UC Irvine
UC Irvine Electronic Theses and Dissertations

Title
Short Wavelength Light-Evoked Responses of Drosophila and Mosquitoes

Permalink
https://escholarship.org/uc/item/0r89t2qk

Author
Baik, Soyeon

Publication Date
2019

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA,
IRVINE

Short Wavelength Light-Evoked Responses of *Drosophila* and Mosquitoes

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Physiology and Biophysics

by

Lisa Soyeon Baik

Dissertation Committee:
Professor Todd C. Holmes, Chair
Associate Professor Francesco Tombola
Professor Craig Montell
Professor Diane O'Dowd
Professor Rongsheng Jin

2019

Portions of Chapter 1 © 2011 Science and 2011 Nature
Portion of Chapter 2 & 3 © 2017 National Academy of Sciences
Portion of Chapter 3 © 2018 PLOS
All other materials © 2019 Lisa Soyeon Baik

DEDICATION

To

my parents

in recognition of their love, sacrifice, and support.

TABLE OF CONTENTS

LIST OF FIGURES ..... **v**

LIST OF TABLES ..... **vii**

ACRONYMS & SYMBOLS ..... **viii**

ACKNOWLEDGMENTS ..... **ix**

CURRICULUM VITAE ..... **xi**

ABSTRACT OF THE DISSERTATION ..... **xvi**

Chapter 1 Introduction **1**
1.1 Significance **2**
1.2 Innovation **3**
1.3 Background and Preliminary Data **3**

Chapter 2 : CRYPTOCHROME-mediated neuronal phototransduction and clock resetting **7**
2.1 Introduction **7**
2.2 Materials and Methods **9**
2.2.1 Transgenic Fly Line Construction 9
2.2.2 Fluorescence Imaging & Quantification 11
2.2.3 Electrophysiology 11
2.2.4 Optics 12
2.2.5 Transparency of Drosophila Cuticles to UV light 12
2.2.6 Locomotor Analysis 13
2.3 Results **13**
2.3.1 CRY mediates electrophysiological response to UV light 13
2.3.2 Generation of tryptophan mutant CRY transgenic flies. 16
2.3.3 Transgenic WT CRY successfully rescues phototransduction in a FAD-specific manner. 19
2.3.4 Tryptophan residue closest to FAD binding site is necessary for CRY-mediated electrophysiological response to UV and blue light 23
2.3.5 Residue changes of tryptophan outside of FAD binding site have minor effects on CRY phototransduction 25
2.3.6 Histidine378 and Cystein416 residues minimally contribute to CRY-mediated electrophysiological light response 30
2.3.7 H378 histidine and W342 tryptophan residues are important for circadian photoentrainment of locomotor activity rhythm. 34
2.4 Discussion **38**

Chapter 3 : Short Wavelength Light-Evoked Behaviors in *Drosophila melanogaster* **41**
3.1 Introduction **41**
3.2 Materials and Methods **44**
3.2.1 Generation of gl^{60j}-$cry^{-/-}$ double mutant line by homologous recombination 44
3.2.2 Immunocytochemistry 44
3.2.3 Light Pulse Arousal 45
3.2.4 Behavioral Phototaxis 45
3.2.5 Locomotor analysis 46
3.2.6 Anticipation index 47

3.2.7 Standard Light Choice Assay 47
3.2.8 Multibeam Light Choice Assay 48
3.3 Results 48
3.3.1 Acute arousal behavioral response to UV light is CRY-dependent. 48
3.3.2 CRY mediates executive choice attributes of positive phototaxis and avoidance behaviors to different intensities of UV light. 54
3.3.3 CRY mediates attraction/avoidance behavior to UV light 57
3.3.4 The circadian clock modulates both the valence and the time-of-day dependent changes of UV light avoidance/attraction behavior. 60
3.3.5 Constant light-induced disruption of the circadian clock abolishes time-of-day dependent changes of UV light avoidance behavior and reveals that CRY and HK regulate the valence of UV light avoidance and attraction. 66
3.3.6 LNv circadian neurons are necessary for normal UV avoidance behavior. 70
3.3.7 The CRY/HK phototransduction pathway modulates the timing and valence of blue and orange light choice behavior. 74
3.3.8 UV light-evoked attraction/avoidance behavioral response is intensity-dependent. 76
3.3.9 High intensity UV light evokes avoidance in a clock-dependent and exposure duration-independent manner, while low intensity UV light evokes fast attraction response in an external opsin photoreceptor-dependent manner. 80
3.3.10 External and internal phototransduction mechanisms modulate distinct time-of-day dependent spatial preference of light environment. 86
3.4 Discussion 89

Chapter 4 : Circadian basis of time-of-day activity and light-evoked attraction/avoidance in day- vs. night-biting mosquitoes 95
4.1 Introduction 95
4.2 Materials and Methods 96
4.2.1 Immunocytochemistry 96
4.2.2 Light Choice Behavior Assay 97
4.3 Results 98
4.3.1 Diurnal and Nocturnal mosquitoes display specie- and sex-specific avoidance/attraction behavior to UV light. 98
4.3.2 Mosquito light environmental preference is wavelength-dependent. 102
4.3.3 PER and PDF staining reveals location of circadian neuronal circuit in diurnal and nocturnal mosquitoes. 104
4.3.4 PER oscillation exhibits opposing phase peaks between diurnal and nocturnal mosquitoes. 110
4.3.5 Constant light exposure disrupts circadian protein expression and clock-modulated avoidance/attraction behavioral responses to UV light. 115
4.4 Discussion 118

Chapter 5 : Conclusion and Final Remarks 120

REFERENCES 123

LIST OF FIGURES

Figure 1–1 CRY mediates electrophysiological response to short wavelength light in the l-LNvs, independent of external photoreceptors. 4
Figure 1–2 High resolution structure of *Drosophila* CRY. 6
Figure 2–1 l-LNv electrophysiological response to UV light is attenuated in flies lacking CRY-based phototransduction. 15
Figure 2–2 *Drosophila* head and eye cuticles transmits UV light. 16
Figure 2–3 Site-directed mutagenesis of *cryptochrome* expressed *in vivo*. 18
Figure 2–4 No mutation (WT CRY) control rescues electrophysiological response to short wavelength light, while *cry-null* shows reduced response during lights on. 22
Figure 2–5 FAD-specific redox inhibitor DPI abolishes WT CRY electrophysiological response to short wavelength light. 23
Figure 2–6 W420 Tryptophan residue is necessary for CRY-mediated electrophysiological response to UV and blue light 24
Figure 2–7 W397Y Tryptophan residue mutation does not affect CRY-mediated electrophysiological response to UV and blue light 27
Figure 2–8 W342 Tryptophan residue is not required for CRY-mediated electrophysiological response to UV and blue light 28
Figure 2–9 W394 Tryptophan residue is not required for CRY-mediated electrophysiological response to UV and blue light 29
Figure 2–10 H378 histidine residue is not required for CRY-mediated electrophysiological response to UV and blue light 32
Figure 2–11 C416 cystein residue is not required for CRY-mediated electrophysiological response to UV and blue light 33
Figure 2–12 Transgenic CRY mutant flies have normal LD and DD phenotypes 35
Figure 2–13 Histidine 378 and tryptophan 342 residues mediate circadian photoentrainment. 37
Figure 3–1 *Drosophila* acute arousal response to UV light is CRY-dependent. 51
Figure 3–2 *Drosophila* CRY-mediated acute arousal response to UV light is not attenuated in mutants lacking *trpA1*. 53
Figure 3–3 Drosophila positive phototaxis behavior towards UV-light is attenuated in mutants lacking CRY- and in mutants lacking external photoreceptors. 55
Figure 3–4 *Drosophila* positive phototaxis behavior towards UV-light is not attenuated in mutants lacking *trpA1*. 56
Figure 3–5 CRY-based phototransduction contributes to UV light avoidance behavior in *Drosophila*. . 59
Figure 3–6 CRY-based phototransduction mediates *Drosophila* choice of light environment. 60
Figure 3–7 Circadian mutants have defective timing of UV light avoidance behavior. 62
Figure 3–8 Circadian mutants have disrupted entrainment to UV light. 64
Figure 3–9 Period expression rescues timing of UV light avoidance behavior. 65
Figure 3–10 Constant light condition induces valence shifts and eliminates time-of-day dependent modulation of UV light avoidance/attraction behavior. 68
Figure 3–11 Constant UV light condition disrupts locomotor activity rhythm. 69
Figure 3–12 LNv -ablated or -silenced flies phenocopy the valence shift from UV light avoidance to attraction seen in $cry^{-/-}$ *and* $hk^{-/-}$ flies. 70
Figure 3–13 LNv circadian cells modulate the valence of UV light avoidance/attraction behavior but not timing. 72
Figure 3–14 LNv ablated flies have defects in locomotor activity profile throughout the day. 73

Figure 3–15 The CRY-mediated phototransduction modulates blue and orange light avoidance/ attraction behavior. 75
Figure 3–16 Internal neuronal photoreceptors are necessary for avoidance behavioral response to high intensity UV light. 77
Figure 3–17 Both external photoreceptors and direct neuronal photoreceptors are necessary for UV light avoidance/attraction behavior. 78
Figure 3–18 UV light-evoked attraction/avoidance behavioral response is intensity-dependent. 79
Figure 3–19 High intensity UV light evokes avoidance modulated by the circadian clock that is independent of duration of light exposure. 82
Figure 3–20 External opsin-based photoreceptors modulate attraction behavior in response to low intensity UV light. 84
Figure 3–21 UV light-evoked attraction/avoidance behavioral response is intensity-dependent. 85
Figure 3–22 High spatiotemporal resolution of preference and activity using multibeam monitors. 87
Figure 3–23 Avoidance/attraction behavior has distinct time-of-day dependent spatial preference. 88
Figure 4–1 UV light-evoked attraction/avoidance responses in diurnal and nocturnal mosquitoes are specie- and sex-dependent..... 101
Figure 4–2 Mosquito light-evoked attraction/avoidance behavior is wavelength-specific. 103
Figure 4–3 Circadian neuronal circuit of diurnal and nocturnal mosquito brains. 107
Figure 4–4 Schematic representation of *Aedes aegypti* and *Anopheles coluzzii* circadian neurons. ... 108
Figure 4–5 PDF neuronal circuit and projections of mosquito brains. 109
Figure 4–6 Diurnal versus nocturnal mosquito PER expression in PDF+ LNv neurons oscillate in anti-phasic manner 111
Figure 4–7 Oscillatory PER expression of *Aedes aegypti* DNs. 112
Figure 4–8 Oscillatory PER expression of *Aedes aegypti* m-ANs. 113
Figure 4–9 Oscillatory PER expression of *Anopheles coluzzii* DNs. 114
Figure 4–10 Oscillatory PER expression of *Anopheles coluzzii* PI neurons. 115
Figure 4–11 Constant UV light exposure disrupts circadian protein expression and clock modulation of attraction/avoidance behavioral responses to UV light in mosquitoes. 117

LIST OF TABLES

Table 1. Mean number of circadian neurons ±SEM (n) in female mosquito brain 108

ACRONYMS & SYMBOLS

♀	Female
♂	Male
A-peak	Afternoon peak
Asn	Asparagine
Clk	Clock (gene/protein name)
CRY	Cryptochrome
CT	Circadian Time
CTT	C-terminal tail
Cys	Cystidine
dCRY	*Drosophila* CRYPTOCHROME
DD	Constant darkness
DPI	Diphenyleneiodonium
DN	Dorsal Neuron
eGFP	enhanced Green Fluorescent Protein
FAD	Flavin Adenine Dinucleotide
FF	Firing frequency
Gl^{60j}	*glass60j*
HB	Hofbauer-Buchner
hid	*head involution defective* (gene name)
His	Histidine
HK	Hyperkinetic
IR	Infrared
Kvα	voltage-gated potassium alpha
Kvβ	voltage-gated potassium beta
l-LNv	large-Lateral Ventral Neuron
LD	light:dark
LL	Constant light
LNd	Lateral Dorsal Neuron
LNv	Lateral Ventral Neuron
m-AN	mid-Anterior Neuron
PDF	Pigment Dispersing Factor
PER	Period (gene/protein name)
Phe	Phenylalanine
PI	pars intercerebralis
Rh7	Rhodopsin 7
rpr	*reaper* (gene name)
SEM	standard error mean
s-LNv	small-Lateral Ventral Neuron
TIM	Timeless (gene/protein name)
Trp	tryptophan
trpA1	Transient Receptor Potential A1
UV	Ultraviolet
WT	wild type
ZT	Zeitgeber Time

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Todd Holmes for the years of guidance and support. I am grateful for all the advice and opportunities you have provided me with. It is thanks to you I have gained the confidence as a writer and presenter, and even more importantly as a scientist. Thank you for teaching me the love for coffee, Tomato Tuesday, and IPA. The stories and wisdom you shared with me are words I will carry with me for the rest of my career. I can't thank you enough for being the amazing mentor that you are.

To the Holmes lab members, current and former, I thank you for sharing your passion for science with me. Spending numerous hours together over the years has been inspiring and intellectually stimulating. I thank you all for contributing to growth of the lab and myself. Logan, Ceazar, and David I am glad I not only had the pleasure of working with you, but to have gained lifelong friends over our times together.

For years of hard work and dedication, I thank my former undergraduate research assistants, especially Yocelyn Recinos and Joshua Chevez. It was not only a joy, but also an honor to have worked with you over the years and witness your growth as academics and scientists. Much of the work highlighted in this thesis would not have been possible without your effort.

I am grateful for the amazing women in science I have the pleasure of knowing. Dr. Joanna Chiu will forever be my mentor and role model- you were the first to teach me the love for flies and you helped me see my dream as an academic professor a reality. UCI's Graduate Dean, Dr. Frances Leslie, I thank you for relentless dedication to supporting graduate students and promoting diversity at UCI. Both of your continual encouragement and advice inspire me to do the same for others.

For successful collaboration, including work in rhodopsin7 and mosquitoes, I thank my collaborators Dr. Craig Montell at UC Santa Babara and Dr. Anand Ray and Tom Gouda at UC Riverside. It has been a pleasure working with you and it truly helped broaden my scientific perspective. Thank you for spending the time to discuss ideas with me and for writing letters of recommendation. I am grateful to have gained mentors and academic supporters through our years of collaborations.

I would like to thank my thesis committee members, Professors Craig Montell, Francesco Tombola, Diane O'Dowd, and Rongsheng Jin, for reading this thesis and providing years of advice. I deeply appreciate the time and insight you have provided.

To my friends, Stephanie, Ann, Honyin, Sarah, Morgan, Joyce, Rian, Clinton, and Tobias, I am blessed to have you all in my life. Thank you for celebrating my achievements with me regardless of its size, and for consoling me through the bad times. I'm thankful for all the food, wine, and beer we've shared together over the years.

To my loving family, I could never have completed this PhD without your support. Even though he won't be able to read nor comprehend this, I'm grateful for all the emotional support my dog, Aston, has provided me with. To my brother Tony and sister(-in-law) Hannah, thank you for always being so understanding and supportive. To my husband, Jeremy, thank you for letting me be the scientist and woman that I am today, even if it means supporting my workaholic tendencies and comforting me through the tough times. To my parents, Dr. Kiho Baik and Hyunsun Baik, all of my success and everything that is good in my life, I owe them all to you. You guys are my biggest inspiration and I have nothing but sincerest respect and love for you both. I want nothing more than to make you proud. Thank you for always believing in me

and for giving me the best chance at life, even when it meant at the cost of your sacrifice. I thank you and I love you. 엄마, 아빠, 존경합니다. 감사합니다. 사랑합니다.

Thank you to National Science Foundation Graduate Research Fellowship Program, ARCS Foundation Scholarship, and UCI Graduate Dean's Dissertation Fellowship for supporting my graduate thesis work. This work was also funded by the National Institute of Health GM102965, GM107405, and GM127102 to TCH. Thank you as well to Proceedings of the Science, Nature, National Academy of Sciences, and PLOS publishing group for their permission to adapt published work in this thesis.

CURRICULUM VITAE

Lisa Soyeon Baik

Education

University of California, Irvine, Irvine, CA

Doctor of Philosophy (Ph.D.)	Biomedical Sciences- focus on Physiology & Biophysics	2013 - 2019

University of California, Davis, Davis, CA

Bachelor of Science (B.S.)	Neurobiology, Physiology & Behavior	2007 - 2011
Bachelor of Arts (B.A.)	Psychology	2007 - 2011

Peer-Reviewed Publications

Baik, L. S., Recinos, Y., Chevez, J. A., Au D., & Holmes, T. C. (2018). Multiple phototransduction inputs integrate to mediate UV light-evoked avoidance/attraction behavior. ***Journal of Biological Rhythms*** (submitted).

Baik, L. S., Recinos, Y., Chevez, J. A., & Holmes, T. C. (2018). Circadian modulation of light-evoked avoidance/attraction behavior in Drosophila. ***PLoS One***, *13*(8), e0201927.

Ni, J. D., **Baik, L. S.**, Holmes, T. C., & Montell, C. (2017). A rhodopsin in the brain functions in circadian photoentrainment in Drosophila. ***Nature***, 545(7654), 340.

Baik, L. S., Fogle, K. J., Roberts, L., Galschiodt, A. M., Chevez, J. A., Recinos, Y., Nguy V., & Holmes, T. C. (2017). CRYPTOCHROME mediates behavioral executive choice in response to UV light. ***Proceedings of the National Academy of Sciences,*** 114(4), 776-781.

Fogle, K. J., **Baik, L. S.**, Houl, J. H., Tran, T. T., Roberts, L., Dahm, N. A., ... & Holmes, T. C. (2015). CRYPTOCHROME-mediated phototransduction by modulation of the potassium ion channel β-subunit redox sensor. ***Proceedings of the National Academy of Sciences***, 112(7), 2245-2250.

Honors and Awards

- Recipient of Graduate Dean's Dissertation Fellowship (2018-2019; $23,555)
- Recipient of National Science Foundation Graduate Research Fellowship Program (NSF GRFP) (2015-2018; $138,000).
- Recipient of ARCS Foundation Scholar Award (2015-2017; $15,000).
- Recipient of Society for Research on Biological Rhythms (SRBR) Biennial Meeting Konopka Excellence Award (2018)
- Recipient of Annual Center for Circadian Biology Symposium at University of California- San Diego Best Poster Award (2016).
- Recipient of Society for Research on Biological Rhythms (SRBR) Biennial Meeting Merit Award (2016).
- Recipient of School of Medicine Travel Award (2016, 2017(x2)).

- Recipient of The UC-Irvine Department of Physiology & Biophysics Most Outstanding Student of the Year Award (2015).
- Recipient of Honorable Mention for National Science Foundation Graduate Research Fellowship Program (2014).
- Recipient of University of California-Irvine Graduate Dean Recruitment Award (2013).
- Recipient of University of California-Davis MIND Institute Volunteer Supervisor Award (2013).

Research Experience

Graduate Student Researcher Aug. 2013-Dec. 2014/ Mar. 2014-Mar. 2019
Department of Physiology and Biophysics, School of Medicine, University of California-Irvine (research advisor: Dr. Todd Holmes)

- Investigation of electrophysiological and behavioral light responses mediated by novel photoreceptors in *Drosophila* and other insects.
- Investigation of neural network underlying the circadian system and phototransduction pathways in *Drosophila* and mosquitoes.
- Characterization of molecular mechanism underlying novel phototransduction mechanisms using an array of transgenic flies.

Graduate Student Researcher (rotation) Jan. 2014-Mar. 2014
Department of Biological Chemistry, School of Medicine, University of California-Irvine (research advisor: Dr. Bogi Andersen)

- Successfully described mechanism of a novel circadian clock modulator, GM129, as a transcriptional repressor through epigenetic regulations, as well as its target clock controlled genes in *Mus musculus*.

Research Assistant, (promoted from volunteer position) June 2011-Aug. 2013
"Molecular Genetics of Circadian Rhythm and Animal Behavior", Department of Entomology, University of California-Davis, (research advisor: Dr. Joanna Chiu).

- Investigated molecular and biochemical changes of circadian clock proteins in *Drosophila melanogaster* with respect to time, with focus on posttranslational modifications, e.g. phosphorylation.
- Applied powerful genetic and proteomic tools to assay changes in the circadian protein-protein interaction network in response to time, genetics, development, and environmental stimuli.
- Successfully optimized Affinity Purification (AP) and Mass Spectrometry (MS) *in vivo* using fly materials to profile interactomes of circadian clock proteins.

Junior Specialist Sept. 2011- June 2013
"EARLY START Lab", MIND Institute (Medical Investigation of Neurodevelopmental Disorders), University of California-Davis Medical Center (research advisor: Dr. Sally Rogers)

- Examined patterns of growth and change in neurodevelopmental disorders, particularly in Autism.
- Coordinated several clinical studies investigating the effects of early diagnosis and treatment in Autism.
- Collected, managed, and performed statistical analysis on large-scale datasets.
- Hired, trained, and supervised up to 10 volunteer research assistants.

Student Research Assistant Oct. 2009- Aug. 2011
"EARLY START Lab", MIND Institute (Medical Investigation of Neurodevelopmental Disorders), University of California-Davis Medical Center (research advisor: Dr. Sally Rogers)

- Performed analysis on impact of interventions designed to improve behavioral outcomes of Autism.
- Managed subject recruitment, scheduling, and behavioral assessments.

Other Publications and Presentations

Select Conference Presentations

Baik, L.S., Recinos, Y., Chevez, J.A., Holmes, T.C. (2018). "Novel photoreceptors entrain the circadian clock and modulate UV light-evoked behaviors in *Drosophila.*" ***Society for Neuroscience 2018***, San Diego, CA, U.S.A.
- Poster presentation

Baik, L.S., Recinos, Y., Chevez, J.A., Holmes, T.C. (2018). "Circadian Modulation of UV Light Avoidance Behavior in *Drosophila*." ***2018 The Society for Research on Biological Rhythm Biennial Conference***, Amelia Island, FL, U.S.A.
- 15 minute research talk, Konopka Excellence Award

Baik, L.S., Recinos, Y., Chevez, J.A., Holmes, T.C. (2017). "Drosophila circadian clock entrainment by novel phototransduction pathways and subsequent modulation of light-evoked behaviors." ***Center for Circadian Biology Fall Workshop on Biological Timing***. La Jolla, CA, U.S.A.
- 30 minute research talk

Baik, L.S., Fogle, K.J., Recinos, Y., Chevez, J.A., Holmes, T.C. (2017). "Circadian modulation of UV light avoidance behavior in *Drosophila*." ***2017 Cold Spring Harbor Laboratory's Neurobiology of Drosophila Meeting***, Cold Spring Harbor, NY, U.S.A.
- 15 minute research talk

Baik, L.S., (2017). "Can you feel the light tonight?". ***UC-Irvine NSF GRFP "Training for Tomorrow" Symposium***, Irvine, CA, U.S.A.
- 5 minute research talk

Baik, L.S., Fogle, K.J., Roberts, L., Galschiodt., A., Chevez, J.A., Recinos, Y., Nguy, V., Holmes, T.C. (2017). "CRYPTOCHROME mediates behavioral executive choice in response to UV light." ***9th Annual UCSD Center for Circadian Biology (CCB) Symposium***, La Jolla, CA, U.S.A.
- Poster presentation

Baik, L.S., Fogle, K.J., Roberts, L., Holmes, T.C. (2016). "CRYPTOCHROME is a direct neuronal ultraviolet light sensor." ***2016 The Society for Research on Biological Rhythm Biennial Conference***, Palm Harbor, FL, U.S.A.
- Poster presentation, Merit Award

Baik, L.S., Fogle, K.J., Roberts, L., Holmes, T.C. (2016). "CRYPTOCHROME is a direct neuronal ultraviolet light sensor." ***8th Annual UCSD Center for Circadian Biology (CCB) Symposium***, La Jolla, CA, U.S.A.
- Poster presentation, Best Poster Award

Baik, L.S., Fogle, K.J., Houl, J.H., Tran, T.T., Roberts, L., Dahm, N.A. , Cao, Y., Zhou, M., Holmes, T.C. (2015). "CRYPTOCHROME-mediated phototransduction by redox sensing and modulation of the potassium ion channels." ***2015 Cold Spring Harbor Laboratory's Neurobiology of Drosophila Meeting***, Cold Spring Harbor, NY, U.S.A.
- Poster presentation

Baik, L.S., Fogle, K.J., Houl, J.H., Tran, T.T., Roberts, L., Dahm, N.A. , Cao, Y., Zhou, M., Holmes, T.C. (2015). "CRYPTOCHROME mediated phototransduction by modulation of the potassium ion channel beta subunit redox sensor." ***26th Orange County Graduate Women in Science Spring Conference***, Irvine, CA, U.S.A.
- 15 minute research talk.

Baik, L.S., Fogle, K.J., Houl, J.H., Tran, T.T., Roberts, L., Dahm, N.A. , Cao, Y., Zhou, M., Holmes, T.C. (2015). "CRYPTOCHROME mediated phototransduction by modulation of the potassium ion

channel beta subunit redox sensor." ***7th Annual UCSD Center for Circadian Biology (CCB) Symposium***, La Jolla, CA, U.S.A.
- Poster presentation

Select Published Abstracts

Baik, L.S., Recinos, Y., Chevez, J.A., Holmes, T.C. (2018). "Novel photoreceptors entrain the circadian clock and modulate UV light-evoked behaviors in *Drosophila.*" ***Society for Neuroscience 2018***, San Diego, CA, U.S.A.

Baik, L.S., Recinos, Y., Chevez, J.A., Holmes, T.C. (2018). "Circadian Modulation of UV Light Avoidance Behavior in *Drosophila*." ***2018 The Society for Research on Biological Rhythm Biennial Conference***, Amelia Island, FL, U.S.A.

Baik, L.S., Fogle, K.J., Recinos, Y., Chevez, J.A., Holmes, T.C. (2017). "Circadian Modulation of UV Light Avoidance Behavior in *Drosophila*." ***2017 Cold Srping Harbor Laboratory's Neurobiology of Drosophila Meeting***, Cold Spring Harbor, NY, U.S.A.

Baik, L.S., Fogle, K.J., Roberts, L., Galschiodt., A., Chevez, J.A., Recinos, Y., Nguy, V., Holmes, T.C. (2017). "CRYPTOCHROME mediates behavioral executive choice in response to UV light." ***9th Annual UCSD Center for Circadian Biology (CCB) Symposium***, San Diego, CA, U.S.A.

