immunoblot detected with PER antibody, This time the exposure was 30 seconds, and it was detected on the same membrane as Figure 8. The collection times were ZT 1, 5, 9, 13, and 17, to better show when PER protein levels peak.

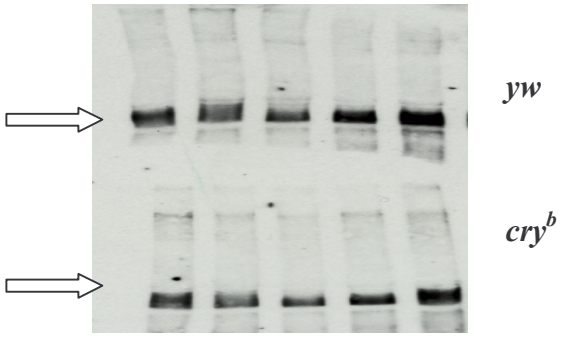

Figure 10: Immunoblot - Membrane Detected with PER antibody (30 second exposure). Top Panel: *yw*; x-axis from left to right: ZT 1, 5, 9, 13, 17. Bottom Panel: *cry^b*; x-axis from left to right: ZT 1, 5, 9, 13, 17.

The previous figures (6-10) support the hypothesis that *cry*^{b} mutant flies would cycle the two important clock proteins, TIM and PER, normally during and after a DD/Temperature cycle. In Figure 6 you can see that in both the *yw* and *cry*^{b} flies TIM is cycling at similar times, reaching a peak near ZT 14. This is good as ZT 13 is the expected peak for this protein because TIM levels begin to rise at early night. As expected in Figure 7, the *tim*^{0} mutant acted as a TIM marker at ZT 10 and 22; TIM was detectable in both the *yw* and *cry*^{b} flies. The cycling also peaked around the expected ZT14.

Figure 8 shows PER accumulation at its peak near ZT 14 and ZT 18, very similar to that of TIM. In figure 9 the PER antibody was fresh so the data came out a bit "dirty", but protein cycling was evident. Figure 10 also supported that PER cycles normally in *cry*^{b} flies, showing when PER protein levels peak between ZT13 and 17. These immunoblot results show the separate proteins cycling within both wild type and *cry*^{b} mutant *Drosophila*, peaking around the expected ZT13.

In conclusion, both Per and TIM oscillate normally under temperature cycles in cryb flies. CRY is therefore not required under these conditions.

LD/Temperature Cycle Entrainment Behavioral Actograms

Behavioral entrainments were conducted to see whether adding a temperature cycle into an LD cycle would cause *cry*^{b} fly activity to shift, favoring the temperature cycle. In figure 11 four different fly types (*yw; cry*^{b}*; glass*^{60j}*; glass*^{60j}*cry*^{b}) were subjected to a 6 hour advanced temperature cycle (see Methods section for full entrainment details).

