

EFFECTS OF LIGHT ON BDBT PUNCTA FORMATION IN THE *DROSOPHILA*  
EYE REVEAL INVOLVEMENT OF BDBT WITH COLOR PREFERENCE  
BEHAVIOR AND DISTINCT NUCLEAR LOCALIZATION PATHWAYS FOR DBT  
AND PER.

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DOCTOR OF PHILOSOPHY

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Brent Nolan, Candidate for the Doctor of Philosophy Degree  
University of Missouri-Kansas City, 2020

ABSTRACT

In this dissertation, the research focus is on elucidating the mechanism behind BRIDE OF DOUBLETIME (BDBT) puncta/foci formation. During this process it was discovered that both the circadian and visual photoreceptors have roles for normal cycles of BDBT foci formation. This BDBT foci cycling is needed for normal changes in subcellular localization of the circadian regulators PERIOD (PER) and DOUBLETIME (DBT) in the *Drosophila* eye. Finally, BDBT is demonstrated to have a role with the eye clock in altering circadian color preference in flies.

The kinase DBT is a regulator of the *Drosophila* circadian clock and phosphorylates PER during the day, targeting PER for degradation. BDBT interacts with DBT, enhancing DBT kinase activity towards PER, and accumulates during the dark of a 12 hr light/12 hr dark cycle in PER- and DBT-dependent cytosolic foci. This accumulation of BDBT is shown here to be constitutively high when flies are raised under constant dark (circadian time: CT), but decreases during constant light conditions (LL), demonstrating a light/dark responsive mechanism.

Analysis of circadian loss of function CRYPTOCHROME (*cry*) mutants and mutants of the RHODOPSIN 1(*ninaE*) visual transduction pathway indicated that disappearance of cytosolic BDBT foci in response to light is dependent upon both the CRY and the RHODOPSIN-1 visual transduction pathways. The ARR1 (*Arr<sup>1</sup>*) and ARR2 (*Arr<sup>2</sup>*) mutants eliminated the wild-type accumulation of BDBT foci under dark conditions in LD and constant darkness (DD) conditions, indicating a novel mechanism in which ARRESTINS are able to regulate accumulation of BDBT cytosolic foci. *Arr<sup>1</sup>* and *Arr<sup>2</sup>* mutants also lead to increased nuclear accumulation of PER protein during stimulated day and night, likely affecting circadian regulation in the *Drosophila* eye.

As arrestins have been shown to affect endocytosis of RHODOPSIN, I examined the effect of overexpression of a temperature sensitive dynamin GTPase in the eye SHIBIRE (SHI) and observed increased membrane vesicle formation at restrictive temperatures. Knock-down of BDBT specifically in the eye was achieved producing a loss of circadian molecular oscillations for both PER and DBT with uncoupling of their sub cellular localizations: PER was constitutively nuclear and DBT was constitutively cytosolic. The loss of eye-specific molecular rhythm was reflected in an altered rhythm of visual preference that has been observed with other loss of circadian function mutations. The research put forth in this dissertation has illustrated the importance of BDBT foci formation to maintaining a functional eye clock in *Drosophila* and novel role where the eye clock can influence behavior.

## APPROVAL PAGE

The faculty listed below, appointed by the Dean of the School of Graduate Studies, have examined a dissertation titled “Effects of Light on BDBT Puncta Formation in the *Drosophila* Eye Reveal Involvement of BDBT with Color Preference Behavior and Distinct Nuclear Localization Pathways for DBT and PER”, presented by Richard “Brent” Nolan, candidate for the Doctor of Philosophy Degree, and certify that in their opinion it is worthy of acceptance.

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## CONTENTS

ABSTRACT.....	iii
LIST OF ILLUSTRATIONS.....	vii
ACKNOWLEDGEMENTS.....	x
Chapter	
1. INTRODUCTION.....	1
2. METHODS AND MATERIALS.....	20
3. BDBT FOCI DATA FOR PUBLICATION	
a. BDBT Foci Formation Oscillate in a Circadian Manner Through a Light/Dark Mechanism. ....	24
b. BDBT Foci Formation is Required for the Timely Sub-cellular Localization of Circadian Regulator Proteins DOUBLETIME and PERIOD and Circadian Color Preference.....	39
4. BDBT FOCI- INVESTIGATIVE AND SUPPLEMENTAL DATA.....	59
5. FUTURE DIRECTIONS.....	82
REFERENCES.....	94
VITA.....	112

## ILLUSTRATIONS

Figure	Page
1.1 Schematic illustrating how oscillations of circadian proteins are regulated by post translational and post transcriptional feedback loops.....	4
1.2 Immunoblot analysis of DBT <sup>K/R</sup> NLS- interactions with BDBT.....	5
1.3 GAL4-UAS binary expression system technique used to study the effects that BDBT foci confers on eye clock proteins. ....	8
1.4 The <i>Drosophila</i> Ommatidium and RHODOPSIN in rhabdomeric structures.....	10
1.5 Model of the <i>Drosophila</i> phototransduction cascade.....	13
3.1 Effects of constant light and darkness after LD entrainment on BDBT foci.....	27
3.2 Immunoblot analysis of BDBT protein levels in WT and ARRESTIN mutant lines.....	28
3.3 Effects of photoreceptor mutants on BDBT foci and PER localization in LD cycles. ....	33
3.4 Effects of photoreceptor mutants on BDBT foci accumulation in LL-7hrs and DD. ....	38
3.5 Effects of temperature sensitive SHIBIRE mutants on BDBT foci and subcellular fractionation experiment. ....	43
3.6 Effects of bdbt-RNAi on PER and DBT localization in the fly eye.....	48
3.7 Immunoblot analysis of PER protein levels in WT and BDBT knockdown flies...	50
3.8 A model for how the circadian clock influences color preference in flies.....	51
3.9 Behavioral assays for color preference in the BDBT knock-down flies and two control genotypes.....	52

3.10 Behavioral assays for color preference in DBT <sup>K/R</sup> mutants using Rh1-GAL4 driver line.....	53
4.1 Effects of Phospholipase C ( <i>norpA</i> ) on BDBT foci formation in constant light and constant dark.....	62
4.2 Effects of RHODOPSIN and ARR2 mutants on BDBT foci formation and ARR1 expression in <i>Drosophila</i> eye.....	63
4.3 Effects of RHODOPSIN and ARR1 mutants on BDBT foci formation and ARR2 expression in <i>Drosophila</i> eye.....	66
4.4 Colocalization staining of BDBT and DBT in <i>Drosophila</i> eye. ....	69
4.5 Co-immunoprecipitations to determine protein-protein interactions with DBT and BDBT.....	72
4.6 Effects of ARRESTIN mutants on DBT post translational modifications.....	74
4.7 Brain expression patterns of GMR-GAL4>UAS-mCD8-GFP and Rh1-GAL4>UAS-GFP in flies.....	77
4.8 Effects of <i>spag</i> -RNAI and <i>dbt</i> -RNAi on BDBT foci formation with GMR-GAL4 driver.....	79
4.9 Table illustrating the effects of eye specific knockdown of BDBT and DBT on period.....	80
4.10 Fluorescent and Differential Interference Contrast Microscopy eye sections to access eye morphology in <i>arrestin</i> mutants.....	81
5.1 BDBT knockdown effects on circadian color preference using Rh1-GAL4 driver.....	91
5.2 DBT knockdown effects on circadian color preference.....	92

5.3 Model for BDBT foci formation.....93

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## CHAPTER 1

### INTRODUCTION

#### **Circadian Rhythms**

Circadian rhythms are affected by environmental cues such as light and temperature, but in their absence an approximately 24 hour rhythm persists (Pittendrigh, 1960). These cues synchronize (or entrain) biological processes promoting oscillations of negative feedback loops between regulatory proteins and transcription factors (Allada et al., 2001). To what extent these biological processes affect our bodies and minds is what drives research in the fields of circadian rhythms and sleep. It is important to mention that although sleep and circadian rhythms are often thought of in close relation that they are distinct processes. This nuance is important in circadian and sleep focused fields as we must be careful not to lump the two together; however, as the two are related it is worth noting that research in one field can influence work in another.

In this thesis, the focus will be upon the effects of the *Drosophila* eye and processes of eye clock as circadian rhythms are strongly influenced by light signaling occurring in the photoreceptor cells. This light signaling influences the circadian rhythms of the eye clock which in turn alters the entrainment of the clock cells in the brain. However, the brain will maintain rhythmic behavior in the absence of light. For example, flies that are completely blind to light through a *glass<sup>60j</sup>;cry<sup>b</sup>* double mutation lose their ability to entrain to a light/dark cycle, but exhibit free running rhythmic behavior of approximately 24 hrs (Helfrich-Forster et al, 2001). The process of how the eyes influence the brain in *Drosophila* is still not well

understood. In this thesis, data will be presented at how disruptions occurring in the eye clock through a BRIDE OF DOUBLE-TIME (DBT) foci generating mechanism disrupt normal function of the eye clock. This dysfunction to the *Drosophila* eye clock also demonstrates behavioral changes through daily regulated color preferences providing a novel pathway for how the eye can influence behavior.

### ***Drosophila* as a Circadian Model**

Circadian rhythms are synchronized to alternating light dark cycles (Zeitgeber time-ZT) through degradation of the PERIOD (PER) and TIMELESS (TIM) proteins. During the evening PER and TIM accumulate in the cytosol where they dimerize, promoting their nuclear translocation. In the nucleus, PER and TIM act as circadian transcriptional regulators repressing their own transcription along with other genes controlled by the CLOCK/CYCLE (CLK/CYC) heterodimer. This repression is relieved during the day as CRYPTOCHROME (CRY), a photoreceptor that dimerizes with TIM upon blue light stimulation, leads to degradation of TIM/CRY in response to light (Ceriani et al, 1999). PER is then phosphorylated by the circadian kinase DOUBLETIME (DBT) targeting it for proteasomal degradation relieving the PER/TIM repression on CLK/CYC controlled genes. In the absence of CRY activity at night, nuclear PER accumulates through another round of night and PER/TIM accumulation (Kloss et al., 1998; Price et al., 1998) (Figure 1.1). There are also components of a secondary feedback loop such as *par domain protein 1* (*pdp-1*) and *vriille* (*vri*) that are regulated through the CLK/CYC transcription factors and are required for a functional *Drosophila* clock (Blau & Yound, 1999; Cyran et al., 2003).

PDP-1 has been shown to be involved with clock output regulation while VRI acts to repress transcription of *Clk* (Benito et al., 2007). Many of these regulatory circadian proteins in *Drosophila* are conserved in mammals making the *Drosophila* circadian clock a useful model for the more complex mammalian clock (Allada et al., 2001).

BDBT is a non-canonical FK506 binding protein initially discovered by the Price lab, which has shown that BDBT can enhance DBT kinase activity towards PER and accumulates during the dark of an LD cycle in PER- and DBT-dependent cytosolic foci in the photoreceptor cells of the fly eye (Fan et al, 2013). In this thesis when we refer to the circadian clock, we will mostly be describing the effects of the *Drosophila* eye clock and not the subset of clock cells found in the fly brain.

BDBT is a novel interactor of DBT that was originally identified as CG17282 through immunoprecipitations of MYC-tagged DBT in Schneider 2 (S2) cells (Fan et al., 2013). BDBT interacts with DBT in vitro, in S2 cells, and in fly heads, and it is essential for normal cycles of PER nuclear accumulation in the small and ventral lateral neurons and circadian behavior. Previous work in the Price lab has shown that the interaction between BDBT and DBT is dependent on BDBT having access to the DBT nuclear localization sequence (NLS) (Figure 1.2). Using polyacrylamide gel electrophoresis (PAGE) analysis, we determined when BDBT is knocked down, DBT undergoes increased post translational modifications. This causes DBT to migrate more slowly on a gel likely due to autophosphorylation of DBT while PER protein levels increase in both hypo- and hyper- phosphorylated states (Fan et al,

## The Fly Circadian Rhythm, Like the Human Rhythm, is Produced by Interlocked **Transcriptional** and **Post-translational** Feedback Loops

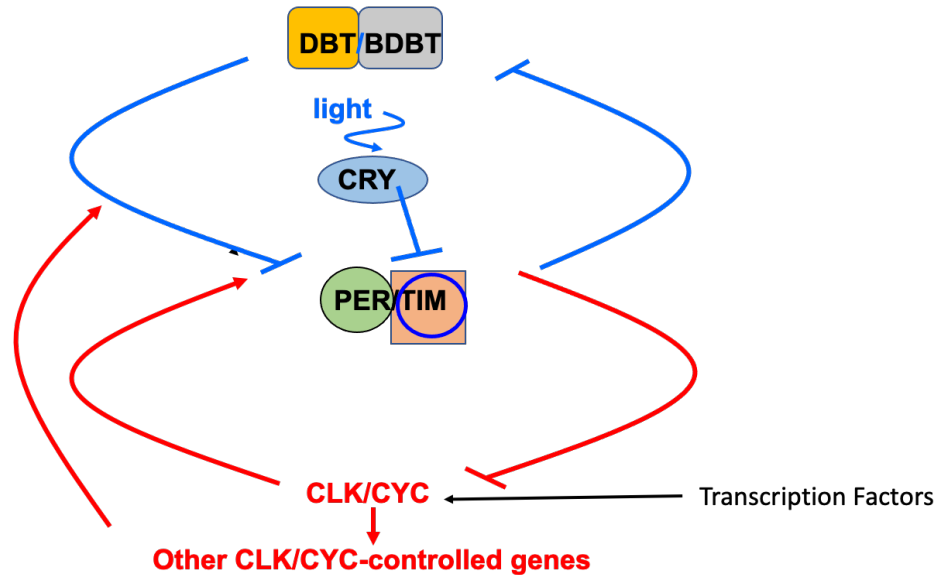
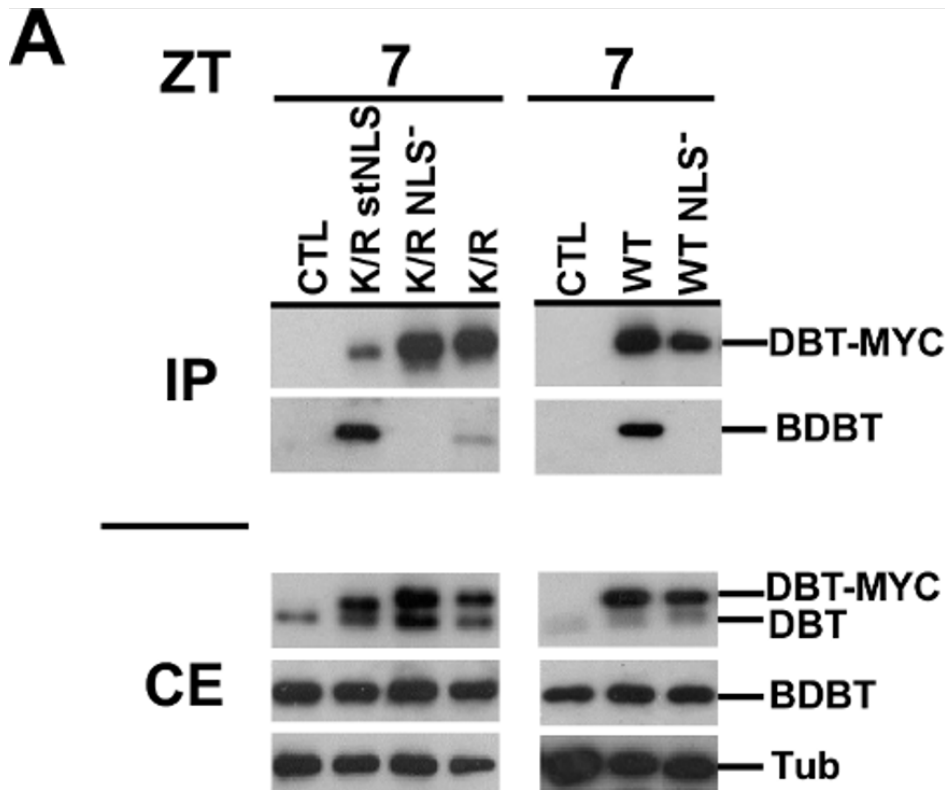


Figure 1.1. Schematic illustrating the general concept of how circadian oscillations are regulated by transcriptional and post translational feedback loops, in which PER and TIM can translocate to the nucleus to repress the transcription factors CLOCK/CYCLE (CLK/CYC) from expressing additional PER and TIM along with other CLOCK/CYCLE dependent genes. This repression is relieved by the response of CRYPTOCHROME (CRY) to blue light which then targets TIM for degradation and thereby allows targeting of PER by the circadian kinase DBT whose activity is enhanced by BDBT.



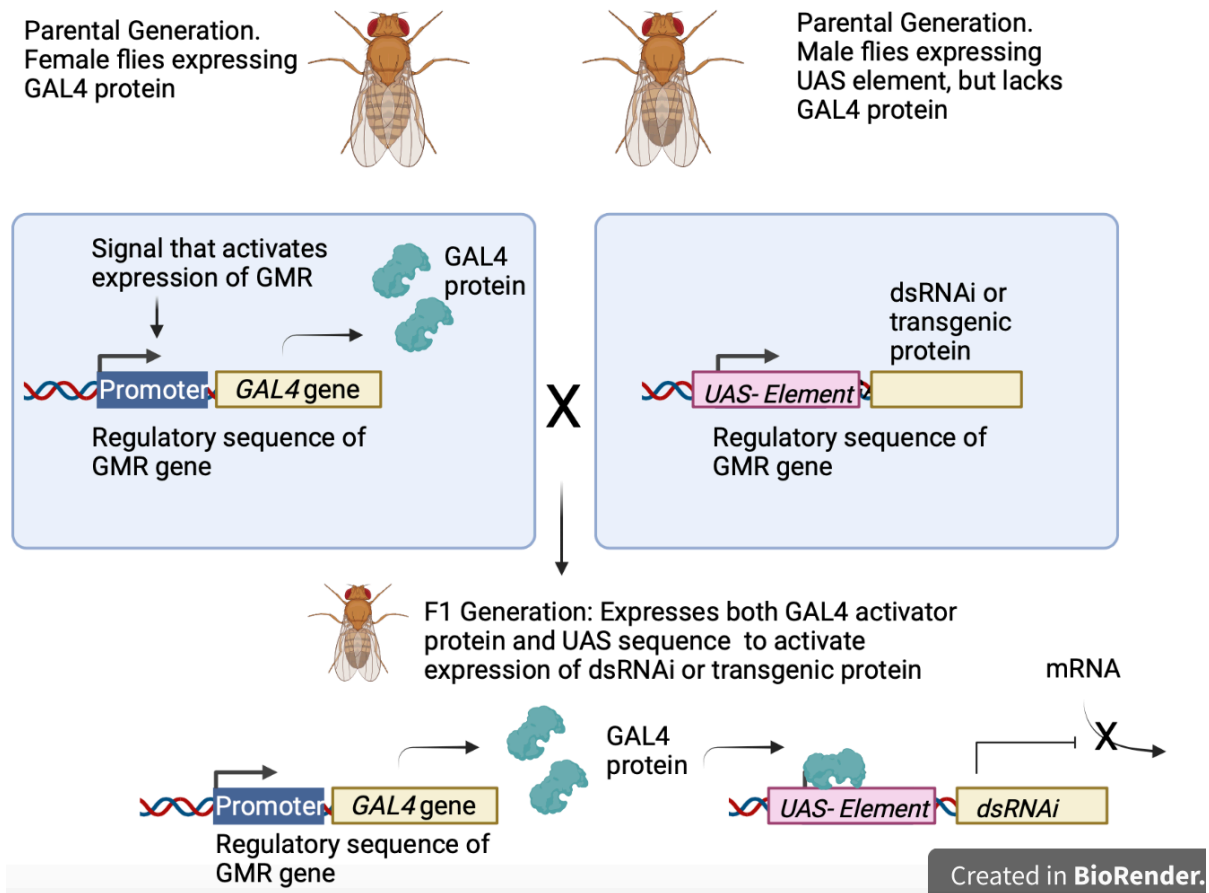
**Figure 1.2. Immunoblot analysis of DBT<sup>K/R</sup> NLS- interactions with BDBT.** Heads were collected at the noted times in a light dark cycle. Crude extracts (CE) and immunoprecipitations (IP) of the indicated MYC-tagged DBT's expressed from a UAS promoter with the *tim*GAL4 driver were run on western blots and probed for DBT, BDBT, and tubulin as a loading control. Immunoprecipitations indicate that BDBT can still interact with DBT<sup>K/R</sup> (A dominant negative DBT mutant) or wild type DBT, but only when the nuclear localization sequence was present, indicating that the NLS- mutation disrupts complex formation between DBT and BDBT (Fan from Venkatesan et al, 2015).

2013). In addition to its interaction with DBT, BDBT may interact with other proteins through a tetratricopeptide repeat (TPR) motif at its C-Terminal end composed of two  $\alpha$  helices (Fan et al., 2013). TPR motifs are known to mediate protein-protein interactions (Zeytuni & Zarivach, 2012) and thereby may promote assembly of multiprotein complexes, thus potentially providing another mode of DBT regulation through BDBT interactions modulating DBT activity towards its substrates.

Besides mediating an effect of DBT protein on PER through increased phosphorylation and degradation of PER, immunofluorescent analysis indicated that BDBT accumulates in cytosolic foci through a temporal mechanism in the fly eye. During the evening from ZT13 and ZT19, BDBT cytosolic foci accumulate to a high point in which they are broadly expressed throughout the photoreceptors before again becoming sequestered in focal streaks at ZT1 and ZT7 in cross sections of the retina (Fan et al., 2013). Expression of BDBT cytosolic foci corresponds to the rhythmic accumulation of nuclear PER at ZT19 and its absence at ZT7 demonstrating oscillating patterns of foci formation between the stimulated middle of the day and night. Utilization of *per<sup>0</sup>* flies and UAS-dcr2;timGAL4>/+;UAS-dbt RNAi:/+ lines led to a failure of BDBT puncta formation in the *Drosophila* photoreceptors at all timepoints, indicating foci accumulation is dependent upon a circadian mechanism involving DBT and PER (Fan et al., 2013).

### **Clock Cells of the Fly Brain and Eye**

Traditionally, clock cells are those identified through expression of circadian targets such as *per* and *tim* clock gene mRNAs and their protein products (Zerr et al, 1990; Ewer et al, 1992). There are approximately 150 neurons that express the



**Figure 1.3. The use of the GAL4-UAS binary expression system technique to study the effects that BDBT foci confers on eye clock proteins.** The GAL4/UAS technique for controlled gene mis- or over-expression in *Drosophila* is achieved by generating a fly transgenic for two separate constructs. In the example shown, one construct consists of the coding sequence of the yeast GAL4 transcription factor fused directly downstream of the GMR binding sequences, which bind the eye-specific *glass* transcription factor to promote the expression of the GAL4 specifically in the fly eye. The second construct consists of GAL4-binding UAS elements which are fused directly upstream to *bdbt*-RNAi-expressing genes. A male fly carrying the GMR-GAL4 transgene expressing the GAL4 protein (see top left panel) produces viable flies with wild type circadian periods, while a fly carrying the UAS-*bdbt* RNAi transgene (shown top right panel) does not express the *bdbt*-RNAi transgene because of the absence of the GAL4 protein and therefore also has wild type rhythms. A mating of these two flies produces progeny flies that express the GAL4 protein which then binds to the UAS element and promotes the expression of *bdbt*-RNAi in the *gmr*-expressing neurons.



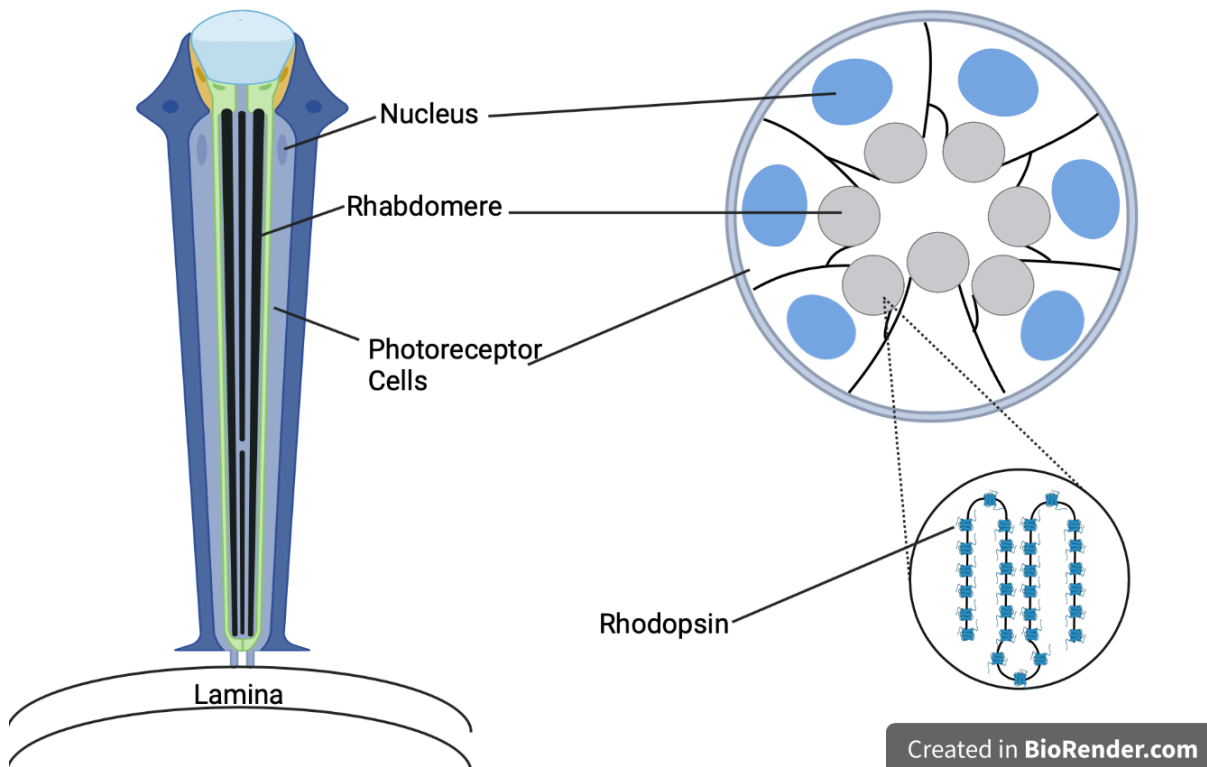
genes required for this molecular clockwork in the adult brain and these neurons have been divided into six groups based on their location and size (Frisch et al., 1994; Kaneko and Hall, 2000; Helfrich-Förster, 2003). These subgroups consist of small and large ventrolateral neurons (LN<sub>Vs</sub>), the dorsolateral neurons (LN<sub>Ds</sub>), and three groupings of dorsal neurons (DN<sub>s</sub>), the DN1s, DN2s, and DN3s. This allowed researchers to isolate groupings of clock cells and target them with a genetic approach using the yeast GAL4-UAS system (Figure 1.3) (Brand and Perrimon, 1993).