Baik, L.S., Fogle, K.J., Roberts, L., Holmes, T.C. (2016). "CRYPTOCHROME is a Direct Neuronal Ultraviolet Light Sensor." ***2016 The Society for Research on Biological Rhythm Biennial Conference***, Palm Harbor, FL, U.S.A.

Baik, L.S., Fogle, K.J., Roberts, L., Holmes, T.C. (2016). "CRYPTOCHROME is a direct neuronal ultraviolet light sensor." ***8th Annual UCSD Center for Circadian Biology (CCB) Symposium***, San Diego, CA, U.S.A.

Baik, L.S., Fogle, K.J., Houl, J.H., Tran, T.T., Roberts, L., Dahm, N.A. , Cao, Y., Zhou, M., Holmes, T.C. (2015). "CRYPTOCHROME-mediated phototransduction by redox sensing and modulation of the potassium ion channels." ***2015 Cold Spring Harbor Laboratory's Neurobiology of Drosophila Meeting***, Cold Spring Harbor, NY, U.S.A.

Roberts, L., Fogle, K.J., **Baik, L.S.**, Leise, T.L., Welsh, D.K., Holmes, T.C. (2015). "Molecular and whole circuit level light control of the Drosophila clockwork and beyond." ***2015 Cold Spring Harbor Laboratory's Neurobiology of Drosophila Meeting***, Cold Spring Harbor, NY, U.S.A.

Ni, J.D., **Baik, L.S.,** Holmes, T.C., Montell, C. (2015). "A New Circadian Photosensor in Flies" ***2015 Chronobiology Gordon Research Conference***, Girona, Spain.

Baik, L.S., Fogle, K.J., Houl, J.H., Tran, T.T., Roberts, L., Dahm, N.A. , Cao, Y., Zhou, M., Holmes, T.C. (2015). "CRYPTOCHROME mediated phototransduction by modulation of the potassium ion channel beta subunit redox sensor." ***7th Annual UCSD Center for Circadian Biology (CCB) Symposium***, San Diego, CA, U.S.A.

Baik, L.S., Lam, V. H., Li, Y. H., Chiu J. C. (2014). "Posttranslational regulation of the Drosophila circadian clock." ***2014 The Society for Research on Biological Rhythm Biennial Conference***, Big Sky, Montana, U.S.A.

Pedagogical Experience

- Teaching Assistant, PHYS290/ Physiology of Ion Channels, University of California- Irvine (Spring 2016).
- Undergraduate Research Assistant Supervisor, Holmes laboratory, University of California-Irvine: Joey Thai (Fall 2014-Winter 2016), Yocelyn Recinos (Winter 2014-Spring 2017), Joshua Chevez (Fall 2015-Summer 2018), Kevin Xie (Spring 2017-Fall 2017), Shafaq Alkhaffaf (Spring 2017).
- Graduate Rotation Student Mentor for: Rommel Santos (Spring 2016), Athena Papasodero (Summer 2016), Rachel Waymack (Fall 2016), David Au (Winter 2018).

- Laboratory Supervisor of work-study undergraduate students, Holmes laboratory, University of California- Irvine: total of 20 students to date (September 2014-March 2019)
- Mentor, UCI Women and Minorities Mentorship Program (2017-2019).
- Undergraduate Research Assistant Supervisor, Rogers laboratory, MIND Institute, University of California-Davis: total of 16 students (Sept. 2011- June 2013)

Key Skills

- Electrophysiology: whole-cell patch clamp electrophysiology in *Drosophila* brain; TEVC in Oocytes.
- Animal Behavior: assays and analysis in *Drosophila*, mosquitoes, mice, and humans.
- Molecular and Cellular Biology/ Protein Chemistry: immunocytochemistry and confocal imaging in *Drosophila* and mosquito brain; cloning and generation of transgenic fly lines; testing protein expression in transgenic flies using SDS-PAGE, Western blotting analysis, and in-gel protein staining techniques; optimizing protein extraction and subcellular fractionation using fly and mouse tissues, and affinity purification for mass spectrometry to decipher protein-protein interaction networks; testing epigenetic regulation of gene expression using qRT-PCR, and ChIP in mouse tissues.
- Statistical Analysis: conducting mathematic computation, and generating tables and graphs using SPSS, R, Sigma Plot, MATLAB, and Microsoft Excel.

Scholarly or Professional Memberships

- STEM Chair of Diverse Educational Community and Doctoral Experience (DECADE, funded by US Government of Education) (2016-2017).
- National Committee Member of Graduate Women In Science (GWIS) (2016-2018).
- Member of American Association for the Advancement of Science (AAAS)/Science Program for Excellence in Science (2016-2019).
- Member/School of Medicine Student Representative of Diverse Educational Community and Doctoral Experience (DECADE, funded by US Government of Education) (2013-2019).
- Member of Orange County Graduate Women In Science (OCGWIS) (2013-2019).

Volunteer and Outreach

- Mentor, UC-Irvine Women and Minorities Mentorship Program (2017-2019).
- Recruiter/Student Representative, California Forums for Diversity in Graduate Education (October 2018).
- Tutor, NSF GRFP Writing Workshop, University of California-Irvine (Oct. 2015, 2016, and 2018).
- Panelist, NSF GRFP Workshop, University of California-Irvine (Sept., 2015, and July 2017).
- Organizer, Department of Physiology & Biophysics Research In Progress Seminar (2014-2016).
- Scientific Advisor, "Ask A Scientist Night" hosted by Irvine Unified School District (2013-2018).

ABSTRACT OF THE DISSERTATION

Short Wavelength Light-Evoked Responses in *Drosophila* and Mosquitoes

By

Lisa Soyeon Baik

Doctor of Philosophy in Physiology and Biophysics

University of California, Irvine, 2019

Professor Todd C. Holmes, Chair

Light is a powerful environmental regulator of insect behavior. My thesis focuses on novel phototransduction mechanisms in insect central brain neurons that directly regulate physiology and behavior, independent of external eyes. I hypothesized that novel short wavelength-activated phototransduction mechanisms modulate complex behaviors such as arousal and decision-making. To test this, I developed and integrated molecular, electrophysiological, imaging, and behavioral approaches. I described mechanisms of a direct neuronal blue- and UV-light sensor, CRYPTOCHROME (CRY), in mediating behavioral responses to short wavelength light, and how CRY couples to membrane voltage-gated K^+ β subunits, HYPERKINETIC. I also found that the previously uncharacterized Rhodopsin7 is a direct photoreceptor uniquely expressed in circadian neurons mediating circadian photoentrainment. I developed several assays to measure light-evoked avoidance/attraction behaviors with high spatial and temporal resolution in flies and mosquitoes. I demonstrated circadian regulation of these behaviors in *Drosophila* at the genetic and neuronal circuitry level. I also show valence control of light-evoked behavioral responses by integration of different light inputs, where nocturnal and diurnal mosquito species have unique, circadian clock-controlled

behavioral preference to different light environments. Further, I identified that circadian neural circuits in the same mosquito species show striking anatomical and transcriptional diversities, which likely underlie observed behavioral differences. I conclude that light is a powerful cue that drives complex insect behaviors.

Chapter 1 Introduction

Many insects, including *Drosophila melanogaster* and mosquitoes use short wavelength to guide their physiology and behavior. Many animals display a strong spectral sensitivity for short wavelength light, such as blue and ultraviolet (UV) light. UV light is a distinct component of sunlight that is crucial to an organism's survival. It has been long assumed that insect responses to UV light are mediated by UV-sensitive opsins expressed in eyes and other external photoreceptors. However, various organisms often express other non-opsin photoreceptors including the blue-light sensitive flavoprotein CRYPTOCHROME (CRY). In the *Drosophila* brain, CRY is expressed in a specific subset of neurons, including a subset of circadian and arousal photoreceptive neurons (1-3). CRY mediates rapid electrophysiological responses to blue and UV light in CRY-expressing l-LNv (4-6). Spectral sensitivity of electrophysiological changes mediated by CRY is largely consistent with the *in vitro* analysis of peak spectral absorbance of purified *Drosophila* CRY proteins, which show major absorbance peaks at 450nm (blue) and 365nm (UV-A) (7, 8). I found that CRY mediates a wide range of behavioral responses to blue and UV light (4, 6, 9). A long-term goal of my thesis was to establish an integrative framework that addresses how different light wavelengths activate classical and non-classical photoreceptors to modulate more complex insect behaviors such as arousal and choice of light environment. Specifically, I investigated short wavelength light activation of CRY along with other light input channels. I also examined the mechanism underlying circadian and other light-evoked behaviors.

1.1 Significance

Mosquitoes are significant diseases vectors that negatively affect human health and the ecosystem at large. Malaria is endemic in 97 countries, with 3.2 billion people at risk, and is spread by mosquitoes such as *Anopheles gambiae,* while *Aedes aegypti*, spreads viruses that cause Dengue, Zika and other diseases. Both of these mosquito species have been introduced into the US within the past 30 years. In 2013, there were about 198 million cases of malaria and estimated 584,000 deaths according to the World Health Organization. Understanding light sensing in mosquitoes is significant as they carry human diseases such as West Nile virus, malaria, yellow fever, dengue fever, and Zika virus. While mosquitoes are mostly considered for their obvious negative impacts on human health, they are also crucial vectors of many diseases that adversely impact other animals (reptiles, birds, agriculture, and other non-human animals). For example, large-scale declines in species abundance of many North American bird populations have occurred in the past 15 years due to West Nile virus carried by *Culex quinquefasciatus* mosquitoes. *Culex quinquefasciatus*-mediated West Nile Virus contributed to extinction of many native bird species in the Hawaiian Islands (10). Adverse effects of mosquitoes on human populations and ecosystems are being accelerated by climate change.

Short wavelength light, including near UV, violet, and blue light modulates a wide range of behaviors in *Drosophila melanogaster* and other insects. The short wavelength modulated behaviors we are studying include light evoked arousal, phototaxis, environmental light choice and circadian entrainment. For UV light, it has been long assumed that opsin-based external photoreceptors are solely responsible for all UV sensing in insects. Recently, I identified CRY as a photopigment that in conjunction with UV opsins underlies the complex behavioral responses to blue light (4) and UV light (6). We also discovered that a previously uncharacterized

Rhodopsin, Rhodopsin 7 (Rh7), in *Drosophila* functions as a violet photoreceptor and contributes to circadian photoentrainment (11).

1.2 Innovation

I developed several behavior assays that measure light choice behavior in flies and mosquitoes with high spatial and temporal resolution. This provides an innovative framework that can be extended further to understanding insect phototransduction and decision-making behaviors. Chapter 2 focuses on defining the molecular mechanism coupling the light activation of CRY to the fast changes in the membrane potential. In Chapter 3, I developed a light choice behavior apparatus that has a high spatial and temporal resolution. I focused on a series of aspects that shape complex decision-making behavior in *Drosophila*, including the light input, circadian network, and the neural circuitry. In Chapter 4, I tested harmful mosquito species to determine their behavioral responses to short wavelength light. I also described a fundamental difference in circadian neuronal network of mosquito species that potentially drive the diurnality versus nocturnality and drive light environmental choice behaviors.

1.3 Background and Preliminary Data

Electrical activity in the LNvs contributes to circadian rhythms (3, 12, 13) and, reciprocally, LNv neuronal firing rate is circadian regulated (14-16). Circadian regulation of firing rate is widely conserved in other invertebrate and vertebrate species (17, 18). For insects, CRY is characterized as the primary photoreceptor for blue light-activated circadian entrainment (19-25). We have shown that CRY mediates rapid electrophysiological responses to short

wavelengths of light in CRY-expressing l-LNv (4-6). The spontaneous neuronal firing rate of l-LNv increases in response to varying intensities of blue light (Fig. 1-1 A, B (5)), but does not respond to orange light (Fig. 1-1 D, E (5)). The l-LNv electrophysiological response to is absent in *cry*-null flies, but is functionally restored by expression of wild type *Drosophila* or Monarch Butterfly *Danaus plexippus* CRY1 in the LNv (Fig. 1-1 F, G (5)). The CRY-mediated light response is still robust even in the absence of all opsin-based external photoreceptors (Fig 1-1 C, E (5)).

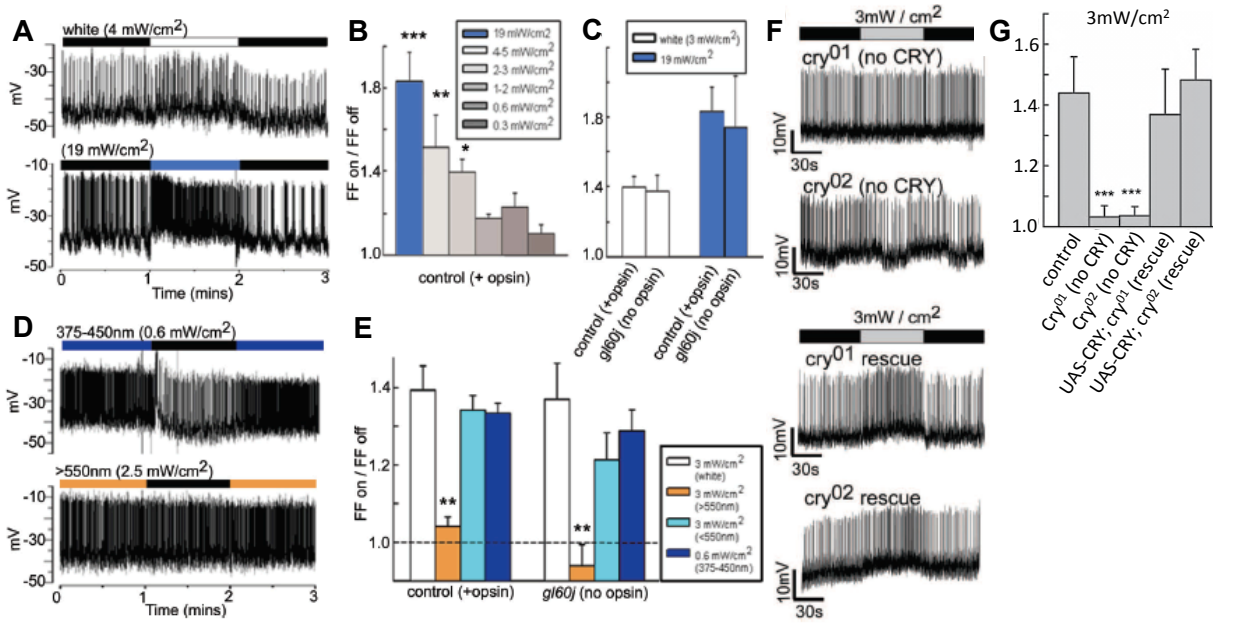

Figure 1–1 CRY mediates electrophysiological response to short wavelength light in the l-LNvs, independent of external photoreceptors.
(A) Upper panel: response of a representative tonic firing cell to 4 mW/cm^2 white light. Lower panel: light response of burst firing cell to 19 mW/cm^2 blue light. Alternating light/dark cycles denoted by white or blue versus black bars above traces. (B) Firing frequency in light/dark varies with light intensity. (C) Genetic ablation of all external opsin-based photoreceptors has no effect on the l-LNv light response. (D) Representative recordings of l-LNv light response evoked by blue-violet and orange-red light. Alternating light/dark cycles denoted by violet, orange, and black bars. (E) Spectral profile of light responses of l-LNv from control vs. null *gl*^{60j} mutant flies that lack all opsin photoreceptors is indistinguishable, but responses to white and blue light of *cry*^{b} hypomorph is significantly reduced. (F) l-LNv FF response evoked by 3 mW/cm^2 white light/dark does not differ between control and LNv specific expression of CRY in *cry*^{01} and *cry*^{02} null genetic background flies. (G) Firing frequency (FF) response evoked by light/dark does not differ between control and LNv specific expression of CRY in *cry*^{01} and *cry*^{02} null genetic background flies. (From Fogle et al., 2011, *Science.*)

In contrast to opsins, CRY proteins use flavin adenine dinucleotide (FAD) as their chromophore. FAD bound to CRY undergoes a series of redox state changes with absorption of specific spectra of light depending on its redox state. We found that CRY-mediated electrophysiological response to blue light requires the activity of its cofactor, Flavin Adenine Dinucleotide (FAD) (4, 5, 14). Consistent with our findings in short wavelength spectral sensitivity of electrophysiological changes mediated by CRY, *in vitro* spectral analysis of purified *Anopheles gambiae* CRY1 and *Drosophila melanogaster* CRY proteins show major absorbance peaks at 450nm (blue) and 365nm (UV-A) in an oxidized baseline redox state of CRY (26). When CRY is blue light activated to a hemi-reduced redox state, CRY absorption spectra shows a single greatly enhanced absorbance peak at 365nm (7, 8, 26, 27). This suggests that hemi-reducing CRY by blue light activation may subsequently significantly increase the CRY responses to UV light, depending on whether the hemi-reduced state of CRY is capable of light response.

CRY belongs to the well-conserved cryptochrome/photolyase superfamily of blue/UV-light sensitive photoreceptors. Photolyase and CRY, while different in its primary functions, shares common structural folds, flavin-based cofactors, and internal photoactivation mechanisms (1, 28). Photoreduction of FAD depends on stepwise electron transfer from the conserved Trp triad of CRY/Photolyase (8, 29). The high-resolution crystal structure of the *Drosophila* CRY protein confirms the highly conserved Trp tetrad electron transfer pathway near the FAD binding pocket (Trp394→Trp342→Trp397→Trp420) (Fig. 1-3 C). In addition to Trp residues, other nearby residues can further protonate or stabilize the flavin. For an example, a conserved His residue of *photolyase* near the flavin active site protonates flavin in the semiquinone state (30).

His378 occupies this conserved His position in *Drosophila* CRY (Fig. 1-3 B). Furthermore, an Asn residue near the flavin in *photolyase* is poised to form a hydrogen bond with the reduced flavin N5, but not the fully oxidized Flavin (31). In *Drosophila* CRY, Cys416 is in the same position as Asn, within the hydrogen bonding distance of both unprotonated N5 and O4 of FAD (Fig. 1-3 B).

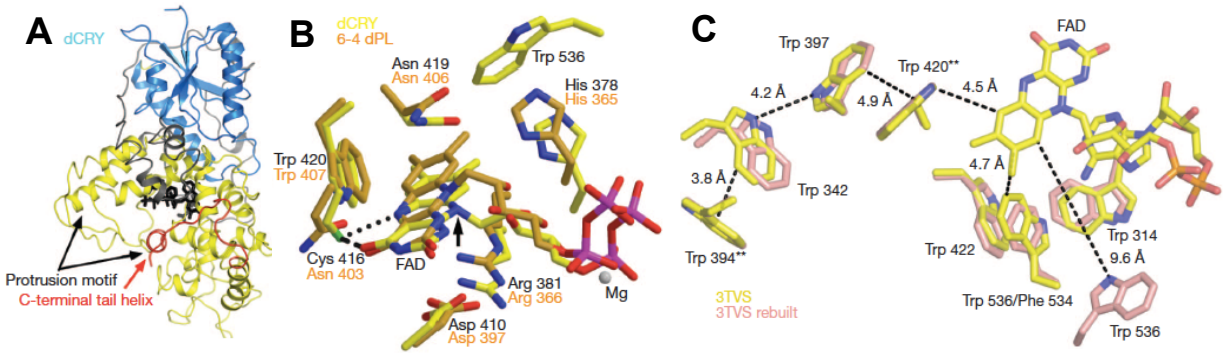

Figure 1–2 High resolution structure of *Drosophila* CRY.
(A) Full-length structure of *Drosophila* CRY (3TVS). N-terminal a/b domain (blue) is coupled to the C-terminal helical domain (yellow) through a long linker (grey). C-terminal helix (red) docks to the photolyase DNA binding cleft besides the flavin (black). **(B)** Cofactor binding region of CRY. Flavin center of CRY (yellow) compared to 6-4 Photolyase (orange). CRY Cys 416 replaces 6-4 photolyase Asn 403 and is in hydrogen bonding distance (dotted lines) of both N5 and O4 of FAD. CRY His 378 replaces semiquinone flavin protonating 6-4 photolyase His 365. (Zoltowski et. al., 2011, *Nature*- original manuscript). **(C)** The corrected CRY Trp residues are shown in 3TVS (yellow) and rebuilt structure (pink). The Trp triad photoreduction pathway includes Trp 420, Trp 397 and Trp 342. Trp 536 is replaced by Phe 534 in the rebuilt structure. Double asterisks denote that the new positions of Trp 394 and Trp 420 are nearly identical to the old. (from Zoltowski et. al., 2011, *Nature*).

Reprinted with permission from:

Fogle KJ, Parson KG, Dahm NA, Holmes TC. (2011) CRYPTOCHROME is a blue-light sensor that regulates neuronal firing rate. *Science* 331(6023):1409-13.
Zoltowski BD, Vaidya AT, Top D, Widom J, Young MW, Crane BR. (2011) Structure of full-length Drosophila cryptochrome. *Nature* 480(7377):396-9.

Chapter 2 : CRYPTOCHROME-mediated neuronal phototransduction and clock resetting

2.1 Introduction

For nearly a century, it has been assumed that insect sensitivity to ultraviolet (UV) light is exclusively mediated by UV-sensitive opsins expressed in eyes and other external photoreceptors. However, organisms express other non-opsin photoreceptors including the blue-light sensitive flavoprotein CRYPTOCHROME (CRY). CRY is a highly conserved and evolutionarily ancient flavoprotein that is widely expressed in both prokaryotes and eukaryotes. Here I demonstrated that CRY mediates the electrophysiological response to UV light, in addition to blue. I also investigated the molecular mechanism coupling the light-activation of CRY to rapid changes in membrane potential of LNv neurons. In *Drosophila melanogaster*, light activation of CRY initiates a relative slow (~1 hr) irreversible process of ubiquitin mediated degradation of the clock protein TIMELESS and CRY itself, thus resetting the circadian clock (5, 14, 15, 32-35). More recently, CRY was discovered to mediate phototransduction shown by rapid membrane depolarization ($\tau \sim 100$ msec) and increased spontaneous action potential firing rate in the lateral ventral neurons (LNvs) in response to blue and UV light (4, 5, 14). The mechanisms for light activated CRY leading to TIM degradation/clock resetting and phototransduction appear to differ based on timing of onset and reversibility/irreversibility following light exposure. Other mechanistic distinctions include for clock resetting, light activated CRY obligatorily binds to TIM (25, 36). In order for light to be conditional for clock resetting, TIM must interact with the CRY C-terminus (22, 24). In contrast, neuronal CRY-mediated phototransduction is independent of TIM interactions and is robust in mutants lacking the CRY C-terminus (5).

Redox state and CRY C-terminus protein conformational changes are among several broad proposals for CRY's light activation mechanism, which are not necessarily exclusive. Purified CRY's light activation in vitro leads to the hemi-reduction of a bound flavin adenine dinucleotide cofactor (7, 26, 27, 37) followed by an intramolecular transfer of flavin's redox state to CRY's protein exterior via Trp triad molecular wire of conserved tryptophan (W) residues (26, 37). These Ws create an electron transfer chain through which FAD is reduced, balanced by the deprotonation of solvent-exposed Ws down the molecular wire (38). In spite of considerable work on animal and plant CRYs, CRY biophysical mechanisms continue to be controversial because such studies almost always use purified protein removed from native cellular environments (26). Alternative proposed mechanisms for both CRYs and related DNA photolyases include light activation focus on C-terminus conformational changes. Further support for a redox based mechanism for CRY light evoked membrane depolarization in *Drosophila* LNv neurons is shown by downstream redox signaling to potassium channel heteromultimeric complexes consisting of redox sensing cytoplasmic potassium beta (Kvβ) HYPERKINETIC (HK) subunits and ion conducting voltage-gated potassium alpha (Kvα) ether-a-go-go family subunits (4). However, the molecular mechanism of redox coupling between CRY and FAD leading to membrane potential changes remains unclear. We hypothesize that photoreduction of CRY-bound FAD leads to intramolecular transfer of flavin's redox state to CRY protein through nearby trp residues. Earlier work examining the light activation mechanism in plant CRYs suggest that trp residues mediate an electron transfer chain through which FAD is reduced, balanced by deprotonation via other nearby residues. Alternatively, light activated CRY may signal through protein conformational state changes, particularly in the CRY protein C-terminal. To distinguish between these possibilities concerning redox and C-terminus

mechanisms for CRY phototransduction in Drosophila neurons and clock resetting, I created transgenic flies expressing mutations of the tryptophan residues in close proximity to the FAD chromophore including one in close proximity to the C-terminus. In *Drosophila* neurons, dCRY evokes a rapid increase in membrane potential and FF (4-6). Using a sensitive measure of whole cell-patch clamp electrophysiology paired with evoked potential analysis, I measured the rapid neuronal response of tryptophan mutant CRYs. Here I hypothesized that the nearby tryptophan residues by the FAD binding site in dCRY is necessary for phototransduction and thus neuronal electrophysiological response to short wavelength light in *Drosophila*.

2.2 Materials and Methods

2.2.1 Transgenic Fly Line Construction

I generated a vector from synthetic DNA (Genescript) containing a full drosophila cryptochrome sequence, in frame with eGFP (Addgene). Point mutations of CRY were introduced into wild type CRY tagged with eGFP in the UAS-pJFRC7 vector (Pfeiffer et al., 2010). Wild-type and mutant CRY-eGFP constructs in the pJFRC7 vector allow for controlled insertion into same genomic location (a specific PhiC31 genomic site. when injected into fly embryos (39), thus allowing for direct experimental comparison of resultant transgenic fly lines. The pJFRC7 vectors containing wild-type control and experimental mutant CRY-eGFPs were injected into fly embryos which were screened for eye color as evidence of successful transgenesis. Resultant transgenic flies were isogenized by backcrossing with w^{1118} flies for a minimum of 6 generations.