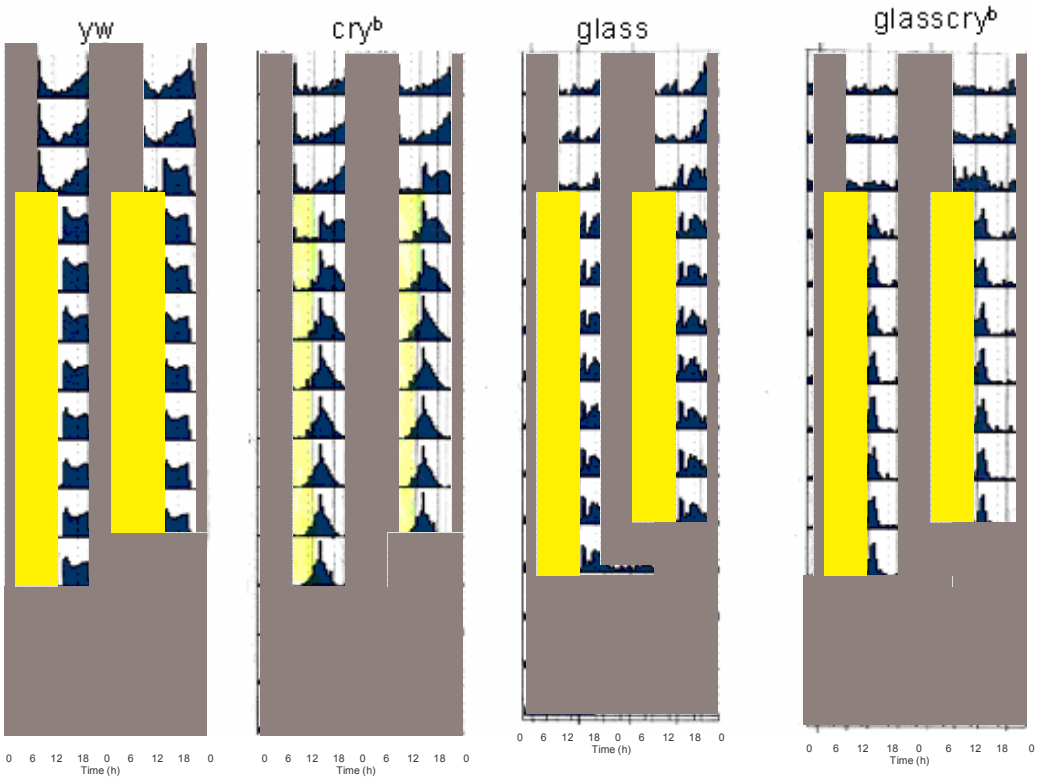

Figure 11: LD/Temperature Cycle Entrainment of wild type *yw,* and mutant *cry*^{b}*; glass*^{60j}*; glass*^{60j}*cry*^{b} flies. The x axis represents time (in hours). The y axis is representative of average fly activity. The gray shaded area is lights off and 25C, until addition of Tcycle: then gray is 20C; white: lights on; yellow: temperature 29C.

Figure 12 represents fly activity after a 6 hour delayed temperature cycle onset to the LD cycle. This entrainment was done to see if the results would be similar to those of the advanced temperature cycle. Only *yw* and *cry*^{b} flies are shown to compare to figure 11.

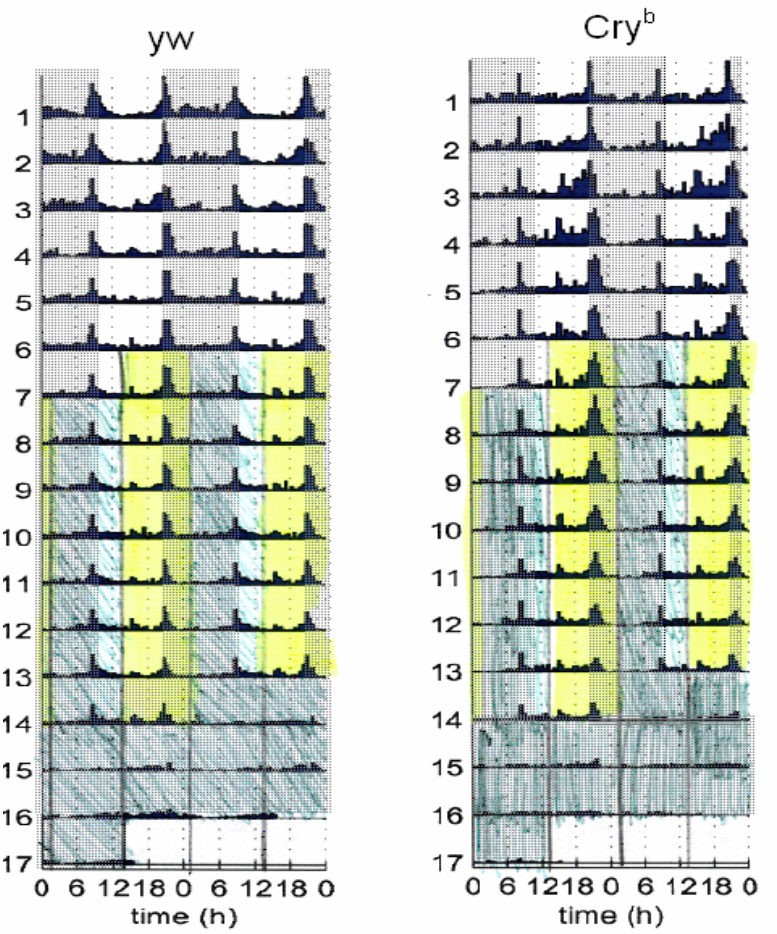

Figure 12: LD/Temperature Cycle Entrainment of *yw* and *cry*^{b} flies. The x axis represents time (in hours). They axis is representative of average fly activity. The gray shaded area is lights off and 25C; white: lights on; yellow: temperature 29C; green: temperature 20C.