In the fly brain, circadian research is often focused on the lateral and dorsal neurons. The lateral neurons reside in both hemispheres of the fly brain in subgroups such as the LN<sub>Vs</sub> and the LN<sub>Ds</sub>. Pigment-dispersing factor (PDF) is a neuropeptide that is expressed in 16 neurons that represent most of the ventral component (LN<sub>Vs</sub>) of these subgroups and is crucial for daily entrainment and locomotor rhythms (Renn et al., 1999; Grima et al,2004; Helfrich-Förster, 1998; Stoleru et al, 2004). This makes all PDF expressing cells circadian clock cells which allows for additional genetic isolation of these PDF clock cells with tools like the *pdf*GAL4 driver. PDF signaling through the s-LN<sub>Vs</sub> is primarily associated with morning as these cells promote morning activity (Choi et al., 2012) while the LN<sub>Ds</sub> are associated with activity in the evening. However, the morning and evening properties to these circadian pacemakers are not rigid and are capable of change when exposed to different environmental conditions (Rieger et al., 2009; Zhang et al., 2010; Peschel and Helfrich-Förster, 2011).

Previously, the effects of BDBT focused on targeting BDBT for knockdown using a *timGAL4 > UAS-dcr2; UAS-bdbt-RNAi* line. This genotype did not reduce BDBT foci or affect PER nuclear localization in the eye, presumably because the knockdown of BDBT was not strong enough in the fly eye. For this project it is necessary to distinguish between the clock cells of the eyes and those of the brain, as the daily accumulation of BDBT foci is only seen in the eye. To do this we utilized the Glass Multiple Reporter (GMR) enhancer, which is strongly expressed in the *Drosophila* eye due to multiple binding sites for the eye-specific transcription factor GLASS, along with Dicer to target knockdown of BDBT in the fly eye.

If BDBT is knocked down in flies using *UAS-dcr2;timGAL4>/bdbt RNAi*, flies exhibited arrhythmic behavior even though we do not see reduced BDBT foci formation and this *bdbt-RNAi* phenotype was able to be rescued with BDBT overexpression and suppressed with DBT overexpression (Fan et al, 2013). Another study from our lab showed that the cochaperone SPAGHETTI (SPAG) also binds to DBT and can act to stabilize DBT levels and activity in *Drosophila* (Fan et al, 2015). RNA polymerase II associated protein-3 (RPAP3) is the mammalian ortholog to SPAG and contains tetratricopeptide repeat domains that have been shown to promote apoptosis induced by various stimuli (Itsuki et al, 2008; Yoshida et al, 2013). These data demonstrating the strong synergistic effects of BDBT and SPAG upon DBT through protein-protein interaction, and the involvement of SPAG

## *Drosophila* Ommatidium



**Figure 1.4. Diagram of the *Drosophila* Ommatidium and RHODOPSIN in rhabdomeric structures.** A cross sectional and top down view of the *Drosophila* Ommatidium as seen in our eye sections. Zoomed sections illustrates RHODOPSIN coating the membrane of the rhabdomeric microvilli.

in apoptosis warranted further investigating these interactions (Yoshida et al. 2013; Fan et al, 2015).

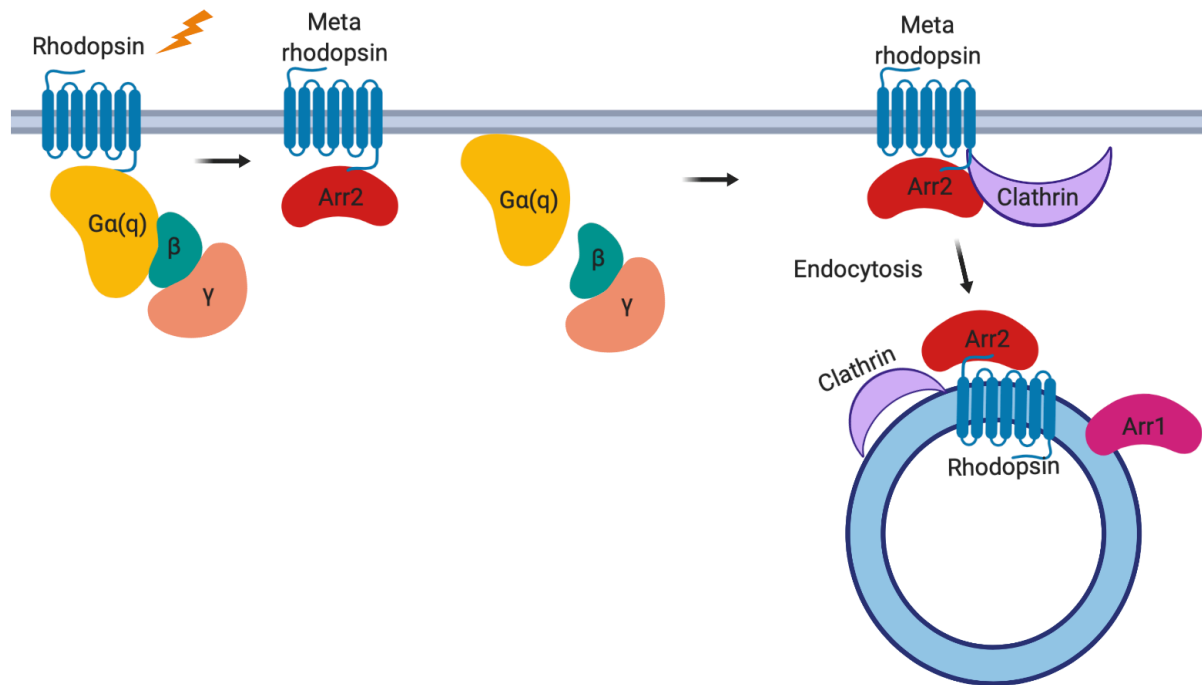
### ***Drosophila* Visual Transduction Pathway**

The *Drosophila* compound eye is made up of roughly 700-750 ommatidia, depending on sex, each consisting of eight core photoreceptor neurons known as the R1-R8 photoreceptors that are involved in image formation and motion detection (Figure 1.4). This system has been extensively studied as the *Drosophila* visual transduction system and is one of the fastest G-protein-coupled signaling cascades allowing for quick response times to sensory information. Over 30 genes involved in this photo-response have been identified, but we chose to focus on RHODOPSIN-1, which is primarily expressed in the R1-R6 photoreceptor cells, and ARR1 and ARR2 which are involved in quenching of RHODOPSIN-1 signaling and endocytosis (Alloway & Dolph, 1999; Satoh et al, 2005). RHODOPSIN-1 is the primary opsin expressed in the fly eye and if BDBT foci formation is dependent up a light/dark mechanism then RHODOPSIN-1 may be involved with this signaling pathway. If BDBT foci are comprised of vesicles than we hypothesize that in ARRESTIN mutants we would see alterations to the formation of BDBT foci such as is seen in RHODOPSIN-1 immuno-positive vesicles (Satoh et al, 2005). This accumulation of BDBT foci may provide insights into how light is driving circadian rhythms in the *Drosophila* eye through a BDBT/DBT interaction beyond the canonical circadian CRY degradation pathway.

RHODOPSIN-1 is a transmembrane protein located in the microvilli structures of photoreceptor cells. These microvilli structures are called rhabdomeres and are

located towards the middle of each ommatidium. Upon blue light stimulation, RHODOPSIN can be converted to meta-RHODOPSIN and trigger a heterotrimeric G-protein signaling pathway where it activates the alpha subunit. This leads to downstream effects such as activation of phospholipase C (PLC), hydrolysis of phosphatidylinositol-4,5- biphosphate into secondary messengers, and activation of TRP and TRPL cation channels (Chyb, Raghu, & Hardie, 1999; Montell & Rubin, 1989; Phillips, Bull, & Kelly, 1992; Hardie & Minke, 1992; Niemeyer et al., 1996). In this signaling cascade PLC is required, and previous analysis of the principal visual PLC gene in *Drosophila*, designated no receptor potential A (*norpA*), showed that it is predominately expressed in rhabdomeres and null mutants of the *norpA* gene show drastically reduced receptor potential through electroretinogram (ERG) activity (Bloomquist et al, 1988).

Upon activation and opening of the TRP and TRPL cation channels there is a cellular influx of  $Ca^{2+}$  to promote an eye specific protein kinase C activity, which can in turn regulate cation channel activity. Besides these levels of regulation, the expression pattern of ion channels also determines the physiological properties of neuronal cells. Trafficking of these ion channels into and out of the plasma membrane is important as this allows for modulating the number of channels at a specific cellular localization. In flies, activation of the photoreceptor and this visual transduction signaling cascade leads to an influx of  $Ca^{2+}$  through TRP channels initiates the translocation of TRPL from the rhabdomere, where cell signaling occurs, to the cell body (Bahner et al, 2002; Meyer et al., 2006). Many of these proteins involved in the eye visual response (including Rh1, TRP channels, and NORPA) are



**Figure 1.5. Model of the *Drosophila* phototransduction cascade.** Illustrated here is how RHODOPSIN is converted to meta-RHODOPSIN upon blue light stimulation. This leads to recruitment of ARR2 to RHODOPSIN to quench signaling and activation of heterotrimeric G-protein signaling cascade. ARR2 can interact with clathrin and form RHODOPSIN-1 immunopositive vesicles along with ARR2 and ARR1.

regulated by interactions in a large multimeric signaling complex mediated by inactivation-no-afterpotential D (INAD), a scaffolding protein (Tsunoda et al., 1997) INAD contains PDZ domains that show light sensitivity, and some of its associations can be disrupted through light by altering a PDZ domain. This occurs through a light dependent disulfide bond under reducing conditions of two cysteine residues (Wang & Montell, 2007). TRP is involved in localizations of the INAD multimolecular complex to the rhabdomeres (Arnold & Clapham, 1999). In addition, INAD can mediate the circadian and visual pathways through interactions with CRY (Mazzotta et al., 2013).

The R1-R6 photoreceptors have axonal projections that project into the first ganglionic layer, also known as the lamina of the fly eye. To better understand this accumulation of BDBT foci we chose to focus on a targeted genetic approach to the RHODOPSIN-1/visual transduction pathway and the CRY/circadian pathway. One caveat worth noting is that some of these visual mutants make these mutant lines subject to neurodegeneration by light exposure (Shieh, 2011) which is why we also focused on experiments in constant darkness to reduce this neurodegeneration.

In addition to these two pathways, we choose to also focus on ARRESTIN mutants as they play a key role in endocytic cell-survival activity, quenching of RHODOPSIN-1 signaling, and recycling of RHODOPSIN-1 in the visual transduction pathway (Wang & Montell, 2007). When RHODOPSIN is converted to meta-RHODOPSIN, ARR2 is in a dephosphorylated state and able to bind to activated RHODOPSIN. Then ARR2 undergoes phosphorylation in a light dependent manner to prevent further signaling (Alloway & Dolph, 1999; Matsumoto & Pak, 1983).

ARR2 is found associated at membranes under all light conditions where it can bind to RHODOPSIN and requires post translational modification to release this interaction.

In flies that are dark-reared, ARR1 is predominantly cytoplasmic and in response to light, ARR1 localizes to RHODOPSIN-1 immuno-positive vesicles along with ARR2. These vesicles do not form in ARR1 mutants indicating a key role in endocytic uptake of RHODOPSIN-1 (Sato et al, 2005). The role of ARR2 in this process is to inhibit RHODOPSIN-1, preventing constant G-protein signaling cascades. Once ARR2 is dephosphorylated it is able to bind to clathrin, which aids in the formation of coated vesicles (Moaven et al., 2013). Since BDBT forms foci that appeared similar to RHODOPSIN-1 immuno-positive vesicles in response to dark conditions our early hypothesis led us to further investigate whether BDBT foci were part of a similar endocytic mechanism involving ARRESTINS.

### **Thesis Overview**

PROJECT: BDBT Foci Formation and their Role in the Eye Clock.

My dissertation is focused on investigating the mechanisms related to circadian dysfunction through BDBT foci accumulation in a light/dark dependent manner. Presented here are the effects of light and dark on the formation of BDBT foci along with a genetic approach to observe the effects of circadian (*cry*) mutants and visual mutants of the RHODOPSIN-1-linked heterotrimeric G-protein coupled signaling pathway on BDBT foci accumulation. Both the circadian CRY and visual RHODOPSIN-1 photoreceptors are required for light-dependent absence of BDBT



foci. These studies also revealed a potential role for dark-mediated ARRESTIN-dependent membrane vesicle generation in the formation of BDBT foci.

As ARRESTINS have been shown to promote endocytosis of RHODOPSIN, we examined the effects of overexpressing a temperature sensitive SHIBIRE (SHI), the *Drosophila* ortholog of the dynamin GTPase in the eye. The dynamin family of proteins mediate plasma membrane fission during clathrin-mediated endocytosis (Doherty & McMahon, 2009), and here we show that at the restrictive temperature, we see strong foci formation, consistent with a role for membrane vesicle formation in the generation of these foci. In addition, a significant fraction of BDBT was associated with the membrane fraction during centrifugation, even in RHODOPSIN-1 mutant (*ninaE*) flies.

Previous work demonstrated that BDBT foci formation is dependent on DBT and PER. This led to examination of how this disruption to BDBT foci in the *Drosophila* eye clock alters other circadian processes such as DBT and PER protein levels, their subcellular localization, and protein-protein interactions. Knock-down of BDBT specifically in the eye was achieved using a GMR-GAL4 > UAS;*dcr2*,UAS:*bdbt*-RNAi fly line, and this produced loss of circadian molecular oscillations for both PER and DBT with uncoupling of their sub cellular localizations; PER was constitutively nuclear and DBT was constitutively cytosolic.

Initial analysis of BDBT foci formation indicated that this mechanism is only occurring in the fly eye as we do not observe BDBT foci accumulating in the brain. Through the data presented in this thesis, our analysis elude how eye-specific molecules like RHODOPSIN-1 and ARRESTINS strongly influence BDBT foci

formation differently under light and dark conditions. RHODOPSIN-1 and ARRESTINS are not found in the fly brain so these data further suggest that these proteins produce an eye specific connection with the clock through BDBT foci formation. This may provide a link between BDBT foci formation and daily changes in subcellular localization that also occur with RHODOPSINS, TRPL channels, and G- $\alpha$  proteins.

Previous work demonstrated that RHODOPSIN-1 is involved with circadian color preference in the fly eye (Lazopulo, et al., 2019) so we investigated what effects would BDBT knockdown in the eye have on circadian color preference. The visual preference results produced a change in the RHODOPSIN-1 dependent color preference behavior suggesting that the *Drosophila* eye clock may communicate with the brain clock to alter circadian visual preferences. Our hypothesis for a BDBT foci mechanism evolved throughout the project as new findings emerged. Initially the focus was to determine how light effects BDBT foci formation and our results demonstrate a key role for BDBT foci formation in the *Drosophila* eye clock and circadian color preference behavior modulated by the fly eye.

### **Collaborators Involved in this Work**

I received a significant amount of help on this project. Angel Le, Carmen Zatezalo, Lauren Francis, Seyyed Mahmoudjafari, Briana Shores, Connor Flathers, Abigail Bowser, Hana Fiedler, and Colleen Bontrager were all undergraduate or high school students who assisted with the cryostat sectioning of fly heads, immunofluorescent procedures, and confocal imaging. Sheyum Syed and Stanislav

Lazopulo from the University of Miami were an enormous help with their work performed on the circadian color preference assays. Jin Yuan-Fan taught me many of the methods used throughout my dissertation as well as performed the work done with our subcellular fractionation experiments. Finally, my advisor, Dr. Jeffrey Price help manage my efforts and contributed to the overall design of these projects and analysis.

## CHAPTER 2

### MATERIALS AND METHODS

#### **Fly Lines and Rearing conditions (LD to DD, LD to LL, LD, DD, DD to LL)**

The following fly lines were used for this study: Wild type (WT) Canton S flies, *cry<sup>out</sup>* (Yoshii et al., 2008), *cry<sup>b</sup>* (Stanewsky et al., 1998), *w<sup>\*</sup>*; *ort<sup>1</sup> ninaE<sup>1</sup>* (Bloomington Drosophila Stock Center line 1946). *w<sup>\*</sup>*; *sr<sup>1</sup> ninaE<sup>17</sup> e<sup>s</sup>* (Bloomington Drosophila Stock Center line 5701), *norpA* mutants (Bloomington Drosophila stock center lines 9047, 9048, 9049 and 9051), *Arr1<sup>1</sup>cn<sup>1</sup> bw<sup>1</sup>* (Bloomington Drosophila Stock Center line 42252), *w<sup>\*</sup>*; *Arr2<sup>3</sup>* (Bloomington Drosophila Stock Center line 42255), *UAS-shi<sup>ts</sup>*, *GMR-GAL4* (Steinhilb et al., 2007), *w<sup>1118</sup>*; *UAS-Dcr-2*; *Df(3L)Ly, sens[Ly-1]/TM3*, *Sb<sup>1</sup>* (Bloomington Drosophila Stock Center line 24645). Rh1-GAL4 (X and III<sup>rd</sup> chromosome insertions; (Lazopulo et al., 2019)), wild type and K/R *UAS-dbt myc-his* (Muskus et al., 2007), *yw*; *UAS-mCD8-GFP* (Bloomington Drosophila Stock Center line 5137), *UAS-bdbt RNAi* (Vienna Drosophila RNAi Center line 100028), *UAS-dbt RNAi* (Vienna Drosophila RNAi Center line 9241), and *UAS-spag RNAi* (Vienna Drosophila RNAi Center line 31253). Our fly food is composed of agar (0.52%), yeast (1.1%), corn meal (5.3%), molasses (6.8%), and 10% Tegosept (0.1%) as an anti-fungal agent. Mutants of the indicated type were harvested at the indicated times in LD cycles (ZT, lights on from 0-12), the second day of constant darkness (DD times are subjective day), the first day of constant light (LL times indicate previous time in LD), after rearing in constant darkness, or after rearing in constant darkness followed by 7 hours of light. For demonstration of the effects of *bdbt* RNAi on foci formation, *GMR-GAL4*; *UAS-dcr* flies were crossed to *UAS-bdbt* RNAi from

the Vienna Drosophila RNAi center, allowing for eye specific BDBT knockdown. For the analysis of UAS-*sh<sup>1s</sup>* expression, flies were raised at 18°C in LD and harvested at ZT19 or raised at 18°C in LD and then elevated to 30°C in LD 7 hours before harvest at ZT19.

### **Immunoblot and Immunoprecipitation Analysis**

For analysis of changes to BDBT, DBT, PER, and TUBULIN protein levels, heads were prepared from flies under their respective rearing conditions, homogenized in 1.1x Laemmli SDS loading buffer (7 µl per head), and then heated for 5 min at 100°C and stored at -80°C. For immunoblot assays, head extracts were subjected to SDS-PAGE, transferred to nitrocellulose, and antigens detected with the appropriate antibodies as described (Fan et al., 2013). Extracts were analyzed on either 5.7% (for PER) or 10% (for DBT, tubulin, and BDBT) SDS-PAGE gels with the ECL procedure (GE Healthcare). The antibodies used were anti-tubulin from the Drosophila Studies Hybridoma Bank (Iowa City, Iowa), rabbit anti-DBT C #91 1:2000 (Muskus et al., 2007), guinea pig anti-BDBT #589 1:5000 (Fan et al., 2013), and rabbit anti-PER (Muskus et al., 2007). Typically, immunoblots of independent experiments were performed three times for each figure. For immunoprecipitations, DBT-MYC was immunoprecipitated with mouse anti-MYC (MMS-150R from Covance Research Products, Berkeley, CA at a concentration of 1:150) or DBT with rabbit anti-DBT antibody. The amount of BDBT in the co-immunoprecipitate was detected with guinea pig anti-BDBT 589, as previously described (Venkatesan et al., 2015).

## **Immunofluorescence Laser Scanning Confocal Microscopy**

For detection of BDBT, DBT, and PER in the eyes, fly heads were collected under their respective rearing conditions and embedded at -80°C in OCT (Ted Pella).

Sections prepared with a cryostat were processed for immunofluorescent detection of BDBT, detected with guinea pig anti-BDBT 589 (1:5000) and goat anti-guinea pig IgG Alexa Fluor 488 or Alexa Fluor 641 (1:1000), detection of DBT, detected with rabbit anti-DBT C (1:2000) and anti-rabbit IgG Alexa Fluor 488 (1:1000), detection of PER, detected with rabbit anti-PER (1/10,000) and goat anti-rabbit IgG Alexa Fluor 568(1/1000), detection of GFP, detected with goat pAB anti-GFP NB100 (1:500) and donkey anti-goat IgG Alexa Fluor 488 (1:1000), detection of PDF, detected with mouse anti-PDF (DSHB) (1:1000) and donkey anti-mouse IgG Alexa Fluor 568 (1:1000), and detection of ARR1 or ARR1, detected with rabbit anti-ARR1 (1:500) or ARR2 (1:1000) (Dolph et al., 1993) and goat anti-rabbit IgG Alexa Fluor 568, by confocal microscopy (Zeiss LSM5) using a 40x water immersion lens, as previously described (Fan et al., 2013). The expression pattern of BDBT (low and broad, fingers, high and broad) or its lack of expression (none), DBT localization (neither streaks nor nuclear, streaks, streaks around nuclei, or nuclear), or PER localization (cytoplasmic, both nuclear and cytoplasmic, nuclear) was tabulated by two observers blinded to the identity of the samples. Both observers produced equivalent results.

## **Subcellular Fractionation**

Fly heads were collected under their respective rearing conditions. 4 tubes were prepared for each sample (crude, homogenate, supernatant, and membrane) as

described (Xu & Wang, 2016). For crude collections, 5 heads from each sample were homogenized in 35 $\mu$ L 1.1X Laemmli SDS buffer and heated at 100°C for 5 min. Next, 20 heads were homogenized in 122 $\mu$ L of lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA) in a 'homogenate' tube then centrifuged at 950 X g for 5 min at 4°C. The soluble fraction was removed from the homogenate tube and added to the membrane tube. Next, 25 $\mu$ L of 1.1 X Laemmli SDS buffer was added to the homogenate pellet and heated at 100°C for 5 minutes. The membrane tube was subjected to 40 min centrifugation (18,500xg) at 4°C. The soluble fraction was removed and added to 'supernatant' tube with 28 $\mu$ L of 5XSDS, homogenized and heated for 5 min at 4°C. Finally, 20 $\mu$ L of 1.1XSDS were added to the membrane tube, homogenized and heated for 5 min at 100°C. A blot was then run to detect BDBT (detected with guinea pig anti-BDBT 589 (1:5000)), DBT (detected with rabbit anti-DBTC (1/2000)) or tubulin (detected with mouse hybridoma anti-tubulin).

### **Behavioral Assays**

The visual preference assays were performed by Stanislav Lazopulo from the Syed lab at the University of Miami as described in their publication (Lazopulo et al., 2019). Briefly, flies of the indicated genotype were placed in glass tubes with three colored sectors (blue, green and red), subjected to a cycle of LD, and activity events and location in each of the three color sectors were determined for several days for each 1 h time interval. The visual preference behavior was averaged for multiple flies of each genotype for the entire time course or for shorter time periods and plotted as a temporal profile of attraction or preference, as shown in the figures.

Circadian activity records were determined in constant darkness after entrainment to 12 h light: 12 h dark cycles as previously described (Muskus et al., 2007). Rhythmic flies were those that produced a single strong peak of activity by chi-square periodogram analysis, and the period at the peak was the period of the free-running rhythm.