Point mutations were introduced to the template vector using QuikChange™ II Site-Directed Mutagenesis Kit (Agilent). Following primers were designed as recommended in the QuickChange™ protocol:

- **W342Y, with codon change of TGG to TAC**: cgttcggcttagcgtacgggatgctcaggcag and ctgcctgagcatcccgtacgctaagccgaacg
- **W397Y, with codon change of TGG to TAC**: gctgcagtccatgctcgtagctctgccacaaacc and ggtttgtggcagagctacgagcatggactgcagc
- **W420Y, with codon change of TGG to TAC**: agctggatacccacatgtagttgccagcgcaga and gtctgcgctggcaactacatgtgggtatccagct
- **W394F, with codon change of TGG to TCC**: gctcccagctctggaacaaaccgccgcg and cgcggcggtttgttccagagctgggagc
- **C416N, with codon change of TGC to AAC:** atccagttgccagcgttgaccgaccaatccgc and gcggattggtcggtcaacgctggcaactggat
- **H4378R, with codon change of CAT to CGC:** gtgttgcgcagcgtgcggtggagccatccc and gggatggctccaccgcacgctgcgcaacac

As quality control, mutant vectors were sequenced before proceeding (Retrogen, Inc.). WT (no mutation) and mutant vectors were injected into *Drosophila* embryo (BestGene) to generate the *UAS-eGFP-CRY (mutation)* transgenic flies. *UAS-eGFP-CRY* (no mutation or mutation) flies were crossed into *cry-null* background and again with *cry24-GAL4* to generate the final transgenic mutant line expressing mutant (or WT control) CRY under *cry*-driver in a *cry-null* background:

- **WT CRY control:** *cry24-GAL4*; *UAS-eGFP-CRY; cry*^{01}

- **CRY(W342Y):** *cry24-GAL4*; *UAS-eGFP-CRY(W342Y); cry^{01}*
- **CRY(W397Y):** *cry24-GAL4*; *UAS-eGFP-CRY(W397Y); cry^{01}*
- **CRY(W420Y):** *cry24-GAL4*; *UAS-eGFP-CRY(W420Y); cry^{01}*
- **CRY(W394F):** *cry24-GAL4*; *UAS-eGFP-CRY(W394F); cry^{01}*
- **CRY(C416N):** *cry24-GAL4*; *UAS-eGFP-CRY(C416N); cry^{01}*
- **CRY(H378R):** *cry24-GAL4*; *UAS-eGFP-CRY(H378R); cry^{01}*

2.2.2 Fluorescence Imaging & Quantification

12:12 LD entrained adult male (3-5 days post-eclosion) fly brains were dissected in 1x PBS solution and mounted in VECTASHIELD (Vector Labs) at ZT23. Brains were imaged with Zeiss LSM 700 confocal microscope. Fluorescence was quantified with ImageJ, and normalized to background fluorescence.

2.2.3 Electrophysiology

Whole-cell current-clamp recordings were carried out from protocols adapted from(5). Adult male (3-5days post-eclosion) fly brains were dissected in external recording solution. l-LNv were subjected to whole-cell current-clamp with external solution: 122mM NaCl, 3mM KCl, 1.8mM $CaCl_2$, 0.8mM $MgCl_2$, 5mM glucose, 10mM HEPES, 7.2 pH, and 250-255mOsm; internal solution: 102mM Kgluconate, 17mM NaCl, 0.085mM $CaCl_2$, 1.7mM $MgCl_2$ (hexahydrate), 8.5mM HEPES, 0.94mM EGTA, 7.2pH, and 232-235mOsm. For DPI recordings, diphenyleneiodonium chloride (Sigma) was added to the external recording solution at 16.8μM concentration.

2.2.4 Optics

Custom-ordered multichannel LED source (Prizmatix/Stanford Photonics, Palo Alto, CA) fitted to the Olympus BX51 WI miscrscope was used for all optics using electrophysiology recordings. LED peak wavelengths are as follows: UV (365 nm) blue (450 nm), and red (630 nm), and all exposures were set to intensity of 150μW/cm^2. Light intensities were determined by a Newport 842-PE Power/Energy meter. Each LED was triggered on and off with TTL pulses programmed by pClamp (Molecular Dynamics) data acquisition software. Each color pule was preceded by minimum 50 sec pre-pulse dark baseline, and there was 95 sec inter-pulse intervals between each light exposure from there on, with 5-10 times of each color exposed per cell. All sweeps containing each light exposure recordings were averaged and baseline was adjusted to pre-pulse signal. Furthermore, Gaussian and Butterworth filters were applied to the averaged signals using the ClampFit 10 software (Molecular Dynamics).

2.2.5 Transparency of Drosophila Cuticles to UV light

The proportion of UV light (365 nm) that is transmitted through head and eye cuticles was measured using a modified version of a protocol previously described in (5). Briefly, adult male flies were dissected in ice cold PBS for removal of head or compound eye cuticle tissue within 3 days of eclosion. Three blue-black light strips were mounted facing downward in a light-tight enclosure to shine UV light on a Li-Cor LI-250A light meter with the Li-Cor Quantum sensor facing upward. The quantum sensor was wrapped in aluminum foil with a small pinhole made in the foil with a diameter smaller than the cuticle samples. The transparency of the cuticles was measured as the amount of 365 nm transmitted through the cuticle tissues divided by baseline measurements of the amount of UV light that passes through a small droplet of PBS covering the pinhole. The amount of light transmitted was measured for 10 compound eye and 7

head cuticle tissue samples. A statistical comparison of the percentage of UV light that passes through eye versus head cuticles was determined using student t-test.

2.2.6 Locomotor Analysis

Locomotor activity of individual flies was measured using the TriKinetics Locomotor Activity Monitoring System via infrared beam-crossing recording total crosses in 15 or 30 min bins. Actograms were generated using Clocklab software. Average activity eduction graphs, % rhythmic flies, and its statistics were measured using FaasX software and Microsoft Excel.

2.3 Results

2.3.1 CRY mediates electrophysiological response to UV light

CRY was traditionally known as a blue photoreceptor, but a spectroscopy study reveals CRY sensitivity to UV light, in addition to blue *in vitro.* Here I tested whether CRY-mediated l-LNv electrophysiological response (firing frequency light on/light off) was observed in response to UV light. Control fly l-LNvs respond to UV light with varying degrees to different intensities (Fig. 2-1 A and E; 365nm LED- low=20 μW/cm^2, intermediate=150 μW/cm^2, high=640 μW/cm^2). The l-LNv response to UV light is significantly attenuated in *cry-null* mutant flies (*cry*^{01}, Fig. 2-1 B, E) and *hyperkinetic*-null mutant flies (*hk*$^{-/-}$, Fig. 2-1 C, E) relative to control (Fig. 2-1 A, E).

To determine if CRY-mediated l-LNv UV light responses are cell-autonomous, I performed genetic rescue experiments. Genetic rescue of LNv-targeted expression of CRY in *cry-null* genetic background cell-autonomously rescues the l-LNv UV light response at low and intermediate intensities, but incompletely for high intensity (Fig. 2-1 D, F). Similarly, LNv-targeted expression of WT-HK in *hk-null* genetic background rescues l-LNv UV light response

at low and intermediate intensities, but again not at high intensity (Fig. 2-1 G). Expression of redox sensor-disabled HK point mutant, HK-D260N, does not rescue l-LNv response to UV light at all intensities (Fig. 2-1 G). Thus, electrophysiological responses to UV light are specifically mediated by light-activated CRY coupled to the membrane via HK redox sensor, consistent with previous findings using blue light (4).

To determine whether opsin-based photoreceptors contribute also to the l-LNv electrophysiological response to UV light, I recorded from the l-LNv neurons of gl^{60j} flies, which lack eyes and other external photoreceptors (and DN1p circadian cells). The l-LNv UV light responses of gl^{60j} flies are qualitatively lower but do not significantly differ from control flies (Fig. 2-1 H, J). The l-LNv UV light response of $glass^{60j}$-$cry^{-/-}$ (gl^{60j}-$cry^{-/-}$) double mutant flies is indistinguishable from that of *cry-null* flies (Fig. 2-1 I, J). These results suggest that CRY mediates electrophysiological responses to UV in the l-LNvs in an opsin-independent manner. gl^{60j}-$cry^{-/-}$ double mutant flies show some residual electrophysiological UV response at higher intensities indicating the presence of a yet-to-be identified third photoreceptor for the l-LNvs, consistent with earlier findings (21). Lastly, we measured the proportion of UV light transmittance through eye and head cuticle tissue using a 365 nm LED light source using procedures described in (5). Eye cuticles are over 85% transparent and head cuticles are nearly 50% transparent to 365 nm UV light (Fig. 2-2).

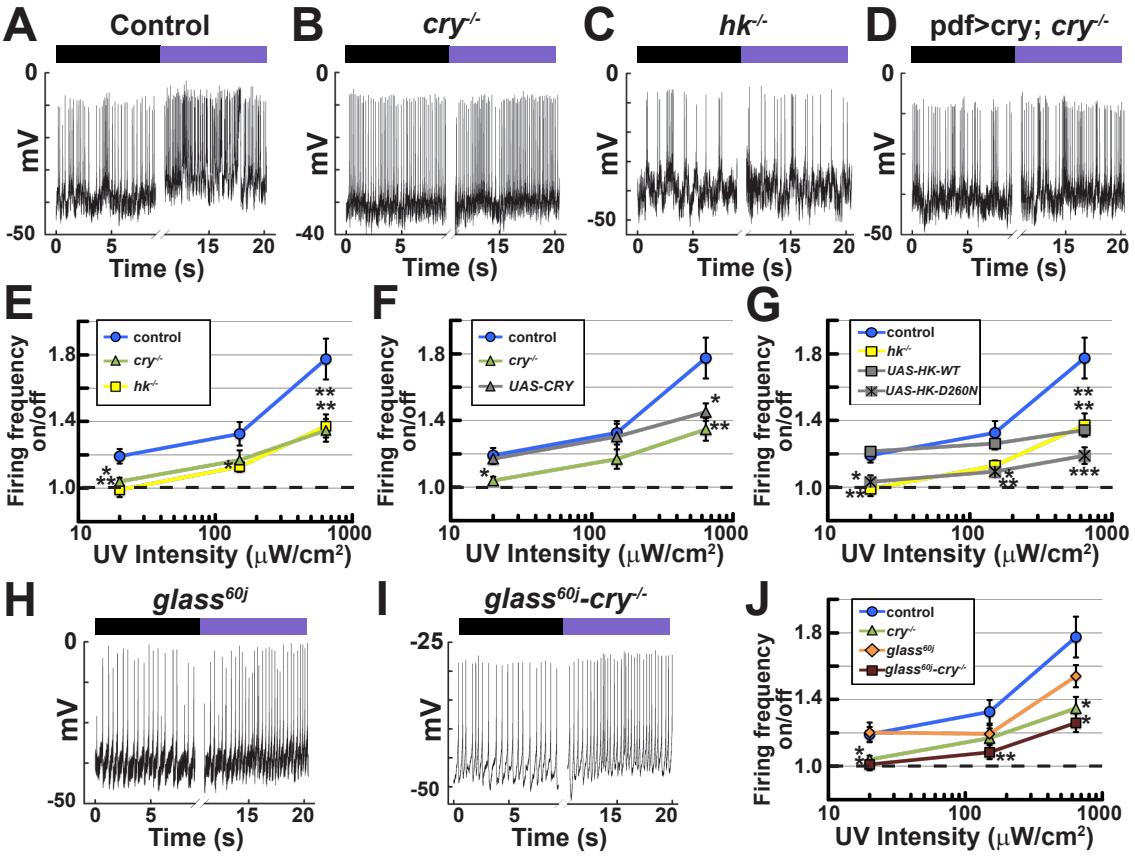

Figure 2–1 l-LNv electrophysiological response to UV light is attenuated in flies lacking CRY-based phototransduction.

(A) Representative trace for control l-LNv UV light response. (365 nm, 640 μW/cm^2, violet bar; lights off, <0.01 μW/cm^2, black bar) (The gap in the x-axis removes <1s wherein a noise transient is caused by manual opening of the shutter to expose the prep to light) vs. representative traces for **(B)** *cry*$^{-/-}$, **(C)** *hk*$^{-/-}$, and **(D)** "cry rescue" flies (*pdf-GAL4*-driven LNv *UAS-CRY* expression in a *cry*$^{-/-}$ background). **(E-G)** Dose response quantification of l-LNv firing frequency (FF) response (FF on/ FF off) to UV light at low (20 μW/cm^2), intermediate (150 μW/cm^2), and high (640 μW/cm^2) intensities. **(E)** Electrophysiological response of control flies increase with increasing intensities of UV light (1.19 ± 0.04, n=17, low; 1.33 ± 0.07, n=15, intermediate; 1.77 ± 0.12, n=15, high intensity). The significantly attenuated UV light responses of *cry*$^{-/-}$ (1.04 ± 0.02, n=17, p=0.01, low; 1.17 ± 0.06, n=15, p=0.129, intermediate; 1.35 ± 0.07, n=15, p=0.005 vs. control, high intensity) and *hk*$^{-/-}$ (0.99 ± 0.04, n=15, p=0.002, low; 1.13 ± 0.03, n=14, p=0.049, intermediate; 1.37 ± 0.07, n=26, p=0.008 vs. control, high intensity) flies do not differ from each other (p=0.622, low; p=0.879, intermediate; p=0.978, high intensity). **(F)** Dose response quantification of FF for control vs. *cry*$^{-/-}$ and "*cry* rescue" flies. Full rescue is achieved at low (1.18 ± 0.03, n=15, p=.99 vs. control) and intermediate intensities (1.24 ± 0.03, n=15; p=.14 vs. control), but is incomplete at high intensity UV light (1.45 ± 0.05, n=15, p=.03 vs. control and p=.68 vs. *cry*$^{-/-}$). **(G)** Dose response quantification of FF for control vs. *hk*$^{-/-}$ and *pdf-GAL4*-driven rescue of wild type HK (*UAS-HK-WT*) or of redox sensor-disabled point

mutant HK (*UAS-HK-D260N*), both in $hk^{-/-}$ genetic background. Wild-type HK rescue flies also achieve rescue at low (1.21 ± 0.03, n=16, p=0.97 vs. control) and intermediate intensities (1.26 ± 0.03, n=16, p=0.732 vs. control), but not at high intensity (1.34 ± 0.04, n=16, p=0.001 vs. control, and p=0.99 vs. $hk^{-/-}$). The redox sensor-disabled point mutant HK-D260N fails to rescue the light response at all UV light intensities (1.03 ± 0.04, n=13, p=0.033, low; 1.09 ±0.03, n=15, p= 0.004, intermediate; 1.19 ± 0.05, n=11, p=<0.001 vs. control, high intensity; p=>0.417 vs. $hk^{-/-}$ all intensities). **(H)** Representative trace for $glass^{60j}$ (gl^{60j}) mutant l-LNv UV light response. **(I)** Representative trace for gl^{60j}-$cry^{-/-}$ double mutant l-LNv UV light response **(J)** Dose response quantification FF for gl^{60j} and gl^{60j}-$cry^{-/-}$ double mutant flies. gl^{60j} flies response do not significantly differ from control (1.20 ± 0.06, n=18, p=0.87, low; 1.19 ± 0.05, n=14, p=0.15, intermediate; 1.54 ± 0.07, n=16, p=0.098 vs. control, high intensity). gl^{60j}-$cry^{-/-}$ double mutant has significantly attenuated UV response compared to control (1.01 ± 0.03, n=20, p=0.002, low; 1.08 ± 0.04, n=32, p=0.003, intermediate; 1.26 ± 0.05, n=28, p=<0.001 vs. control, high intensity) and do not differ from $cry^{-/-}$ response (p=0.499, low; p=0.252, intermediate; p=0.157 vs. $cry^{-/-}$, high intensity).

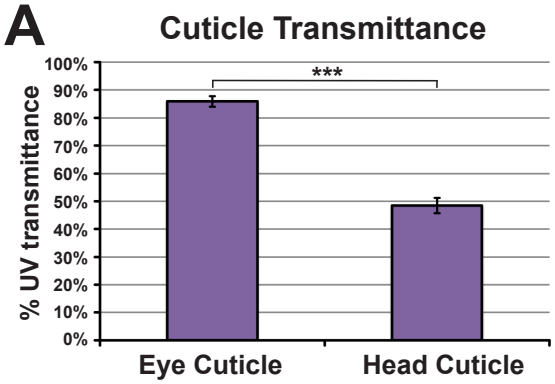

Figure 2–2 ***Drosophila*** **head and eye cuticles transmits UV light.**
(A) The proportion of 365 nm (UV) light transmitted through cuticle tissues was calculated as the amount of UV light transmitted through either eye (n=10) or head (n=7) cuticle tissues divided by baseline measurements of transmittance through a droplet of PBS.

2.3.2 Generation of tryptophan mutant CRY transgenic flies.

I generated transgenic UAS- fly lines to express CRY single point tryptophan mutants, then targeted wild type and mutant CRY expression in CRY expressing neurons in a *cry-null* mutant fly genetic background. The targeted expression of wild type and mutant CRY was monitored by a fusion protein consisting of CRY and enhanced-GFP (eGFP), which also informed protein expression levels. Because tryptophan has a large, bulky ring structure, we individually replaced tryptophan residues with other large ring residues (tyrosine or

phenylalanine) rather than alanine to prevent collapse of the CRY structure. I targeted four different tryptophan residues nearby the FAD binding site of CRY that likely play a role in electron transfer: W342, W397, W420, and W394 shown in the *Drosophila* CRY high resolution structure and ranked from furthest to closest to the FAD binding. To measure the response of mutant CRY in the absence of native CRY, I crossed the wild type and mutant CRY transgenics into a *cry-null* genetic background and expressed the wild type and mutant CRY-eGFP by crossing with *cry24-GAL4* driver. Expression of all CRY transgenics was confirmed by confocal imaging of freshly dissected transgenic adult fly brains, and quantified for equivalency of level of expression (Fig. 2-3 C-D). The W342Y CRY protein expression levels are lower compared to WT CRY, suggesting instability caused by the W342Y mutation (Fig. 2-3 D). All other mutant CRYs express at levels comparable to that of WT CRY (Fig. 2-3D).

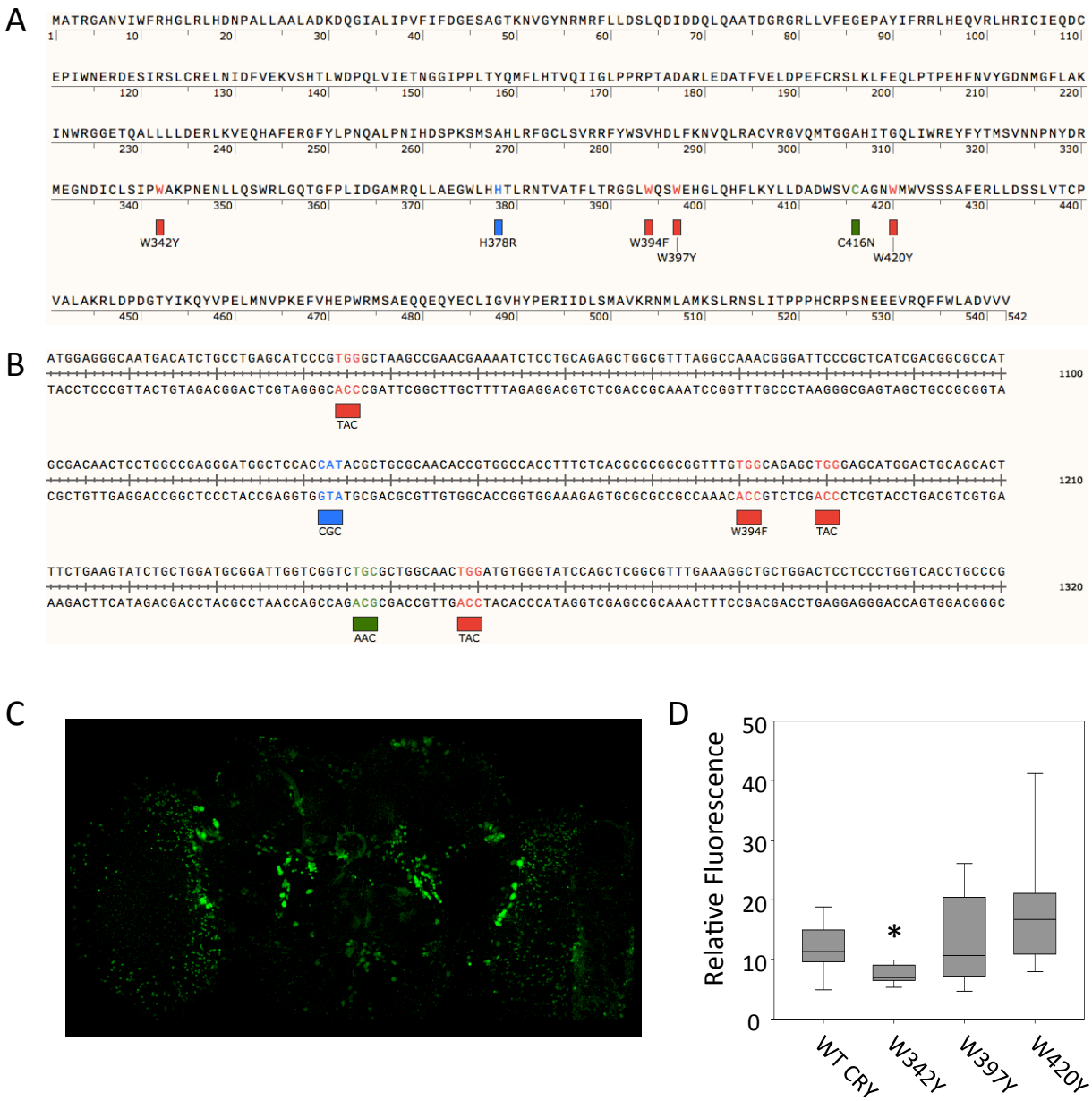

Figure 2–3 Site-directed mutagenesis of *cryptochrome* expressed *in vivo*.
(A) Codon sequence of cryptochrome showing sites of sequence change in tryptophan to tyrosine residues. **(B)** Protein sequence of *Cryptochrome* (*Cry*) indicating (red) sites of mutagenesis. **(C)** Confocal imaging confirming expression of *eGFP*-tagged transgenic *Cry* in a adult transgenic fly brain. **(D)** Quantification of relative fluorescence level of no mutation control (*WT Cry*) or transgenic point-mutant *Cry* expressions in the adult brain.

2.3.3 Transgenic WT CRY successfully rescues phototransduction in a FAD-specific manner.

Here I used a highly sensitive evoked potential method to capture membrane potential changes using whole cell patch-clamp in *ex vivo* preparation of adult fly brains. Light responses were recorded from large-lateral ventral neurons (l-LNv) of transgenic flies expressing positive control WT or tryptophan mutant CRYs. The evoked-potential protocol consists of a baseline pre-stimulus recording of membrane potential changes followed by 5 seconds of LED light exposure of ultraviolet (UV), blue stimuli, or red light negative control stimuli under current-clamp mode at a fast rate. Light stimuli were administered via TTL trigger, then signal averaged by time-lock based on the light stimulus onset and offset in repetitive manner (minimum 5 light stimuli per cell per color, 5-29 cells). Based on empirical recording data, I employed a 90 second interval between light stimuli to achieve recovery of membrane potential baseline state. The evoked potential protocol averages out individual action potentials and random electrical fluctuations to provide a kinetically robust membrane voltage light response (5).

The phototransduction responses recorded in l-LNv include those from positive control transgenic flies expressing WT CRY and negative control responses from *cry-null* flies. Responses to UV (365 nm LED, 150μW/cm^2) and blue (450 nm LED, 150μW/cm^2) were measured at the known spectral absorbance peaks at the redox based oxidation state (FAD^{ox}) and light evoked hemi-reduced state ($FAD^{\bullet -}$) of CRY. Additionally, the l-LNv light response to red light (630 nm LED, 150μW/cm^2) was measured. The CRY red light response is both a negative spectral control, based the assumption based on earlier biophysical experiments using purified CRY protein that *Drosophila* CRY does not reach a light induced fully reduced state, as well as an open experimental question given the possibility that CRY *in vivo* may reach the $FADH^{\bullet}$ reduced state which absorbs red light well past 650 nm. These alternatives can be resolved by

comparing the red light responses of WT CRY and *cry-null* neurons. The UV light response recorded from wild type CRY fly l-LNv neurons shows a rapid and long lasting increase in membrane potential (Fig. 2-4 A). The UV light evoked rapid membrane potential increase is followed by a slow decrease that takes almost a full minute to return to baseline even after the light is turned off. The UV light evoked increase in membrane potential increase in FF during UV light stimulus (Fig. 2-4B). The increase FF in response to UV light shows a surprisingly sustained response. Post UV light FF is maintained for 10-20 sec after UV light is turned off and returns to baseline FF within 30-60 sec (Fig. 2-4 C). WT CRY flies respond to blue light stimulus with rapid increase in membrane potential followed by slow return to baseline membrane potential (Fig. 2-4 D). Blue light also evokes an increase in FF in flies expressing WT CRY, with sustained FF increase, returning to baseline FF within a min (Fig. 2-4 E-F). In contrast, red light causes a measurable but lower membrane evoked potential change compared to UV or blue (Fig. 2-4 G). The red light-evoked response is not as long lasting in WT CRY-expressing flies as compared to the UV or blue light responses (Fig. 2-4 G). Red light evokes minimal FF change during and the red light pulse, but the post-stimulus probability of firing increases during the 10 seconds following the red light stimulus (Fig. 2-4 H-I). These results are surprising because *Drosophila* CRY is reported not red light sensitive based on *in vitro* absorbance measurements using purified CRY protein. Alternatively, the sensitive light evoked potential assay may reveal opsin based eye photoreceptor responses (40), although sustained light responses lasting up to 10 seconds duration post stimulus would not be an expected feature of opsin phototransduction.

Diphenyleneiodonium (DPI) is a flavin-specific redox inhibitor that acutely blocks CRY-mediated electrophysiological response to blue light (4). To confirm that blue light evoked

responses in membrane potential change in WT CRY-expressing l-LNv is in fact mediated by CRY, I tested for blue light response following administration of DPI. Presence of DPI severely diminished blue light response in WT CRY-expressing l-LNvs, indicating that electrophysiological light response seen in WT CRY-expressing flies is in fact mediated by the transgenic WT CRY (Fig. 2-5).