A third LD/Temperature Cycle entrainment was done, but with a ramping temperature cycle. This was tested to see if having the temperature slowly increases from 20ºC to 29ºC (and back down again) in relation to the lights on and off would affect the fly activity and their ability to respnd to the temperature cycles (data not shown).

In figure 11, the *cry*^{b} mutant activity was surprising. The activity under the first constant 25ºC in LD seemed relatively normal, although it was a bit weaker in morning and evening activity peaks compared to the wild type. Then as the temperature cycle was added in, there was a very noticeable shift in peak activity, and the *cry*^{b} flies looked to be adopting the phase of the temperature cycle over that of LD. Releasing the flies into constant conditions at the end of the entrainment (constant darkness at 20ºC) gave proof that the flies were in fact synchronized to the temperature cycle and not to the LD. This contrasts with wild type flies; they did not shift their phase to temperature, but maintained that of the LD cycle. For these *yw* flies, there was only a gradual shift in activity with the onset of the temperature cycle. There also appeared to be a lot of masking going on, although there was a slight decrease as the temperature cycle duration lengthened.

Results similar to *cry*^{b} were seen with the double mutant *glass60*^{j}*cry*^{b} flies. The activity in LD followed no pattern, as the flies are both visually blind and lack the photoreceptive CRY. Therefore, they don't seem to possess any LD anticipation. Although the activity during the temperature cycling seemed masked, the flies appeared to adopt the phase of the temperature cycle. Like *cry*^{b} the flies showed synchronization to the phase after release into constant conditions.

The *glass*^{60j} fly activity did not appear to favor temperature over LD, and there was no noticeable shift. This data was comparable with that of the wild type flies. Activity of these flies was a bit skewed compared to *yw*, probably because they have CRY but they are blind to the light. The last few days in DD looked very similar to the first few days of activity where there seemed to be no peak of morning activity. This also seemed very different than the *cry*^{b} in DD at 20ºC.

In summary these results indicate that CRY is important for the proper integration of light and temperature input. In its absence, flies become more sensitive to temperature.

Figure 12 was the delayed temperature cycle onset with LD. When compared to figure 11, both the wild types show similar entrainment, with their phase matching that of the LD cycle. Whether the temperature cycle came 6 hours before or after the start of the LD did not make a difference in wild type. Figure 12 however did not show similar results than figure 11 for cry^b. As seen in the figure the cry^b flies remained entrained to the phase of the LD cycle, similar to *yw* flies. Thus, the directionaliry of the TC compared to the LD cycle is important to uncover the function of CRY for the integratinn of light and temperature inputs.

DISCUSSION

Cry^{b} mutants cannot cycle TIM and PER correctly in the photoreceptor cells under LD conditions, and the flies are unable to reset their circadian clocks when subjected to light pulses. Through a series of TIM and PER specific immunoblots, the hypothesis was tested that TIM and PER proteins would cycle normally in DD/Temperature cycles in the *cry*^{b} mutants. As seen in the immunoblot figures (6-10) TIM and PER clearly cycle in the mutant flies, probably as well as in *yw* wild type. These data show that *cry*^{b} flies are entrainable to temperature in the absence of light, and the clocks located in the peripheral tissue are able to function normally under these conditions.

It was proposed that CRY could play a role in the negative feedback loop in the peripheral tissues (like those probed in my immunoblots). If this is the case then it is conceivable that transcription would be more severely affected than the protein levels. There is also the possibility that the oscillations occurring are normal under temperature cycles, but they disappear once released into constant conditions faster in the *cry*^{b} flies compared to wild type. This hypothesis requires further testing by measuring the molecular rhythms in temperature entrained flies that have been returned to constant temperature conditions.

In the behavioral entrainments it was fascinating to see the *cry*^{b} flies adopt the phase of the temperature cycle over that of LD, similar to the completely blind mutant *glass60*^{j}*cry*^{b} flies. Therefore a novel defect in the entrainment response of *cry*^{b} flies has been identified. The flies are more strongly influenced by the temperature cycles, and have

more difficulty synchronizing to light. The behavioral results also suggest that wild type flies could require both CRY and a visual input to properly synchronize their behavior.

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