### **Statistics**

For analysis of the BDBT, DBT and PER expression patterns determined by immunofluorescence, experimental data from blinded scores of at least three experiments by one observer were pooled and subjected to either a Kruskal-Wallis nonparametric ANOVA with multiple comparisons of mean ranks for all groups or a Manny-Whitney U test (with continuity correction). The Statistica software package was used for this analysis



## CHAPTER 3

# BDBT FOCI FORMATION OSCILLATE IN A CIRCADIAN MANNER THROUGH A LIGHT/DARK MECHANISM

### Introduction

A novel interactor of the circadian kinase DBT has been identified as CG17282 or BRIDE OF DOUBLETIME through immunoprecipitations of MYC-tagged DBT in S2 cells (Fan et al., 2013). BDBT interacts with DBT in vitro, in S2 cells, and in fly heads, and it is essential for normal cycles of PER nuclear accumulation and circadian behavior. BDBT is a non-canonical FK506-binding protein that, in addition to its interaction with DBT, may interact with other proteins through a tetratricopeptide repeat (TPR) motif at its C-terminal end composed of two  $\alpha$  helices. TPR motifs mediate protein-protein interactions (Zeytuni & Zarivach, 2012) and thereby may promote assembly of multiprotein complexes, thus potentially providing another mode of DBT regulation to modulate its activity towards its substrates.

Besides BDBT mediating an effect of DBT protein on PER protein levels producing elevated hypo- and hyper-phosphorylated PER, our immunofluorescent analysis indicated that BDBT accumulates in cytosolic foci through a temporal mechanism. During the evening from ZT13 to ZT19, BDBT cytosolic foci accumulate to a high point in which they are broadly expressed throughout the photoreceptors before again becoming sequestered in foci forming streak like patterns at ZT1 and ZT7 in the outer part of the retina (Fan et al., 2013). Broad

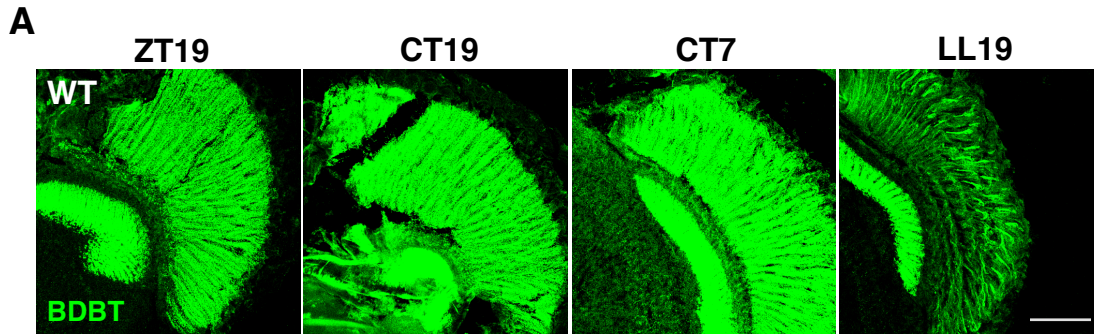
expression of BDBT cytosolic foci in the photoreceptors corresponds to the rhythmic accumulation of nuclear PER at ZT19 and a loss of BDBT foci at ZT7 when PER is cytosolic and PER protein levels are low. Utilization of a *per<sup>0</sup>* flies and UAS-*dcr2;timGAL4>/+;UAS-dbt* RNAi:/+ lines to examine BDBT puncta, led to a complete loss of BDBT puncta formation in the *Drosophila* photoreceptors at both timepoints, indicating foci accumulation is dependent upon a circadian mechanism involving DBT and PER (Fan et al., 2013). Presented here are the effects of light on the formation of BDBT foci and a genetic approach to observe the effects of circadian (*cry*) mutants and visual mutants of the RHODOPSIN-1 (Rh1)-linked heterotrimeric G-protein coupled signaling pathway on BDBT foci accumulation. The studies suggest a role for dark-mediated membrane vesicle generation in the formation of foci and reveal a role for BDBT in circadian co-transport of DBT and PER to nuclei in the eye. Also, how a BDBT-dependent eye clock is shown to affect a circadian change in color preference in the eye.

## Results

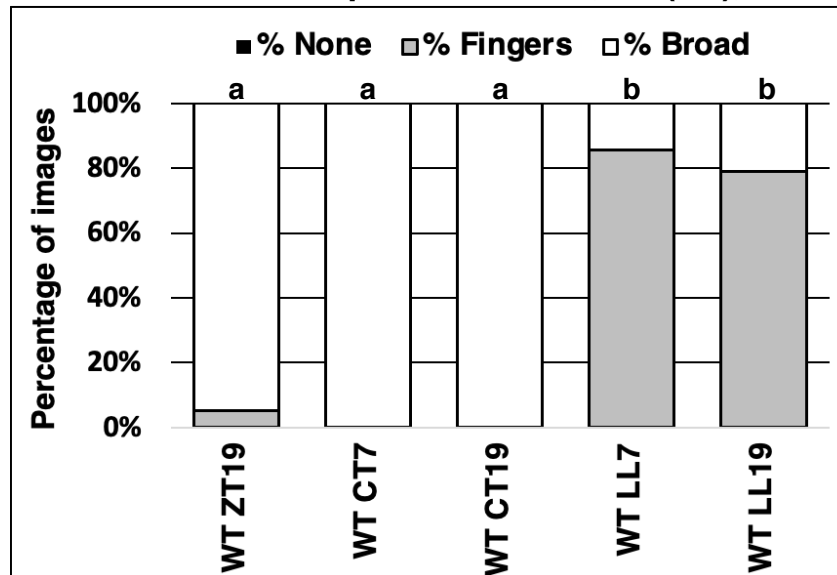
### **BDBT Foci are Constitutively High in the Dark and Low in Constant Light**

*Canton S* flies were raised under circadian time (CT) to analyze the subcellular localization of BDBT foci in photoreceptor cells, where PER protein is also highly expressed. CT flies are entrained to 12 hours lights on/12 hours lights off cycles and then moved to constant darkness and collected under dark conditions at CT7 and CT19 on the second day after termination of LD. This allows us to determine if BDBT foci formation is a circadian process or a light driven process. Eye sections

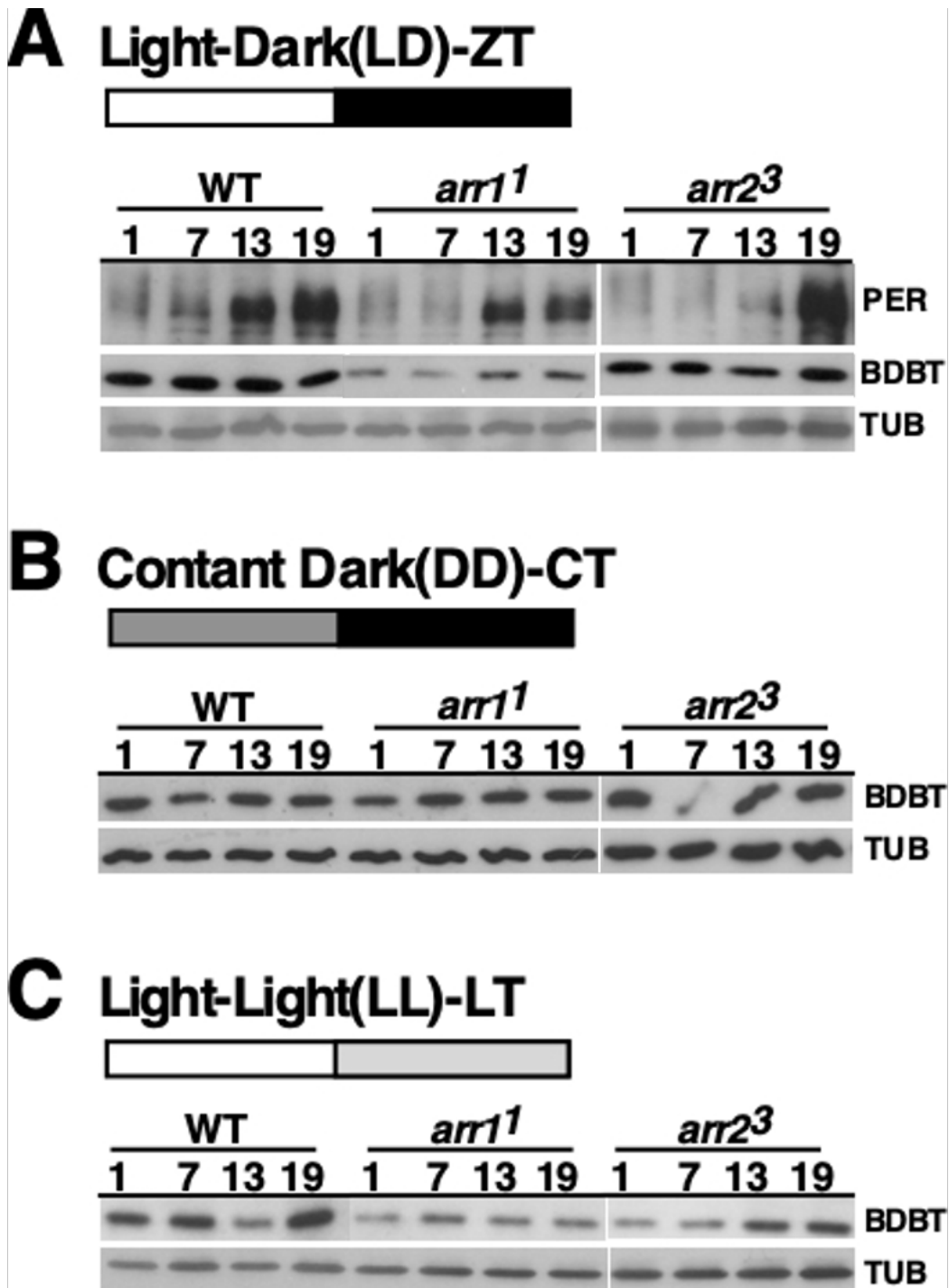
were prepared through a cryostat where heads are embedded in random orientations per our methods. This results in fly sections occurring through different planes of the eye, producing eye sections of different sizes and degrees of damage to the eye. Here we present images that produce laminar immunofluorescence and photoreceptors projecting in a 'spoke-like' pattern, but the size and degree of intactness will vary. Eye sections were prepared for immunofluorescent detection using an antibody against BDBT with confocal microscopy. Interestingly, under constant darkness BDBT foci accumulation remained consistently high at both CT7 and CT19 (Figure 3.1; LL-7 is equivalent to ZT7 conditions shown in the next figure). Flies were also collected in LL-7 hr and 19 hrs, whereby they were maintained in constant light for 7 hours and 19 hours after the termination of the final dark period of LD starting at ZT0. In LL conditions, the abundance of foci was restricted to streaks or 'finger' projections in the retina and remained relatively low at all times of day, forming 'finger' like projections (Figure 3.1). Immunoblots demonstrated that BDBT protein levels did not oscillate regardless of puncta formation and remained constant throughout these conditions (Figure 3.2). This apparent constancy of BDBT protein may be due to BDBT being expressed throughout the brain and these are whole head extracts including BDBT protein found in the fly brain as well. Detection of BDBT by immunoblot analysis of head extracts may not be specific enough to pick up spatially restricted changes within the eye. However, the lack of foci under lighted conditions and broadly expressed foci throughout the cytosol of the photoreceptors in constant darkness suggest that BDBT foci accumulation is dependent upon darkness and prevented by light. If BDBT foci are modulated by



**B** WT BDBT Foci Expression in LD, DD(CT), & LL



**Figure 3.1. Effects of Constant Light and Darkness after LD Entrainment.** (A) Wild type Canton S flies were harvested at the indicated times in LD (ZT, lights on from 0-12), during the second day of constant darkness (DD times are subjective circadian times), or during the first day of constant light (LL times indicate the previous time in LD). (B) Heads were sectioned and probed with anti-BDBT antibody, and the location of BDBT (broadly throughout the eye or in fingers on the outside) or its lack of expression (none) were tabulated for multiple sections after detection by confocal microscopy. A Kruskal-Wallis nonparametric analysis ( $H(4, N=63) = 40.5$ ) followed by multiple comparisons of mean ranks for all groups showed that sections of flies from darkness (a) differed significantly from those of flies from light (b) no matter what the phase or duration of the prior light exposure regime had been ( $P < 0.06$ ), while there were no statistically significant differences within the a or b groups, indicating that foci accumulation is constitutively suppressed by light and activated by darkness. Scale bars represent 50 microns.



**Figure 3.2. Immunoblot Analysis of BDBT Protein Levels.** WT (*CantonS*), ARR1(*arr1<sup>1</sup>*) and ARR2 (*arr2<sup>3</sup>*) mutants were analyzed. Heads were collected at respective timepoints and analyzed for PER, BDBT, and Tub as a loading control. (A) In a light/dark cycle (ZT), (B) Constant Darkness (CT), and (C) Constant Light (LL). BDBT levels do not show oscillating expression patterns as is seen with PER.

light and dark, their accumulation may provide insights into how light is driving circadian rhythms in the *Drosophila* eye through a BDBT/DBT interaction beyond the canonical circadian CRY degradation pathway. To determine the general nature of this dark-dependent effect, BDBT foci were again analyzed through a set of fly lines mutant for circadian (CRY) and visual (Rh1, ARR1, ARR2) proteins.

### **Analysis of BDBT Foci in Circadian *cry* and Visual *ninaE* Mutants**

As CRY serves as the circadian clock photoreceptor (Emery et al., 1998; Stanewsky et al., 1998) it appeared a likely candidate to be transmitting light signals to BDBT. To determine whether CRY is having an effect on BDBT foci formation, two CRY visual mutants were raised and collected under LD conditions. *Cry<sup>out</sup>* consists of a 1490-bp deletion resulting in a loss of function allele (Yoshii et al., 2008), whereas *cry<sup>b</sup>* is a strongly hypomorphic allele consisting of the point mutation D410N, which is highly conserved among class I photolyases. The *cry<sup>b</sup>* mutation produces a missense mutation at a conserved flavin-binding residue, which alters the integrity of the protein, thereby leading to degradation (Stanewsky et al., 1998). Eye sections were then incubated with anti-BDBT antibodies to compare any changes in BDBT foci to the expression patterns of *WT* via immunofluorescence confocal microscopy. All sections stained for BDBT underwent scoring by observers blinded to sample identity as described in methods, with sections scored as those with no foci, with broad but weak foci, with fingers or with broad and strong foci. The strong *cry<sup>out</sup>* mutant, which results in a non-functional eye clock, did not exhibit light-dependent decreases in BDBT foci at ZT7. However, the hypomorphic *cry<sup>b</sup>* mutant

did exhibit some loss of BDBT foci (Figure 3.3). These results and the results obtained in figure (Figure 3.4) are inconclusive in determining whether there are strong differences between the *cry<sup>out</sup>* and *cry<sup>b</sup>* mutants. However, it is clear that *cry* mutants reduce the sensitivity of the foci to light. The *cry<sup>out</sup>* results are consistent with effects of circadian *per* and *dbt* mutants on foci formation (Fan et al., 2013).

To look at the effects of RHODOPSIN-1 (*ninaE*; the major *Drosophila* RHODOPSIN expressed in photoreceptors R1-R6) mutants on BDBT foci accumulation two different *ninaE* mutants were employed. RHODOPSIN-1 is also activated maximally by green light leading to a visual transduction signal that may play a role in the light dependent disappearance of BDBT foci. *NinaE<sup>1</sup>* and *ninaE<sup>17</sup>* are both loss of function alleles containing a Q251Stop mutation and a large deletion of the 5' region, producing no detectable *ninaE* transcripts, respectively (O'Tousa et al., 1985). Both the *ninaE<sup>1</sup>* and *ninaE<sup>17</sup>* mutants led to a significant increase of BDBT foci formation during light, when BDBT foci are normally low, while also producing elevated levels of foci accumulation in DD (Figure 3.3). These two *ninaE* mutants appear to disrupt the disappearance of BDBT foci during light, or alternatively it is possible that wild type Rh1 acts to inhibit BDBT foci accumulation in response to light and this inhibition is lost in the *ninaE<sup>1</sup>* and *ninaE<sup>17</sup>* mutants. In summary, these results indicate that both the circadian and visual photoreceptors are needed for reduced BDBT foci during light in an LD cycle.

## Effects of *arrestin* Mutants on BDBT Foci Formation

After observing that *ninaE* mutants are able to produce strong phenotypic effects on the formation of BDBT foci (figure 3.3), next we looked at what affect additional interactors of the *ninaE* signaling pathway on this foci formation. As previously mentioned, the visual ARRESTINS, ARR1 and ARR2, mediate endocytosis of Rh1 from the rhabdomere into the cytosol of the photoreceptors and to quench Rh1 signaling (Dolph et al., 1993; Satoh & Ready, 2005). Without normal quenching of RHODOPSIN in response to light we wanted to determine how this would affect BDBT foci formation. A strong loss of function mutation for each was examined. *Arr1*<sup>1</sup> contains a DNA insertion resulting in approximately ten percent of wild type ARR1 protein levels (Dolph et al., 1993). In the *arr1*<sup>1</sup> mutant, BDBT foci formation was completely abolished during LD in both light and dark conditions, producing a significant phenotype opposite that of the *ninaE* mutants (Figure 3.3). This suggests that BDBT foci may be regulated through an ARR1 endocytic or membrane vesicle generating mechanism, and without sufficient ARR1, aggregation of BDBT to foci is impaired. The lack of BDBT foci accumulation is similar to the results seen in earlier studies where *Arr1* mutants lead to no RHODOPSIN-1-immunopositive vesicles budding from the rhabdomere (Satoh & Ready, 2005) suggesting a role in endocytosis that involves both circadian and visual transduction pathways.

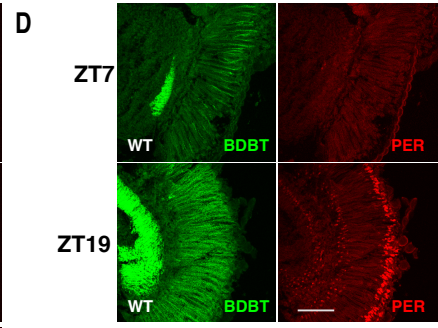
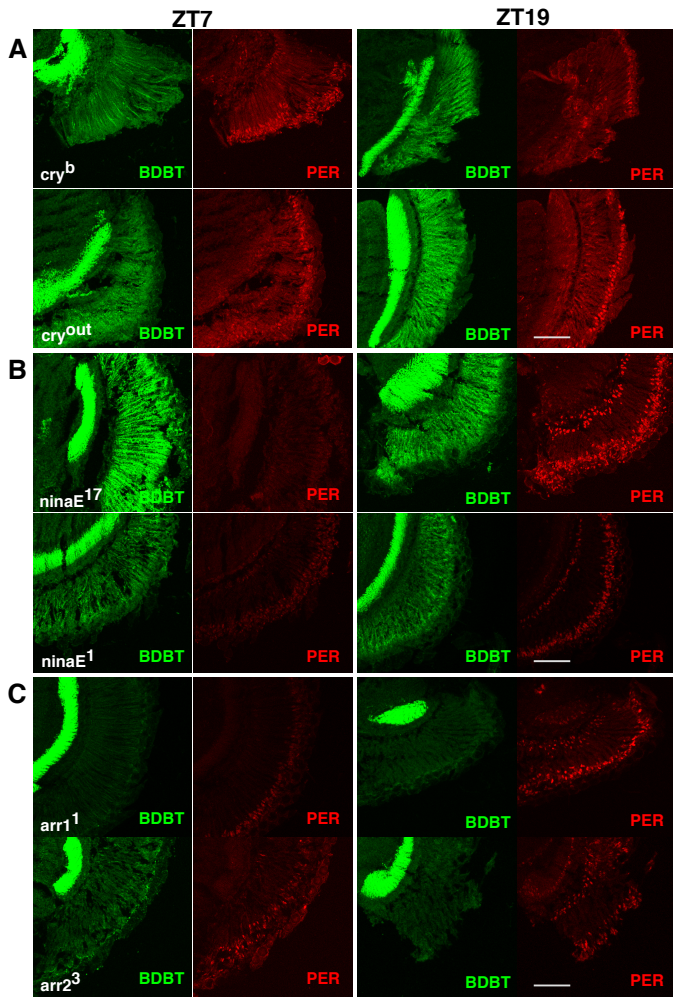
This experiment was then repeated with the *Arr2* line (*Arr2*<sup>3</sup>) to investigate whether BDBT foci require just ARR1 or also ARR2. *Arr2*<sup>3</sup> contains a single amino



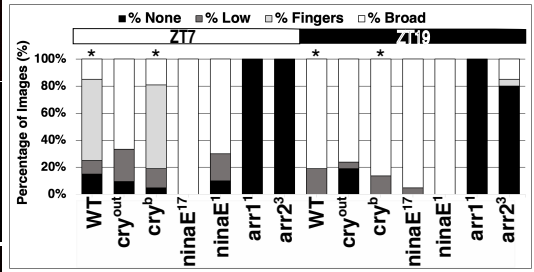
acid change (V52D) producing a loss-of-function mutant with less than one percent of ARR2 protein (Dolph et al., 1993). When the *arr2<sup>3</sup>* mutants were subjected to a LD cycle, broadly expressed BDBT foci mostly failed to form in the dark (Figure 3.3). Previously, ARR1 and ARR2 have been observed to show some redundancy in their function for RHODOPSIN-1 signaling (Satoh & Ready, 2005). These data suggest an ARRESTIN-dependent mechanism is necessary for the proper formation of BDBT foci, but it appears to affect the dark phase of the LD cycle rather than the light phase (the opposite of the light transduction phase where ARRESTINS show effects on RHODOPSIN-1). However, since BDBT foci are low during the day in WT flies we cannot rule out an effect on the light phase as well.

### **BDBT Foci in Genotypes Raised in Constant Darkness or Constant Darkness Followed by 7hrs of Light**

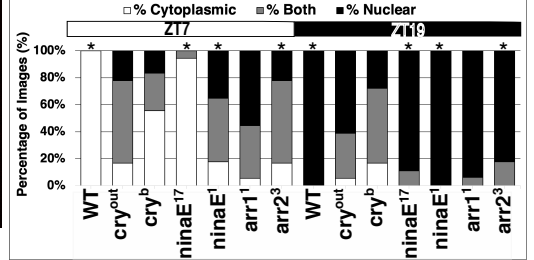
Several of the mutants with affected RHODOPSIN-1-mediated signal transduction lead to light-dependent neurodegeneration in the eye – in particular those affecting ARR2 (Wang & Montell, 2007) - and so it was important to rule out light-dependent neurodegeneration as a cause of their effects on BDBT foci formation. Therefore, we raised flies entirely in the dark or in the dark followed by 7 hours of light, and then assessed BDBT foci. In constant darkness, BDBT was mostly broad and highly expressed in Canton S wild type flies, while after 7 hours of light the foci were low in level or localized to fingers (Figure 3.4). In *cry<sup>out</sup>* mutant flies, there was no depression of high broad expression by constant light, while in



**E** BDBT Foci Expression in WT vs Photoreceptor Mutants LD



**F** PER Expression in WT vs Photoreceptor Mutants LD



### Figure 3.3. Effects of Photoreceptor Mutants on BDBT Foci and PER

**Localization in LD Cycles.** Wild type Canton S flies and mutant lines were harvested at the indicated times in LD (ZT, lights on from 0-12). Heads were sectioned and probed with anti-BDBT and anti-PER antibodies, and the location of BDBT (broadly throughout the eye at high levels, fingers on the outside, broad low expression, or no expression) and PER (nuclear, cytosolic, or on the outside of the eye around nuclei) were tabulated for multiple sections after detection by confocal microscopy. (A) Effects of Cryptochrome mutants on BDBT foci accumulation and PER localization at ZT7 and ZT19. The strong *cry<sup>out</sup>* mutant did not exhibit light-dependent decreases in BDBT foci at ZT7, while the hypomorphic *cry<sup>b</sup>* mutant did exhibit loss of foci. (B) Effects of *ninaE* (Rh1) mutants on BDBT foci accumulation and PER localization at ZT7 and ZT19. Both the *ninaE<sup>1</sup>* and *ninaE<sup>17</sup>* mutants led to a significant increase of BDBT foci formation during light, when BDBT foci are normally low, while also producing normally elevated levels of foci accumulation at night. (C) Effects of ARRESTIN mutants on BDBT foci accumulation and PER localization at ZT7 and ZT19. Both *Arr1<sup>1</sup>* and *Arr2<sup>3</sup>* led to significant decreases in BDBT foci accumulation and decreases to cytosolic PER. (D) The expression pattern of BDBT foci in eye sections was scored by observers blinded to sample identity for ~20 images in each group. The relative percentages showing no foci (none), low levels of broad expression, foci localized to the outer area of the retina (fingers), or high broad expression are shown. The samples labeled with “\*” showed statistically significant differences with the same genotype at the other time point (ZT7 vs ZT19) with  $P < 0.001$ , by a Mann Whitney U test (with continuity correction). (E) PER localization in eye sections was scored by observers blinded to sample identity for ~18 images in each group. The relative percentages showing cytosolic, localization to the outside of the retina around the nucleus, or nuclear are shown. The samples labeled with “\*” showed statistically significant differences with the same genotype at the other time point (ZT7 vs ZT19) with  $P < 0.03$ , by a Mann Whitney U test (with continuity correction). The scale bar indicates 50  $\mu\text{m}$ .