I tested for evoked potential light responses from l-LNv of *cry-null* negative control flies. *cry-null* flies do not show increase in membrane potential change to blue and UV light. Instead, *cry-null* l-LNvs show a rapid hyperpolarization of membrane potential during UV and blue light exposures, followed by gradual depolarization (Fig. 2-4 A, D). In contrast to WT CRY-expressing l-LNvs, l-LNvs lacking *cry* completely lack FF increase during UV and blue light stimuli (Fig. 2-4 B, E). Accompanying the timing of gradual depolarization following hyperpolarization, *cry-null* l-LNvs do show increase in FF after UV and blue lights are turned off, and this increase in FF is comparable to that of a WT CRY-expressing l-LNv (Fig. 2-4 C, F). *cry-null* l-LNvs lack significant membrane potential change during and after red light exposure (Fig. 2-4 G). FF change during and after red light exposure in *cry-null* l-LNv is indistinguishable from that of WT CRY-expressing l-LNvs (Fig. 2-4 H, I). In summary, expression of transgenic WT CRY successfully rescues electrophysiological responses to UV and blue light. Increase in membrane potential and FF observed in WT CRY flies is significantly different than that of *cry-null* flies specifically in response to UV and blue light.

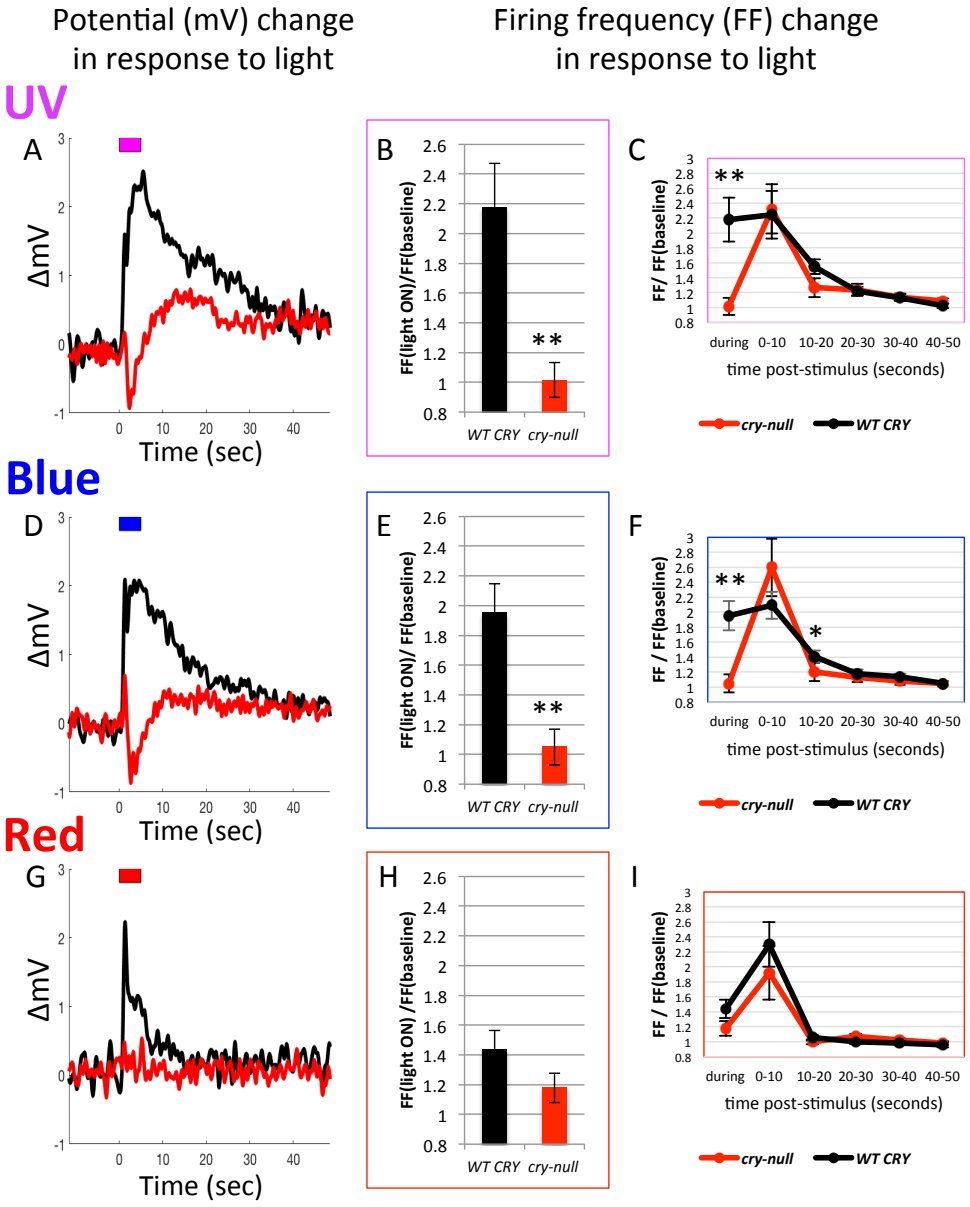

Figure 2–4 No mutation (WT CRY) control rescues electrophysiological response to short wavelength light, while *cry-null* shows reduced response during lights on.
l-LNv electrophysiological light responses of no mutation (WT CRY) control (black) vs. *cry-null* (red) flies, in response to UV (365 nm LED, 150μW/cm^2) **(A-C)**, blue (450 nm LED, 150μW/cm^2) **(D-F)**, or red (630 nm LED, 150μW/cm^2) **(G-I). (A, D, G)** shows average changes in membrane potential in respect to light stimuli for WT CRY control (black) and *cry-null* (red). **(B, E, H)** Firing frequency (FF) change (during light on)/ FF (dark baseline) for WT CRY (black) and *cry-null* (red). **(C, F, I)** FF change over time, during and after light stimuli/ FF (dark baseline) for WT CRY (black) and *cry-null* (red).

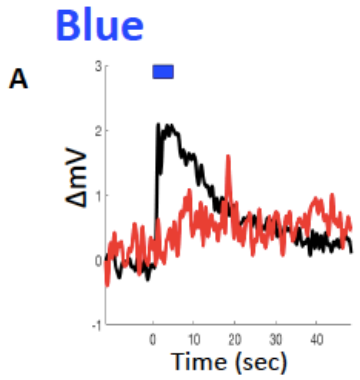

Figure 2–5 FAD-specific redox inhibitor DPI abolishes WT CRY electrophysiological response to short wavelength light.
(A) l-LNv electrophysiological light responses of changes in membrane potential for no mutation (WT CRY) control (black) in standard external solution vs. administration of DPI (red) blue light stimuli (450 nm LED, 150μW/cm^2).

2.3.4 Tryptophan residue closest to FAD binding site is necessary for CRY-mediated electrophysiological response to UV and blue light

The crystal structure of drosophila CRY revealed the trp-tetrad residues that may function as an electron transfer chain to mediate CRY phototransduction (37, 41). Of the four identified tryptophan residues, W420 is located the closest to the FAD-CRY interaction site. Mutation at W420 *in vitro* shows a highly defective phototransduction response by CRY, accompanied by slower electron transfer kinetics compared to WT CRY (42). Here I test for rapid electrophysiological evoked potential response in W420Y mutant CRY. Compared to WT CRY, W420Y CRY shows defective increase in l-LNv membrane potential during and following UV and blue light exposures (Fig. 2-6 A, D). W420Y CRY has significantly lower FF increase compared to WT CRY during the UV and blue light stimuli (Fig. 2-6 B, E). Similar to *cry-null* flies, FF change after UV or blue light exposure in not significantly different compared to FF change of WT CRY-expressing flies (Fig. 2-6 C, F). W420Y CRY-expressing l-LNv does not show much response to red light exposure (Fig. 2-6 G-I). I conclude that W420, tryptophan

residue closes to CRY's FAD interaction site, is necessary for CRY-mediated phototransduction response to UV and blue light.

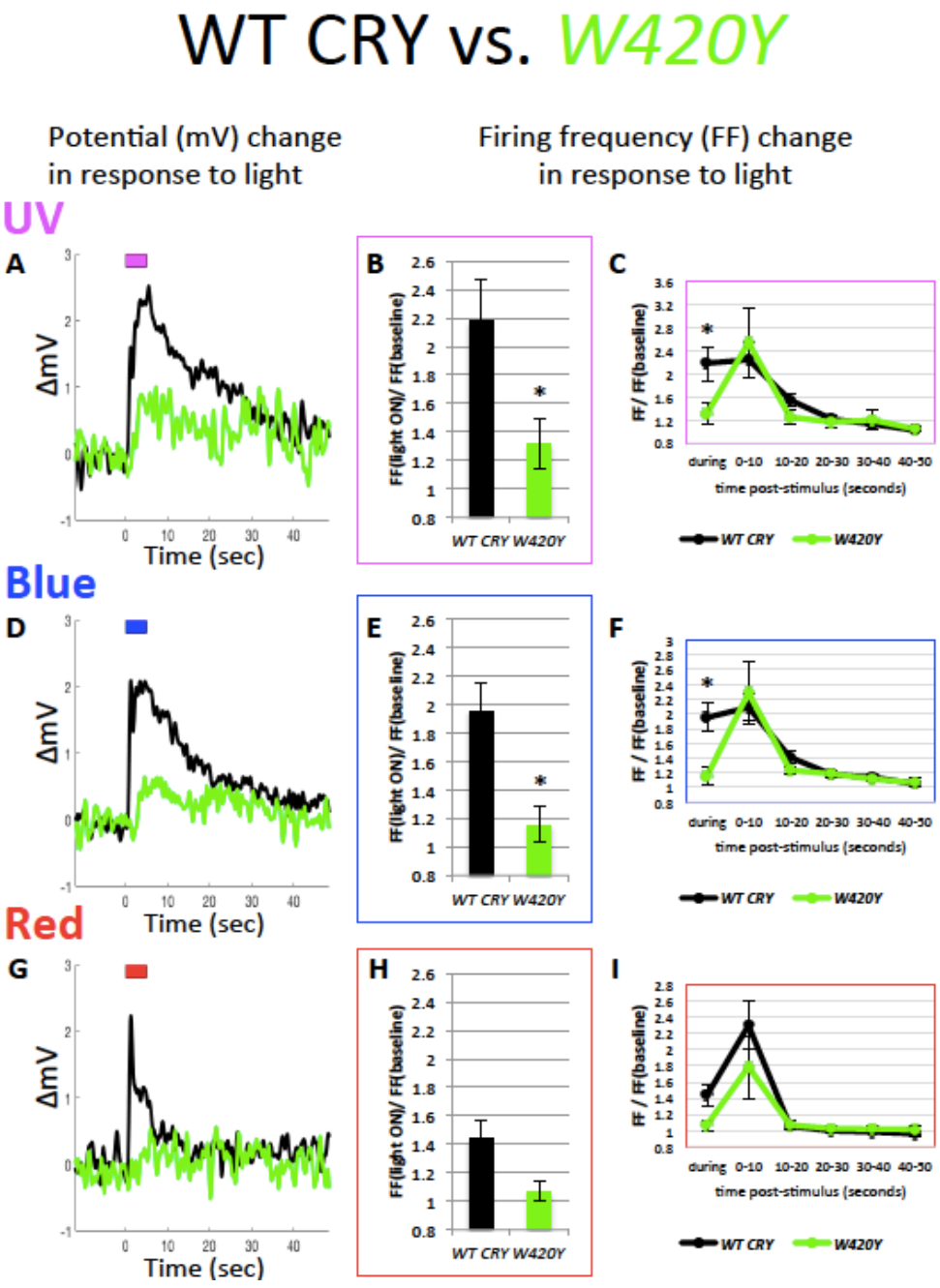

Figure 2–6 W420 Tryptophan residue is necessary for CRY-mediated electrophysiological response to UV and blue light

l-LNv electrophysiological light responses of no mutation (WT CRY) control (black) vs. W420Y mutant CRY-expressing (green) flies, in response to UV (365 nm LED, 150μW/cm^2) **(A-C)**, blue (450 nm LED, 150μW/cm^2) **(D-F)**, or red (630 nm LED, 150μW/cm^2) **(G-I). (A, D, G)** shows average changes in membrane potential in respect to light stimuli for WT CRY control (black)

and *W420Y* CRY mutant (green). **(B, E, H)** Firing frequency (FF) change (during light on)/ FF (dark baseline) for WT CRY (black) and W420Y CRY mutant (green). **(C, F, I)** FF change over time, during and after light stimuli/ FF (dark baseline) for WT CRY (black) and W420Y CRY mutant (green).

2.3.5 Residue changes of tryptophan outside of FAD binding site have minor effects on CRY phototransduction

After W420, W397, W342, then W394 tryptophan residues follow in sequence within the dCRY trp-tetrad. *In vitro,* W420 mutation results in detrimental loss of photoreduction activity of CRY, but not W397, W342, or W394 mutations (Lin 2018). To test this *in vivo*, I recorded from l-LNv of transgenic fliesexpressing W397Y, W342Y, or W394F mutant CRYs. W397Y CRY-expressing flies show significant increase in membrane potential during and following UV light exposure (Fig. 2-7 A). FF of W397Y CRY-expressing l-LNv is increased during UV light stimulus, and the level of FF increase is compared to that of WT CRY-expressing l-LNvs (Fig. 2-7 B). FF change in W397Y CRY mutant following UV light exposure is also similar to that of WT CRY flies (Fig. 2-7 C). Similar to UV light, membrane potential and FF increase during and after blue light stimulus in W397Y CRY mutant flies. Blue light response of W397Y CRY mutant flies is indistinguishable from that of WT CRY transgenic flies (Fig. 2-7 D-F). Membrane potential and FF changes during and after red light stimulus in W397Y CRY mutants is also indistinguishable from that of WT CRY transgenic flies (Fig. 2-7 G-I). W342Y CRY mutant flies show increase in membrane potential and FF in response to UV and blue light, and the response is indistinguishable from that of WT CRY transgenic flies (Fig. 2-8 A-F). Red light evoked changes in W342Y CRY mutant fly membrane potential and FF is also indistinguishable from that of WT CRY transgenic flies (Fig. 2-8 G-I). Overall, W397 and W342 intermediate residues do not seem to significantly contribute to electrophysiological light response by CRY.

The W394 tryptophan residue is located the furthest away from the CRY FAD binding site. Extended light exposure leads to a conformation of CRY that causes displacement of C-terminal tail (CTT) (43, 44). *In vitro*, W394F mutation of CRY has been shown to reduce light-induced conformational changes (42). Here I tested for rapid light response *in vivo* for W394F CRY mutant transgenic flies. I found that W394F CRY mutation does not significantly affect the rapid electrophysiological membrane potential response to UV, blue, or red light and the response is similar to that of WT CRY (Fig. 2-9 A, D, G). FF change during and after light stimulation is comparable to that of WT CRY-expressing l-LNvs for UV, blue, and red stimuli (Fig. 2-9 B-C, E-F, H-I). I conclude that the redox potential of W394 does not affect the electrophysiological phototransduction mechanism of CRY *in vivo*.

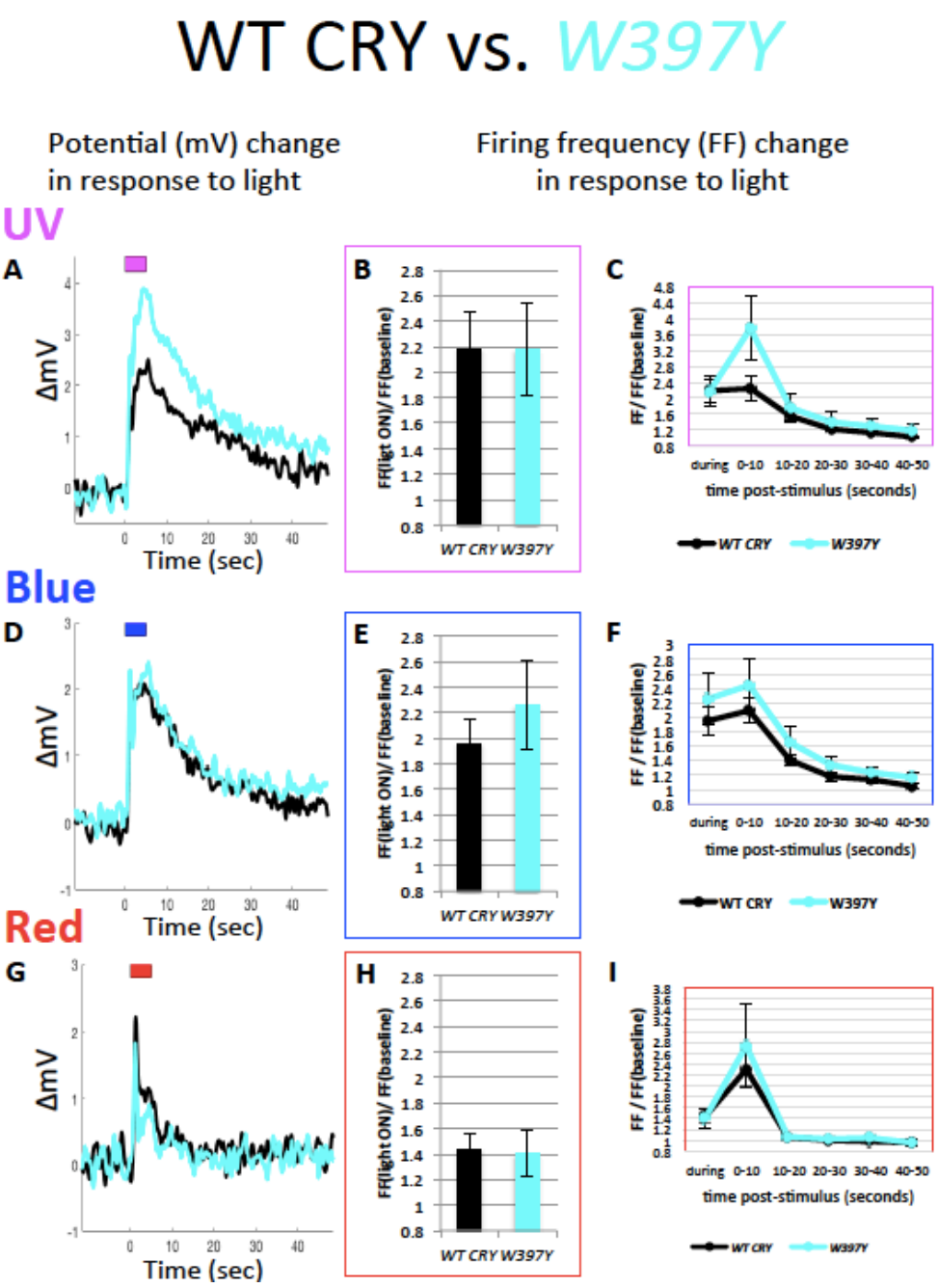

Figure 2–7 W397Y Tryptophan residue mutation does not affect CRY-mediated electrophysiological response to UV and blue light

l-LNv electrophysiological light responses of no mutation (WT CRY) control (black) vs. *W397Y* mutant CRY-expressing (cyan) flies, in response to UV (365 nm LED, 150µW/cm^2) **(A-C)**, blue (450 nm LED, 150µW/cm^2) **(D-F)**, or red (630 nm LED, 150µW/cm^2) **(G-I). (A, D, G)** shows average changes in membrane potential in respect to light stimuli for WT CRY control (black) and *W397Y* CRY mutant (cyan). **(B, E, H)** Firing frequency (FF) change (during light on)/ FF (dark baseline) for WT CRY (black) and *W397Y* CRY mutant (cyan). **(C, F, I)** FF change over time, during and after light stimuli/ FF (dark baseline) for WT CRY (black) and *W397Y* CRY mutant (cyan).

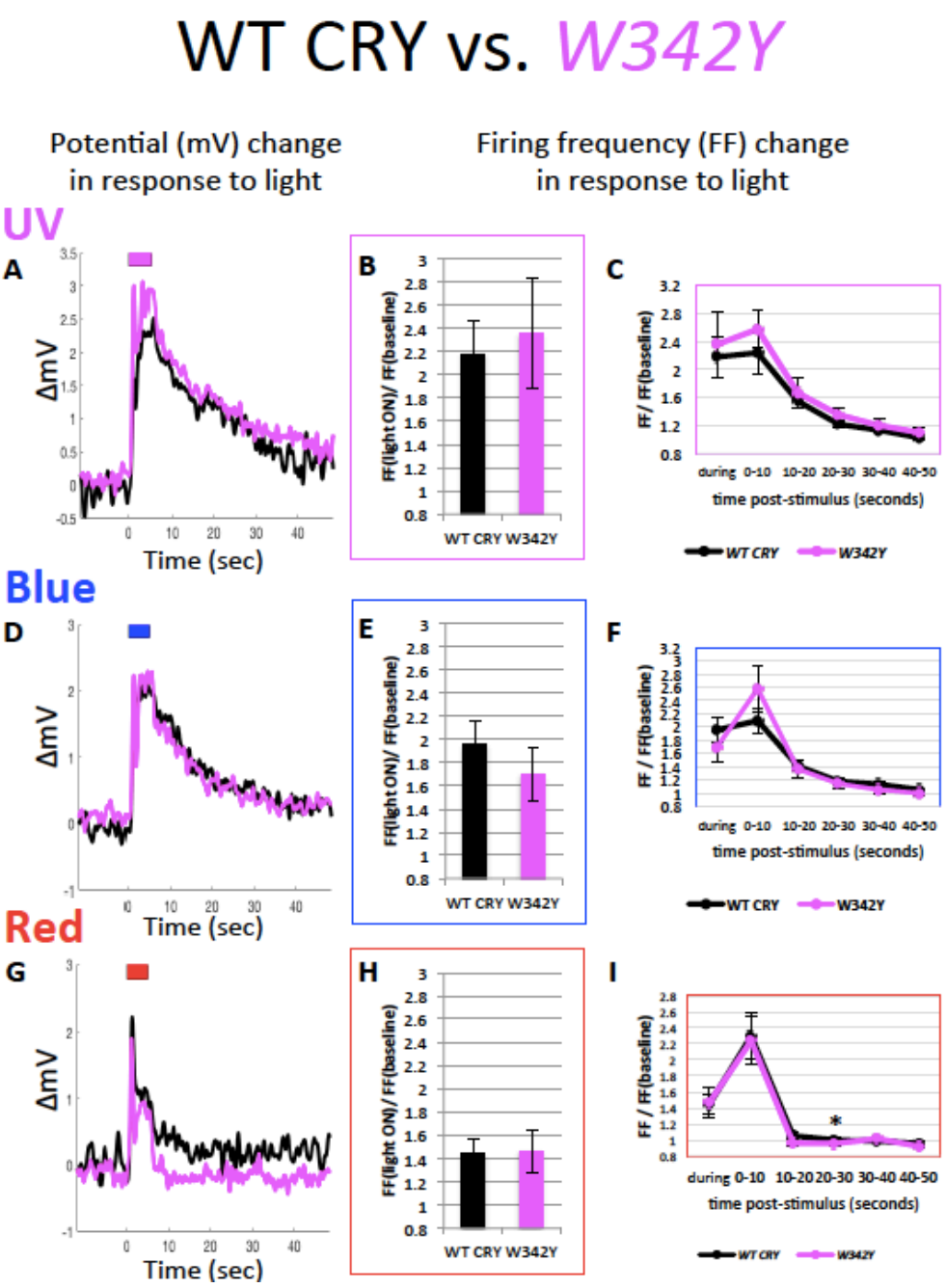

Figure 2–8 W342 Tryptophan residue is not required for CRY-mediated electrophysiological response to UV and blue light

l-LNv electrophysiological light responses of no mutation (WT CRY) control (black) vs. *W342Y* mutant CRY-expressing (r magenta) flies, in response to UV (365 nm LED, 150µW/cm^2) **(A-C)**, blue (450 nm LED, 150µW/cm^2) **(D-F)**, or red (630 nm LED, 150µW/cm^2) **(G-I). (A, D, G)** shows average changes in membrane potential in respect to light stimuli for WT CRY control (black) and *W342Y* CRY mutant (magenta). **(B, E, H)** Firing frequency (FF) change (during light on)/ FF (dark baseline) for WT CRY (black) and *W342Y* CRY mutant (magenta). **(C, F, I)** FF change over time, during and after light stimuli/ FF (dark baseline) for WT CRY (black) and *W342Y* CRY mutant (magenta).