*cry<sup>b</sup>* flies there was a weak effect of light, which produced slightly reduced levels of BDBT foci compared *cry<sup>b</sup>* flies there was a weak effect of light, which produced slightly reduced levels of BDBT foci compared with DD, but the levels were still elevated and the difference not statistically significant (Figure 3.4). The two *ninaE* mutants produced no reduction in foci with 7 hours of light, and the foci were broad and of high intensity under both conditions (Figure 3.4). Likewise, as in LD, the *Arr1<sup>1</sup>* and *Arr2<sup>3</sup>* mutants produced almost no eye sections with broadly distributed foci in DD or after 7 hours of light, although there were fingers of foci in *Arr1<sup>1</sup>* mutants that were not seen in LD (Figure 3.4). Therefore, the absence of effects of light on BDBT foci in photoreceptor mutants and the absence of broadly distributed foci in *Arr* mutants were found under both LD conditions as well as transient light vs DD conditions. This suggest that these results with BDBT foci formation are not due to light-dependent neurodegeneration and are due to disruptions in the signaling mechanism for light. However, the *ninaE* mutations drive developmental changes in rhabdomere structure, and *Arr1* mutations produce progressive neurodegeneration in the eye after eclosion. While the collections were performed on young flies to minimize progressive neurodegeneration, we cannot rule out that some of these effects on BDBT foci are produced by neurodegenerative effects of the mutants.

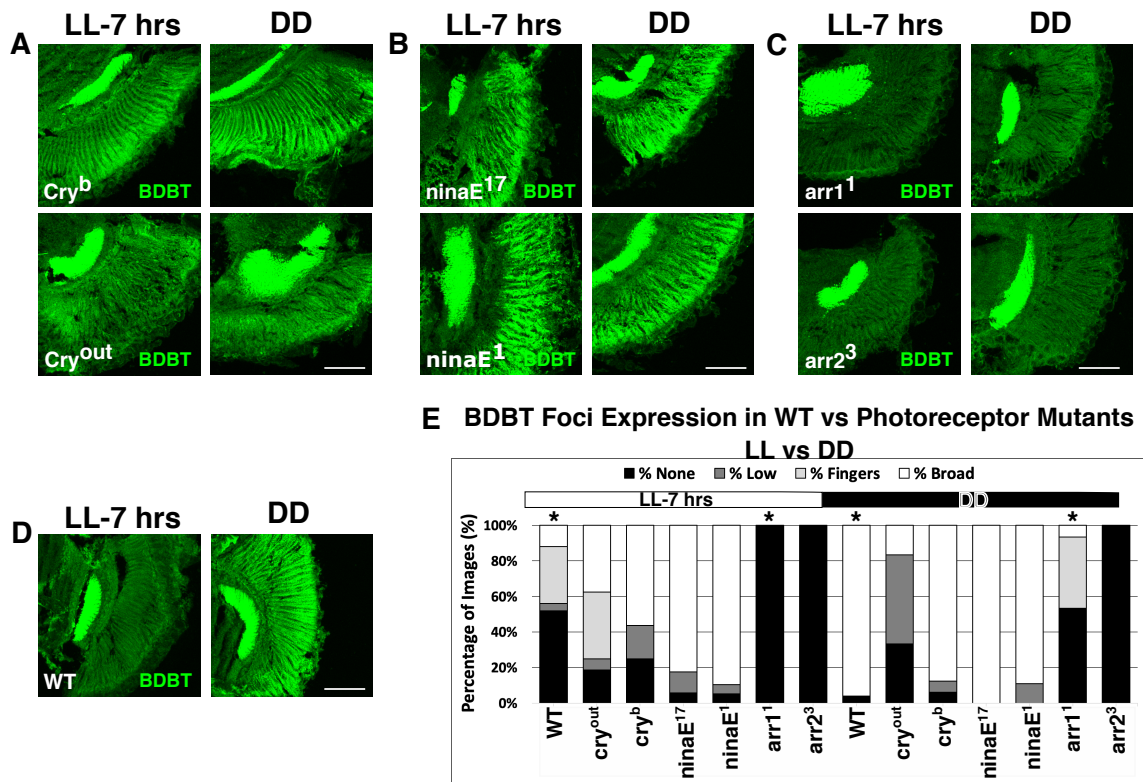
## Discussion

The formation of ARRESTIN- and dark-dependent BDBT foci demonstrates a mechanism in which light input signals are being regulated through dual inputs from the circadian and visual pathways in the *Drosophila* eye. Our finding that WT BDBT

foci formation requires ARRESTINS and dark conditions is of interest since the other well studied ARRESTIN function involving RHODOPSIN - inactivation of photoreceptor signals via interactions with RHODOPSIN- is a light-dependent rather than a dark-dependent process (Dolph et al., 1993). However, several proteins involved with phototransduction are regulated through mechanisms involving the translocation into and out of the rhabdomeres of photoreceptors, and this movement is thought to be involved in the adaptations of eyes to both light or dark by altering light sensitivity. For example, in contrast to ARRESTINS, in the dark a  $G\alpha$  protein translocates to the rhabdomere in advance of the RHODOPSIN response, and within a few minutes of light exposure it moves to the cell bodies (Cronin et al., 2004; Kosloff et al., 2003). Likewise, the transient receptor potential- like (TRPL) cation channel moves to the cell body with light exposure and into the rhabdomeres in the dark, again demonstrating the importance of translocation in these signaling pathways in both light and dark (Bahner et al., 2002). This movement of TRPL channels occurs in the membrane, and previous work showed that ARR2 was required for the dark-mediated movement (Cronin et al., 2006). Alternatively, an additional study found that light-mediated transport of TRPL out of the rhabdomere was prevented in a double *Arr1; Arr2* mutant, implicating ARRESTINS in both light and dark dependent roles (Meyer et al., 2006).

In dark conditions both ARR1 and ARR2 are primarily located in the cytosol at times at which BDBT foci are also accumulating (Alloway & Dolph, 1999). When RHODOPSIN is converted to M-RHODOPSIN in response to blue light, ARR2 is recruited to the rhabdomere within 5 minutes of light exposure to deactivate further

RHODOPSIN/ $G\alpha$  protein signaling (Ranganathan & Stevens, 1995; Satoh & Ready, 2005). Release of ARR2 requires phosphorylation of ARR2 by CAMKII (Alloway & Dolph, 1999; Kahn & Matsumoto, 1997) and ARR1 may also play a role in deactivating ARR2 signal quenching. Termination of RHODOPSIN quenching is significant in *Arr2* mutants, but not *Arr1* mutants. However, deactivation of quenching is more severe when both *Arr1* and *Arr2* are mutated, indicating a coupling effect (Dolph et al., 1993), similar to the one previously mentioned with translocation of TRPL channels. If ARR2 requires ARR1 to release from RHODOPSIN and must be released to form BDBT foci, this codependence could explain why BDBT foci fail to form without either ARR1 or ARR2. In the *ninaE* mutants assayed here, which produce no functional transcripts of *RHODOPSIN-1*, we saw increased BDBT foci during LL/DD and LD cycles. A possible explanation could be that without RHODOPSIN for ARR2 to deactivate ARR2 is more freely available to generate BDBT foci through an endocytic mechanism. This model would explain the dark-dependent role for ARR2 in BDBT foci formation, since it would act to deactivate RHODOPSIN under lighted conditions and then serve a role in the formation of BDBT foci under dark conditions, providing a junction between circadian and visual transduction signaling. Alternatively, *RHODOPSIN-1* could signal the elimination of BDBT foci in response to light. Either mechanism will be of interest because not only does this elucidate a light-dependent process, but it provides additional information for neuronal signaling processes that might have more broad applications beyond just the circadian and visual fields.



**Figure 3.4. Effects of Photoreceptor Mutants on BDBT Foci Accumulation in LL-7hrs and DD.** Heads were harvested from flies raised in constant darkness or raised in constant darkness and subjected to 7 hrs of light. Heads were sectioned and probed with anti-BDBT antibodies, and the location of BDBT (Broadly throughout the eye at high levels, in fingers, broadly throughout the eye at low levels) or its lack of expression (none) was tabulated for multiple sections after detection by confocal microscopy. (A) BDBT detected in *cry<sup>out</sup>* and *cry<sup>b</sup>* mutants. (B) BDBT detected in Rh1 mutants (*ninaE<sup>17</sup>* and *ninaE<sup>1</sup>*) (C) BDBT detected in *arr1<sup>1</sup>* and *arr2<sup>3</sup>* mutants (D) BDBT detected in wild type flies. (E) Data from several experiments were pooled and plotted here. Asterisks over the lanes indicate a statistically significant difference between dark-reared and light-pulsed flies of the same genotype with  $P < 0.05$ , by a Mann-Whitney U test (with continuity correction). The *cry* and *ninaE* mutants retained relatively high levels in response to light, while the *arr* mutants were low in both light and dark. The scale bar indicates 50  $\mu\text{m}$ .

# BDBT FOCI FORMATION IS REQUIRED FOR THE TIMELY SUBCELLULAR LOCALIZATION OF CIRCADIAN REGULATOR PROTEINS DOUBLETIME AND PERIOD

## Results

### ***Arr1* and *Arr2* Mutant Effects on PER Localization**

As previously reported (Fan et al., 2013), BDBT foci fail to form in *per<sup>0</sup>* mutants as well as in knockdown of the PER kinase DBT using UAS-*dcr2;timGAL4>/+;UAS-dbt* RNAi:/+ lines. Since a functional circadian clock appears necessary for BDBT foci accumulation we next wanted to observe if the absence of BDBT foci formation caused by ARRESTIN mutants in turn is associated with the circadian regulator PER. In some of our eye sections in which we detected BDBT we also detected PER with a different fluor (Alexa Fluor 568 instead of 488; eg, the representative examples shown in (Figure 3.3). In *WT* flies, using immunofluorescence, PER remains expressed throughout the cytosol at low levels at ZT7 during the day, and then at ZT19 PER localizes to the nuclei of the photoreceptors (Figure 3.3). Confocal microscopy was employed to examine PER expression in the photoreceptor cells in our *cry*, *ninaE* and *arrestin* mutants. As previously shown (Dolezelova et al., 2007; Stanewsky et al., 1998), the *cry* mutants blunt the oscillations of PER, with moderate and equivalent nuclear localization at both ZT7 and ZT19 (Figure 3.3; 7 types of photoreceptors on the outside of the eye and one on the inside), because the eye clock is not entrained in the *cry* mutants. By contrast, the *ninaE* mutants retain robust oscillations of PER localization, with high



levels of nuclear PER during the night and low levels during the day (Figure 3.3), as RHODOPSIN-mediated light signaling does not entrain eye PER (Zerr et al., 1990). However, in the *Arr1*<sup>1</sup> mutant PER was mostly localized to the nuclei of the photoreceptors during ZT7 and ZT19 (Figure 3.3). This change to PER subcellular localization in *Arr1*<sup>1</sup> mutants does not alter rhythmicity as activity assays show these flies remain rhythmic (not shown). PER was also found to be more nuclear in *arr2*<sup>3</sup> mutants than our *WT* controls, but the changes observed were not significant, and PER was found in the cytosol as well as the nuclei of photoreceptor cells in this mutant (Figure 3.3).

These results demonstrate that not only are BDBT foci dependent upon visual ARRESTIN proteins, but in the absence of functional ARR1, the absence of these foci is associated with increased PER nuclear localization, thereby disrupting oscillations of circadian clock proteins in the fly eye. BDBT foci accumulation and PER spatial changes are likely not due to changes in protein levels as DBT and BDBT levels remain constant and PER levels still oscillate in fly heads of both *WT* and ARRESTIN mutants, although we see lower BDBT protein levels in *Arr1*<sup>1</sup> mutants (Figure 3.2), these effects on PER could potentially be downstream of the effects on BDBT. This experiment was repeated three times and there were no oscillating patterns to BDBT protein levels observed. We did observe decreased protein levels at different times of day in *Arr1*<sup>1</sup> mutants between the three experiments.

## **BDBT Foci are Eliminated with Activation of Vesicle Uptake Pathways**

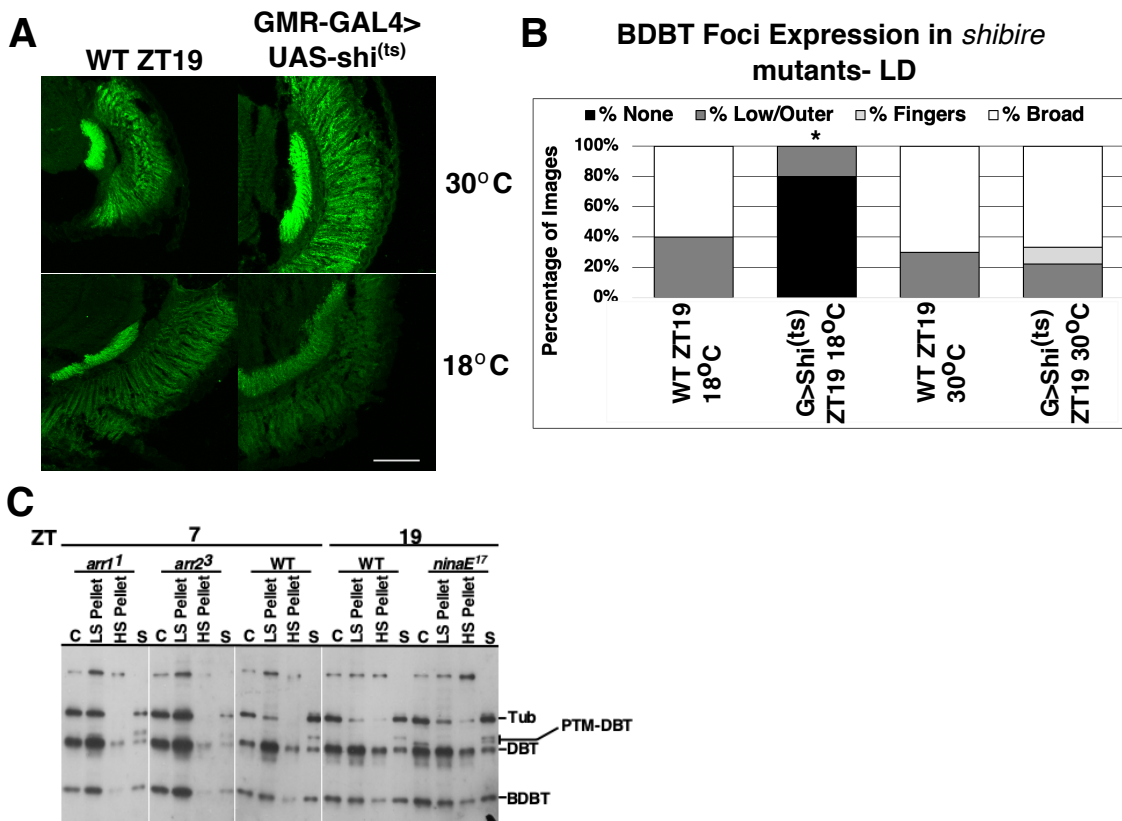
To better understand whether membrane vesicle generation has a role in BDBT foci formation we utilized a temperature sensitive dynamin GTPase (SHI<sup>ts</sup>). *Shi<sup>ts</sup>* is a dominant negative allele that can be expressed at a permissive temperature of 18°C and when exposed to the restrictive temperature of 30°C synaptic transmission is inhibited (Van der Bliek & Meyerowitz, 1991; Kitamoto, 2001). SHI<sup>ts</sup> mutants have also demonstrated to be involved with endocytic pathways in *Drosophila* hemocytes (Guha et al., 2003). These pathways could affect intercellular signaling pathways through receptor-mediated endocytosis and/or recycling of membrane proteins that undergo changes to their subcellular localization in a light dependent manner and we investigated these processes with BDBT foci formation. To target *shi<sup>ts</sup>* to the peripheral eye clock we employed the GMR-GAL4 driver. BDBT foci were scored by observers blinded to sample identity for distribution and levels at ZT19 in flies raised continuously at 18°C or raised to 30°C for 7 hours before collection at ZT19 in LD. The elevated levels at ZT19 were largely suppressed in flies raised at 18°C but not in flies raised at 30°C for the final 7 hours before collection (Figure 3.5).

We hypothesized at the permissible temperature of 18°C we would observe a WT phenotype at ZT19 of broad BDBT foci expression, but we observed a strong decrease in BDBT foci. Previous work in *Drosophila* using the GMR-GAL4>UAS-*shi<sup>ts1</sup>* line showed that at the restrictive temperature *per* was low and stable throughout the day suggesting that *shi<sup>ts</sup>* can disrupt the clock by reducing transcription of *per* and *vri* leading to reductions in PER levels (Kilman et al., 2009). *Per<sup>0</sup>* mutants also led to reduced BDBT foci (Fan et al., 2013) and therefore

suggests that these reductions to PER may lead to reductions of BDBT foci in *shi<sup>ts</sup>* mutants at 30°C. Our results of broad BDBT foci expression in *shi<sup>ts</sup>* mutants at the restrictive temperature at 30°C are instead consistent with the previous results demonstrating membrane vesicles being stalled at the membrane. It is plausible that the increase in BDBT foci formation at the restrictive temperature is due to BDBT be restricted to foci at the membrane and that the effects on *per* transcription are not seen due to this membrane paralysis occurring before *per* levels are lowered. Further analysis of these mutants during a day timepoint and exploring PER expression in the eye will help provide additional information to this process.

To further support the idea that BDBT is found localized to the membrane of photoreceptor cells we performed a centrifugation experiment to look at different cellular fractions. In our centrifugation experiment in which membrane fragments were pelleted, a substantial fraction of BDBT and DBT were pelleted with the membrane fractions at both ZT7 and ZT19 in wild type flies, *Arr* mutants, and the strong loss of function *rhodopsin* mutant (*ninaE<sup>17</sup>*; Figure 3.5), while tubulin was predominantly found in the supernatant.

These results are consistent with localization of BDBT and DBT to membrane vesicles that do not include RHODOPSIN-1. Note that our immunofluorescence analysis of BDBT foci also shows high levels of these foci in the *ninaE<sup>17</sup>* mutant, consistent with the prediction that Rh1 can negatively regulate these foci in response to light but is not needed to be taken up to form them in the dark. The lack of change with time and in the *Arr* mutants is surprising given the strong effects of these on



**Figure 3.5** (A) WT or GMR-GAL4>UAS-shi<sup>ts</sup> flies were raised at 18°C in LD and collected at ZT19 or raised at 18°C in LD and raised to 30°C for 7 hours before collection at ZT19 to inactivate SHI<sup>ts</sup>. Heads were collected and processed for BDBT detection, and these samples were then imaged for BDBT localization (A). The images were scored by observers blinded to sample identity for no expression, broad but low or outer only expression, fingers, or broad high expression, and the results are plotted in panel B. Overexpression of SHI at the functional temperature (18°C) lowered the detection of BDBT foci at ZT 19. C) Wild type, *Arr1*<sup>1</sup>, *Arr2*<sup>3</sup>, or *ninaE*<sup>17</sup> mutant flies were raised in LD, and heads were collected at ZT7 or ZT 19. Crude extracts were prepared from these (labeled “C”), or the extracts were subjected to brief low speed centrifugation (LS) followed by extended high speed centrifugation (HS), and each of these pellets was resuspended. The supernatant remaining was also electrophoresed (S). While all components are found in the low speed pellet, which presumably consists of unlysed cells, tubulin is found mostly in the supernatant while DBT and BDBT are found in both the high-speed pellet and supernatant, consistent with some localization to the membrane for both DBT and BDBT. The distribution does not change with time or genotype, but much of the detection is in brain tissue that is not involved in circadian rhythms or photoreception. Scale bars indicate 50 μm.

BDBT foci formation, but BDBT and DBT are expressed extensively in the fly brain in many cells that are not regulated by the circadian clock or light, and these results may include membrane localization in these cells.

### **Effects of BDBT RNAi in the Eye on DBT and PER Localization and Eye Circadian Rhythms**

As previously mentioned, BDBT foci formation has been shown to require circadian regulators like DBT and PER protein (Fan et al., 2013). If our *Arr* mutants can disrupt the formation of BDBT foci and lead to changes in PER spatial localization, we hypothesized that DBT and PER are likely being affected as well through these same processes by knock-down of BDBT. Our previous attempts to knock down BDBT in the eye with the *tim*GAL4 driver were not successful (Fan et al., 2013). The *timeless* gene is repressed at night due to PER/TIM repression on the transcription factors CLK/CYC and this repression may be affecting the efficacy of the BDBT-RNAi. To achieve stronger knock-down we used the UAS-GAL4 system to drive expression of UAS-*bdbt*-RNAi through the Glass Multiple Reporter (GMR) enhancer, which is strongly expressed in the *Drosophila* eye, along with Dicer to knockdown expression of BDBT in the fly eye. Male flies that did not inherit the GMR-GAL4 driver (UAS-*dcr*/UAS-*bdbt* RNAi) were used as our wild-type positive control, and female flies with the genotype GMR-GAL4>UAS-*dcr*/UAS-*bdbt* RNAi were the *bdbt*-RNAi knock-downs. Sections were stained with anti-DBT (Figure 3.6), or with anti-BDBT and anti-PER (Figure 3.6). BDBT showed a persistent oscillation in the eye, but the numbers of eyes with broad/high level expression were lower at all time points, showing persistent knock-down in levels (Figure 3.6). As we

previously observed in the lateral neurons (Fan et al., 2013), this knock-down produced high levels of nuclear PER at all times of day rather than the wild type oscillation in the controls (Figure 3.6). The high levels of nuclear PER were accompanied by higher than wild type PER levels at all times in the head (Figure 3.7).

Finally, we observed exactly the opposite effect on DBT nuclear localization. In the *GMR-GAL4>/+; UAS-dcr2/UAS-bdbt* RNAi lines DBT protein failed to localize to the nuclei in photoreceptor cells at any of the four timepoints (ZT1, 7, 13 and 19) while it was predominantly nuclear along with PER at ZT1 and 19 in the wild type control. Instead, DBT was observed in streaks expressed throughout the rhabdomeres at all time points in the knock down flies (Figure 3.6). The only time point at which similar streaks were observed in wild type controls was at ZT13 prior to nuclear localization, suggesting that these streaks might be precursors to nuclear localization.

These results indicate that proper BDBT foci formation in WT flies plays a role in DBT and PER localization within the *Drosophila* eye. Potentially it needs to be recruited away from this nuclear localization sequence (NLS) to deactivate the DBT kinase and allow its interactions with importins, thereby producing conditions for nuclear transport of DBT.

### **Knock-down of BDBT in the Eye Alters the Normal Circadian Profile of Color Preference**

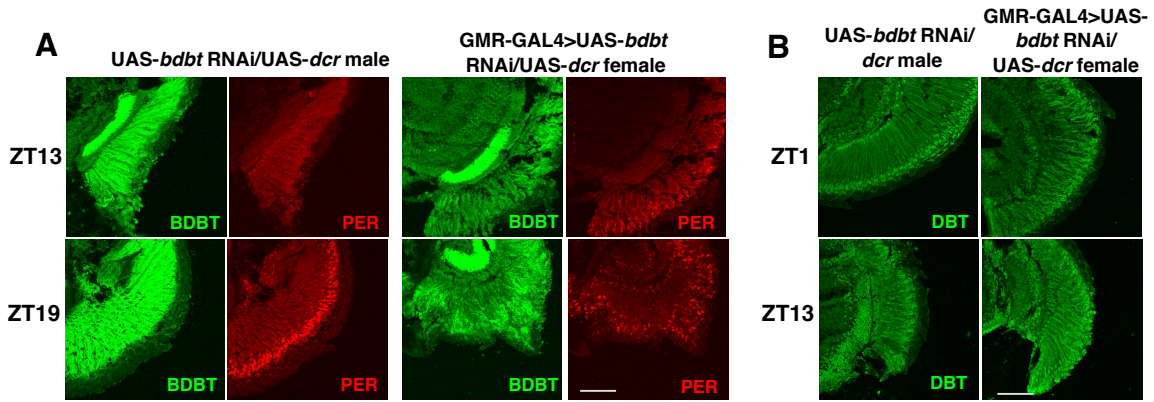
Work by two of our collaborators had previously shown that *Drosophila* exhibits a change in color preference that is regulated by the circadian clock and the

Rh1 of the eye, as well as extra-retinal photoreceptors (Figure 3.8) (Lazopulo et al., 2019). Therefore, we assessed the effect of *bdbt*-RNAi knock down in the eye on this change in color preference. Wild type controls showed the normal profile, with high preference for green light in the morning and evening, equal preference for green and red light in the middle of the day and the wild type avoidance of blue light during the day (Figure 3.9). The GMR-GAL4>UAS-*dcr*/UAS-*bdbt* RNAi flies showed no dip in green preference in the middle of the day and avoidance of blue light (detected by photoreceptors outside the eye) at all times of day (Figure 3.9). The results are similar to those obtained in other clock mutants and argue for altered rhythmicity in the Rh1-expressing photoreceptors, which have been shown to respond to green light and to be needed for the green preference during the day (Lazopulo et al., 2019). The loss of eye-specific molecular rhythm using the GMR-GAL4 driver was reflected in an altered rhythm of visual preference that has been observed with other loss of circadian function mutations. These results suggest that an eye specific clock mechanism is able to drive behavior in the fly brain; however the mechanism is still not understood.

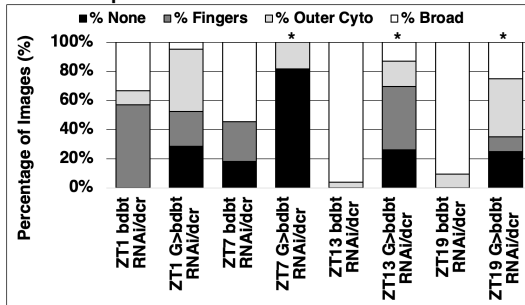
Next, we to determine the consequences of disruptions to the circadian eye clock using a different eye driver to isolate our approach even more. To do this a RHODOPSIN-1-GAL4 driver line was utilized along with a DBT mutant responder line. DBT<sup>K/R</sup> is a catalytically dominant negative protein and a Rh1-GAL4 > UAS-DBT[k38r] line was used to overexpressed DBT<sup>K/R</sup>. What our collaborators observed was a similar phenotype to our GMR-GAL4>UAS-*dcr*/UAS-*bdbt* RNAi flies, with no dip in green color preference during the day (Figure 3.10). Our collaborators also

looked at these flies in activity assays without the color preference and saw that when DBT<sup>K/R</sup> was overexpressed in the eyes the flies progressively lost their ability to anticipate the stimulated day until they could no longer do so displaying no signs of activity until lights were turned on (Figure 3.10). This suggests that strongly arrhythmic eyes lose the circadian control of visual preference even if the brain is still rhythmic. Overall, the data in this chapter show that BDBT is necessary for co-transport of DBT and PER into the nucleus and suggest that this process is regulated by a dark-dependent membrane vesicle formation process and that an eye dependent clock can affect a circadian change in color preference in the eye.

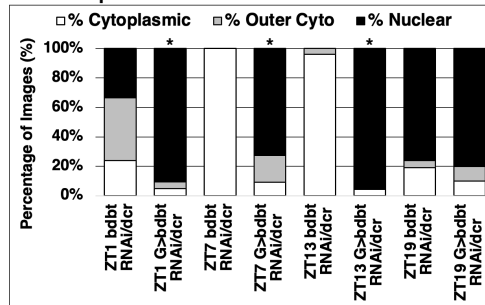




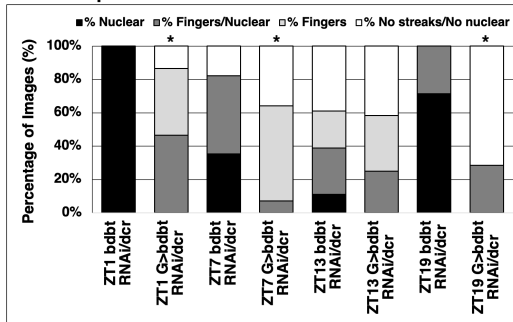
**C** BDBT Expression in UAS-*bdbt*-RNAi/UAS-*dcr* lines



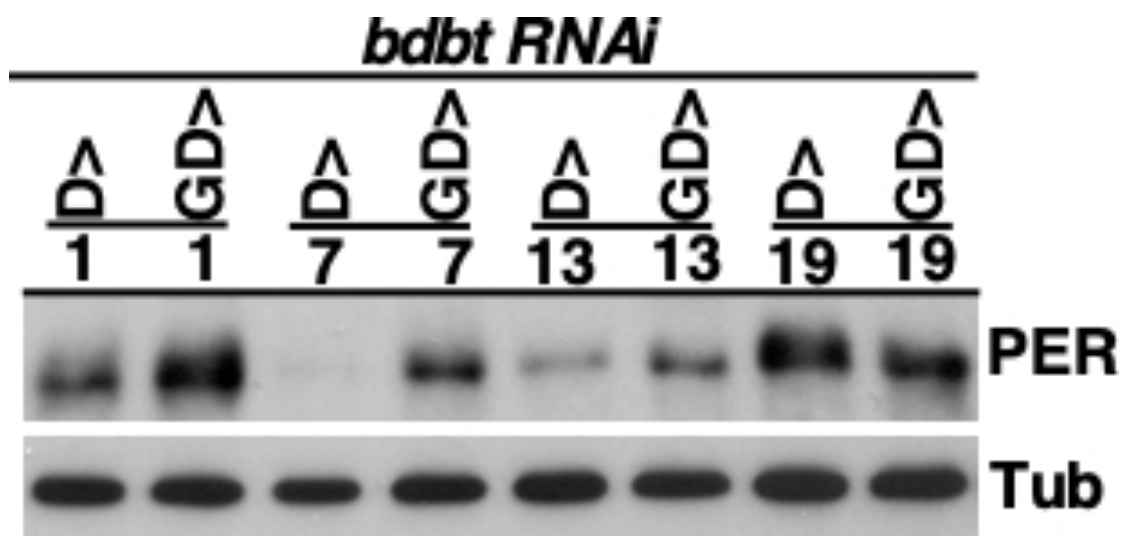
**D** PER Expression in UAS-*bdbt*-RNAi/UAS-*dcr* lines



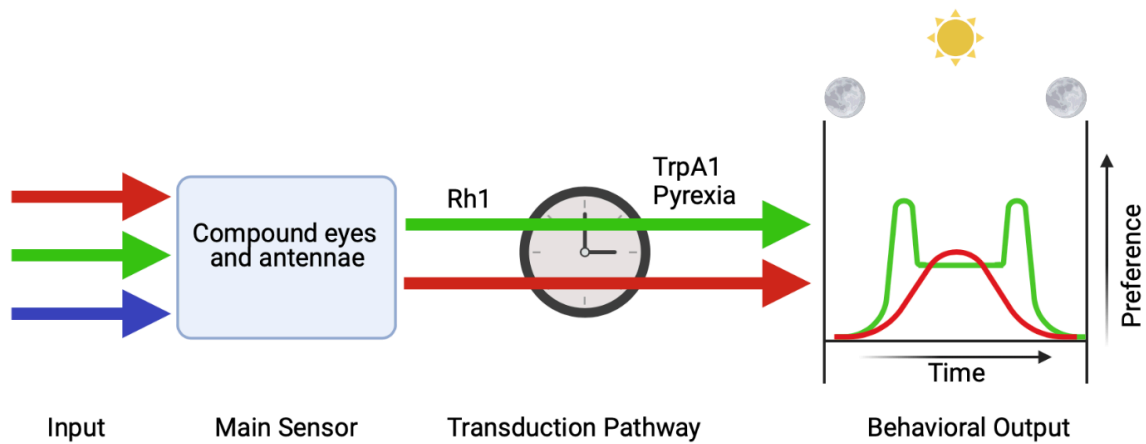
**E** DBT Expression in UAS-*bdbt*-RNAi/UAS-*dcr* lines



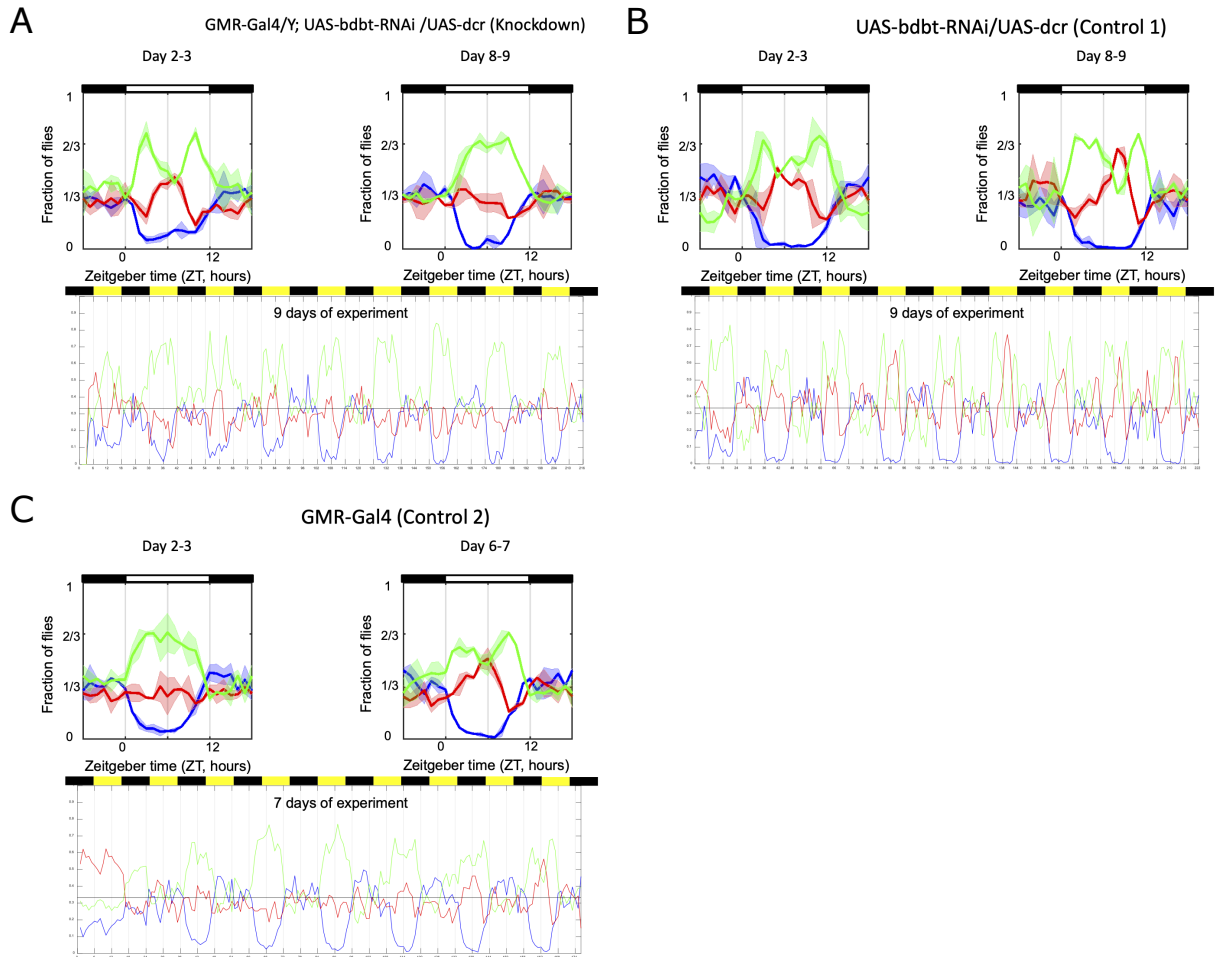
**Figure 3.6. Effects of BDBT RNAi on PER and DBT Localization in the Fly Eye.** Fly heads were collected at ZT1, ZT7, ZT13, and ZT19, and cryosections were prepared for immunofluorescent detection of BDBT, DBT, and PER. (A) Representative confocal Z stacks of GMR-GAL4>UAS-*bdbt*-RNAi/UAS-*dcr* female (knock-down) and UAS-*bdbt* RNAi/UAS-*dcr* male control photoreceptors at ZT13 and ZT19 show elevated BDBT foci in UAS-*bdbt* RNAi/UAS-*dcr* flies, but decreased BDBT foci in GMR-GAL4>UAS-*bdbt*-RNAi/UAS-*dcr* flies as well as increased nuclear PER in the absence of BDBT foci at ZT 13 in the knock-down flies. (B) DBT subcellular localization in UAS-*bdbt* RNAi/UAS-*dcr* control flies demonstrate wild type nuclear localization at ZT1 and “fingers” at ZT13. BDBT knockdown in GMR-GAL4>UAS-*bdbt*-RNAi/UAS-*dcr* flies leads to significant changes in DBT subcellular localization at ZT1, with fingers and no nuclear localization. (C) Graph illustrating the percentage of images scored by observers blinded to sample identity for BDBT foci expression, (D) PER localization, and (E) DBT localization. An asterisk over a bar indicates a knock-down sample that has a statistically significant difference ( $P < 0.05$ ) from the same timepoint for the wild type control by a Mann-Whitney U test (with continuity correction). Scale bar indicates 50  $\mu\text{m}$ .



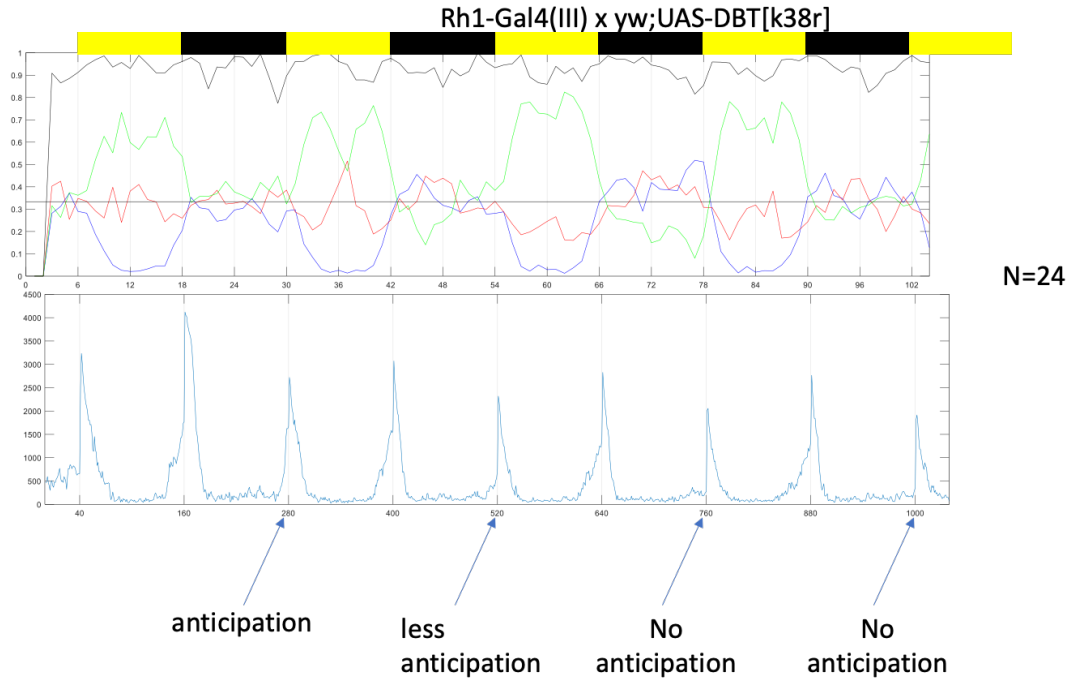
**Figure 3.7.** Immunoblot Analysis of PER in *bdbt*-RNAi (GMR-GAL4 > UAS *bdbt*-RNAi/UAS *dcr*) and WT (UAS-*dcr*/UAS *bdbt* RNAi). PER levels increase during the day (ZT1 & ZT7) and at ZT13 when *bdbt* is knocked down.



**Figure 3.8. A Model for How the Circadian Clock Influences Color Preference in Flies.** Previous data published by our collaborators suggest that Rh1 is involved in green and red light color preference. As Rh1 produces strong phenotypic effects on BDBT foci formation we wanted to determine what effects(if any) BDBT knockdown has on circadian color preference.



**Figure 3.9. Behavioral Assays for Color Preference in the BDBT Knock-Down Flies and Two Control Genotypes.** Flies were entrained and their color preference measured through modified activity assays over the course of 7-9 days. Older BDBT knock-down flies do not exhibit the wild type drop in green preference or rise in red preference during the middle of the day. Color preference assays were performed by our collaborators in the Syed lab.



**Figure 3.10. Behavioral Assays for Color Preference in DBT<sup>K/R</sup> Mutants using RHODOPSIN-1 Driver.** Flies were entrained and their color preference measured through modified activity assays over the course of 7-9 days. Overexpression of DBT<sup>K/R</sup>, a catalytically inactive dominant negative protein, under a RH1-GAL4 driver resulted in decreasing/no anticipation of stimulated day while also increasing green color preference during the day. This suggests that strongly arrhythmic eyes lose the circadian control of visual preference even if the brain is still rhythmic. Color preference and activity assays were performed by our collaborators in the Syed lab.

## Discussion

ARRESTINS are involved with the generation of endocytic vesicles through interactions with CLATHRIN cages that coat these vesicles (Kiselev et al., 2000) and once endocytosed these vesicles then can fuse with early endosomes. If ARRESTINs generate BDBT foci via endocytosis, the next stages post BDBT foci formation are of interest to determine if BDBT is then degraded through a lysosomal pathway, recycled back towards the membrane, or if it gets released and becomes cytosolic. Since ARRESTIN mutants eliminate BDBT foci formation it is possible that BDBT foci are comprised of endocytic or other membrane vesicles. Moreover, when we expressed a temperature-sensitive SHIBIRE ( $SHI^{ts}$ ), the *Drosophila* DYNAMIN ortholog that cleaves vesicles from the membrane, at the restrictive temperature of 30°C BDBT foci were comparable to wild type controls, further supporting a membrane vesicle mechanism. These results may explain the role of SHIBIRE and endocytosis in the clock previously described for both *Drosophila* (Kilman et al., 2009; Wulbeck et al., 2009) and mammals (Deery et al., 2009). Not all of the effects of membrane vesicle formation and endocytosis involve secretion of known circadian neuropeptides, since these effects include reduced PER levels with  $SHI^{ts}$  overexpression. These reductions happens in both WT and *pdf<sup>0</sup>* flies, thereby demonstrating an independence from PDF (Kilman et al., 2009). Moreover, control of endocytosis by circadian rhythms and sleep is involved in regulation of the blood brain barrier in flies (Cuddapah et al., 2019), and the human DBT orthologs CK1 $\delta/\epsilon$  have been shown to interact with GAPVD1, a guanine nucleotide exchange factor

(GEF) that is involved in endocytosis (Hunker et al., 2006). CK1 $\delta/\epsilon$  regulates endocytosis of GAPVD1 through phosphorylation (Guillen et al., 2020).

As briefly mentioned in the introduction the proteins involved in the eye visual response (including Rh1, transient receptor potential (TRP) channels, and No-receptor-potential A (NORPA)) are regulated by interactions in a large multimeric signaling complex mediated by INAD, a scaffolding protein consisting of PDZ domains that are light sensitive, and some of these associations are disrupted by light (Wang & Montell, 2007). Moreover, INAD can mediate an interaction of the circadian and visual pathways because it also binds to fly CRY in a light-dependent manner, and *cry* mutants exhibit impaired visual behavior (Mazzotta et al., 2013). NORPA is a phospholipase that interacts with INAD and has been shown to be involved in the activation and deactivation of phototransduction through RHODOPSINS.

In this dissertation we chose not to focus on NORPA as other non-canonical phototransduction pathways involving RHODOPSINS have been shown to contribute to circadian clock resetting in the absence of NORPA, because *norpA* mutants can still be entrained to LD cycles, although more slowly than WT flies (Ogueta et al., 2018, 2020; Zerr et al., 1990), and because our initial results with analysis of *norpA* mutants provided conflicting results (see next chapter). The *Drosophila* homolog of human FKBP52 (previously known as FKBP59) was identified through a yeast two-hybrid screen as an interactor with the INAD signaling complex (Goel et al., 2001), implicating further roles for FKBP59. BDBT foci might be involved in any of these light-dependent changes; however, their formation in the



*ninaE*<sup>17</sup> mutant, which produces degeneration of rhabdomeric structures (Pinal & Pichaud, 2011), suggests that their formation in this mutant likely occurs outside the rhabdomere and may be involved with a different membrane vesicle process.

The formation of BDBT foci has a significant effect on the subcellular localization of the circadian regulators DBT and PER as well as the circadian rhythm of visual color preference and based on these phenotypes these foci appear to negatively regulate DBT's activity. In the absence of light, BDBT foci accumulate constitutively (Figure 3.1), and in PER and DBT mutants they are eliminated, demonstrating that a functional circadian clock is necessary for foci accumulation (Fan et al., 2013). It's possible that BDBT foci accumulation recruit BDBT away from DBT, indicating a potential mechanism to expose the DBT-NLS, and in wild type flies this dissociation is followed by nuclear localization of DBT. This is consistent with our finding that BDBT binds to the DBT-NLS, needed for nuclear localization of DBT (Venkatesan et al., 2019; Venkatesan et al., 2015). In photoreceptor nuclei the DBT kinase can target PER for degradation through phosphorylation. BDBT foci peak at time when nuclear PER is highest, and this release from the DBT-NLS may allow DBT to translocate to the nucleus via interactions at the NLS with nuclear importins, and therefore it eventually accumulates to a peak in the early morning around ZT2 (Kloss et al., 2001). Both the high levels and nuclear localization of PER are thought to be produced by low DBT activity (Cyran et al., 2005; Muskus et al., 2007), suggesting that the BDBT foci down-regulate DBT at these times by eliminating the interaction with BDBT and thereby produce these effects on PER. This mechanism could also explain why we also see increased nuclear PER and cytosolic DBT in the

absence of BDBT foci such as in ARRESTIN mutants and in BDBT eye specific knockdown. Independent movement to nuclei of two other circadian components (PER and TIM) that likewise associate in a complex has also been shown (Syed et al., 2011).

There are several important additional examples of FKBP's involved in regulation of subcellular localization. BDBT has recently been observed to contribute to different subcellular localization of planar polarity components via DBT (Strutt & Strutt, 2020). Other FKBP's (FKBP51, FKBP52, and FKBPL) have been shown to regulate the nuclear localization of the glucocorticoid receptor (GR) (Fries et al., 2017). FKBPL has been shown to bind to dynamitin, a dynein motor protein (McKeen et al., 2008), and FKBP52 is able to bind to dynein while mediating nuclear localization of GR (Davies et al., 2002). In mammalian cells the DBT ortholog CK1 $\delta$  plays a role in the phosphorylation of tau, thereby modulating tau/microtubule binding (Li et al., 2004). A speculative model postulates that after BDBT dissociates in foci from DBT, then DBT can be moved to the nuclei via interactions with importin and microtubules. Together these results suggest that light-regulated generation of vesicles at a cell membrane is somehow regulating nuclear localization of DBT and PER through BDBT.

The color preference assays produced intriguing results with both our GMR-GAL4>UAS-*dcr*/UAS-*bdbt* RNAi and the UAS-*dbt*<sup>K/R</sup> dominant negative mutant. Expression driven in the eye through both these circadian proteins appear to produce arrhythmicity in the color preference assay. Also, the Rh1-GAL4> UAS-*dbt*<sup>K/R</sup> appears to produce an altered photic response in the locomotor assay as flies

lose their ability to anticipate the day. (Figure 3.10). This suggests some communication with the brain clock through input signals from the eye through a novel process.

## CHAPTER 4

### BDBT FOCI- INVESTIGATIVE AND SUPPLEMENTAL DATA

The work presented in the previous chapters is being organized into a submission for publication as I prepare this dissertation. However, there are additional areas we investigated that I have yet to discuss such as protein-protein interactions, colocalization through immunofluorescence, and post translational modifications of clock proteins. These data presented in this chapter are from experiments that lacked desired specificity; or these results were not pursued extensively to focus on other areas of interest. Regardless, it seems worthwhile to share these results for future investigators to this field.

#### **Effects of Phospholipase C (*norpA*) on BDBT Foci Formation in Constant Light and Constant Dark.**

When we started looking at the effects of visual mutants on BDBT foci formation we initially screened multiple mutants of the visual transduction pathway. One of the genes of interest is *norpA*, which encodes the phospholipase C (PLC) that is the principal target of RHODOPSIN signaling in the eye in response to light. It was of interest to determine if the reductions of BDBT foci mediated in response to light by Rh1 (*ninaE*) were due to the actions of the *norpA* phospholipase C. As mentioned in previous chapters, PLC is predominantly found expressed in rhabdomeres of the fly eyes, and mutants of the *norpA* gene show drastically reduced receptor potential (Bloomquist et al, 1988). When we initially performed our experiments looking at BDBT foci formation in constant light or constant dark we

looked at four mutants of PLC (*norpA*<sup>33</sup>, *norpA*<sup>35</sup>, *norpA*<sup>36</sup>, and *norpA*<sup>45</sup>). The *norpA*<sup>33</sup> mutants consist of a premature stop codon, *norpA*<sup>35</sup> has a R361C point mutation, *norpA*<sup>36</sup> is a loss of function mutant with premature stop codon, and *norpA*<sup>45</sup> is a hypomorphic allele with a premature stop codon (UAG 748) that has shown some ERG activity while the former *norpA* mutants produce virtually no ERG activity (Pearn et al., 1996). What we observed regarding BDBT foci formation is that BDBT is still able to accumulate in dark and disappears during lighted conditions in three of our four *norpA* mutants (Figure 4.1). The *norpA*<sup>45</sup> mutants produced broad expression under lighted conditions, but as this mutant can still transduce signals these results could be due to a phase shift or another unknown interaction with the visual transduction pathway. As our other *norpA* mutants still showed oscillations in BDBT we choose to focus our efforts on the stronger phenotypes seen in the RHODOPSIN and ARRESTIN mutants, but this does not rule out a role for PLC in this process, particularly since there are two other PLC's with which RHODOPSINS have also been shown to interact (PLCD and PLC 21C (Ogueta et al, 2018-2020; Elsaessr et al, 2010).