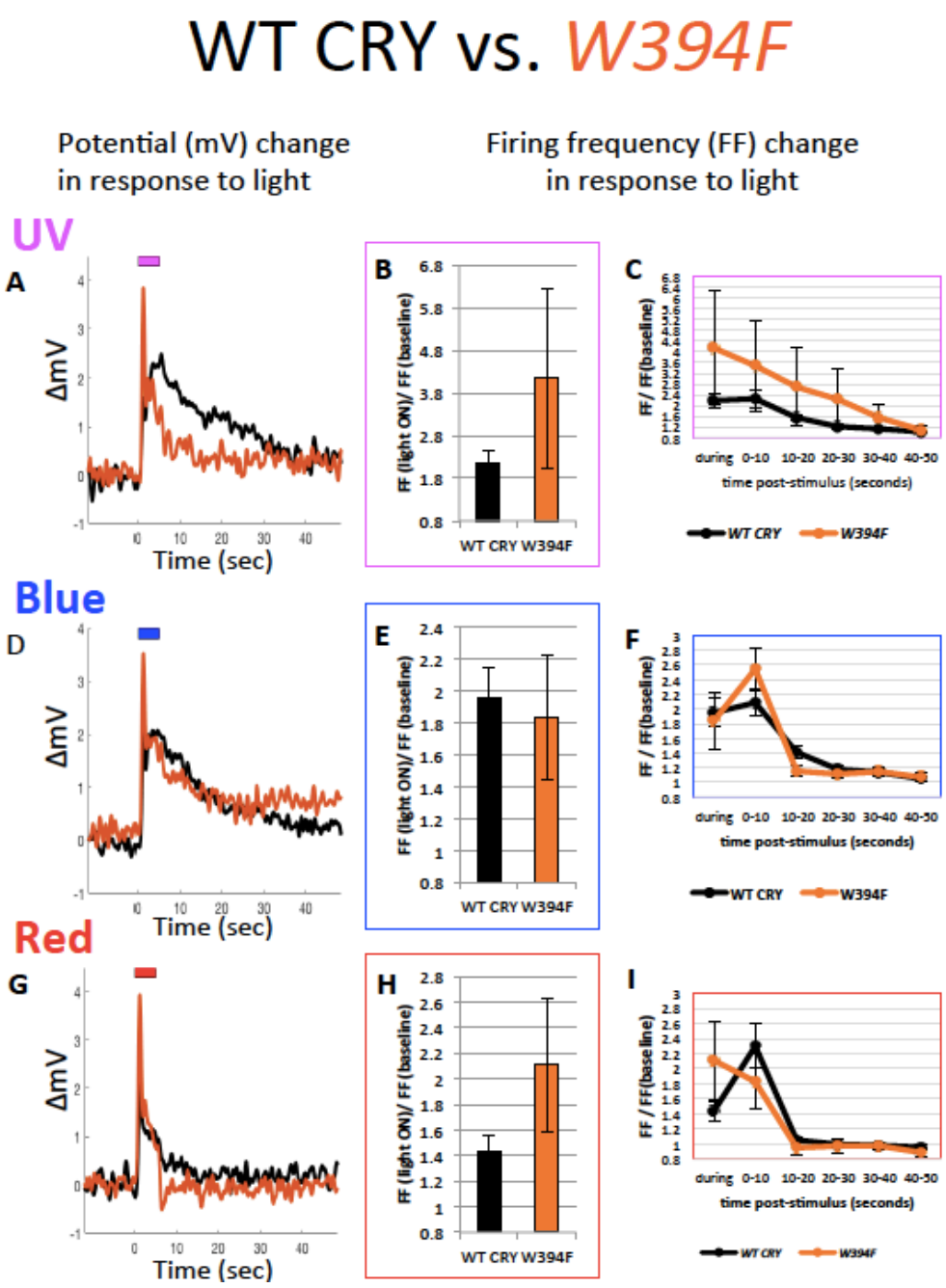

Figure 2–9 W394 Tryptophan residue is not required for CRY-mediated electrophysiological response to UV and blue light

l-LNv electrophysiological light responses of no mutation (WT CRY) control (black) vs. *W394F* mutant CRY-expressing (orange) flies, in response to UV (365 nm LED, 150μW/cm^2) **(A-C)**, blue (450 nm LED, 150μW/cm^2) **(D-F)**, or red (630 nm LED, 150μW/cm^2) **(G-I). (A, D, G)** shows average changes in membrane potential in respect to light stimuli for WT CRY control (black) and *W394F* CRY mutant (orange). **(B, E, H)** Firing frequency (FF) change (during light on)/ FF (dark baseline) for WT CRY (black) and *W394F* CRY mutant (orange). **(C, F, I)** FF change over time, during and after light stimuli/ FF (dark baseline) for WT CRY (black) and *W394F* CRY mutant (orange).

2.3.6 Histidine378 and Cystein416 residues minimally contribute to CRY-mediated electrophysiological light response

In addition to the conserved Trp residues, the *Drosophila* CRY structure shows a conserved Histidine378 near the FAD binding site. The 6-4 photolyase analogue of His378 facilitates the protonation of FAD in a semiquinone state (30). Protonation by H378 may be playing an important role in destabilizing the flavin active site interaction with the flexible c-terminal tail (CTT) by influencing the neighboring residues (37). His (pK_a=6.02) protonates much more readily than Arg and Lys (pK_a=12.48 and 10.79, respectively). H378 may be playing be protonating the flavin in semiquinone state, making it less photoactive.

While protonation by His378 may drive the flavin out of semiquinone state, other residues may work conversely to stabilize the semiquinone state. For instance, the Asn403 residue near the flavin in *photolyase* is poised to form a hydrogen bond with protonated flavin N5, but does not interact with the unprotonated flavin (31). The *Drosophila* analog residue, Cys416 in the same position, is within the hydrogen bonding distance of both unprotonated N5 and O4 of FAD (37). The thiol group of Cys416 can stabilize the negative charge of N5 and O4, preventing further protonation of the semiquinone FAD.

To test the importance of H378 and C416 in electrophysiological light response *in vivo*, I recorded from l-LNv of transgenic flies expressing H378R or C416N mutant CRYs. H378R CRY-expressing l-LNvs membrane potential change looks similar to that of WT CRY-expressing l-LNvs in response to UV and blue light stimuli (Fig. 2-10 A, D). FF of H378R CRY mutant flies are lower compared to WT CRY flies during UV and blue light stimuli, but rapidly increases 0-10seconds post-stimuli (Fig. 2-10 B-C, E-F). H378R mutant CRY l-LNv have

slightly lower membrane potential and indistinguishable FF compared to WT CRY l-LNv during and after red light exposure (Fig. 2-10 G-I). Transgenic C416N CRY mutant flies show lower membrane potential compared to WT CRY-expressing flies during and after UV, blue, and red light stimuli (Fig. 2-11 A, D, G). During UV and blue light exposure, C416N mutant CRY-expressing l-LNvs have significantly lower FF compared to WT CRY-expressing l-LNvs during UV or blue lights on, but is comparable or higher post-stimuli (Fig. 2-11 B-C, E-F). FF change during and post red light stimuli are comparable between WT CRY and C416N mutant CRY-expressing flies (Fig. 2-11 H-I).

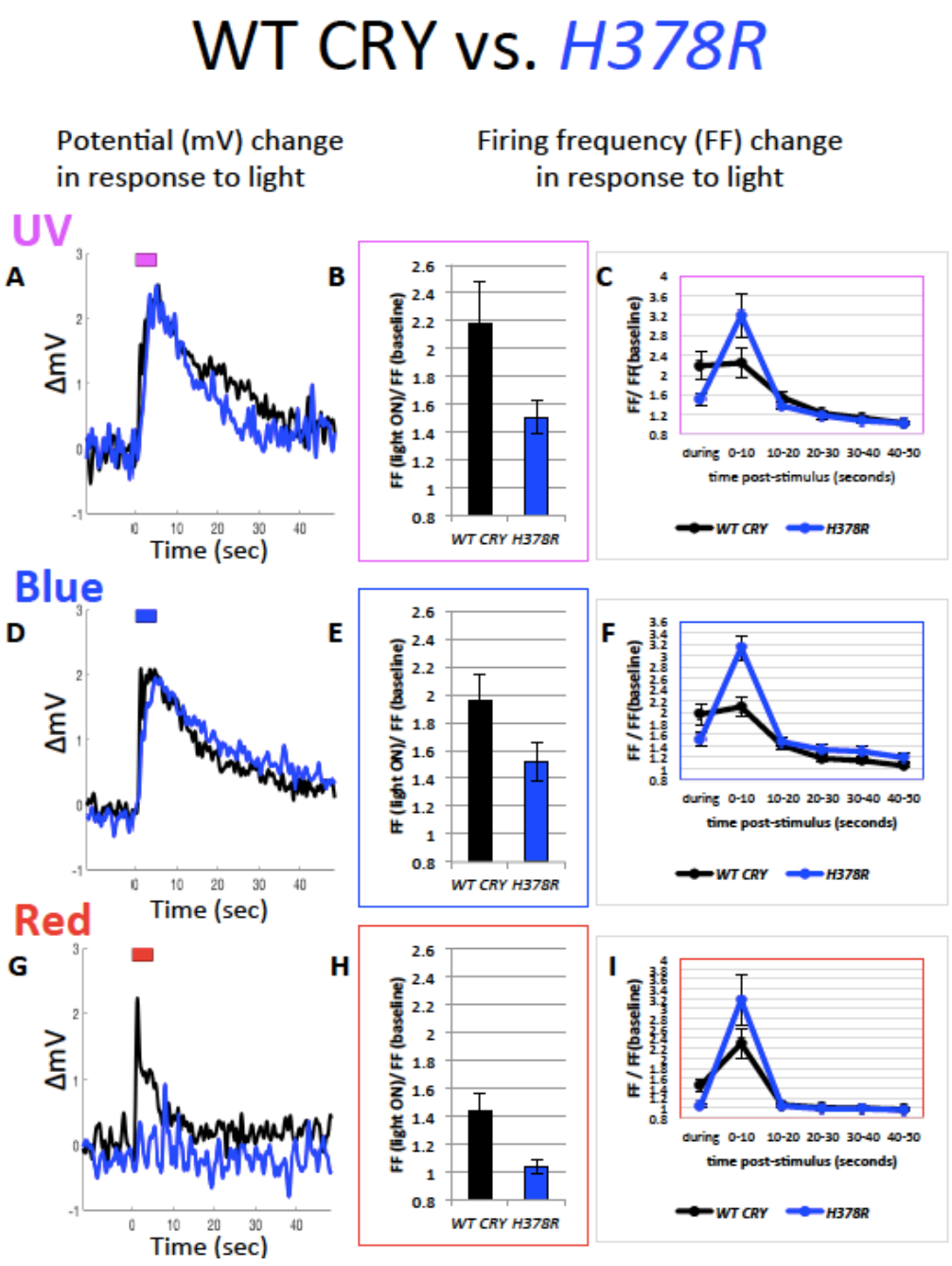

Figure 2–10 H378 histidine residue is not required for CRY-mediated electrophysiological response to UV and blue light

l-LNv electrophysiological light responses of no mutation (WT CRY) control (black) vs. *H378R* mutant CRY-expressing (blue) flies, in response to UV (365 nm LED, 150µW/cm^2) **(A-C)**, blue (450 nm LED, 150µW/cm^2) **(D-F)**, or red (630 nm LED, 150µW/cm^2) **(G-I). (A, D, G)** shows average changes in membrane potential in respect to light stimuli for WT CRY control (black) and *H378R* CRY mutant (blue). **(B, E, H)** Firing frequency (FF) change (during light on)/ FF (dark baseline) for WT CRY (black) and *H378R* CRY mutant (blue). **(C, F, I)** FF change over time, during and after light stimuli/ FF (dark baseline) for WT CRY (black) and *H378R* CRY mutant (blue).

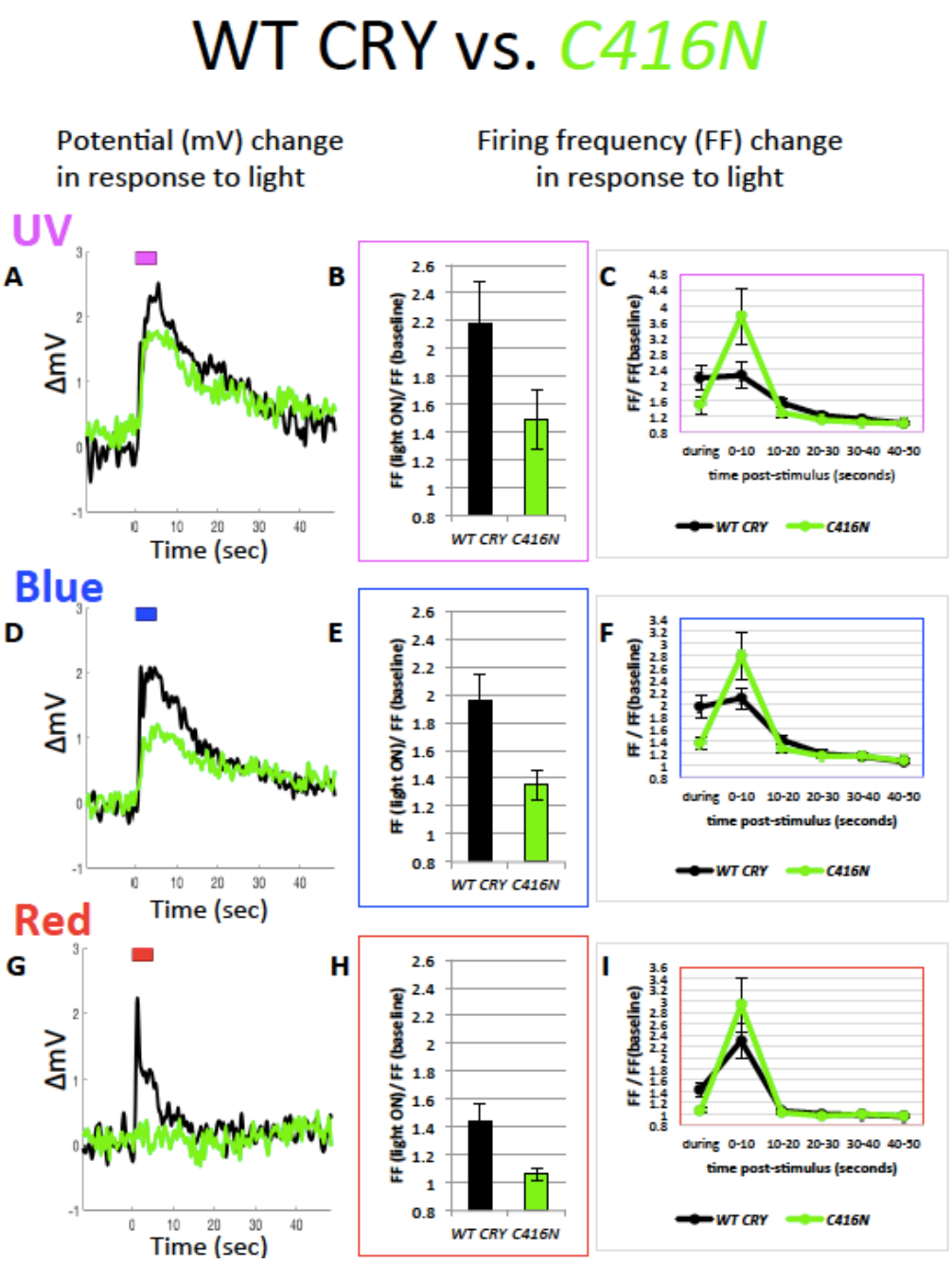

Figure 2–11 C416 cystein residue is not required for CRY-mediated electrophysiological response to UV and blue light

l-LNv electrophysiological light responses of no mutation (WT CRY) control (black) vs. *C416N* mutant CRY-expressing (green) flies, in response to UV (365 nm LED, 150µW/cm^2) **(A-C)**, blue (450 nm LED, 150µW/cm^2) **(D-F)**, or red (630 nm LED, 150µW/cm^2) **(G-I). (A, D, G)** shows average changes in membrane potential in respect to light stimuli for WT CRY control (black) and *C416N* CRY mutant (green). **(B, E, H)** Firing frequency (FF) change (during light on)/ FF (dark baseline) for WT CRY (black) and *C416N* CRY mutant (green). **(C, F, I)** FF change over time, during and after light stimuli/ FF (dark baseline) for WT CRY (black) and *C416N* CRY mutant (green).

2.3.7 H378 histidine and W342 tryptophan residues are important for circadian photoentrainment of locomotor activity rhythm.

CRY is a key modulator for photoentrainment of the circadian clock. One the key features of a functional circadian clock is that an animal that is entrained under light:dark conditions (LD), can maintain rhythmicity even under constant darkness (DD). In contrast, constant light exposure (LL) disrupts the circadian clock in many animals, including *Drosophila*, and the animal becomes arrhythmic (45, 46). However, *cry-null* flies' locomotor activity remain rhythmic in LL (47, 48). Here I tested whether the transgenic CRY mutant flies have circadian behavioral defects under LD, DD, and LL conditions. I found that both control transgenic WT CRY-expressing flies (positive control) and *cry-null* flies (negative control) entrain to standard 12hr:12hr LD condition and maintain rhythmicity in DD (Fig. 2-12 A-B, H, I). Similarly, all tested transgenic CRY mutant flies show normal entrainment and rhythmicity in LD and DD (Fig. 2-12 C-I). This is expected as *cry-null* mutant flies normally do not show circadian abnormalities in locomotor rhythm under LD nor DD conditions. I note that CRY(C416N) mutant flies show a clear delayed onset of the evening peak immediately upon entering DD, but without a long periodicity which is unexpected (Fig. 2-12 G). This may be due to the degradation rate of CRY during lights on that no longer exists in DD. The degradation rate of CRY can be tested using proteomic approaches, such as protein purification followed by Western Blot analysis.

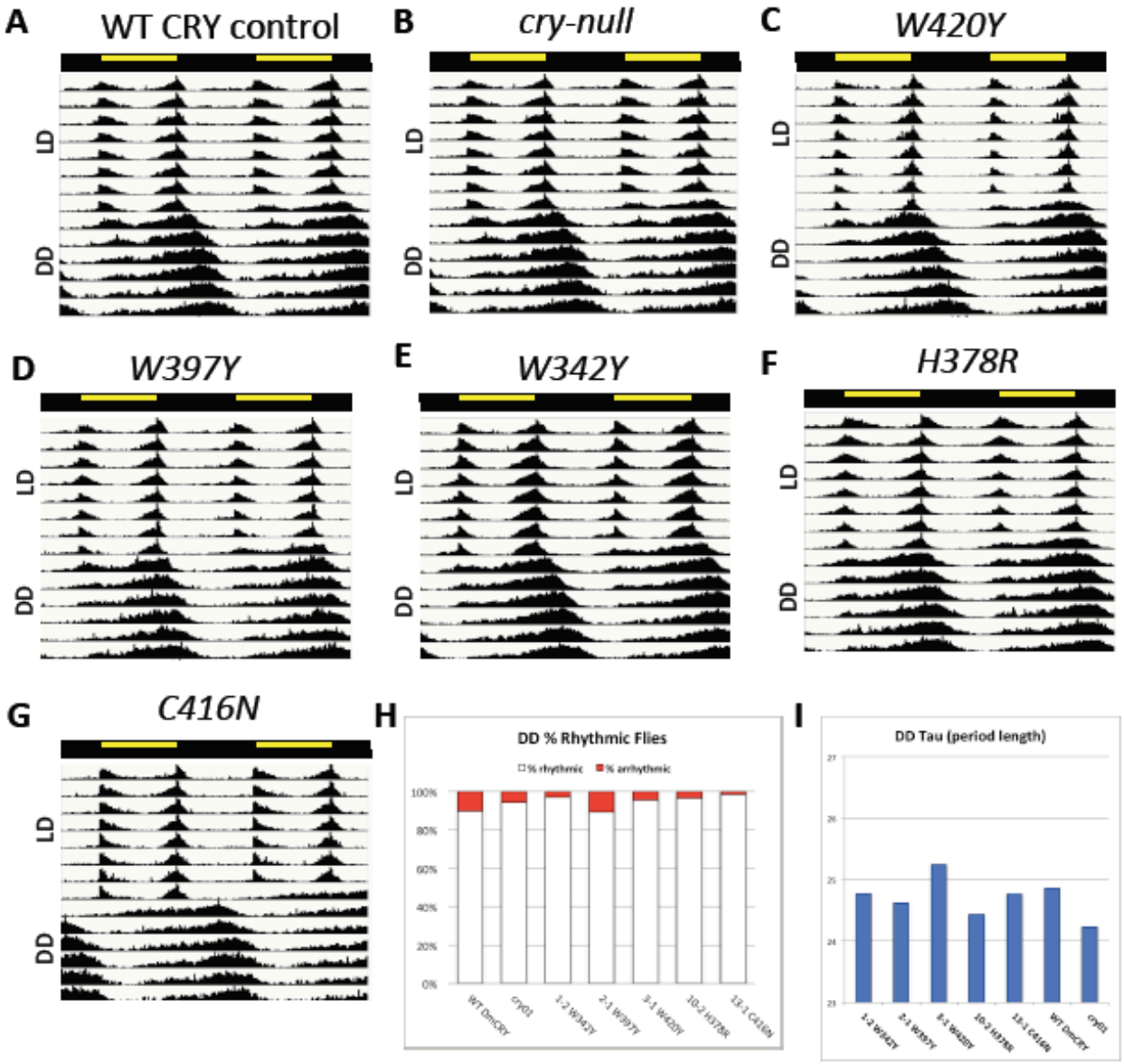

Figure 2–12 Transgenic CRY mutant flies have normal LD and DD phenotypes
(A-G) Representative double-plotted actogram in standard 12hr:12hr light:dark (LD) followed by constant darkness (DD) for WT CRY positive control **(A)**, *cry-null* **(B)**, and CRY(W420Y) **(C)**, CRY(W397Y) **(D)**, CRY(W342Y) **(E)**, CRY(H378R) **(F)**, and CRY(C416N) **(G)** transgenic mutant flies. **(H)** Percentages of rhythmic and arrhythmic flies in DD. **(I)** Period length of rhythmic flies in DD.

I confirm that *cry-null* flies maintain rhythmicity even in LL (Fig. 2-13 B, H). Transgenic flies expressing WT CRY successfully rescued the arrhythmic locomotion phenotype in LL, indicating that this is a functional WT CRY (Fig. 2-13 A, H-I). Similar to WT CRY control, transgenic fly mutants for two tryptophan residues closest to the CRY-FAD binding site,

CRY(W420Y) and CRY(W397Y), do not show LL locomotor defect and are arrhythmic (Fig. 2-13 C-D, H). CRY(C416N) mutant flies also show arrhythmicity under LL (Fig. 2-13 G-H). In contrast, CRY(W342Y) mutant flies show rhythmicity of locomotor behavior with long period of nearly 27hrs under LL condition (Fig. 2-13 E, H-I). Transgenic CRY(H378R) mutant flies also maintain rhythmicity under LL (Fig. 2-13 F, H-I).

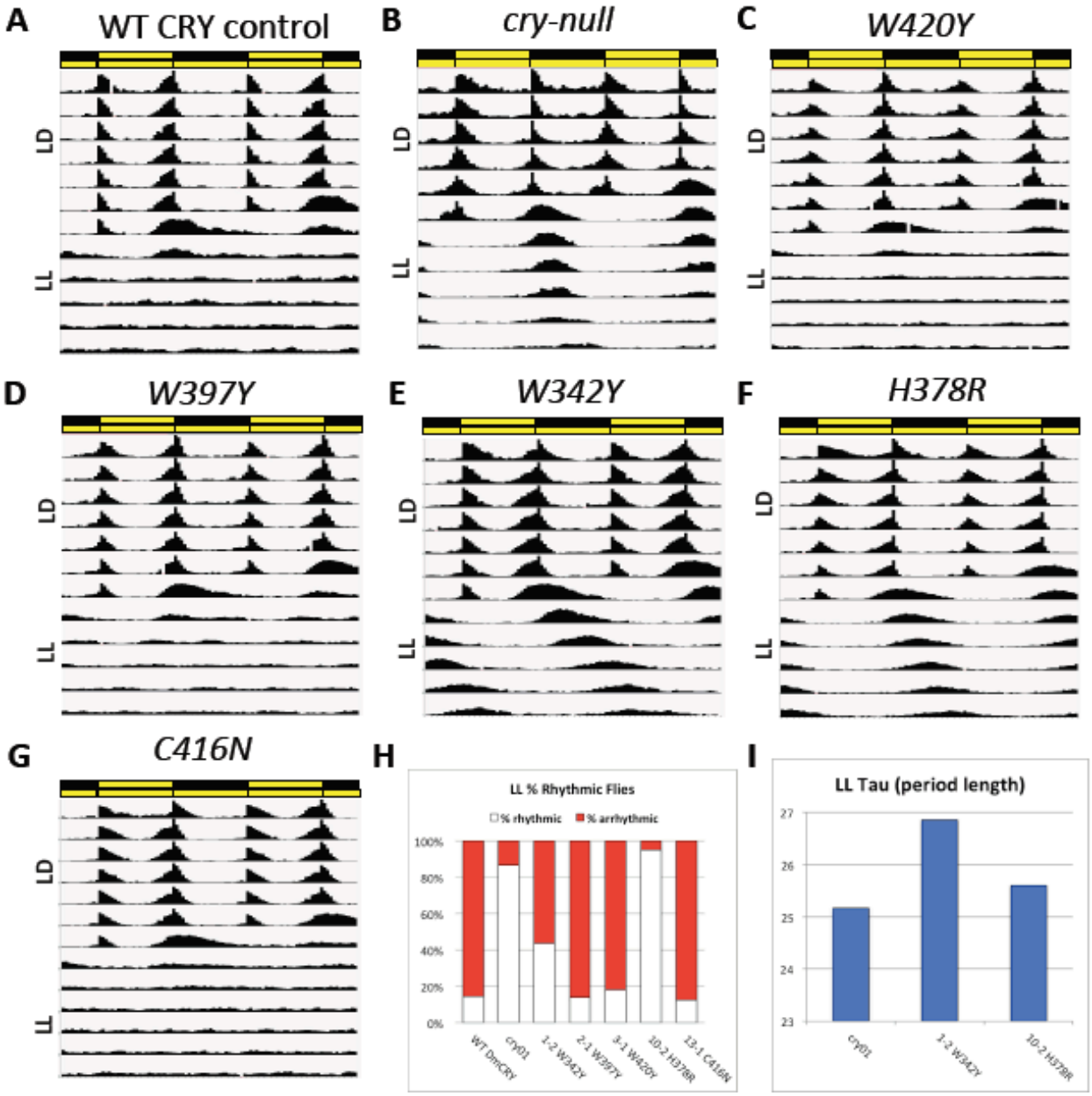

Figure 2–13 Histidine 378 and tryptophan 342 residues mediate circadian photoentrainment.

(A-G) Representative double-plotted actogram in standard 12hr:12hr light:dark (LD) followed by constant light (LL) for WT CRY positive control **(A)**, *cry-null* **(B)**, and CRY(W420Y) **(C)**, CRY(W397Y) **(D)**, CRY(W342Y) **(E)**, CRY(H378R) **(F)**, and CRY(C416N) **(G)** transgenic mutant flies. **(H)** Percentages of rhythmic and arrhythmic flies in LL. **(I)** Period length of rhythmic flies in LL.

2.4 Discussion

The results above show that both CRY- and opsin-based photoreceptors contribute to UV light sensing and behaviors. The l-LNv electrophysiological UV light responses increase monotonically with increasing UV light intensity. The l-LNv electrophysiological response to UV light is severely attenuated in *cry*- and *hk-null* mutants along with qualitative decreases seen in gl^{60j} mutants (Fig. 2-1). There is a small residual l-LNv electrophysiological light response even in gl^{60j}-$cry^{-/-}$ double mutants (Fig. 2-1 J), suggesting that there is another short wavelength light photoreceptor yet to be identified. Subtleties in our data suggest potential circuit level effects for encoding light. Gene replacement rescue experiments in *cry-null* and *hk-null* backgrounds show intensity-dependent degrees of rescue, for which rescue is complete for lower light intensities but incomplete for higher light intensities (Fig. 2-1 F and G). This may be due to the fact that the genetic rescue is limited to the LNv, not all neurons that ordinarily express CRY.