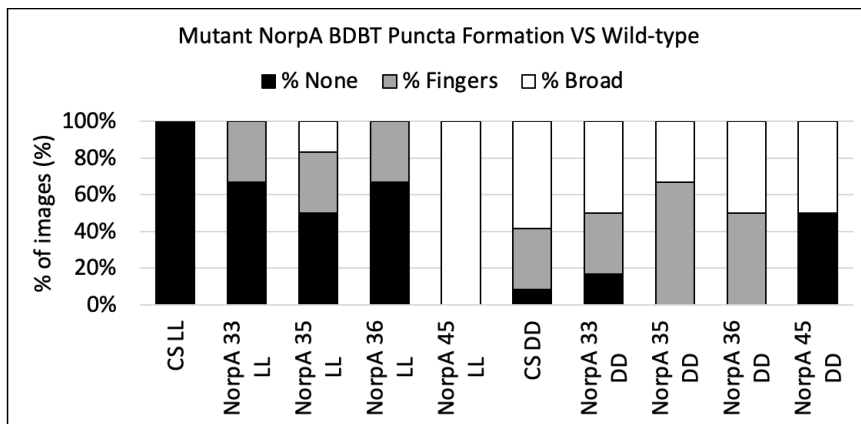
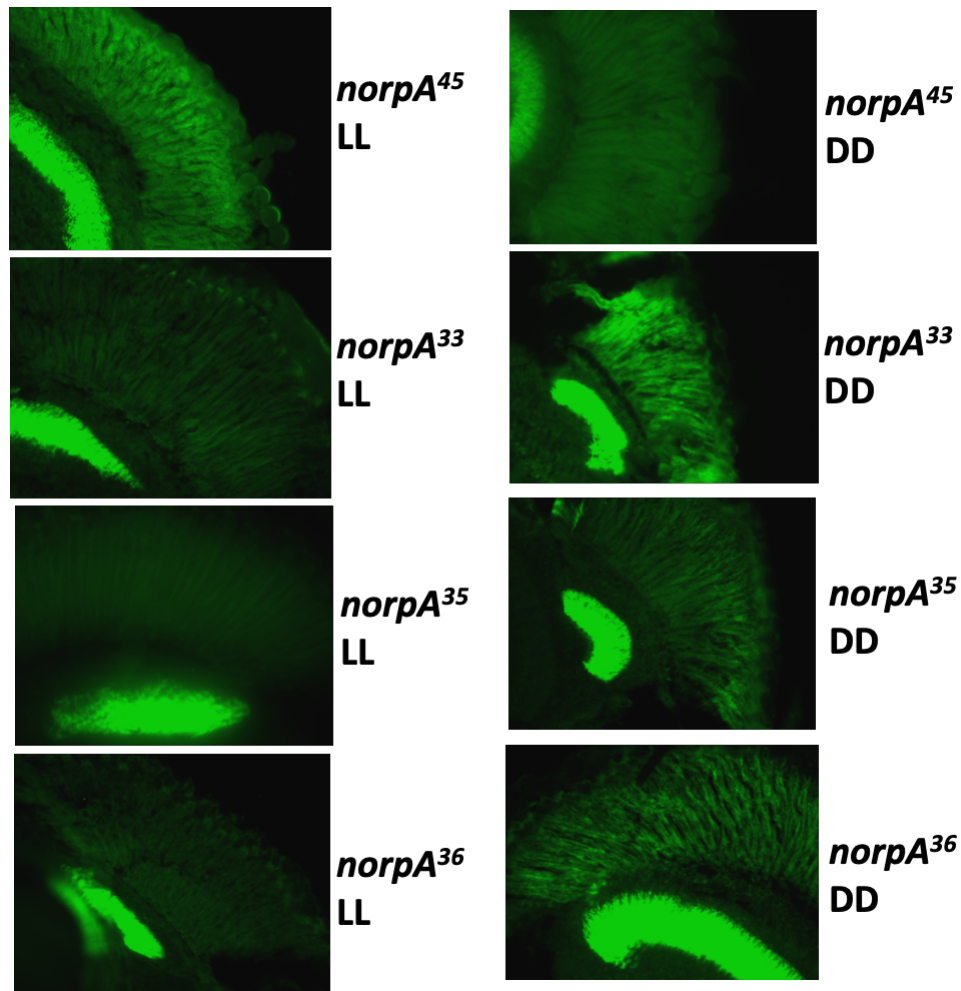
### **Effects of RHODOPSIN-1 and ARR2 Mutants on BDBT Foci Formation and ARR1 Expression in *Drosophila* Eye.**

After discovering that BDBT foci fail to accumulate in ARRESTIN mutants but are constitutively broadly expressed in *RHODOPSIN-1* mutants, we stained eye sections for both BDBT and ARR1 antibodies thanks in part to Dr. Patrick Dolph for donating our lab ARRESTIN antibodies (Alloway & Dolph, 1999). Knowing that

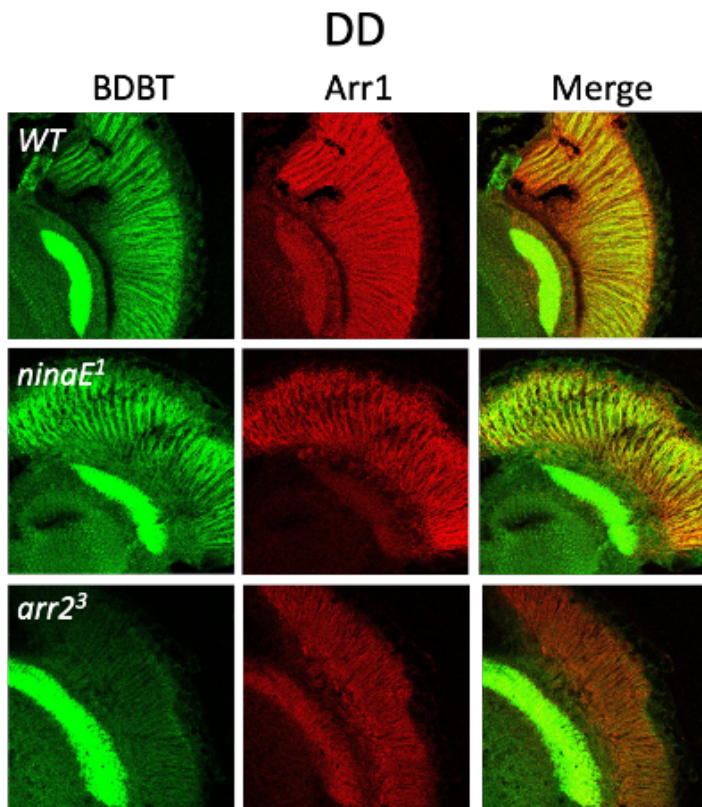
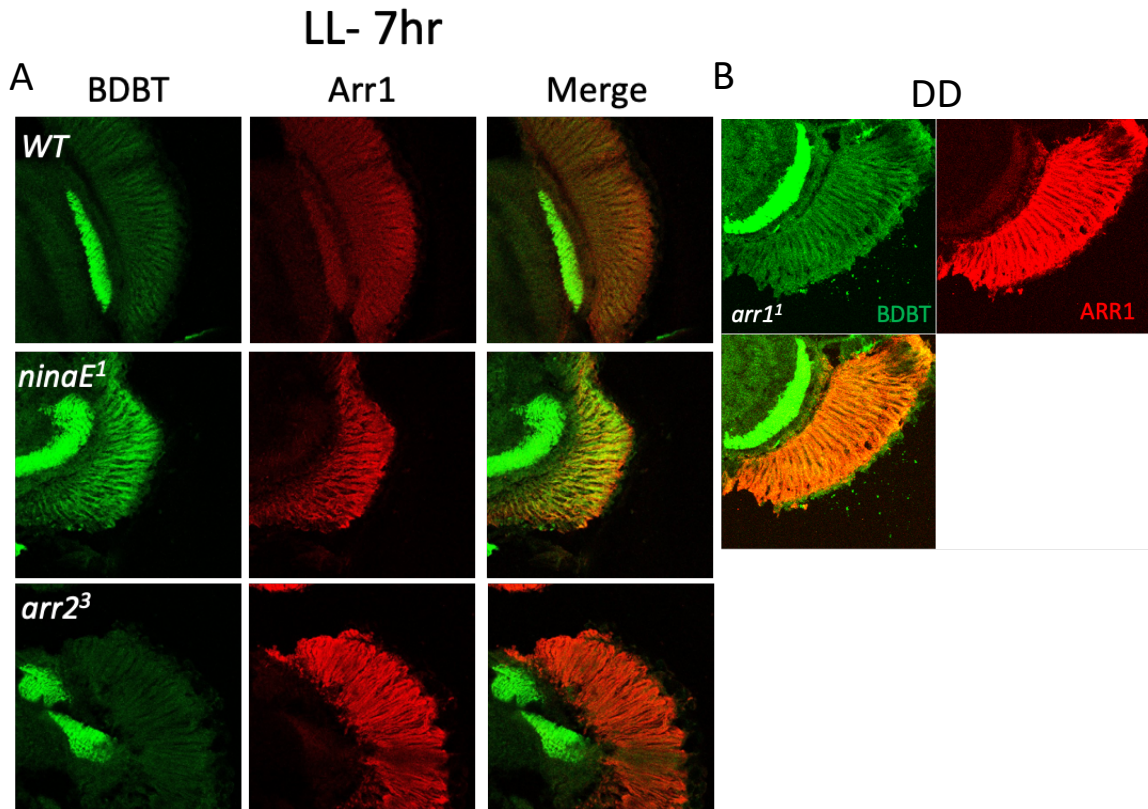
ARRESTINS are involved with the endocytic pathways we thought there may be some strong colocalization between the two. Our results were able to repeat the phenotypes we see with WT, RHODOPSIN, and ARRESTIN mutants, and ARR1 showed strong overlap with BDBT foci formation (Figure 4.2A) as it is expressed broadly through the photoreceptors of the fly eye. However, in our *Arr1* mutant that we used as a negative control there was still strong ARR1 expression in the fly eye (Figure 4.2B). One possibility for this could be because *Arr1*<sup>1</sup> contains a DNA insertion resulting in approximately ten percent of wild type ARR1 protein levels (Dolph et al., 1993) and still allows some detection. Due to a lack of a reduced signal in the negative control and strong expression in WT and mutant lines we realized we would need a different approach to confirm localization. A future ambition is to utilize the Satoh's lab technique for obtaining cross sections of *Drosophila* ommatidia and examining BDBT and ARR1 colocalization with immunofluorescence from the top of the ommatidia instead of the side. This technique would provide additional insights by allowing us to see if BDBT foci formation is occurring in the cell bodies or at the rhabdomere.

### **Effects of RHODOPSIN-1 and ARR1 Mutants on BDBT Foci Formation and ARR2 Expression in *Drosophila* Eye**

Next, I performed the same experiment, but this time looking at ARR2 expression in the eye along with BDBT foci. The results demonstrated repeatable effects for BDBT foci formation seen in these mutants as well as strong ARR2



**Figure 4.1. Effects of Phospholipase C (*norpA*) on BDBT Foci Formation in Constant Light and Constant Dark.** Analysis of *norpA* mutants revealed that BDBT foci are still able to accumulate in a light-dependent manner with decreased foci during constant light (7hrs LL) and broader expression in constant dark. *NorpA*<sup>45</sup> also shows broad expression during the day; however, this *NorpA* mutant has been shown to still produce ERG activity and is not a loss of function allele.





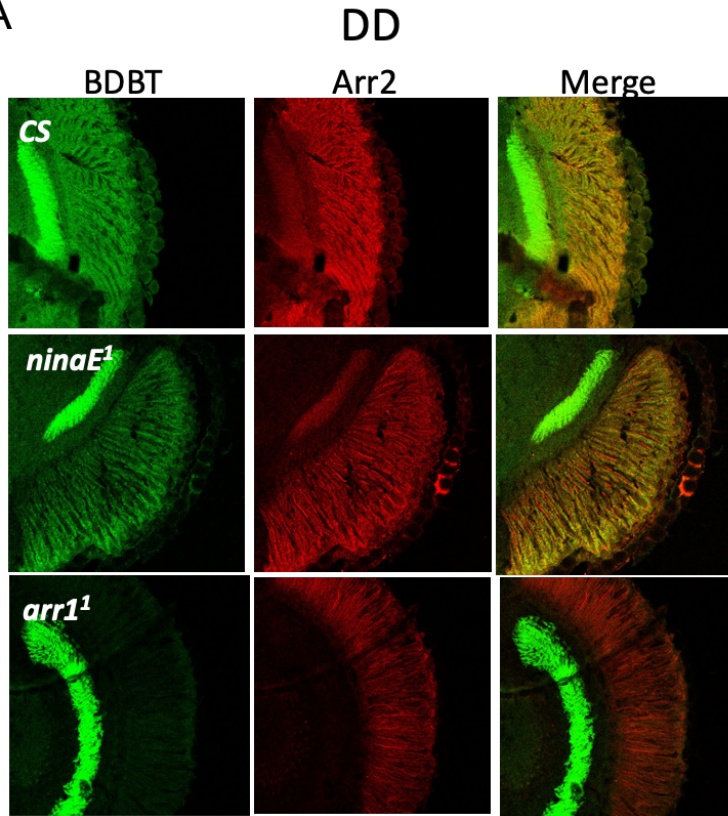
**Figure 4.2. Effects of RHODOPSIN-1 and ARR2 Mutants on BDBT Foci Formation and ARR1 Expression in *Drosophila* Eye.** (A) Representative images of eye sections for WT, *ninaE*<sup>1</sup>, and *arr2*<sup>3</sup> mutants probed with anti-BDBT and anti-ARR1 in constant dark (DD) or 7 hrs constant light (LL). BDBT foci expression was as expected and ARRESTIN expression is found strongly colocalized and expressed broadly through the eye. (B) ARR1 mutants still produced large amounts of ARR1 protein in the fly eye demonstrating a lack of specificity.

expression throughout the photoreceptors. BDBT and ARR2 showed strong overlap between eye sections indicating that they may both be involved in this vesicular trafficking (Figure 4.3). Our *Arr2<sup>3</sup>* mutant, produced only background levels adding to our confidence of the ARR2 antibody. The zooms of the eye sections not only demonstrate the strong overlap between BDBT and ARR2 expression, but an additional phenotype where ARR2 forms ‘finger’ like projections as well in the *ninaE* mutant. These ‘finger’ like projections of ARR2 observed do not seem to affect the colocalization seen in the eye between BDBT and ARR2 but they produce a new mis-localization of ARR2. This mis-localization and formation of ARR2 ‘fingers’ may be due to the accumulation of excessive available ARR2 in the absence of RHODOPSIN at the membrane. While this BDBT/ARR2 overlap offered some evidence of possible vesicular localization colocalization would be better determined if done through ommatidial cross sections in parallel with the surface, as eye sections are not clear and may be interpreted subjectively.

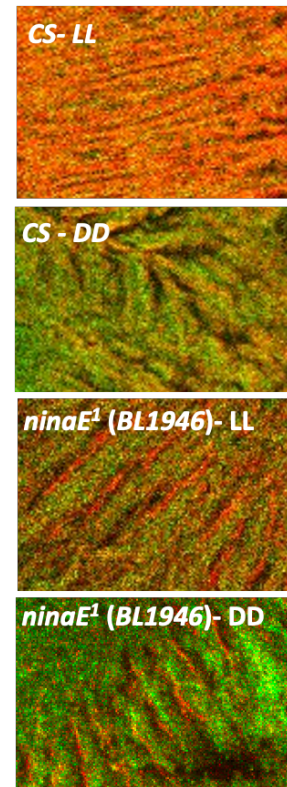
### **BDBT and DBT Colocalization**

Based on our results demonstrating that when we knocked down BDBT with an eye specific GMR-GAL4 driver line we see changes to DBT subcellular localization (Figure 3.6) and knowing that BDBT interacts with the DBT-NLS (Figure 1.2) but does not move with DBT to the nucleus, we checked to see if we could observe any lack of colocalization of DBT and BDBT expression in the photoreceptors. We employed a far-red secondary antibody this time so that we could co-stain eye sections with DBT (green) and BDBT (red). In figure 4.4 we show

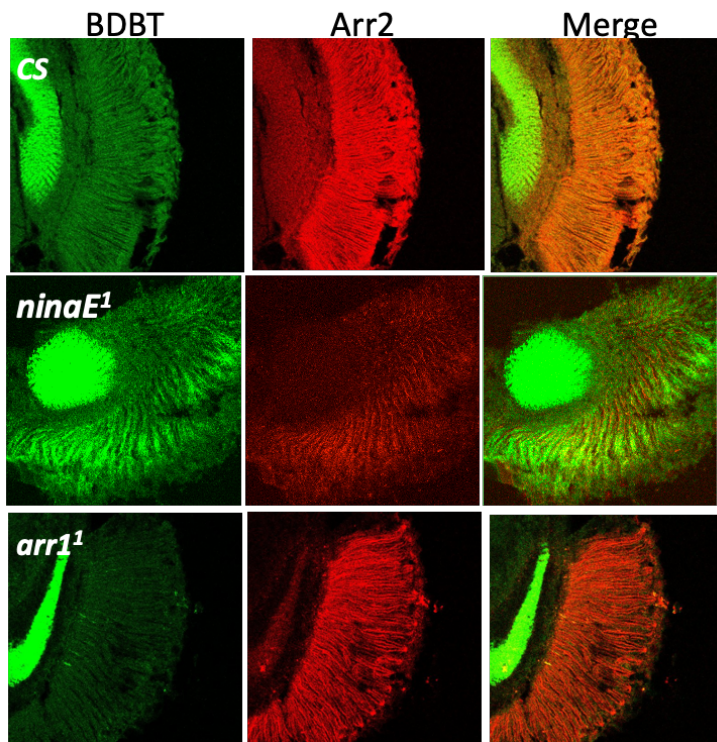
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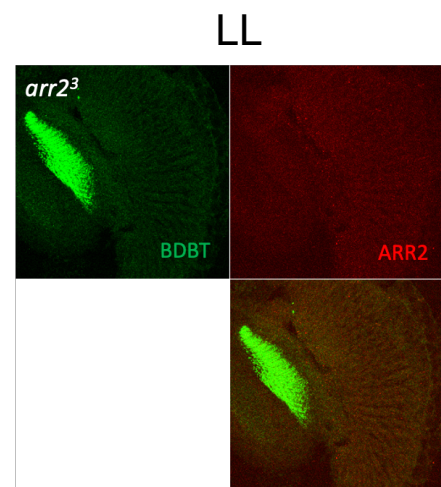
B



LL- 7hr



C

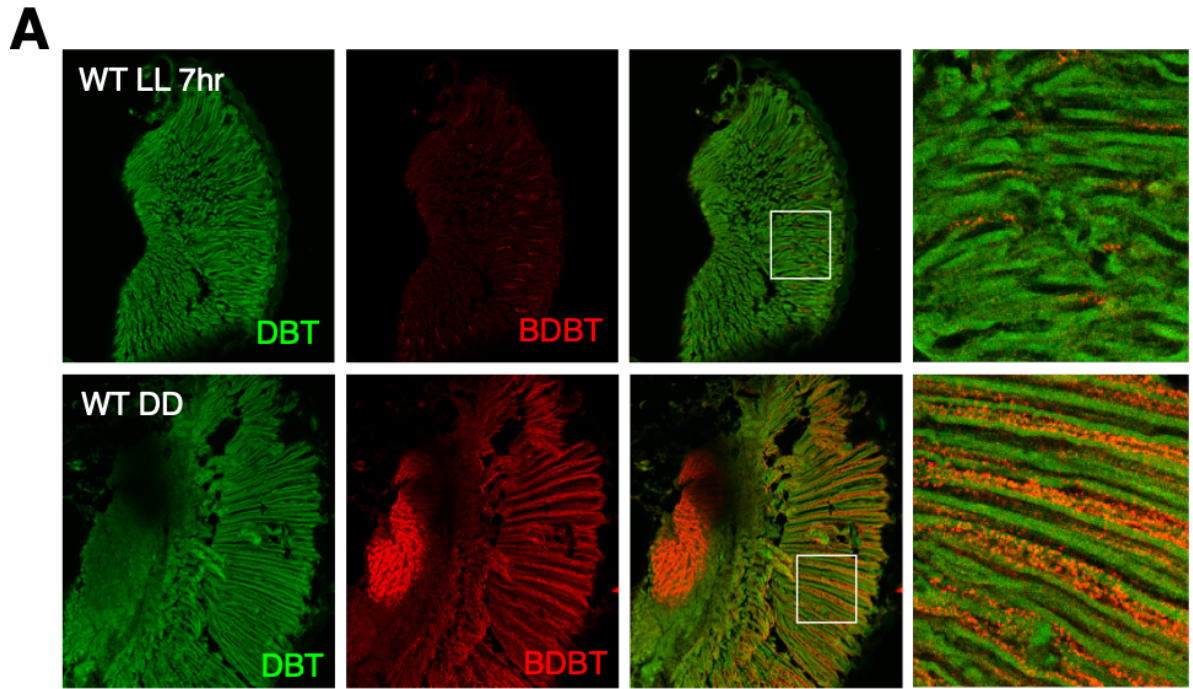


**Figure 4.3. Effects of RHODOPSIN-1 and ARR1 Mutants on BDBT Foci Formation and ARR2 Expression in *Drosophila* Eye.** (A) Representative images of eye sections for WT, *ninaE<sup>1</sup>*, and *Arr1<sup>1</sup>* mutants probed with anti-BDBT and anti-ARR2 in 7 hrs constant light (LL-7) or constant dark (DD). BDBT foci expression was as expected and ARRESTIN expression is found strongly colocalized and expressed broadly through the eye. (B) Zoomed in images of illustrated images for RHODOPSIN and WT flies showing changes to colocalization. (C) Our negative control.

representative images from one set of experiments comprised of a constant darkness or 7 hr constant light after constant darkness. What we saw is that DBT does not localize to foci in the eye, but as both DBT and BDBT are expressed throughout the photoreceptor there is overlap in signal. One could argue that although the colors overlap BDBT foci are separate from the more intense DBT expression we see in DD, but we cannot rule out that DBT is not present at lower quantities in the overlapping expression. This lack of colocalization fits with our model for BDBT to be recruited away from DBT upon foci formation. We hypothesize that DBT is inhibited from nuclear localization during the late day and early evening, but is recruited away from the DBT-NLS during foci formation in the early evening/night. This allows DBT to translocate to the nucleus by possibly reacting with nuclear importins and to reach a peak in nuclear localization in the early morning around ZT2. The reduced interaction with DBT would also be predicted to reduce the kinase activity of DBT towards PER, thereby allowing PER to accumulate and move to the nucleus. These results warrant further investigation through repeat experiments, but if done with ommatidia cross sectioning parallel to the surface of the fly eye these results could be confirmed either way with more confidence.

### **Co-immunoprecipitation Experiments to Look at Protein-Protein Interaction**

Upon discovering the BDBT phenotypes for foci expression and lack of expression in ARRESTIN mutants we revisited prior data from the Price lab. Mass spectrometry had been performed previously to identify proteins that interacted with



**Figure 4.4. Colocalization Staining of BDBT and DBT in *Drosophila* Eye.** Representative eye sections for WT flies stained with anti-DBT (Green) and anti-BDBT (Red) (A). Fly heads were collected after being raised in constant darkness (DD) or stimulated with 7-hrs of constant light (LL 7-hr).

DBT-myc tagged protein, and this analysis led to the discovery of BDBT by the Price lab (Fan et al, 2013). When we revisited the mass spectrometry data, we realized that ARR1 was also pulled down with DBT-myc (Fan & Keightley, unpublished data), and this led me to perform a series of co-immunoprecipitation experiments to determine if ARRESTINS interacted directly with BDBT or DBT. First, I drove expression of tagged versions of DBT and BDBT in the eye by crossing GMR-GAL4 female virgins to either UAS-*dbt-Flag* or UAS-*dbt-myc* male flies. Progeny were aged for approximately one week, entrained for at least three days to a light dark cycle, and heads were collected at ZT1, ZT7, and ZT19. With our GMR-GAL4>UAS-*dbt-FLAG* and TIM-GAL4>UAS-*dbt-FLAG* flies we did not see a difference in ARR1 levels when compared to our negative controls (not shown). This may be due to a lack in interaction between ARR1 and BDBT as we were only able to detect background levels of ARR1. However, we were able to confirm that in our GMR-GAL4>UAS-*dbt-myc* and TIM-GAL4>UAS-*dbt-myc* flies ARR1 is able to be co-immunoprecipitated with DBT (Figure 4.5) and detected through immunoblot analysis. We also probed immunoblots consisting of our IP's for ARR2, but did not see anything above our negative control background levels (not shown).