Here I generated transgenic flies expressing mutant CRY with residue changes of tryptophan that likely play a role in the electron transfer phototransduction mechanism by CRY. I administered a highly sensitive assay with rapid acquisition rate and precisely time-locked administration of LED light stimuli. Using this method, I demonstrated that the CRY-mediated phototransduction has distinct electrophysiological signatures in circadian neurons; rapid and sustained increase in membrane potential and FF in response to short wavelength light exposures (Fig. 2-4). Interestingly, *cry-null* l-LNv initially shows decrease in membrane potential and delayed increase in FF after short wavelength or red light exposures (Fig. 2-4). Similar to *cry-null,* WT CRY-expressing l-LNv also shows small and delayed increase in FF following red light exposure and this FF change is indistinguishable from that of *cry-null* flies (Fig. 2-4 I). This

suggests there may be a red light-sensitive, non-CRY mediated phototransduction input to the l-LNvs, such as red sensitive opsins from the eyes.

Tyrosine and phenylalanine have much lower redox potential and slower electron transfer compared to tryptophan. Mutation of a large bulky ring structure tryptophan to another large and bulky ring residue, tyrosine or phenylalanine, reduces redox efficiency between CRY-FAD, while preventing structure of CRY from possibly collapsing. *In vitro,* mutation of W420Y, but not other tryptophan-triad's tryptophan residues, in CRY has much slower electron transfer kinetics and reduced photoreduction compared to WT CRY, but does not completely abolish the phototransduction (42). Here I observe a similar phenotype *in vivo*. Transgenic W420Y CRY mutant flies do not mimic the change in membrane potential in response to light seen in *cry-null* mutant flies. Instead, W420Y CRY-expressing flies have significantly diminished membrane potential change. W420Y CRY mutant flies also show significantly lower increase in FF, similar to *cry-null*.

W394F mutation *in vitro* lacked light-dependent degradation, but this is a much slower process compared to fast photoreduction. *In vitro* data suggests that W394 is important for CRY conformational change and release of CRY's c-terminal tail (CTT) from the FAD-CRY interaction site. Photoreduced CRY undergoes a conformational change releasing the CTT, targeting core circadian protein TIMELESS for degradation (44). It is clear that CTT is important for the slow response of CRY such as circadian photoentrainment, but our results suggests that the slow conformational change involving CTT may not be necessary for rapid neuronal potential changes in response to light. I propose the possibility that there may be multiple light-activated mechanisms of CRY, a slow mechanism such as photoentrainment, and a fast mechanism such as rapid photoreduction. This is supported by the results that the CRY-

mediated neuronal light response is robust even during the daytime when CRY expression is low (4, 5). It is possible that some residual CRY is present near the membrane versus CRY important for slow molecular clock mechanism. Furthermore, W342Y and H378R CRY mutant flies show behavioral entrainment defect under LL, but does not show defect in rapid electrophysiological light response (Figs. 2-13). These results suggest that W342 and H378 residues of CRY are important for circadian entrainment. W342 residue has been suggested to play a role in interaction with the CTT of CRY and conformational change important for circadian photoentrainment (42). H378 also has been suggested to play a role in modulating the FAD binding site interaction with the CTT of CRY by influencing the neighboring residues upon protonation (37). This suggests that the slow circadian photoentrainment is mediated by conformational change of CRY that modulates interaction between CRY-FAD binding site and CRY's CTT. There has been a long-standing controversy regarding the phototransduction and circadian entrainment mechanism by CRY. Here I show *in vivo* that CRY likely has separate mechanisms for rapid electrophysiological neuronal response to light versus slow circadian photoentrainment. I also show that electron transfer by nearby tryptophan residue mediates the rapid neuronal light response. In contrast, slow circadian photoentrainment is mediated by conformational change of CRY, specifically involving interaction of flexible CTT with FAD-CRY interaction site.

Reprinted with permission from:

Baik LS, Fogle KJ, Roberts L, Galschiodt AM, Chevez JA, Recinos Y, Nguy V, and Holmes TC. (2017) CRYPTOCHROME mediates behavioral executive choice in response to UV light. *Proceedings of the National Academy of Sciences of the United States of America* 114(4):776-781.

Chapter 3 : Short Wavelength Light-Evoked Behaviors in *Drosophila melanogaster*

3.1 Introduction

The ability to anticipate and adapt to daily environmental changes is critical for survival (4, 6, 9, 32, 49, 50). Many insects show strong behavioral responses to short wavelength light. Light can serve as either a repellent or an attractive signal for an animal's behavior, depending on intensity and spectra. Many insects exhibit an innate spectral attraction to low intensity UV light as shown by phototaxis behavioral assays (51-53). In contrast, high intensity UV light induces aversive behavior, particularly in larvae and egg-laying females (54, 55), and reduces mating activity in adult male *Drosophila* (56). Rhythmic short wavelength light avoidance is crucial for avoiding heat, low humidity, and peak ultraviolet (UV) radiation at midday and thus minimizes a range of hazards from desiccation at the organism level to DNA damage at the molecular level. This is important particularly for ectotherms like *Drosophila* that must maintain their internal body temperature through behaviorally choosing different temperature environments (57). Pittendrigh proposed that before the development of Earth's oxygen rich atmosphere, which blocks UV light, the evolution of circadian systems was driven by the need to escape from the harmful effects of UV radiation (58).

Here I show that flies display both attraction and avoidance to short wavelength light. Attraction to light (positive phototaxis) is a crucial escape behavior. In contrast, avoiding short wavelength light minimizes desiccation, UV-induced DNA damage, and excessive heat exposure. I show that adult flies exhibit a circadian-modulated avoidance to chronic high intensity UV light over a 12 hour period that coincides with the peak of light intensity and heat in the middle of the day (9). In contrast, low intensity light evokes immediate attraction to light at

light onset within minutes (6, 52, 53, 59). This suggests UV light-evoked attraction/avoidance responses may depend on intensity and/or duration of exposure. However, it is unclear which phototransduction channels modulates these two opposite behavioral responses to light stimuli, and their time-of-day variance in response. The question still remains, how an animal spatially navigates and chooses between different light environments. Here I comprehensively examine the external and internal factors influencing an animal's spatiotemporal choices in response to different light environments. External factors include the spectral quality and quantity of light, while internal factors include circadian timing and different phototransduction signaling channels (external opsins in eyes and other structures and internal neuronal CRYPTOCHROME and RHODOPSIN 7) in flies. I examined contribution(s) of (1) duration of exposure vs. circadian time-of-day, (2) intensity, and (3) different phototransduction mechanisms on UV light-evoked attraction/avoidance behavior.

In *Drosophila*, short wavelength light is processed through several phototransduction mechanisms: (i) External opsin-based photoreceptors, and direct neuronal phototransduction pathways: (ii) Cry/Hk-based phototransduction, and (iii) internal rhodopsin, Rh7-mediated phototransduction (4, 5, 11, 60-63). All three phototransduction pathways mediate circadian entrainment to light (11, 19-21, 32, 60, 64-72). Both Cry/Hk- and Rh7-mediated phototransduction evokes rapid increase in neuronal action potential firing and resting membrane potential (4-6, 11). This suggests that integration of multiple phototransduction inputs may be crucial in coordinating a complex light-evoked behavior, such as avoidance and attraction.

Cry, the primary circadian light sensor in *Drosophila*, evolved from ancient short wavelength light-activated DNA repair enzymes. CRY signaling modulates multiple behavioral responses to UV light. In *Drosophila,* the short wavelength light-sensitive flavoprotein CRY

mediates acute arousal and rapid positive phototaxis responses (4, 5). CRY also mediates temporally slower circadian entrainment and adult light avoidance behavior responses to short wavelength light (6, 19, 20). These behavioral responses correspond biophysically to the absorbance spectra of CRY in its baseline flavin adenine dinucleotide (FAD) oxidized state with two major peaks at 365 nm (UV) and 450 nm (blue) (7, 27, 29). Activated CRY mediates blue and UV light-evoked changes in electrophysiological action potential firing rate and resting membrane potential in lateral ventral circadian neurons (LNv) coupled by a voltage-gated potassium beta subunit (Kvβ) called HYPERKINETIC (HK) (4-6, 14). HK is a redox sensor that translates redox biochemical signals into changes in membrane electrical potential (4, 6, 73, 74). Light-modulated behaviors are driven by the modulation of membrane excitability in contributing neurons, such as the LNvs (3, 4, 32). CRY-expressing large lateral ventral neurons (l-LNvs) mediate acute behavioral arousal responses to blue light containing spectra (32-35). Arousal and circadian functions are not strictly segregated between the LNv subsets as the small LNvs (s-LNvs) contribute also to arousal (33), and clock cycling is robustly altered in the l-LNv in response to light entrainment cues (75, 76).

In the *Drosophila* brain, approximately 150 pacemaker neurons of the circadian circuit are defined by their expression of ~24 hr cycling PER and TIM (77), of which approximately half express CRY, including the pigment dispersing factor (PDF)-positive lateral ventral neurons (LNv) (1, 2). LNvs show the most rapid white light responses among circadian neurons measured by *period-luciferase* whole circuit dynamic imaging (75). Clock neuron electrical firing is circadian regulated and their electrical activity drives behaviors (14-16, 78). Together, these findings suggest that light-evoked behaviors may be under circadian regulation.

I set out to test whether *Drosophila* long-term (hours) behavioral UV light responses are circadian regulated based on an earlier observation that wild-type *Drosophila* exhibit a peak of UV light avoidance behavior at midday under conditions of constant UV light intensity. Peak of UV avoidance in midday coincides with siesta rest in adult flies and flies show preference sleeping in dark environment over light environment during sleep (79). The peak of midday avoidance coincides with peak UV light intensity in natural environments (6). Earlier work shows larval acute (minutes) light avoidance behavior depends in part on subsets of circadian pacemaker neurons and circadian genes (80, 81).

3.2 Materials and Methods

3.2.1 Generation of gl^{60j}-$cry^{-/-}$ double mutant line by homologous recombination

I generated the gl^{60j}-$cry^{-/-}$ double mutant line (same as gl^{60j}-cry^{01}) by standard recombination genetic crosses of gl^{60j} (Bloomington Stock Center, BL 509) and the cry^{01} line provided by Jeff Hall (48). I verified *glass* and *cry* loss in our gl^{60j}-cry^{01} double mutant line by phenotype, genetic markers, and PCR.

3.2.2 Immunocytochemistry

Brains were dissected in 1X PBS, fixed in 4% paraformaldehyde (PFA) for 30min, washed 3X 10min in PBS-Triton-X 1%, incubated in blocking buffer (10% Horse Serum-PBS-Triton-X 0.5%) at room temperature before incubation with mouse α-PDF C7, monoclonal (1:10,000) and rabbit α-PER, polyclonal (1:1,000) antibodies overnight in 4^{o}C. Brains were washed 3X 10min in PBS-Triton-X 0.5% then incubated in goat α-mouse-Alexa- (1:500) and goat α-rabbit-Alexa-594 (1:500) secondary antibodies in blocking buffer overnight in 4^{o}C. Brains were washed 5X 15min in PBS-Triton-X 0.5% before mounting in Vectashield mounting

media (Vector Laboratories). Microscopy was performed using Zeiss LSM700 confocal microscope.

Behavioral Experiments

3.2.3 Light Pulse Arousal

Flies were entrained for a minimum of three days to a normal Light-Dark (LD) 12:12 cycle. During the dark phase, they were given three consecutive 5-minute pulses of UV (365 nm, 3 mW/cm^2) or orange (595 nm, 7 mW/cm^2) at ZT 18, 19, and 20, for three nights. Activity during all of these pulses and for the subsequent 40-minutes were binned in five-minute intervals and averaged. Statistical tests between genotypes at each five-minute bins were conducted by t-test. Sleeping flies, defined by inactivity for five minutes preceding the pulse, were assessed for the percentage that woke up during the pulse. Active flies were assessed for the change in their activity during and after the pulse; changes were expressed as activity of bin/activity during baseline.

3.2.4 Behavioral Phototaxis

An electrophoresis system photo-documentation hood (15 x 20 cm, Fisher Biotech) was converted into a light-tight chamber to hold a population of flies. A DAM2 *Drosophila* Activity Monitor (32 channels with dual infrared beams, Trikinetics) was mounted to the front of the chamber and sealed with a glass cover on the outer face. For each experiment, a population of 40 adult flies was directly placed into the chamber. w^{1118}, cry^{01}, and gl^{60j} flies were independently tested. The entire apparatus was then placed in a light-sealed enclosure where the flies were left to habituate for 30 minutes before starting the recording. To capture time-of-day effects for

phototactic responses, behavioral activity was recorded from CT 21 to CT 3. An LED light source was turned on for 5 cycles of 5 min pulses followed by 55 minutes of constant darkness. The LED light source was either: (1) a Prizmatix UHP-Mic-LED-595 (595nm) or (2) a Prizmatix Mic-LED-365, High Power UV LED (365 nm). The light intensity for both LED sources was set to 3 $\mu W/cm^2$. Due to the narrow collimated light emission of these two light sources, they were reflected off an inner surface of the enclosure so that their light emissions covered all 32 channels of the activity monitor. Data was collected by the DAM2 *Drosophila* Activity Monitor to be analyzed in 1-minute bins. Excel was used for quantitative analysis of average population activity in response to the light pulses relative to baseline measurements of activity in darkness. Statistical significance was determined using t-test.

Light Intensity – Phototaxis

- Raw (directly in front of LED) = 6 mW/cm^2
- Reflected (on behavior monitors) = 90 $\mu W/cm^2$
- Through glass (within chamber) = 3 $\mu W/cm^2$

3.2.5 Locomotor analysis

Locomotor activity of individual flies was measured using the TriKinetics Locomotor Activity Monitoring System via infrared beam-crossing recording total crosses in 15 or 30 min bins. Actograms were generated using Clocklab software. Average activity eduction graphs, % rhythmic flies, and its statistics were measured using FaasX software and Microsoft Excel. For UV LD and LL experiments, Philips TL-D Blacklight UV source with narrow peak wavelength of 365 nm and intensity of 400 $\mu W/cm^2$ was used.

3.2.6 Anticipation index

Morning anticipation index was calculated using the Harrisingh/Individual index. For individual fly, fraction of activity during the 3 hours before ZT0 was compared to the activity level through the six hours before ZT0 (82).

3.2.7 Standard Light Choice Assay

Standard Trikinetics activity monitors were modified to remove the center barrier in order to accommodate 15 cm-long, 5 mm-diameter glass tubes (Trikinetics, Waltham, MA). Two air holes were drilled into the tube equidistant from the ends, which were both plugged with fly food on both sides, and sealed with paraffin wax after the fly was introduced to the tube. Flies were entrained in the tubes for 3 days in white light 12:12 hr LD and three days in UV light, uncovered, Continuing LD schedule in UV light (UV light on during ZT0-12), one half of the monitor was covered with cardboard, providing the flies with a choice of a shaded environment during the 12 hours of UV light. Philips TL-D Blacklight UV source with narrow peak wavelength of 365 nm and intensity of 400 $\mu W/cm^2$ was used for high intensity, and 10 $\mu W/cm^2$ was used for low intensity by using neutral density filters. Activity for each fly in the UV-exposed versus shaded side of the tube was averaged over 10 days and statistical preferences were determined by t-test. Locomotor activity of individual flies was measured using the TriKinetics Locomotor Activity Monitoring System via infrared beam-crossing recording total crosses in 1 min bins. % activity and statistics were measured using Microsoft Excel and Sigma Plot. For constant light (LL) light choice assay, the protocol was modified as follows: when one-half of the monitors were covered, instead of 12h:12h UV light (365 nm, 400 $\mu W/cm^2$):dark, the UV light was constantly left on. To determine acute light responses distributed throughout the

day, acute 15 min pulses of UV light (365 nm, 400 μW/cm^2 for high intensity pulses or 10μW/cm^2 for low intensity pulses) throughout the 12 hr day on hourly intervals instead of 12h:12h UV light:dark. Each genotype was tested over five separate behavioral runs with >76 flies total per genotype.

3.2.8 Multibeam Light Choice Assay

I adapted the standard LD Light choice assay using Trikinetics MultiBeam Monitors. Two monitors were aligned parallel to each other with each tube containing a single fly measured by both monitors simultaneously. One of the two monitors was covered, providing the flies with a choice of a shaded environment (covered monitor) versus UV-exposed environment (uncovered monitor) during the 12 hours of UV light in daytime (ZT0-12). Each of the infrared beams are spaced every 3 mm. By using two monitors, each fly had 17 infrared beams on each light exposed- and on shaded-side with a total of 34 beams per fly. Locomotor activity, preference percentile, and statistics were measured using Microsoft Excel and Sigma Plot. Data are represented as mean ± S.E.M. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3.3 Results

3.3.1 Acute arousal behavioral response to UV light is CRY-dependent.

CRY is expressed in circadian and arousal neurons (1-3, 5), including l-LNvs, which mediate acute arousal behavioral responses to blue light at physiological intensities that transmit the head and eye cuticles (4). I measured acute behavioral responses to 5-minute pulses of 365 nm UV (3 mW/cm^2) or 595 nm orange (7 mW/cm^2) LED light in the middle of the subjective night at ZT18, ZT19, and ZT20 for three consecutive nights in control and *cry*$^{-/-}$ mutants, as well

as *norpA*^{P24} and *gl*^{60j} mutant flies, which respectively have defects in opsin phototransduction in the eyes and the ocelli, and external photoreceptor development. A representative averaged behavioral actogram of control flies (n=32) responding to UV light pulses is shown (Fig. 3-1). I examined flies that were asleep immediately prior to the light pulse (32, 33). The percentage of flies that awaken in response to 365 nm UV light is significantly lower in *cry*$^{-/-}$, *norpA*^{P24}, and *gl*^{60j} mutant flies compared to control (Fig. 3-1). In response to 595 nm orange light, the percentage of flies that awakened was comparable between control and *cry*$^{-/-}$ flies. However, a significantly higher percentage of *norpA*^{P24} flies awakened, while significantly lower percentage of *gl*^{60j} flies awakened in response to orange light relative to controls, indicating that the *norpA*^{P24} and *gl*^{60j} flies differ in their light responses (Fig. 3-1 B). Recently, the *Drosophila* Transient Receptor Potential A1 (TRPA1) channel has been implicated as a H_2O_2-sensitive high intensity UV (600 mW/cm^2 – 5,000 mW/cm^2) sensor (83). I tested acute arousal responses of *trpA1* null (*trpA1*^{1}) flies to UV light (3 mW/cm^2) and orange light (7 mW/cm^2). The acute arousal response of *trpA1*^{1} flies to either UV or orange light pulses is indistinguishable from that of control flies (Fig. 3-2), indicating that acute arousal responses to UV light pulse is not mediated by TRPA1 at the light intensities tested.

I also examined the behavioral responses of flies that were awake immediately prior to the light pulse. During the UV light pulse (just after 0 min), awake control flies show increased arousal activity, while awake *cry*$^{-/-}$ flies remain relatively inactive during the UV light pulse, then show a delayed response minutes later following the UV light pulse (Fig. 2C). CRY-mediated acute arousal activity is specific to UV light, as the *cry*$^{-/-}$ response to orange light does not differ from that of control flies (Fig. 3-1 D). Acute arousal activity of awake *norpA*^{P24} flies in response to UV light pulse is indistinguishable from that of control flies (Fig 3-1 E). *norpA*^{P24} flies show

increased acute arousal responses during the orange light pulse, but following the orange light pulse, their activity does not differ from control flies (Fig. 3-1F). gl^{60j} awake flies do not respond to either UV nor orange light pulses (Fig. 3-1 E and F). This suggests acute arousal behavioral response to UV may be modulated by DN1 cells and/or Hofbauer-Buchner (HB) eyelet, functionally defective in the gl^{60j} mutants, but not in $norpA^{P24}$ mutants (21, 84-87).

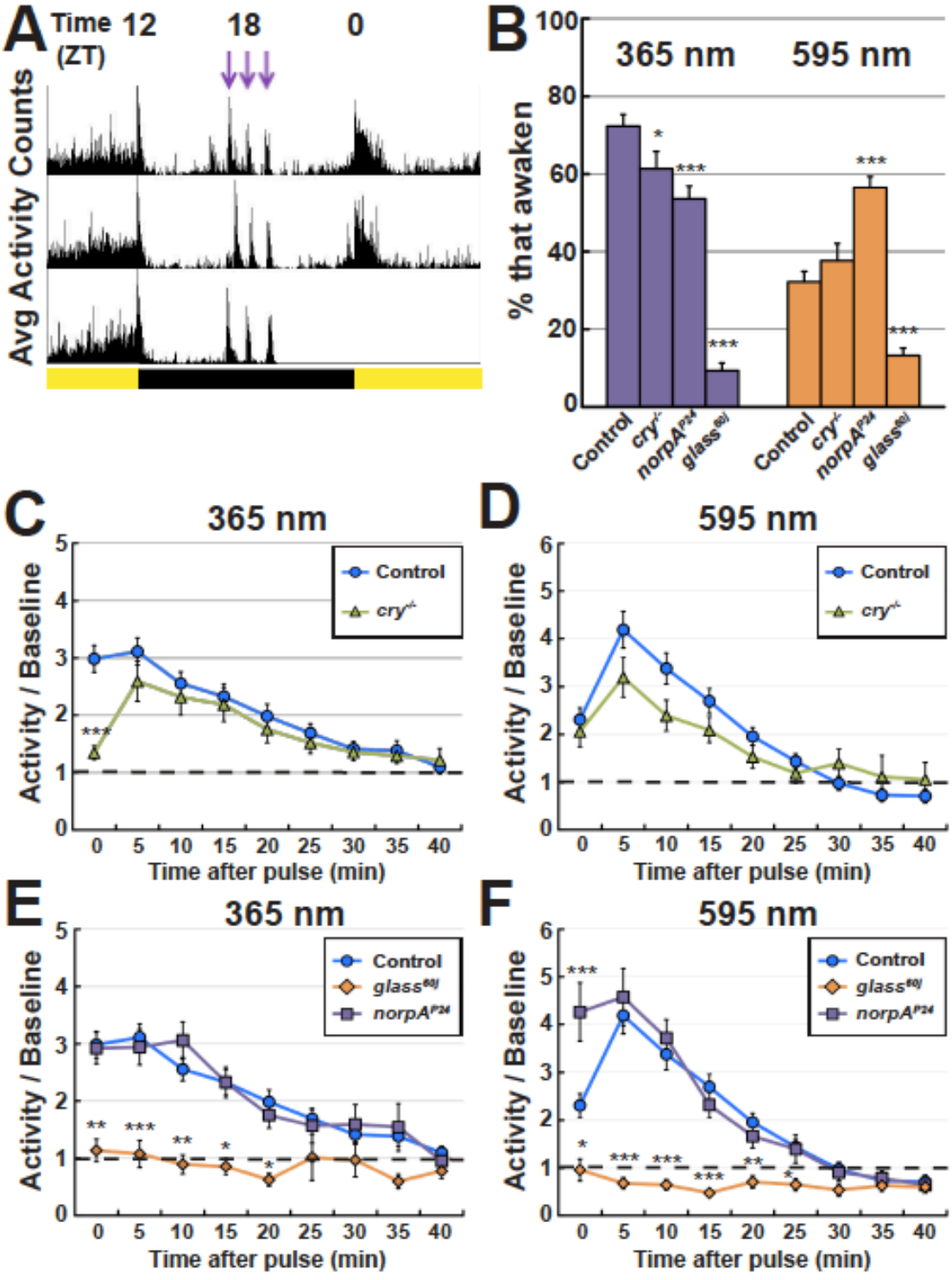

Figure 3–1 ***Drosophila*** **acute arousal response to UV light is CRY-dependent.**
(A) Representative averaged double-plotted actogram of n=32 flies given three 5-minute light pulses (365 nm, 3 mW/cm^2) during three consecutive nights. Flies respond acutely to light pulses but remain entrained to the LD 12:12 environmental cues. **(B)** Percentage of sleeping flies which awaken during the light pulse for UV (365 nm, 3 mW/cm^2) and orange light (595 nm, 7 mW/cm^2). Compared to control flies (0.72 ± 0.03, n=384 flies for UV), significantly lower

percentage of *cry*$^{-/-}$ flies (0.61 ± 0.04, n=192 flies, p=0.039 vs. control) awaken in response to UV light pulse. Percentage of sleeping *cry*$^{-/-}$ flies that awaken during orange light pulse does that differ from that of control flies (0.32 ± 0.03, n=224 flies for control; 0.38 ± 0.04, n=128 flies, for *cry*$^{-/-}$; p=0.264 *cry*$^{-/-}$ vs. control). Both *norpA*^{P24} and *gl*^{60j} flies have significantly lower percentage of flies that awaken in response to UV light pulses (0.54 ± 0.03, n=192 flies, p=<0.001 for *norpA*^{P24} vs. control; 0.09 ± 0.02, n=64 flies, p=<0.001 for *gl*^{60j} vs. control). Significantly higher percentage of *norpA*^{P24} flies awaken (0.56 ± 0.03, n=160 flies, p=<0.001 for vs. control), while significantly lower percentage of *gl*^{60j} flies awaken in response to orange light pulses (0.13 ± 0.02, n=64 flies, p=<0.001 vs. control). **(C-F)** Time course of activity of awake flies during and after UV **(C and E)** or orange **(D and F)** light pulse. Each point on the graph represents a bin of 5-minutes, with the first bin collected during the pulse. **(C)** During the UV light pulse, control flies show a dramatic increase in arousal activity (activity/baseline is 2.98 ± 0.23, n=384 flies) while *cry*$^{-/-}$ flies remain relatively inactive (1.35 ± 0.12, n=192 flies, p=<0.001 vs. control), only responding after the pulse (*cry*$^{-/-}$ vs. control, p>0.213 for all bins, after the light pulse). **(D)** *cry*$^{-/-}$ and control fly activities do not differ during and after the orange light pulse (n=128 flies, *cry*$^{-/-}$ vs. control, n=224 flies, p>0.064 for all bins). **(E)** Activity of awake *norpA*^{P24} flies does not differ from that of control flies (n=192 flies, *norpA*^{P24} vs. control, n=384 flies, p>0.174 for all bins). *gl*^{60j} flies show a significantly lower arousal response both during and after the UV light pulse (n=64 flies, *gl*^{60j} vs. control, p<0.010 for bins during and 5- 20 minutes after the light pulse). **(F)** *gl*^{60j} flies show a significantly lower arousal response during and after orange light pulse (n=64 flies, *gl*^{60j} vs. control, n=224 flies, p<0.018 for bins during and 5-25 minutes after the light pulse). *norpA*^{P24} flies have significantly higher activity than control flies during the orange light pulse (n=160 flies, *norpA*^{P24} vs. control, p=<0.001), but does not differ in activity following the orange light pulse (*norpA*^{P24} vs. control, p<0.332 for all bins, after the light pulse).