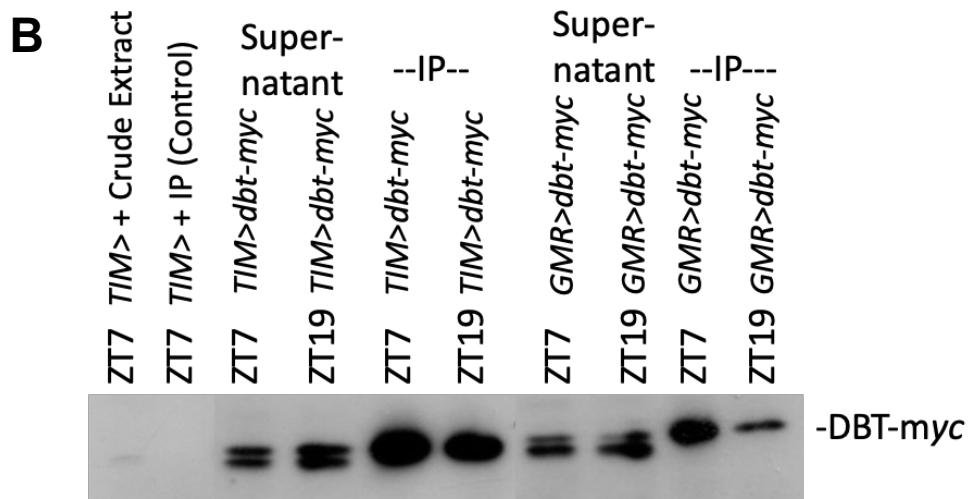
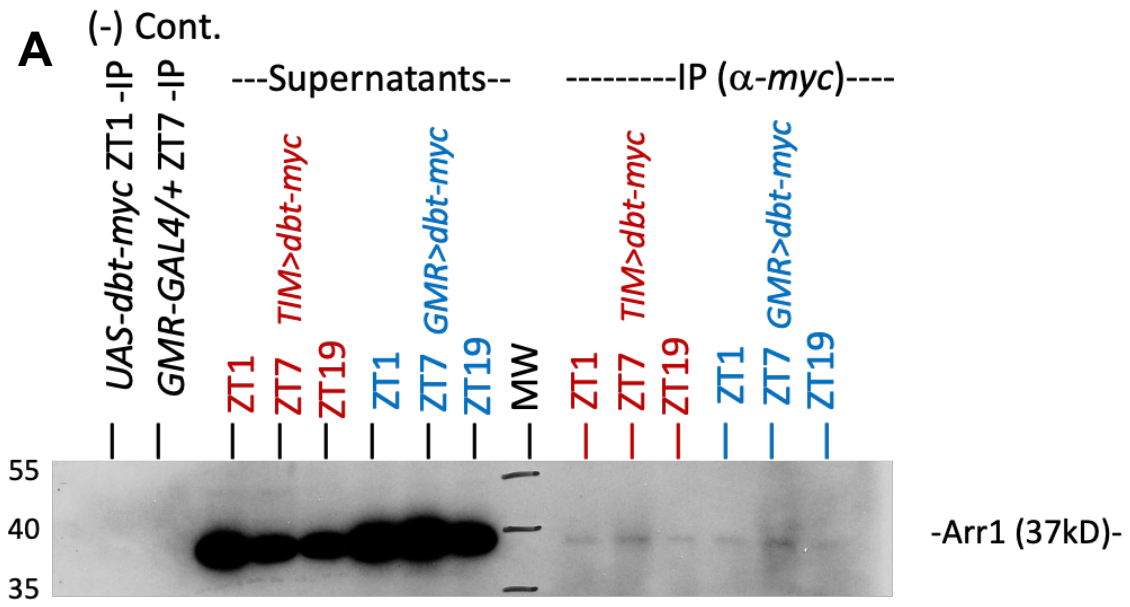
Next, I wanted to see if I could see a change in DBT and BDBT protein interactions through immunoprecipitations in ARRESTIN mutants since ARRESTINS had shown to disrupt BDBT foci formation (Figure 3.3). I performed immunoprecipitations in WT, *Arr1*<sup>1</sup>, and *Arr2*<sup>3</sup> flies collected at ZT1, ZT7, ZT13, and ZT19 and used anti-DBT to do my pull downs. At all timepoints in WT and ARRESTIN mutants DBT was able to pull down equal amounts BDBT indicating that

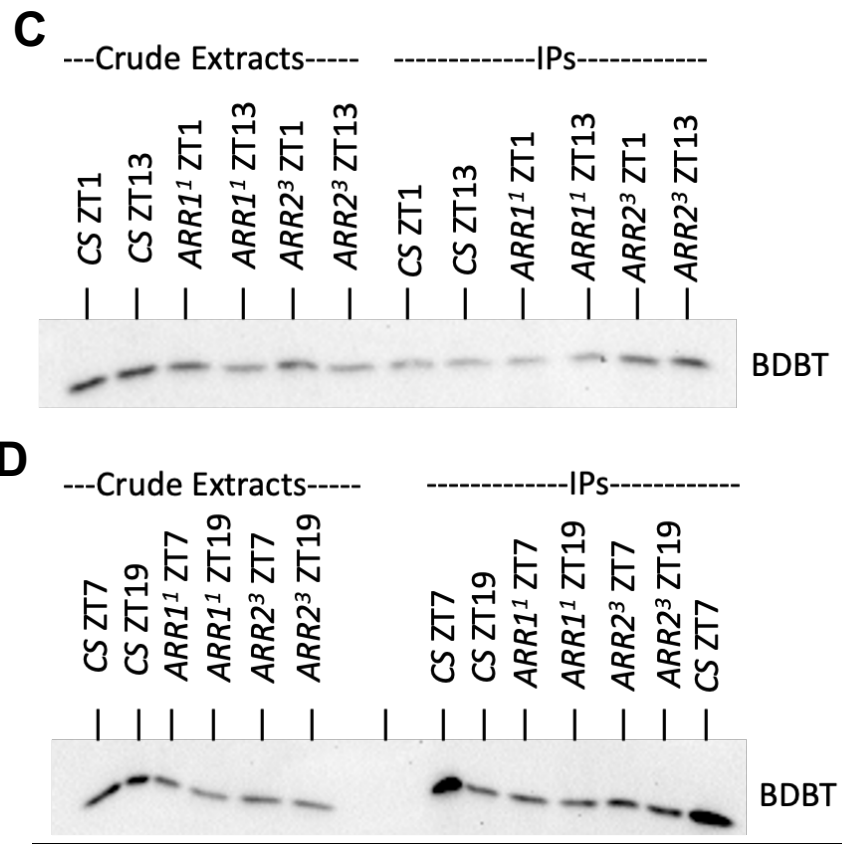
DBT-BDBT protein interactions are likely not being affected (Figure 4.5). One caveat to this experiment is that our IP's target all DBT, which is also expressed throughout the brain, and our immunoblot analysis may not be specific enough to detect differences between BDBT/DBT interactions occurring in the eye.

### **Effects of ARRESTIN Mutants on DBT Post Translational Modifications.**

Since ARR1 is able to interact with DBT through protein-protein interactions I decided to follow up on some earlier results witnessed in our lab. Previously, we had seen post translationally modified DBT migrating more slowly on western blots in an *Arr1*<sup>2</sup> mutant line that is heterozygous for ARR1 (Fan, not shown). I decided to repeat this experiment at four time points ZT1, ZT7, ZT13, and ZT19 in WT flies and the stronger homozygous *arr1*<sup>1</sup> mutant as well as in *arr2*<sup>3</sup> mutants and perform western blots to analyze both DBT and BDBT. In the BDBT blots we did not see any changes to BDBT mobility or any additional bands between WT flies and ARRESTIN mutants (not shown). However, I did notice in my first two replicates of this experiment that in *Arr1* mutants there was a slower migrating form of DBT that showed up at ZT1 and ZT7 in the first experiment and then at all time points in the second replication (Figure 4.6). This post translational modification is not seen in WT flies indicating that it is likely a product of not having ARR1 available. Better understanding of this DBT-PTM could provide insights into how ARR1 is involved in circadian regulation of the fly eye beyond BDBT foci formation.

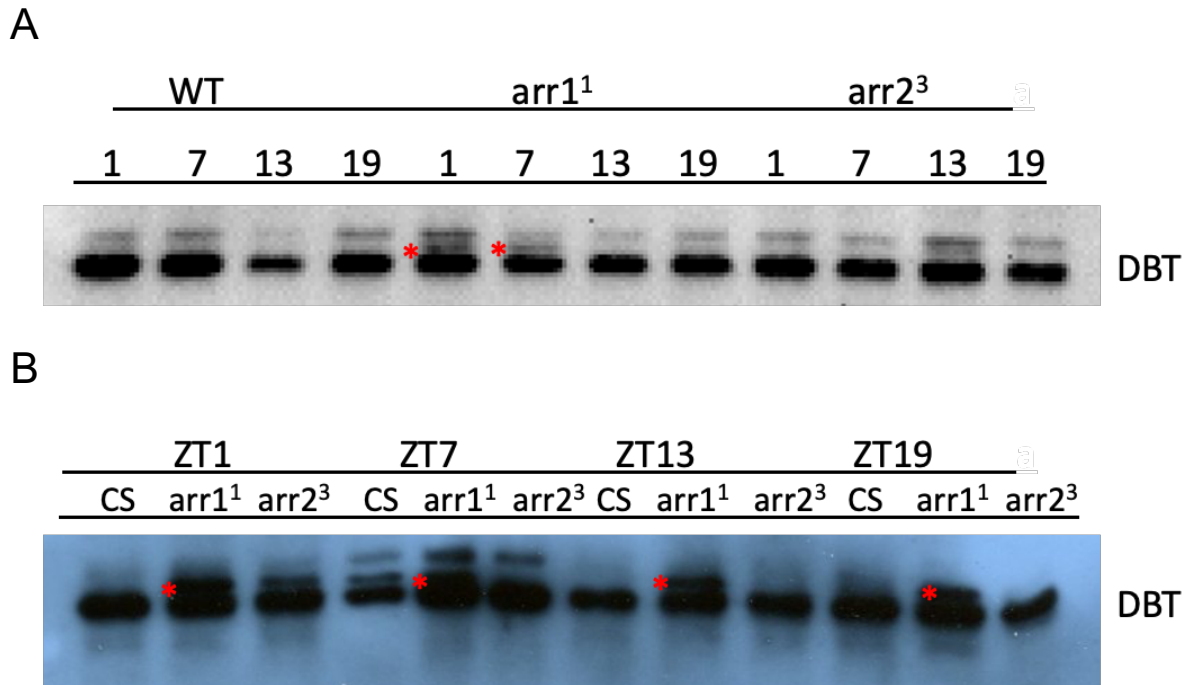






**Figure 4.5. Co-immunoprecipitations to Determine Protein-Protein Interactions.**

(A) UAS-dbt-myc male flies were crossed to either TIM-GAL4 or GMR-GAL4 female virgins. Flies were entrained for at least three days before collections. Driver and responder lines were used as negative controls to compare to supernatants and IPs. IPs were performed by using anti-myc-beads and analyzed through immunoblot analysis and probed for anti-ARR1. ARR1 was able to be precipitated with DBT-myc demonstrating protein-protein interaction. (B) Immunoblot probed with anti-DBT. Immunoprecipitations demonstrate that both endogenous DBT and DBT-myc was found in the supernatant, but only DBT-myc is found in the IPs of both GMR and *tim*GAL4 drivers and no DBT-myc was detected in our negative control (C and D). Co-immunoprecipitations were performed on *WT*, *Arr1*<sup>1</sup>, and *Arr2*<sup>3</sup> flies using anti-DBT for pull downs and probed with anti-BDBT using immunoblot analysis at ZT1, ZT7, ZT13, and ZT19. BDBT protein levels in Co-IP's varied slightly between different timepoints but repeat experiments did not confer these changes to BDBT protein levels.



**Figure 4.6. Different Migrating Forms of DBT in ARRESTIN Mutants.** Flies were collected as previously described and heads collected at ZT1, ZT7, ZT13, or ZT19. DBT migrates at three different distinct bands. The slowest migrating band and WT type band are seen in WT and both ARRESTIN mutants. Another migrating form of DBT is seen most predominantly in *Arr1<sup>1</sup>* mutants (indicated by ‘\*’) in our first experiment (A) and is then seen at all time points in our replicate experiment (B).

## Brain Expression Patterns of GMR-GAL4>UAS-mCD8-GFP and Rh1-GAL4>UAS-GFP in Flies

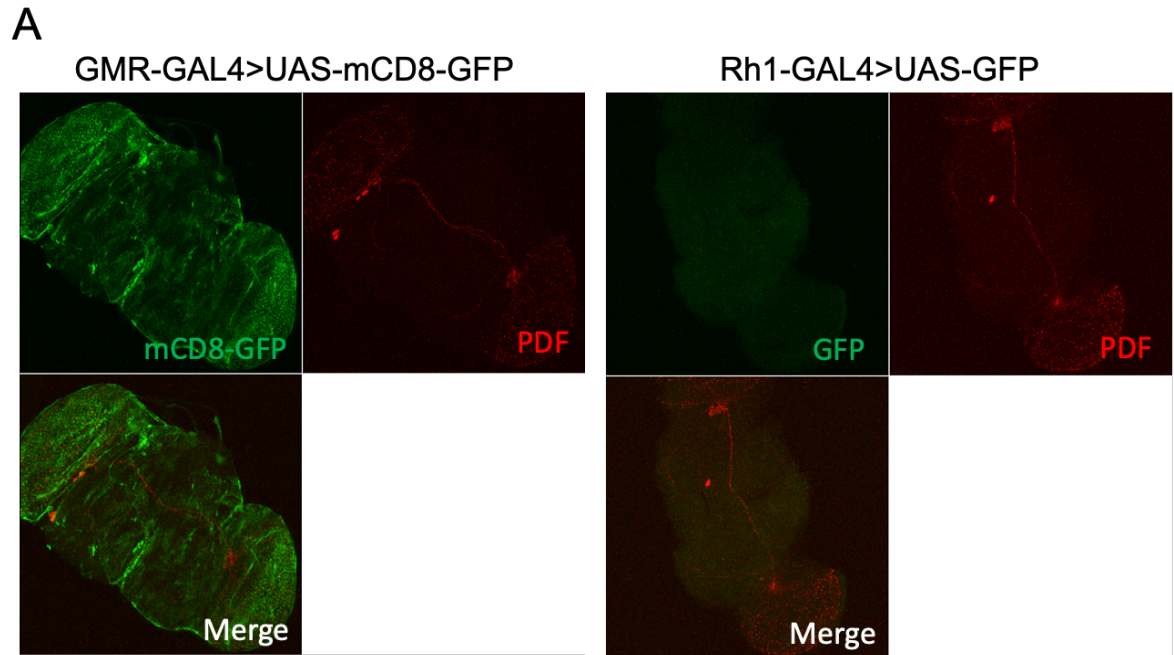
To better understand the effects in our BDBT knockdown experiments where our collaborators analyzed circadian color preference, we decided to confirm that our GMR-GAL4 and Rh1-GAL4 drivers were indeed limited to the fly eye. I stained brains of GMR-GAL4>UAS-mCD8-GFP (mCD8 labels neuronal membranes) flies with anti-GFP and anti-PDF to ensure there was no co-expression in the PDF clock cells (Figure 4.7). I also did immunofluorescence staining on brains of Rh1-GAL4>UAS-GFP with anti-GFP and anti-PDF to confirm RHODOPSIN-1 is not expressed in the brain (Figure 4.7) to help investigate why our GMR driver line has circadian color preference effects with BDBT knockdown, but the Rh1 driver does not. What I observed is that GMR can be found in the brain within the optic lobes and possibly in other brain tissue but is not found in the PDF expressing clock cells such as the small and large ventral lateral neurons. This does raise additional scrutiny to possibly off target expression in the brain not localized to the eye. It makes sense that GMR can be found in the optic lobes as some of the axonal projections from the fly retina extend into the brain and its possible that this expression, due to stronger expression of GMR-GAL4 in the eye than expression of Rh1-GAL4 in the eye, is what produces the stronger effects seen in the GMR-GAL4 driver line. In the brain sections with the RH1-GAL4 driver I detected no signal with anti-GFP. Next, we want to compare expression in the fly eye between these two different drivers as well as look at eye sections in Rh1-GAL4>UAS-*bdbt*-RNAi;UAS-*dcr* flies. If the RH1-GAL4 driver can knockdown BDBT foci formation in the fly eye

and alter DBT and PER localization then it increases our confidence that the effects to altered subcellular localization to PER and DBT that we previously saw in our BDBT knockdown flies (Figure 3.6) is due to knockdown in the eye and not related to cells that express GMR in the fly brain.

### **Effects of *spag*-RNAi and *dbt*-RNAi on BDBT Foci Formation with GMR-GAL4**

#### **Driver.**

Previously, it had been shown that in UAS-dcr2;timGAL4>/+;UAS-dbt RNAi:/+ lines that BDBT foci failed to form (Fan et al, 2013), indicating foci formation is a DBT dependent process. We decided to analyze our GMR-GAL4 driver line with *dbt*-RNAi and *spag*-RNAi to see what affects this may produce on BDBT foci formation when we target our knockdowns to the fly eye. We decided to look at SPAGHETTI due to its ability to stabilize DBT and promote DBT activity (Fan et al, 2015). I stained eye sections for anti-BDBT as well as anti-PER to confirm that we still see nuclear PER during the stimulated day when we knockdown DBT (Figure 4.8). What we witnessed is that *spag*-RNAi had no effect on BDBT foci formation or PER localization during the day or night when compared to our controls. PER was found constitutively nuclear in our *dbt*-RNAi flies at both day and night as we predicted, but we did not see the same effects with our GMR-GAL4 driver on BDBT foci formation as previously seen when *dbt*-RNAi expression was driven with our timGAL4 driver. This experiment has only been performed once and should be repeated, but the results are of interest. If knockdown of DBT in the eye is not enough to affect BDBT foci formation, but knockdown in TIMELESS clock cells can

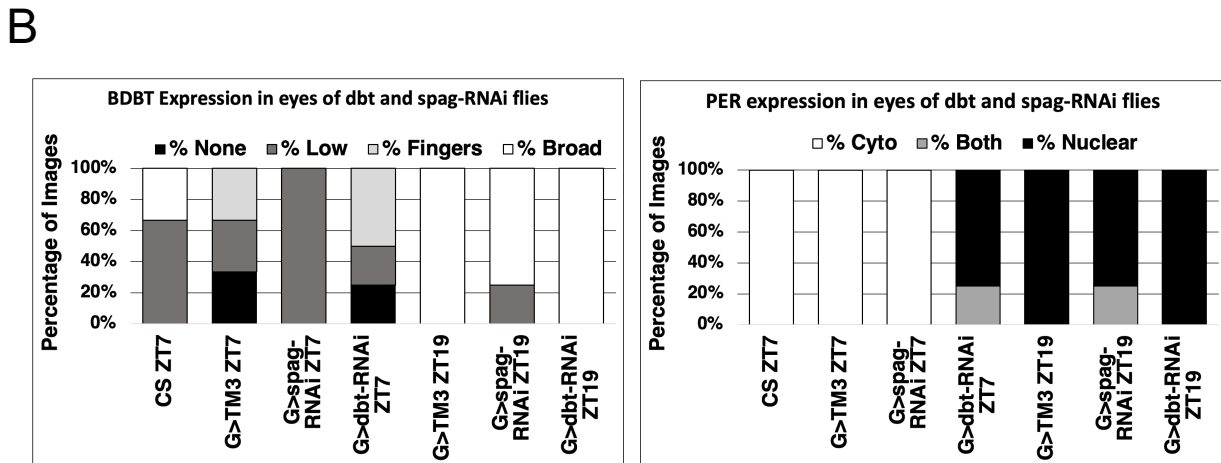
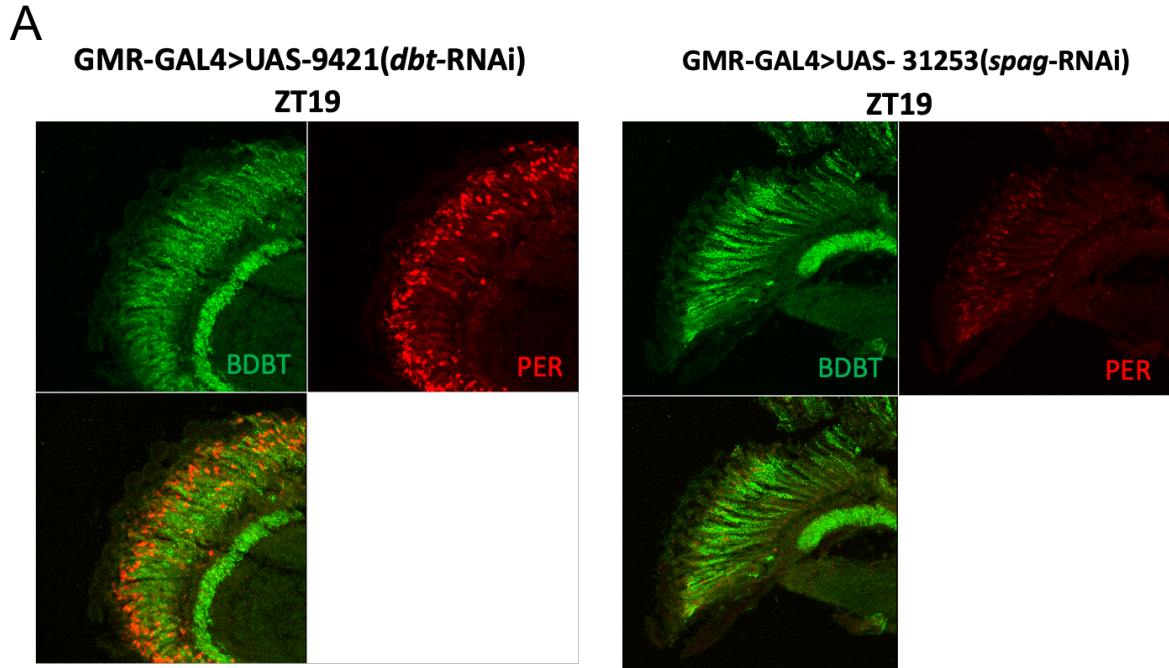


**Figure 4.7. Brain Expression Patterns of GMR-GAL4>UAS-mCD8-GFP and Rh1-GAL4>UAS-GFP in Flies.** (A) Brain sections were stained with anti-GFP and anti-PDF to determine expression patterns. Neither GMR or RH1 are expressed in the PDF expressing neurons confirming that the activity from these driver lines is not occurring in the circadian PDF<sup>+</sup> neurons.

abrogate BDBT foci in the eye this indicates that a DBT involved mechanism in the brain is potentially signaling BDBT foci formation in the eye. As this dissertation has focused predominantly on the effects that the eye can have on other circadian mechanisms the reverse is also of importance and should be further investigated.

### **Effects of Eye Specific Knockdown of BDBT and DBT on Circadian Period.**

To determine how strong the knockdown effects in the eye are on periodicity I performed a set of locomotor assays. Once again using our GMR-GAL4 driver to target expression I crossed GMR-GAL4 flies to either UAS-100028 (*bdbt*-RNAi) or UAS-9241 (*dbt*-RNAi) flies. The results including the average period plus the standards error of the mean are listed in Figure 4.9. Flies in both BDBT and DBT RNAi lines remained rhythmic with wild type periods. It does not appear that knockdown of BDBT or DBT in the *Drosophila* eye is strong enough to generate arrhythmicity while the brain is still rhythmic. It is also worth noting to future investigators that the GMR-GAL4 driver may generate off target effects that require additional scrutiny.



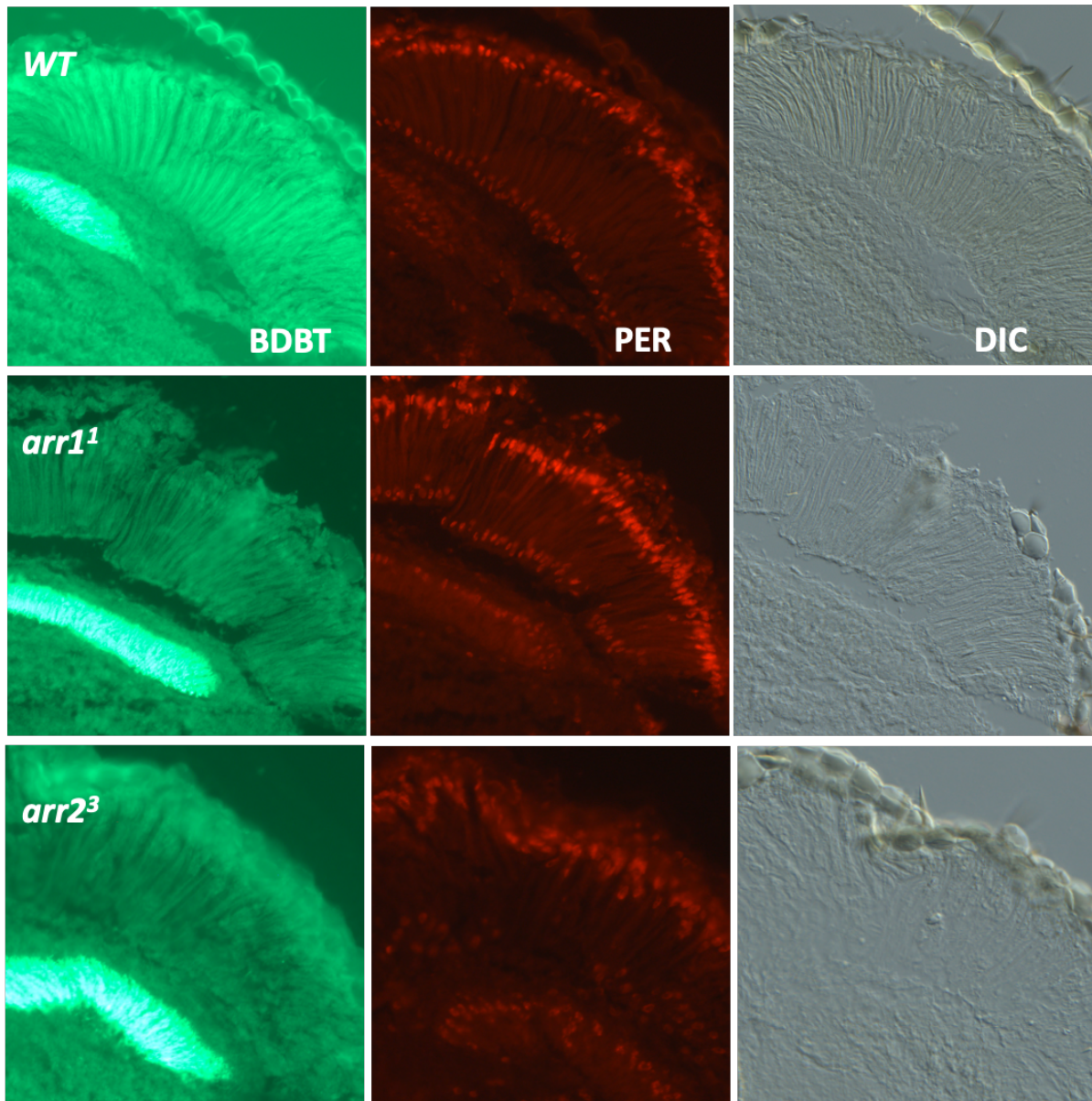
**Figure 4.8. Effects of *spag*-RNAi and *dbt*-RNAi on BDBT Foci Formation with GMR-GAL4 Driver.** (A) Eye sections were collected in WT, GMR>TM3 (Balancer control), GMR-GAL4>UAS-*dbt*-RNAi, and GMR-GAL4>UAS-*spag*-RNAi flies at ZT7 and ZT19 and probed with anti-BDBT and anti-PER. (B) Blind scoring results indicating that neither *dbt* or *spag* knockdown in the eye has an effect on BDBT foci formation. DBT knockdown in the eye is still able to produce a strong effect on PER as seen previously during the day and night.