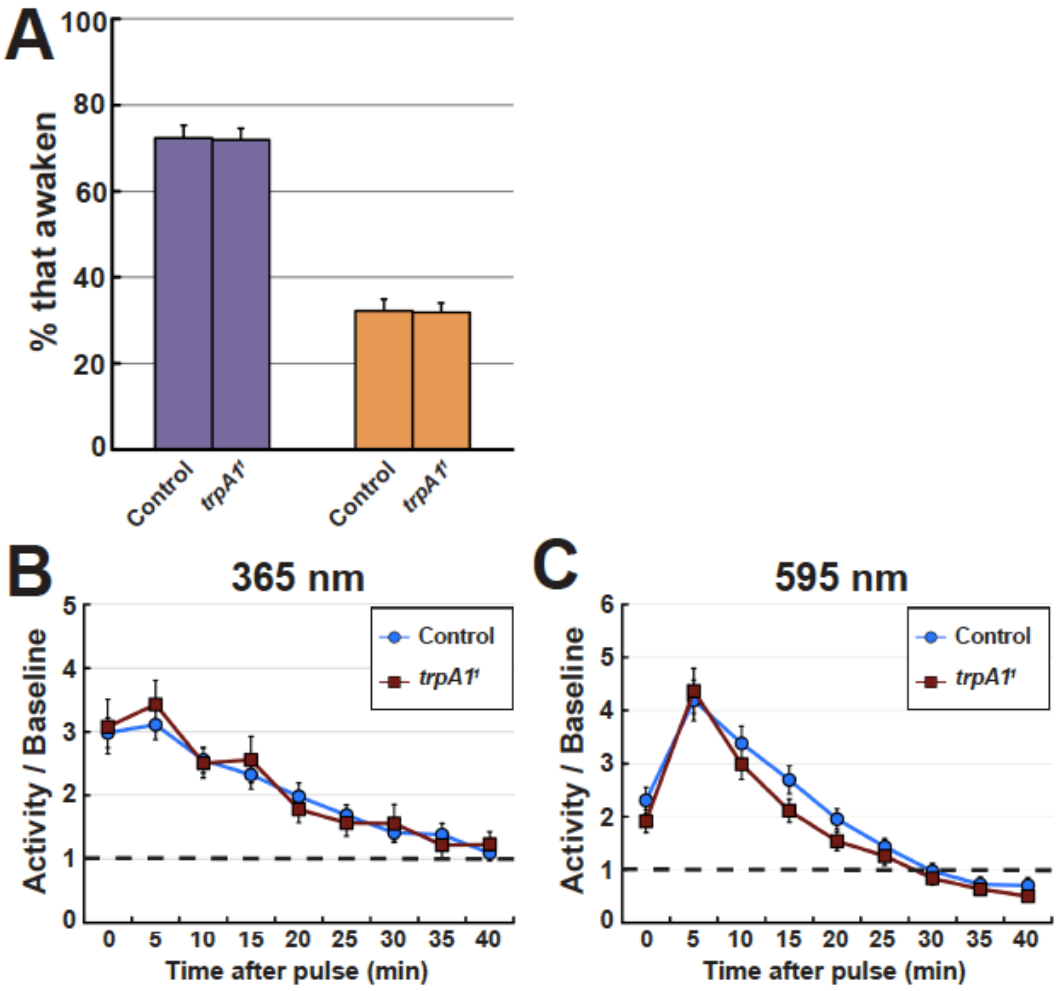

Figure 3–2 *Drosophila* CRY-mediated acute arousal response to UV light is not attenuated in mutants lacking *trpA1*.
(A) Percentage of sleeping flies which awaken during the 5-minute light pulse for UV (365nm, 3 mW/cm^2) and orange light (595nm, 7 mW/cm^2). Percent of sleeping *trpA1*^{1} flies that awaken in response to UV (0.72 ± 0.03, n=192 flies, p=0.928 vs. control) or orange (0.32 ± 0.02, n=192 flies, p=0.919 vs. control) light does not differ from that of control flies. **(B)** Time course of activity of awake control and *trpA1*^{1} flies during and after UV light pulse does not differ from each other (all p>.481 vs. control). **(C)** Time course of activity of awake control and *trpA1*^{1} flies during and after orange light pulse does not differ from each other (all p>.115 vs. control).

3.3.2 CRY mediates executive choice attributes of positive phototaxis and avoidance behaviors to different intensities of UV light.

Positive phototaxis behavior of adult male flies in response to very low intensity UV light (3 μW/cm^2 365 nm LED, 5-minutes per exposure) was measured using a retrofitted Trikinetics DAM5 *Drosophila* Activity Monitor attached to a light-tight chamber holding a population of 40 flies (Fig. 3-3A). Positive phototaxis is measured by increased activity levels (counts/min) from flies migrating to the light-transparent activity monitor in the front (Fig. 3-3 A). Wild-type control flies show robust attraction in response to 5-minute pulses of very low intensity UV light (Fig. 3-3 B and D). Positive phototaxis to UV light is significantly attenuated in $cry^{-/-}$ flies compared to control flies (Fig. 3-3 B and D). Interestingly, control flies choose to linger in the previously light-exposed region following the light-pulse long after the light has been turned off, compared to $cry^{-/-}$ flies which leave the previously light-exposed region quickly (Fig. 3-3 D). This suggests that CRY potentially mediates aspects of executive choice, specifically in choosing to linger in a previously light-exposed region, in addition to simple acute sensory function (Fig. 3-3 B and D). Both $norpA^{P24}$ and gl^{60j} flies show little attraction towards very low intensity UV light (Fig. 3-3 B and F). Thus, external photoreceptors have a primary acute sensory role for UV phototaxis while CRY modulates the magnitude and duration of the response. $trpA1^{1}$ mutant flies do not exhibit attenuated positive phototaxis in response to 5-minute pulses of very low intensity UV light, but surprisingly show significantly higher positive phototaxis compared to control flies (Fig. 3-4 A and C). Orange light (3 μW/cm^2 595nm LED, 5-minutes per exposure) fails to evoke strong positive phototaxis (Fig. 3-3 C, E, G).

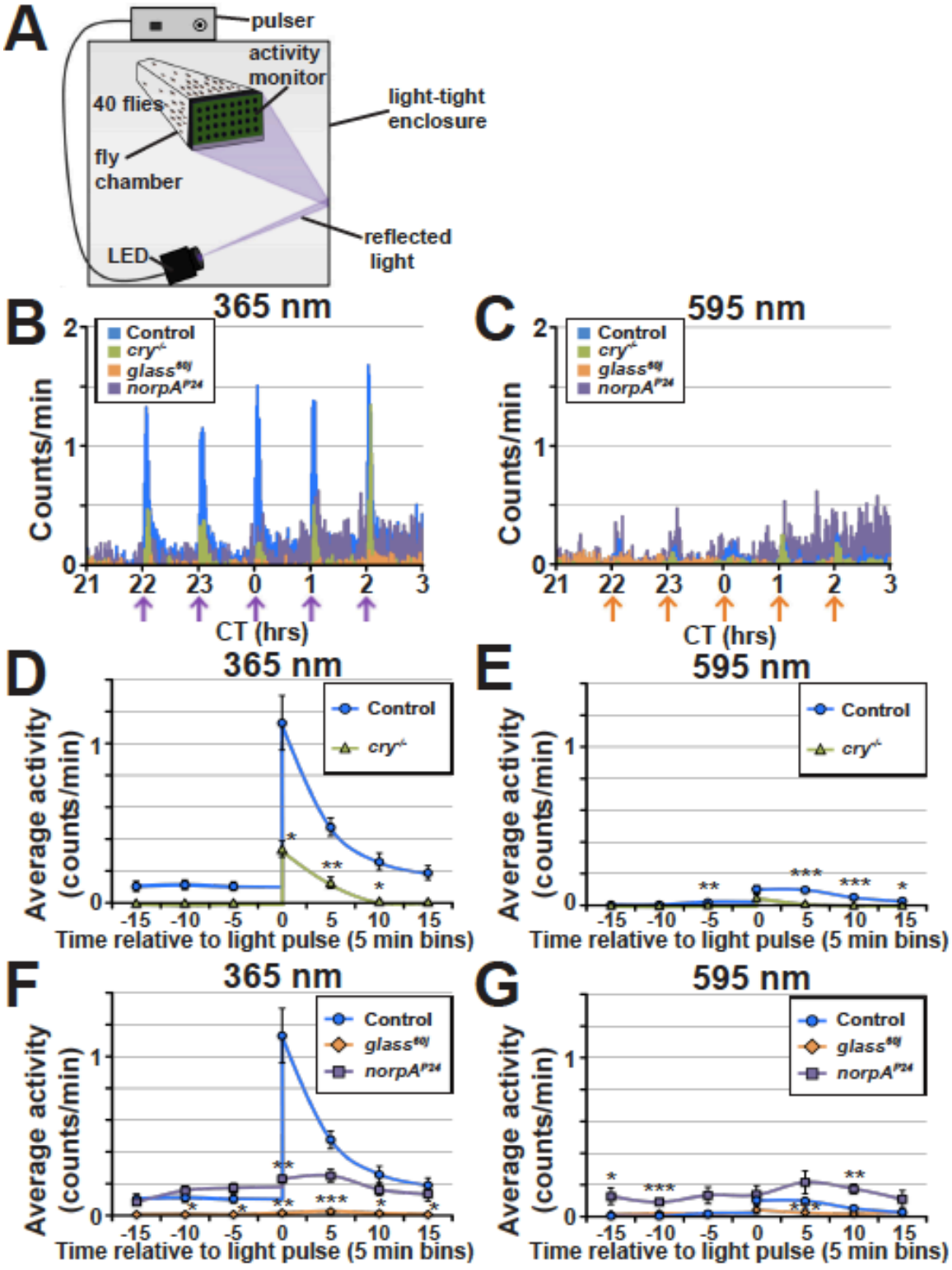

Figure 3–3 Drosophila positive phototaxis behavior towards UV-light is attenuated in mutants lacking CRY- and in mutants lacking external photoreceptors.
(A) A DAM2 *Drosophila* Activity Monitor (32 channels with dual infrared beams, Trikinetics) was mounted to the front of the light-tight chamber holding a population of 40 flies, and sealed

with a glass cover on the outer face. **(B)** Average phototaxis activity counts per minute towards a very low intensity UV light pulse (365 nm, 3 µW/cm2, five exposures of 5-minute light indicated by violet arrows) followed by 55-minute of darkness starting at CT 21 to CT 3 for control (9 experimental repeats, n=40 flies/experiment), *cry*$^{-/-}$ (3 experimental repeats, n=40 flies/experiment), *norpA*^{P24} (4 experimental repeats, n=40 flies/experiment) and *gl*^{60j} flies (4 experimental repeats, n=40 flies/experiment). **(C)** Average phototaxis activity counts per minute towards five 5-minute orange light pulse (595 nm, 3 µW/cm^2, indicated by orange arrows) followed by 55-minute of darkness starting at CT 21 to CT 3 for control (4 experimental repeats, n=40 flies/experiment), *cry*$^{-/-}$ (5 experimental repeats, n=40 flies/experiment), *norpA*^{P24} (3 experimental repeats, n=40 flies/experiment), and *gl*^{60j} flies (6 experimental repeats, n=40 flies/experiment). **(D-G)** Average phototaxis activity in 5-minute bins relative to the UV **(D and F)** or orange **(E and G)** light pulses averaged from panels **(B-C)**.

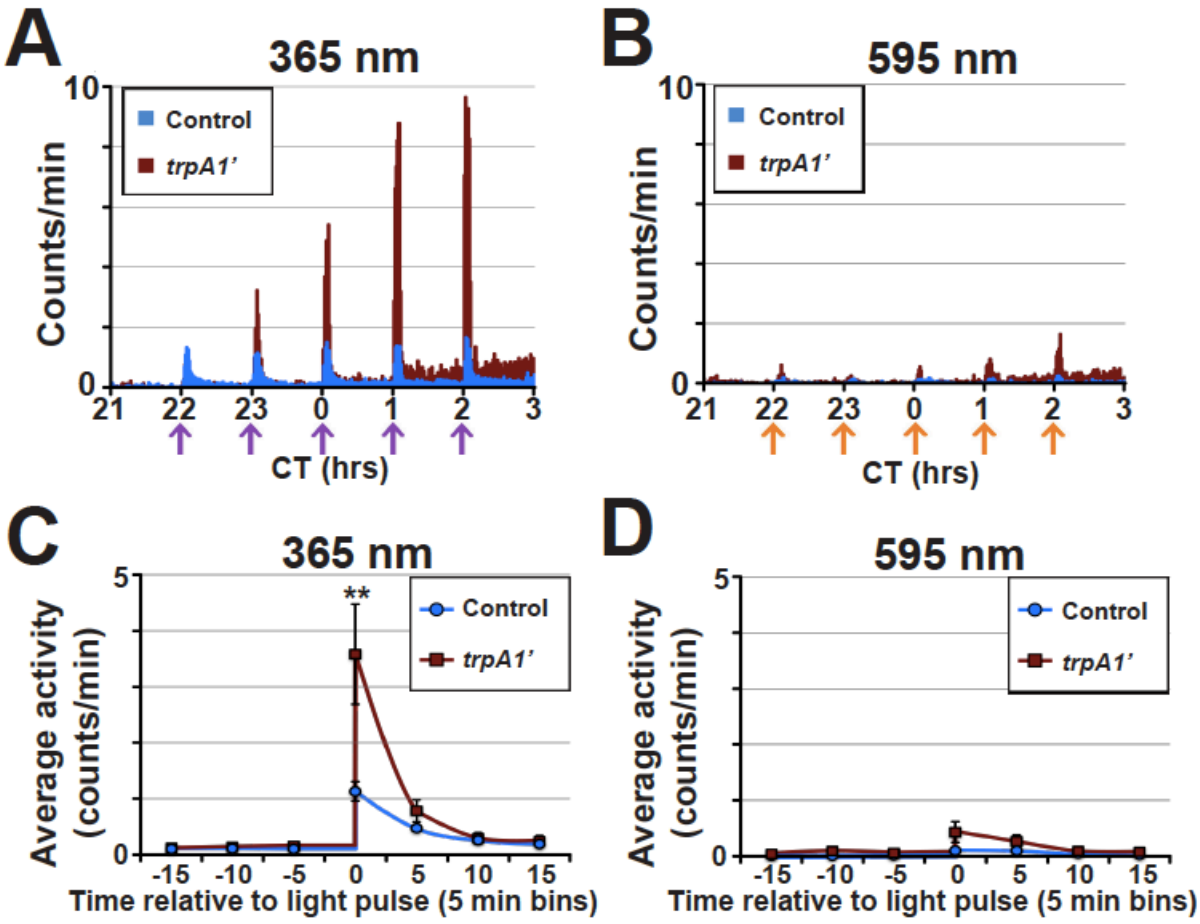

Figure 3–4 *Drosophila* positive phototaxis behavior towards UV-light is not attenuated in mutants lacking *trpA1*.

(A) Average phototaxis activity counts per minute towards a very low intensity, five 5-minute UV light pulses (365 nm, 3 µW/cm^2, indicated by violet arrows) followed by 55 minute of darkness starting at CT 21 to CT 3 for control (9 experimental repeats, n=40 flies/experiment) and *trpA1*^{1} flies (4 experimental repeats, n=40 flies/experiment). **(B)** Average phototaxis activity counts per minute towards a very low, five 5-minute orange light pulse (595 nm, 3 µW/cm^2, indicated by orange arrows) followed by 55 minute of darkness starting at CT 21 to CT 3 for

control (4 experimental repeats, n=40 flies/experiment) and *trpA1*^{1} flies (3 experimental repeats, n=40 flies/experiment). **(C-D)** Average phototaxis activity in 5-minute bins relative to the UV **(C)** or orange **(D)** light pulses averaged from panels **(A-B)**.

3.3.3 CRY mediates attraction/avoidance behavior to UV light

CRY potentially contributes to executive choice evoked by UV light. To test this directly, I measured behavioral avoidance responses to high intensity UV light (400 μW/cm^2). A modified Trikinetics *Drosophila* Activity Monitor system was fitted for long tubes with two infrared photobeams separated by 8.4 cm that measure locomotor activity at different zones for a single fly, with food and air holes placed equally on either side of the long tube to prevent food and air spatial preferences (Fig. 3-5A). Adult male flies were 12:12 LD-entrained in standard white light (3 days) followed by 12:12 LD entrainment in UV light (3 days). Then an opaque screen was placed covering one side of each long tube so that half the length of the tube was exposed to high intensity UV light while the other half of the tube was shaded thus blocking all direct UV light (Fig. 3-5 B). Each infrared photobeam on either side of the long tube allows us to measure the fly's choice of locomotor activity either in the zone of the tube exposed to high intensity UV light or in the zone of the tube shaded from direct UV light. To measure potential time-of-day effects, high intensity UV light was on for 12 hours matching the entrained daytime (ZT0-12) followed by all lights off (ZT 12-24). This presented flies with a choice between activity in the high intensity UV light exposed environment versus escape to the covered environment shaded from direct UV light at all times during daytime for 10 days (Fig. 3-5 B).

Control and *gl*^{60j} flies significantly avoid UV light and strongly prefer to be in the shaded environment, including during the mid-day (Fig. 3-5 C-E). The *gl*^{60j} flies are not as effective as control flies for UV avoidance but show the same pattern of avoidance. In contrast, *cry*$^{-/-}$ and *hk*$^{-/-}$ flies significantly prefer the high intensity UV light environment over the shaded environment

during the daytime, particularly during the early morning and all afternoon hours, and exhibit significantly attenuated avoidance behavior to high intensity UV light compared to controls at all times of day (Fig. 3-5 C and D). To control for potential olfactory cues deposited by flies during daytime activity, I analyzed for environmental preference for both sides of the monitor (ZT 12-24) when the UV light is off. No differences in preferences are detected between all four genotypes during subjective nighttime (Fig. 3-5 F). However, on an hour-by-hour basis, $cry^{-/-}$ and $hk^{-/-}$ flies show small but significant preferences for the covered side during all of the night (Fig. 3-6 B and C). Similarly, control and gl^{60j} flies show small but significant preferences for the covered side during half or nearly half of the night (Fig. 3-6 A and D). This nighttime activity might reflect residual olfactory cues left during daytime activity or differences in food quality on the covered side. Thus results show clearly that CRY and its downstream redox sensor HK mediate choice in avoidance behavior in response to high intensity UV light during day. This territorial preference does not extend in the absence of UV light.

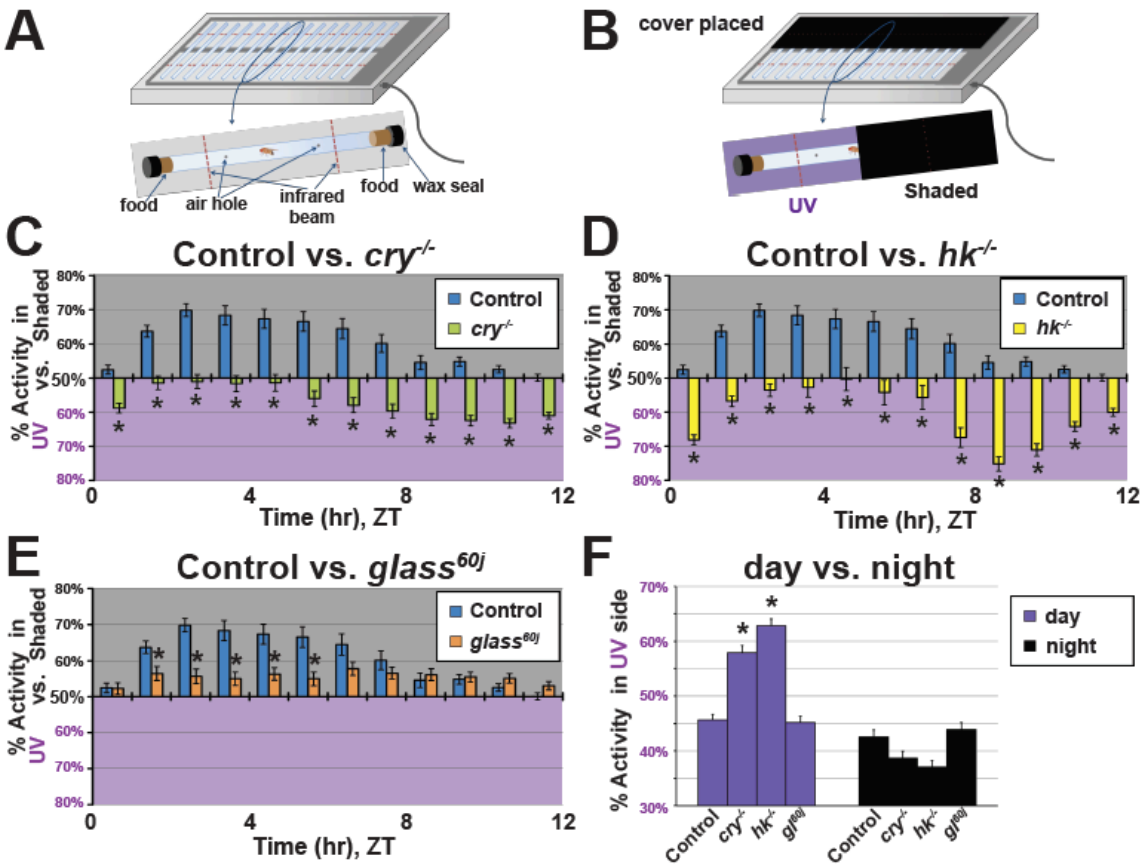

Figure 3–5 CRY-based phototransduction contributes to UV light avoidance behavior in *Drosophila*.

(A) Diagram of the "light choice" apparatus. Standard Trikinetics *Drosophila* activity monitors were modified to fit behavior tubes of 2x length, which has food and air hole on both sides of the tube. Flies are entrained in standard 12:12 white light LD without any cover. 12:12 White light LD was then replaced by 12:12 UV light LD. **(B)** Half of the monitor is then covered with cardboard to provide flies a choice between UV light exposed (400 μW/cm^2) and shaded environments. UV light was turned on only during the entrained daytime (ZT0-12). **(C-E)** Preference for UV-exposed vs. shaded environment is measured by percent of activity in each environment over total amount of activity for each ZT. Gray-shade indicates shaded environment preference (light avoidance), and violet-shade indicates UV environment preference. **(C)** $cry^{-/-}$ flies have a significant defect UV light avoidance behavior at all times of the day compared to control flies, and prefer the UV environment over the shaded ($cry^{-/-}$, n=78 vs. control, n=76, all $p<0.05$) **(D)** Similarly, $hk^{-/-}$ flies also have a significant defect in UV light avoidance behavior at all times of the day compared to control flies, and prefer the UV environment over the shaded ($hk^{-/-}$, n=77 vs. control, all $p<0.05$). **(E)** Mutant flies lacking all opsin-based external photoreceptors (gl^{60j}) show significantly less UV avoidance compared to control flies only during the midday, ZT1-6 (gl^{60j}, n=76 vs. control, all $p<0.05$). **(F)** Average percent activity in UV-exposed environment during the day vs. night. $cry^{-/-}$ and $hk^{-/-}$ flies have significantly higher activity in the UV-exposed environment during the day compared to control flies ($p<0.05$). Daytime percent activity in UV-exposed environment of gl^{60j} flies does not significantly differ from control. Percent activities in UV-exposed environment for $cry^{-/-}$, $hk^{-/-}$, and gl^{60j} flies during the nighttime do not differ from control.

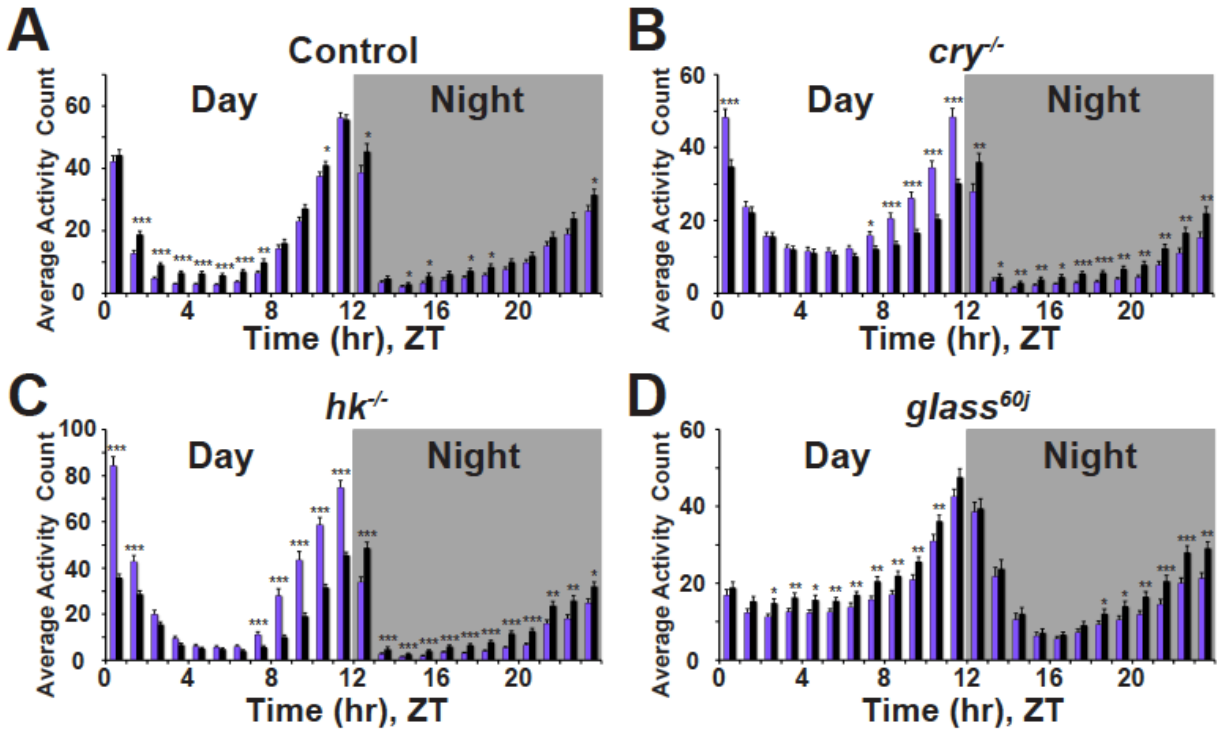

Figure 3–6 CRY-based phototransduction mediates *Drosophila* choice of light environment.
Average activity count over ZT time in the UV-exposed environment (365 nm, 400 μW/cm^2, violet bars) vs. the shaded environment (black bars) are shown. The UV light was on during the daytime (ZT0-12) and off during the nighttime (ZT12-24; shaded gray on the graphs) **(A)** Control flies prefer the shaded environment over the UV-exposed one during the mid-day (n=76) **(B)** cry$^{-/-}$ flies (n=78) and **(C)** *hk*$^{-/-}$ flies both lack the preference for shaded environment in the mid-day, and prefer the UV-exposed environment at other times of the day. (n=77) **(D)** *gl*^{60j} flies choose the shaded environment over the UV-exposed one during the mid-day (n=76), similar to control flies.