A

Genotype	Average Period $\pm$ SEM	Percent Rhythmic (n)
GMR-GAL4 Males	23.4 $\pm$ 0.1	68.75% (16)
UAS-100028 ( <i>dbt</i> -RNAi)	24.8 $\pm$ 0.1	92.3% (13)
UAS-9241 ( <i>dbt</i> -RNAi)	23.6 $\pm$ 0.01	90.0% (11)
GMR-GAL4 > UAS-100028 ( <i>dbt</i> -RNAi)	23.7 $\pm$ 0.2	85.7% (14)
GMR-GAL4 > UAS-9241 ( <i>dbt</i> -RNAi)	23.5 $\pm$ 0.1	86.67% (15)

**Figure 4.9. Table Illustrating the Effects of Eye Specific Knockdown of BDBT and DBT on Period.** GMR-GAL4 was used for eye specific knockdown in UAS-100028 (*dbt*-RNAi) and UAS-9241 (*dbt*-RNAi) flies and their period analyzed. (A) Period was extended in both GMR-GAL4>UAS-100028 flies as well as in our GMR-GAL4 controls. It appears that eye specific knockdown of BDBT is not sufficient to drive arrhythmicity as seen with previous *tim*GAL4 drivers.



**Figure 4.10. Fluorescent and Differential Interference Contrast Microscopy eye sections to assess eye morphology in *arrestin* mutants.** Canton S flies (WT) were compared to our *arrestin* mutant lines at ZT19 of a LD cycle for changes to eye morphology. We did not notice any observable changes in eye morphology in the differential interference contrast (DIC) images and fluorescent detection of BDBT and PER matched our other experimental results with confocal analysis.

## CHAPTER 5

### FUTURE DIRECTIONS

The research put forth in this dissertation has illustrated the importance of BDBT foci formation to maintaining a functional eye clock in *Drosophila*. Overall, we have been able to determine that the formation of ARRESTIN and dark-dependent BDBT foci demonstrates a mechanism in which light input signals are being regulated through crosstalk between circadian and visual pathways in the *Drosophila* retina. This BDBT foci formation is potentially comprised of endocytic or other membrane vesicles and with reduction of these BDBT foci the circadian regulators DBT and PER undergo changes to their normal subcellular localization in the eye while the brain remains rhythmic. Finally, we saw that disruption to the *Drosophila* eye clock through BDBT or DBT is able to alter normal circadian color preference even while the fly brain remains rhythmic. However, our DBT eye-specific mutants also show that this rhythmicity may be disrupted by loss of anticipation to the morning light, demonstrating a novel process in which targeted expression to the eye disrupts normal fly activity. Each of these new discoveries brings about new questions to be answered and where we see the future directions of this project going.

Now that we know that normal cycles of BDBT foci formation require both CRY, RH1 and ARRESTINS, thus connecting the circadian and visual pathways, respectively, we want to understand these individual processes more in detail. In Figure 3.3, we showed that CRY is necessary for normal light-dependent reduction of BDBT foci and knowing that CRY regulates TIM levels in response to light

(Stanewsky et al., 1998) it is of interest to assess what role TIM has in this process by looking at BDBT foci formation in loss of function *tim<sup>o</sup>* mutants or in GMR-GAL4 > UAS-TIM flies to drive overexpression of TIM in the fly eye. If CRY regulates BDBT foci reductions by reducing TIM levels in light, then I would predict that *tim<sup>o</sup>* mutants should express constitutively low levels of BDBT foci, while overexpression of TIM should elevate BDBT foci. Another approach to investigate the circadian process would be to look at the effects of a CRY mutant protein that lacks its C-terminus. The C-terminus domain of CRY has been shown to repress CRY activity towards TIM in the dark leading to constitutively low TIM protein levels under dark conditions in the mutant lacking the C terminus. (Dissel et al., 2004, Busza et al., 2004). If CRY operates through a TIM-dependent pathway then we expect that this mutant would constitutively depress BDBT foci. While these approaches focus on the involvement of CRY in the circadian process, alternatively the C-terminal domain of CRY has also been shown to be involved in a light-mediated interaction between CRY and the rhabdomeric scaffolding protein INAD. Absence of this interaction is predicted to reduce visual transduction and to elevate BDBT foci (Mazzotta et al, 2013) – the opposite prediction to one produced by a TIM-dependent effect.

Our *ninaE* mutants indicated that in the absence of RHODOPSIN-1, broad expression of BDBT foci formation persisted during both light and dark conditions. Whether these effects on BDBT foci require phospholipase C activity (the downstream target of RHODOPSIN for visual transduction) or just more abundant ARRESTIN protein is of interest. In dark conditions both ARR1 and ARR2 are found primarily in the cytosol around the same time BDBT foci are accumulating.

Normally, during the day when RHODOPSIN is converted to M-RHODOPSIN in response to blue light, ARR2 moves to the rhabdomere within 5 minutes of exposure to light to quench further RHODOPSIN/G $\alpha$  protein signaling. (Sato & Ready, 2005; Ranganathan & Stevens, 1995). Without translated RHODOPSIN-1 protein in *ninaE* mutants to quench during the day ARR2 may be more freely available to generate BDBT vesicles - hence why we see broad expression of BDBT foci at all times and under light/dark, constant light, and constant dark conditions. ARR2 requires phosphorylation by Calcium/calmodulin-dependent protein kinase II (CAMKII) to be released from RHODOPSIN (Kahn & Matsumoto, 1997; Alloway & Dolph, 1999), and RHODOPSIN-1 vesicles failed to form in ARR1 mutants (Sato & Ready, 2005), suggesting ARR1 is also required. If ARR1 is necessary for the release of ARR2 from RHODOPSIN, then this would also support the idea that this codependence may be why there is a loss of BDBT foci expression in both mutants. This dark-dependent model for ARR2 which posits that during the day ARR2 quenches RHODOPSIN/G $\alpha$  protein signaling and at night it generates BDBT foci could explain why BDBT foci fail to form in *Arr2*<sup>3</sup> and *Arr1*<sup>1</sup> mutants as the endocytic process is being disrupted.

Another possibility for the increased broad expression of BDBT foci is that RHODOPSIN is required to signal the elimination of BDBT foci through photosensitivity to light. Work we did with strong loss of function *norpA* (PLC) mutants, which is a primary target of RHODOPSIN signaling, show that these mutants were still able to reduce BDBT foci during lighted conditions make it an unlikely pathway. However, the GTPase Rac2 has been shown to be necessary for

the normal termination of the photo response. With loss of Rac2, ARR2 movement was severely impaired demonstrating that light-induced translocation of ARR2 to the rhabdomere occurs through a non-canonical RHODOPSIN/Rac2 pathway that may involve Phospholipase D (Elsaesser, Kalra, & Montell, 2010). Determining how Rac2 mutants affect BDBT foci formation would be of further interest. First, we can determine if Rac2 has a role with BDBT foci formation, as it may hamper the ability for ARR2 to translocate to BDBT, and second it may disrupt the translocation of other circadian clock proteins as we see in *Arrestin* mutants (Figure 3.3).

Our finding that BDBT foci formation requires ARRESTINS and dark conditions is of interest since the other well studied ARRESTIN function involving RHODOPSIN quenching is a light-dependent rather than a dark-dependent process (Dolph et al. 1993). During the phototransduction process several proteins are regulated through translocation from the rhabdomeres to the cell body and this translocation has been thought to be due to adaptations to light and dark sensitivity. The  $G\alpha$ -protein has been shown to move to the rhabdomere prior to the RHODOPSIN response and within only minutes of light exposure  $G\alpha$ -protein returns back to the cell bodies (Kosloff et al., 2003; Cronin, Diao, & Tsunoda, 2004). Also, the TRPL cation channel moves to the cell body in response to light exposure and then back into the rhabdomeres in the dark and this dark-mediated movement requires ARR2 for proper translocation (Bahner et al., 2002; Cronin, Lieu, & Tsunoda, 2006). We would like to investigate these  $G\alpha$ -protein and TRPL cation channels involved in phototransduction to address whether their movement is affected in our BDBT knockdown lines. As well as determining if BDBT foci

formation affect their localization and vice versa it would also be worth looking into how these disruptions to the eye clock affect visual sensitivity which can be measured through an electroretinogram.

As mentioned, we know that in the absence of RHODOPSIN-1, such as in our *ninaE* mutants, BDBT foci continue to form and through utilizing a temperature sensitive SHI mutant we were able to indicate that BDBT foci are possibly comprised of endocytic or other membrane vesicles. Overexpression of a functional dynamin (SHI), a GTPase that aids in removing clathrin coated vesicles from the membrane (Gonzalez-Bellido et al., 2009), at the permissive temperature of 18°C leads to increased endocytosis and decreased BDBT foci formation. At the higher restrictive temperature where SHI becomes inactive, we saw increased BDBT foci. This suggests that BDBT foci formation is likely occurring at the membrane before cleavage occurs. However, we would like to expand on these findings to look at other time points to determine if suppression of BDBT foci is affected by overexpression of functional SHI at other time points, or if overexpression of the inactive SHI at high temperature prevents disappearance of BDBT foci during the day.

If we are able to gain further data supporting that BDBT vesicles are released from the membrane it would be of interest to determine if these are vesicles from part of a lysosomal/autophagosomal pathway. The effect of BDBT foci formation on DBT nuclear localization could be due to an association with aggresomal particles or other proteins involved with trafficking along microtubules as we have seen significant changes to subcellular localization of DBT with reduced BDBT foci (Figure

3.6). If this is the case, the formation of BDBT foci could be negatively regulating DBT activity and positively regulating DBT nuclear localization by regulation of a trafficking process. We showed in constant light that broad expression of BDBT foci is eliminated (Figure 3.1) while PER is also targeted for degradation (Price et al., 1995), presumably due in response to increased DBT and BDBT activity (Fan et al., 2013). If BDBT foci are negatively regulating DBT activity at night it is possible that degradation through this foci formation could provide new insights for an endocytic, lysosomal, or autophagosomal mechanisms that is able to input signals to the circadian clock. By utilizing different biomarkers for lysosomal/autophagosomal degradation we can determine if BDBT associates with these vesicles in a temporal manner and at what stage along the process association occurs or if this process occurs through a separate mechanism.

Of further interest would be understanding how BDBT foci formation is leading to changes in the subcellular localization of the circadian regulators PER and DBT. In the previous paragraph I discussed how this could be in relation to possible affects BDBT foci formation has on DBT activity. However, these changes to subcellular localization could be a direct consequence of the above mentioned lysosomal/autophagosomal targeting of BDBT/DBT or part of another cell trafficking process altogether. In our wild type flies, broad expression of BDBT foci peaks around ZT19 when PER is initially found strongly localized in the nuclei and as DBT begins building up in the nuclei until its peak around ZT2 (Kloss et al., 2001; Fan et al., 2013). When we knocked BDBT down using GMR-GAL4>UAS-dcr in the eye DBT failed to localize to the nuclei while PER is found to be constitutively nuclear



(Figure 3.6). These elevated levels of nuclear PER are thought to occur due to decreased DBT activity against PER (Muskus et al., 2007; Cyran et al., 2005). This suggest that accumulation of BDBT foci may be required for the temporal subcellular regulation of these clock proteins. These foci could be acting to reduce DBT activity through a lysosomal/autophagosomal pathway allowing PER to avoid phosphorylation by DBT and targeted proteasomal degradation or this may be occurring in the cell body separate of this pathway.

Our findings that BDBT binds to the DBT-NLS (Venkatesan et al., 2019; Venkatesan et al., 2015), which is required for normal nuclear localization and phosphorylation of nuclear PER, suggests that BDBT may need to be recruited away from DBT at night. This would allow DBT to be available to be translocated to the nucleus via interactions of its NLS with nuclear importins. This BDBT foci-controlled release of the DBT-NLS could be what allows PER to build up initially in the nuclei at ZT19 before DBT eventually accumulates to a nuclear peak in the early morning around ZT2 to target PER for degradation. If this proposed model is how this mechanism works, it would also explain why in our eye specific BDBT knockdown mutants PER is more nuclear at all times of day and DBT is more cytosolic (Figure 3.6). Possibly without BDBT available to enhance DBT activity, DBT is subject to autophosphorylation and cytosolic localization during the day allowing PER to remain predominantly nuclear and to repress expression of CLK/CYC controlled genes (Fan et al., 2015; Kloss et al., 2001).

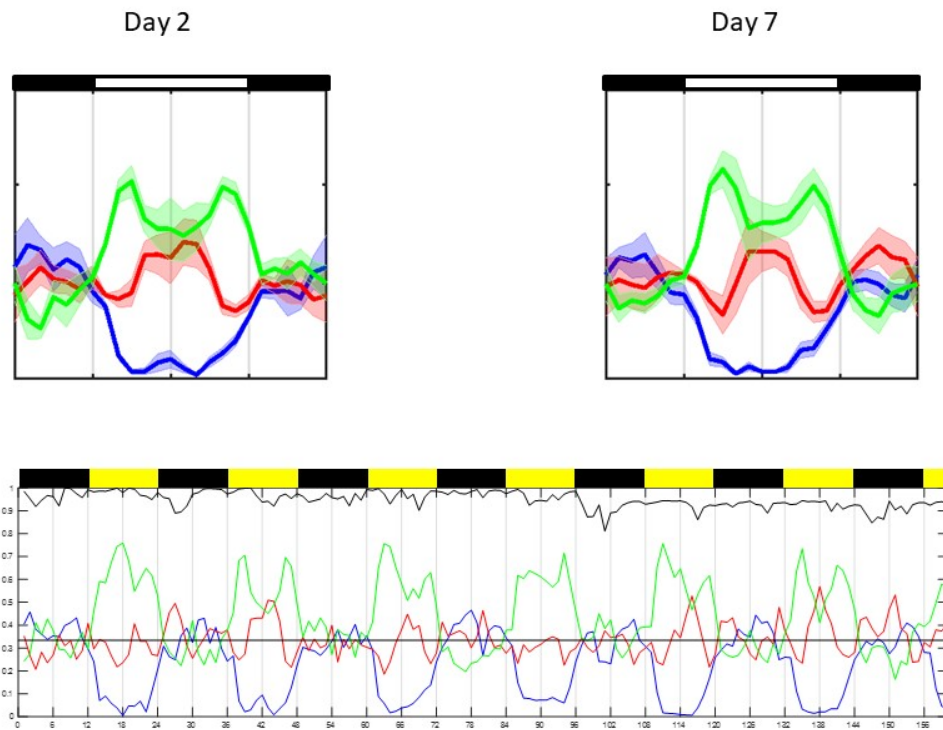
Another target of interest to investigate for the light mediated oscillations of BDBT, PER and DBT in the eye is an ankyrin repeat-containing protein (DAnkrd4).

Recently it has been shown to interact with BDBT and contributes to planar cell polarity by stimulating DBT (Strutt & Strutt, 2020). Initial analysis in our lab of *DAnkrd4*-RNAi lines show that flies expressing these RNAi's with *timGAL4>UAS-dcr* exhibit arrhythmic behavior (Not shown). To further look at these knockdown lines, using our GMR-GAL4 eye-specific driver, we could elucidate novel interactions involving *DAnkrd4* for the BDBT foci formation/expression pattern, PER and DBT localization via confocal microscopy, and BDBT/DBT interactions through co-immunoprecipitations in fly heads.

The final area of research that we would like to pursue further is in relation to the circadian color preference in the fly eye. Our initial BDBT knockdown experiment utilizing the GMR-GAL4 driver led to changes in green circadian color preference during the middle of the day along with a decrease for red color preference. However, when we tried this same BDBT knockdown with a *Rh1-GAL4>UAS-dcr; UAS-dbt*-RNAi we did not see the same changes to color preference (Figure 5.1). First, we would like to determine if the difference between the GMR-GAL4 and Rh1-GAL4 drivers is due to GMR expression outside of the eye or if the RH1 driver is not strong enough to drive knockdown of BDBT foci. We suspect that Rh1-GAL4 is not strong enough to knock down BDBT in the eye because it does produce a similar effect to GMR-GAL4 on color preference when driving the stronger *UAS-dbt<sup>K/R</sup>* effector protein (Figure 3.10). When our collaborators looked at the color preference in *Rh1-GAL4 > UAS-dcr; UAS-dbt*-RNAi they saw some decrease towards green circadian color preference in the middle of the day, but not as strong as in our *GMR-GAL4>UAS-dbt*-RNAi/*UAS-dcr* lines (Figure 5.2). Moreover, *GMR-GAL4> UAS-*

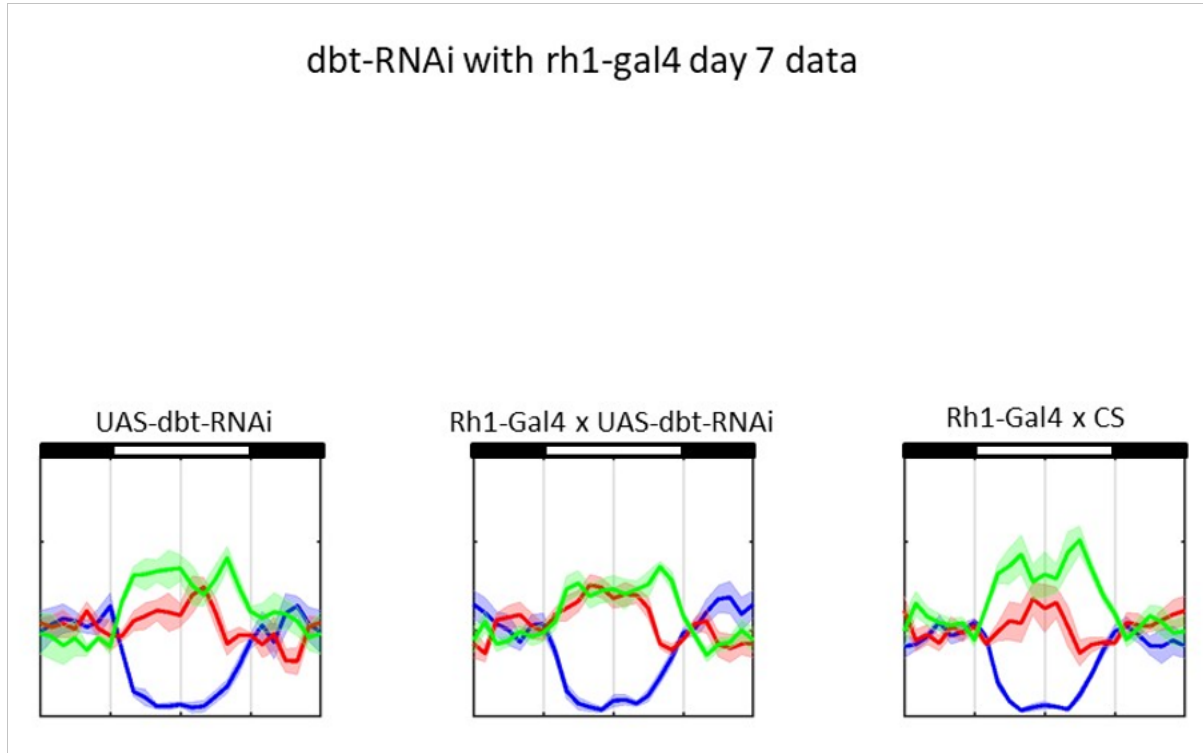
*dbt* RNAi flies are still rhythmic (figure 4.9), and GMR-GAL4 is not expressed in the LNv (Figure 4.7), suggesting that its effects are not mediated in the brain neurons that control circadian behavior. Immunofluorescent analysis of eye sections in both knockdown lines to see how BDBT foci formation/expression are affected is needed. The overall goal to this future endeavor will be to determine if this visual preference is produced solely by an effect on the retinal clock and to what extent the eye regulates this behavior. It seems likely that the eye clock is communicating with the brain clock to regulate behavior, and this line of research is an entirely novel direction for the field.

UAS-bdbt-RNAi /UAS-dcr ; Rh1-Gal4

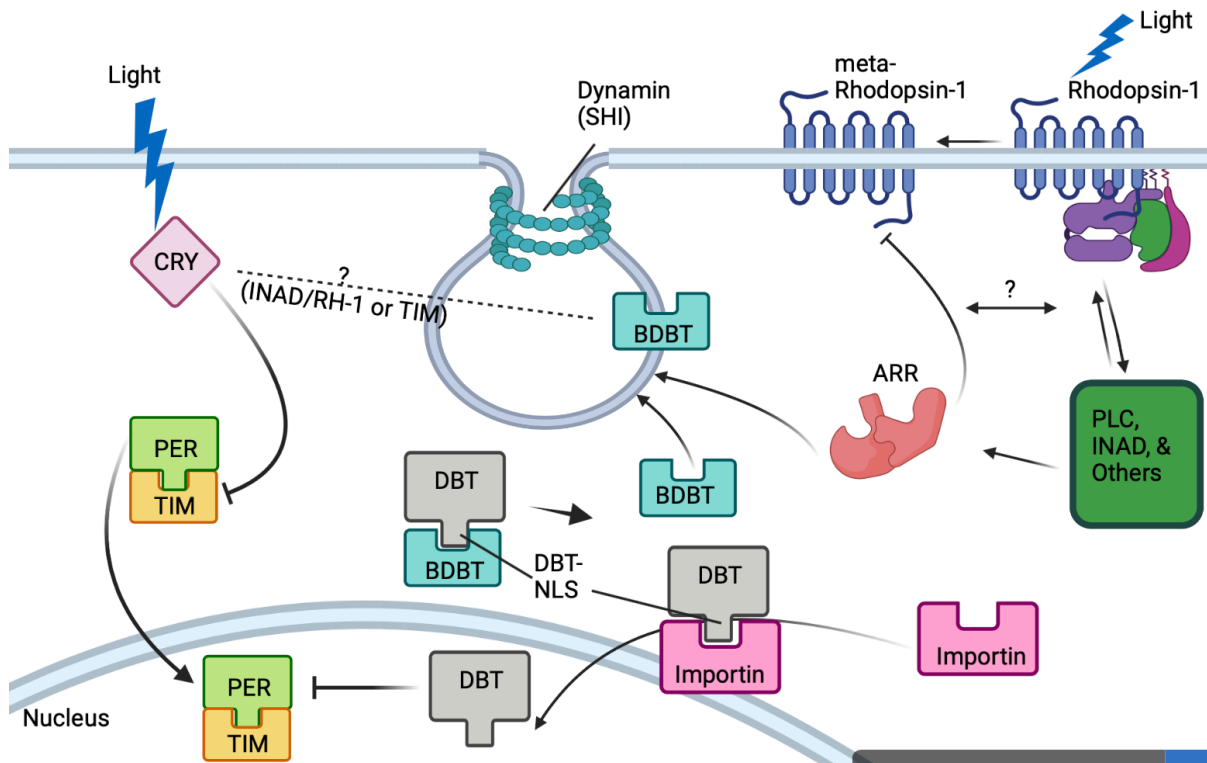


**Figure 5.1. Knockdown of BDBT in Rh1-GAL4 Flies.** BDBT knockdown does not exhibit the same decrease to green color preference as seen in our GMR-GAL4 lines. Future studies will determine if this is due to Rh1-driver not being strong enough or circadian preference is altered in GMR expressing cells outside the fly eye.

dbt-RNAi with rh1-gal4 day 7 data



**Figure 5.2. Knockdown of DBT on Circadian Color Preference.** We see some loss of green color preference in DBT knockdown flies in our initial experiments. We'd like to repeat this experiment with an additional *UAS-dcr* element to increase knockdown efficacy and observe these effects on circadian color preference.



**Figure 5.3. Model for BDBT foci formation.** Illustrated here are the different pathways involved with BDBT foci formation. Light dependent effects on BDBT foci in CRY mutants suggest that CRY is possibly acting through either TIM regulation or through the INAD/RHODOPSIN-1 signalplex leading to increased BDBT foci during the day. ARRESTINS are required for BDBT foci formation at night and this may occur through visual transduction signaling using PLC or through a light/dark mechanism where ARR2 quenches RHODOPSIN signaling during the day and forms BDBT foci at night. BDBT interacts with the DBT-NLS and we hypothesize that recruitment of BDBT to foci may be releasing the DBT-NLS allowing nuclear localization at night through importins.

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