3.3.4 The circadian clock modulates both the valence and the time-of-day dependent changes of UV light avoidance/attraction behavior.

To test the hypothesis that adult UV light avoidance behavior is circadian regulated, I measured this behavior in mutant flies lacking core circadian genes. Control *w*^{1118} flies show normal entrainment in standard 12h:12h UV light:dark (LD) followed by sustained rhythmic activity in constant darkness (DD) (Fig 3-7 A and 1D, and Fig. 3-8 A and E). Circadian clock mutants lack rhythmic behavior in DD (45) (Fig. 3-7 B-D, Fig. 3-8, and Fig. 3-9 A, 2D). Clock

gene null mutants tim^0, clk^{OUT}, and per^0 (all in the w^{1118} genetic background) have defects in DD rhythms, entrainment, and LD morning/evening anticipation behavior (Figs 3-7 B-D, 2A, 2D, and Fig 3-8 B-D and F-H). These features of anticipatory behavior and rhythmicity of locomotor activity in DD are indicators of a functional clock. Behavioral UV light avoidance in adult control flies is absent in the beginning of the morning, steadily rises and peaks during the midday, and gradually decreases approaching simulated "dusk" (ZT9-12) (Fig 3-7 E; (6)) in response to constant UV light levels throughout the 12hr day. Clock gene null mutants tim^0, clk^{OUT}, and per^0 flies all show defective time-of-day dependent modulation UV avoidance exhibited by controls (Figs 3-7 E-F and 3-9 E-F). Circadian mutant tim^0 flies not only fail to show the midday UV avoidance peak, but also shift their behavioral valence to strong attraction to the UV light-exposed environment over the shaded environment during the first hour of the day, then subsequently show mostly weak attraction to the UV light-exposed environment after ZT1 (Fig 3-7 E). Mutant clk^{OUT} and per^0 flies exhibit an even stronger valence shift to UV light attraction throughout the entire day along with defective time-of-day dependent modulation of UV avoidance compared to wild-type controls (Figs 3-7 F and 3-9 E-F).

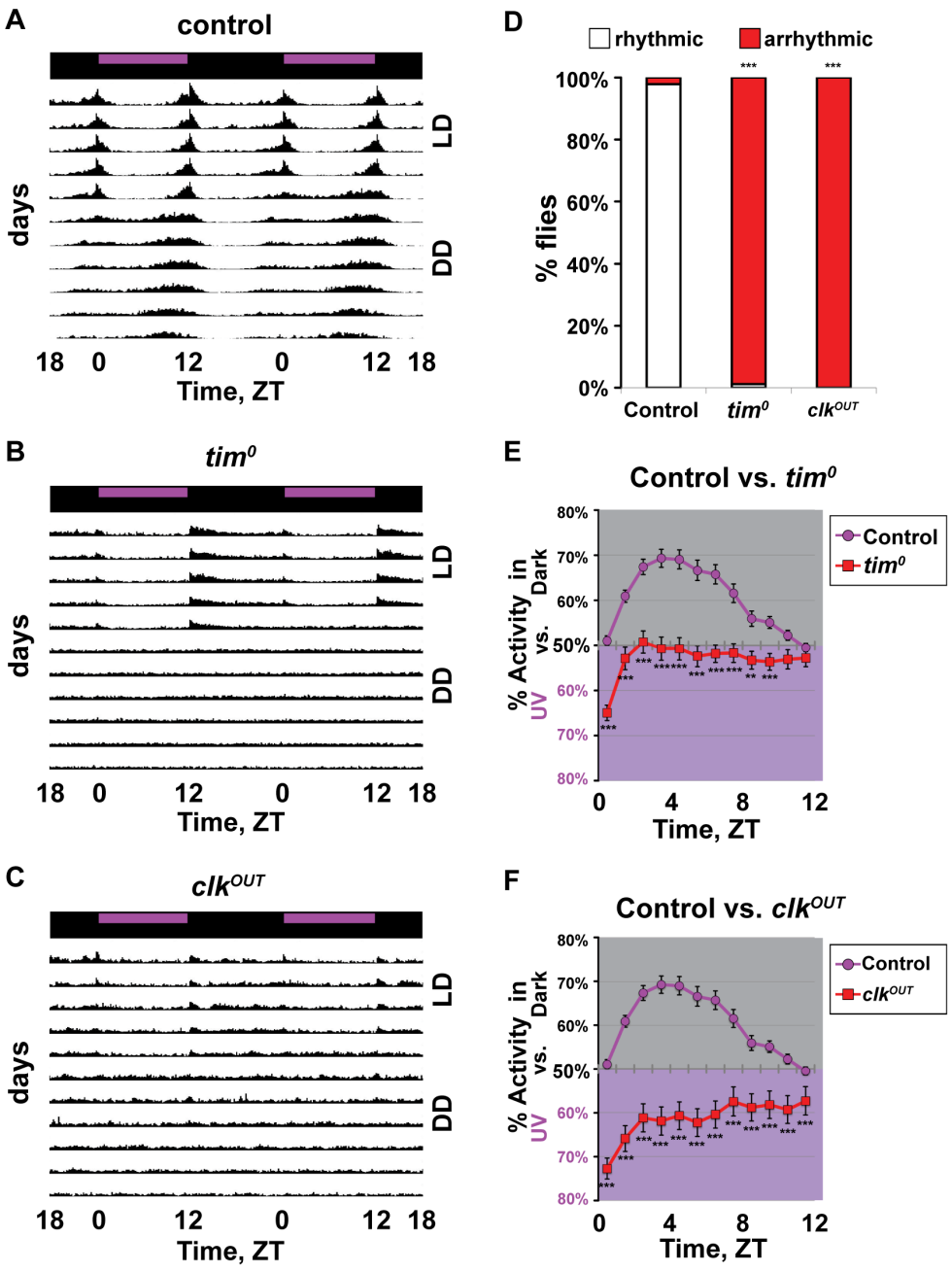

Figure 3–7 Circadian mutants have defective timing of UV light avoidance behavior. **(A-C)** Representative double plotted actogram in standard 12h:12h UV (365 nm, 400 μW/cm^2) light: dark (LD) followed by constant dark condition (DD). **(A)** Control (w^{1118}; n= 32 flies) flies have normal entrainment in LD and maintains rhythmicity in DD condition. Circadian mutants **(B)** tim^0 (n= 30 flies) and **(C)** clk^{OUT} (n= 30 flies) on the other hand show defective entrainment in LD and are arrhythmic in DD. **(D)** Percentages of rhythmic and arrhythmic flies in DD (control, n=95 flies; tim^0, n=89 flies; clk^{OUT}, n=60 flies). **(E-F)** UV avoidance behavior measured by preference for shaded environment vs. UV-exposed environment (365 nm, 400 μW/cm^2) calculated by percent of activity in each environment over total activity for each ZT. **(E)** tim^0 flies (n= 71 flies) show significant attenuation of avoidance and defective time-of-day dependent modulation of UV avoidance. **(F)** clk^{OUT} flies (n= 66 flies) show a significant valence shift from

UV avoidance to strong UV attraction and defective time-of-day dependent modulation of UV avoidance. Data are represented as mean ± S.E.M. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. control.

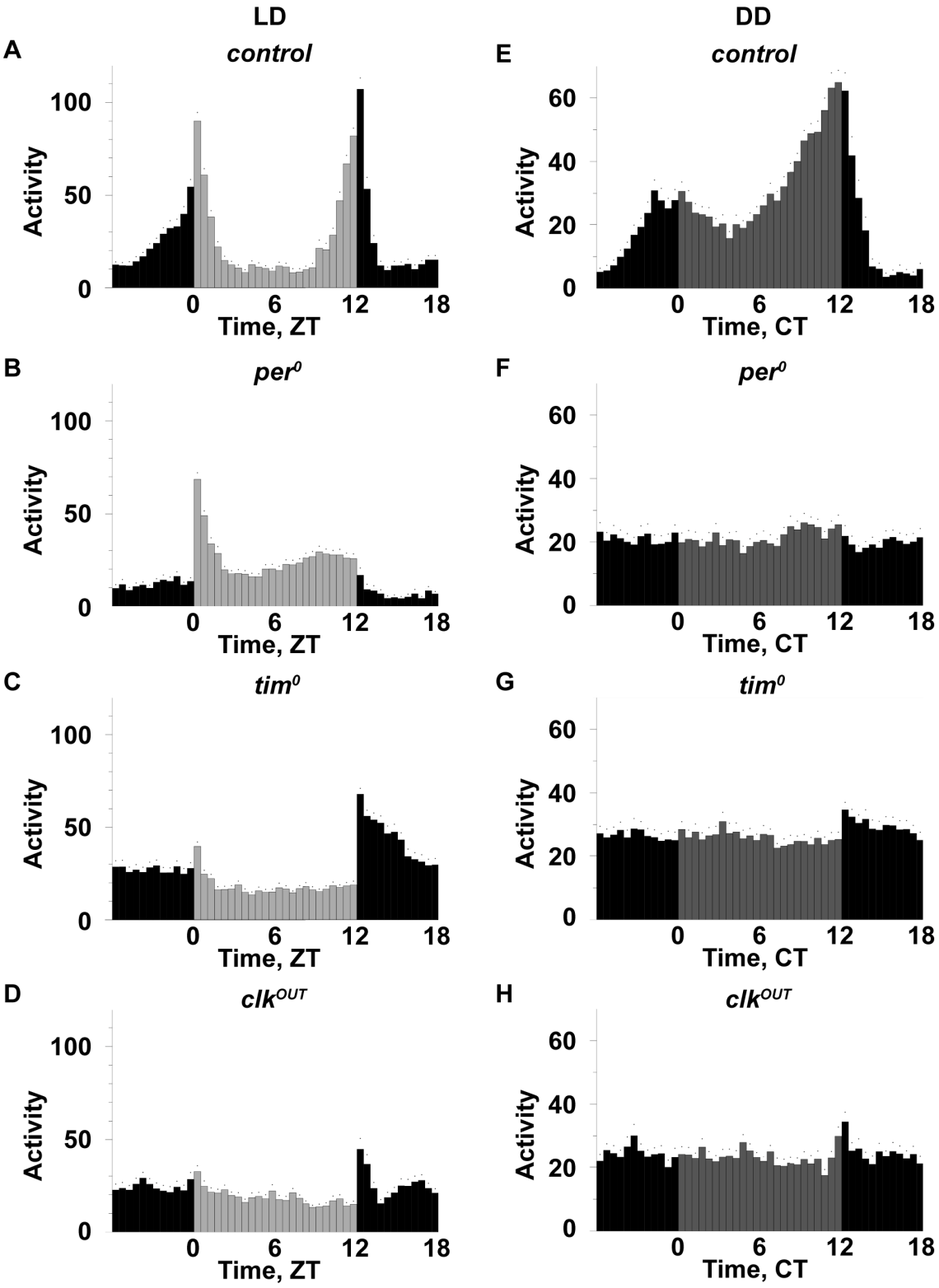

Figure 3–8 Circadian mutants have disrupted entrainment to UV light.
(A-D) Representative average activity plot in standard UV (365 nm, 400 μW/cm^2) light: dark 12:12 LD (5 days). **(A)** Control (n= 32 flies) flies entrain to UV light LD, but **(B)** *per*^{0} (n= 30 flies), **(C)** *tim*^{0} (n= 30 flies), and **(D)** *clk*^{OUT} (n= 30 flies) have defective entrainment in LD. (E-

H) Average activity plot in constant darkness (DD) (5 days) that followed UV LD. **(E)** control (n= 32 flies) maintain rhythmicity, but **(F)** *per*^{0} (n= 30 flies), (G) *tim*^{0} (n= 30 flies), and (H) *clk*^{OUT} (n= 30 flies) flies are arrhythmic in DD.

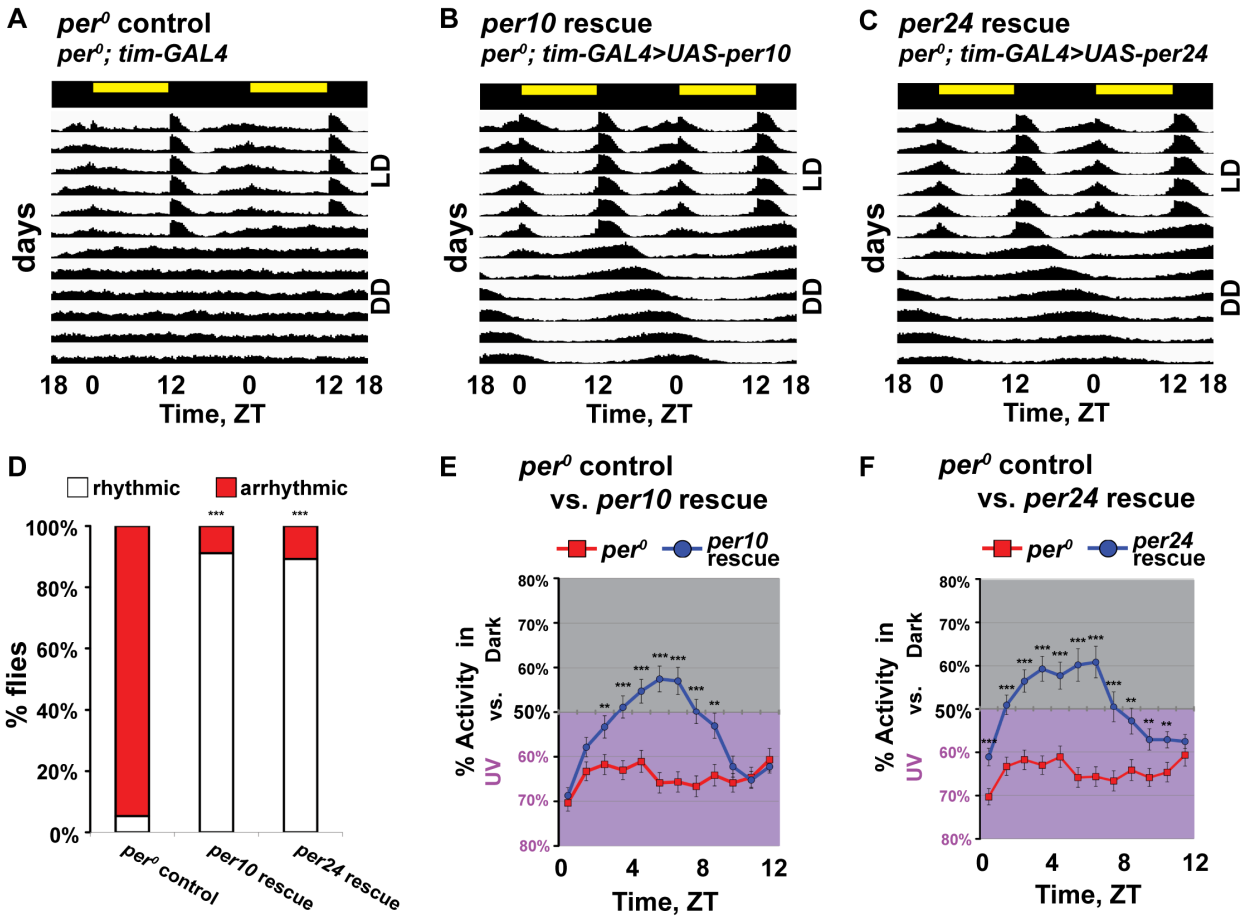

Figure 3–9 Period expression rescues timing of UV light avoidance behavior.
(A-C) Representative double plotted actogram in standard 12h:12h white light: dark (LD) followed by constant dark condition (DD). **(A)** *Period-null* (driver-only) control (*per*^{0}; *tim62-GAL4;* n= 32 flies) flies have defective entrainment in LD and are arrhythmic in DD. Flies with *tim-GAL4* driven expression of *per* in *per-null* genetic background, **(B)** per10 rescue (*per*^{0}; *tim62-GAL4 / UAS-per10*; n=32 flies) and **(C)** per24 rescue (*per*^{0}; *tim62-GAL4 / UAS-per24*; n=32 flies) show normal entrainment and maintains rhythmicity in DD. **(D)** Percentages of rhythmic and arrhythmic flies in DD (*Period-null* driver-only control, n=96 flies; per10 rescue, n=160 flies; per24 rescue, n=160 flies). **(E-F)** UV avoidance behavior measured by preference for shaded environment vs. UV-exposed environment (365 nm, 400 μW/cm^{2}) calculated by percent of activity in each environment over total activity for each ZT. *Period-null* control flies (n=69 flies) show significant attenuation of avoidance and defective time-of-day dependent modulation of UV avoidance. **(E)** per10 rescue (*per*^{0}; *tim62-GAL4 / UAS-per10*; n=104 flies) and **(F)** per24 rescue (*per*^{0}; *tim62-GAL4 / UAS-per24*; n=61 flies) flies have midday peak and time-of-day dependent modulation of UV avoidance. Data are represented as mean ± S.E.M. *p < 0.05; **p < 0.01; ***p < 0.001 vs. *period*-null control.

To determine whether valence and midday peak of avoidance to UV light along with rhythmicity in DD can be rescued, *tim-GAL4* driven genetic rescue of *per* expression in *per-null* mutant background was tested for light environmental choice assay. I confirmed that *tim-GAL4* driven expression of either of two *UAS-per* lines (*UAS-per10* or *UAS-per24*) in the per^0 genetic background rescues normal LD entrainment and rhythmicity in DD (Fig 3-9 B-D). Unlike per^0 driver-only negative control flies, flies with genetic rescue of *per10* or *per24* exhibit time-of-day dependent modulation of UV light avoidance/attraction behavior, including a midday peak of UV light avoidance followed by a gradual decrease approaching simulated "dusk" (ZT9-12) (Fig 3-9 E-F). The valence for UV avoidance is rescued in the midday but does not fully rescue to the wild-type control level (Figs 3-7 and 3-9 E-F). Together, our results show that the molecular circadian clock modulates time-of-day dependent changes of UV light avoidance/attraction behavior to elicit a peak of UV avoidance in the midday.

3.3.5 Constant light-induced disruption of the circadian clock abolishes time-of-day dependent changes of UV light avoidance behavior and reveals that CRY and HK regulate the valence of UV light avoidance and attraction.

Constant light (LL) disrupts the circadian clock in many wild-type animals (88, 89). Mutant $cry^{-/-}$ flies' locomotor activity remain rhythmic in LL (Fig 3-11) (47, 48). Thus, exposure to LL provides an environmental means to render the clock arrhythmic without the use of genetic mutants. I tested wild-type flies for UV light (365 nm, 400 μW/cm^2) avoidance/attraction under LL using the light choice assay(6), along with LL-exposed mutant flies that lack molecular and structural components of light input pathways ($cry^{-/-}$, $hk^{-/-}$, and $glass^{60j}$). All flies tested share the w^{1118} genetic background. In the circadian-disrupting LL condition, all flies completely lack time-of-day dependent changes in UV avoidance/attraction behavior (Fig 3-10). Control wild-

type flies exposed to LL lack not only time-of-day dependent modulation of UV light avoidance and show no preference or weak preference for shade throughout the day as compared to the integrated activity under LD conditions for which the circadian clock is intact (Fig 3-10A). Similarly, *glass*^{60j} flies lack time-of-day dependent modulation of UV light avoidance throughout the daytime and show weak preference of shade throughout the day with no significant differences from control at any time point tested (Fig 3-10B). In contrast, mutant *cry*$^{-/-}$ flies lack time-of-day dependent modulation and show significantly greater preference than the control flies for the high intensity UV-exposed environment at all times tested, consistent with their loss of avoidance in standard LD light choice assay (Fig 3-10C, and Fig 3-12A). Mutant *hk*$^{-/-}$ flies also show significantly greater preference than the control flies to UV light at all times (Fig 3-10 D) in contrast to neutral to slight UV avoidance exhibited by control and *glass*^{60j} flies (Fig 3-10 A-B). The steady value of avoidance/attraction seen for each genotype under LL resembles its trough of avoidance/attraction oscillation seen in LD. The LL-evoked clock disruption eliminates the circadian "filter" that underlies time-of-day dependent modulation of UV light avoidance behavior. When the circadian system is disrupted environmentally via constant light exposure, *cry*$^{-/-}$ and *hk*$^{-/-}$ mutants show constant level of attraction to UV light-exposed environment at all times of day. This suggests an attraction/avoidance valence that is dependent on CRY/HK, and independent of the circadian function. Together these results suggest the surprising finding that the clock itself by multiple measurements contributes to UV light avoidance/attraction valence and reveals the inhibitory signal produced by the UV light activated CRY/HK pathway that alters the valence between light-evoked avoidance and attraction.

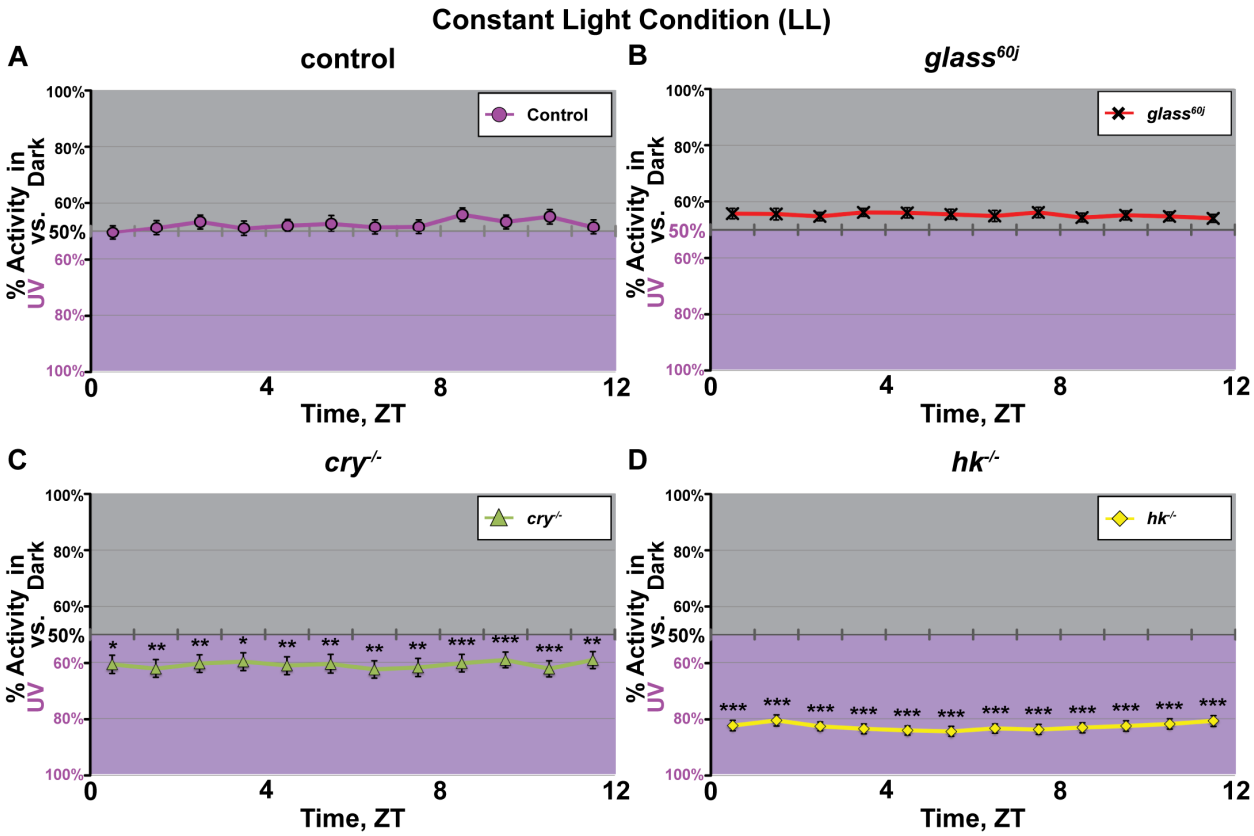

Figure 3–10 Constant light condition induces valence shifts and eliminates time-of-day dependent modulation of UV light avoidance/attraction behavior.

(A-D) UV avoidance/attraction behavior measured by preference for shaded environment vs. UV-exposed environment (365 nm, 400 μW/cm^2,) calculated by percent of activity in each environment over total activity for each circadian time (CT) in constant light condition (LL). All genotypes tested show valence shifts from UV light avoidance to neutrality or attraction, lack "midday peak" of avoidance behavior, and show no time-dependent modulation in the degree of avoidance/attraction at any time of the day. **(A)** Control (w^{1118}; n= 45 flies) and **(B)** $glass^{60j}$ (n= 46 flies) flies show neutrality or slight avoidance, slightly preferring the shaded environment over the UV-exposed environment (all are not significant compared to control). In comparison, **(C)** $cry^{-/-}$ (n= 43 flies) and **(D)** $hk^{-/-}$ (n= 46 flies) flies exhibit strong attraction to the UV-exposed environment at all times of the day. Data are represented as mean ± S.E.M. *p < 0.05; **p < 0.01; ***p < 0.001 vs. w^{1118} control.

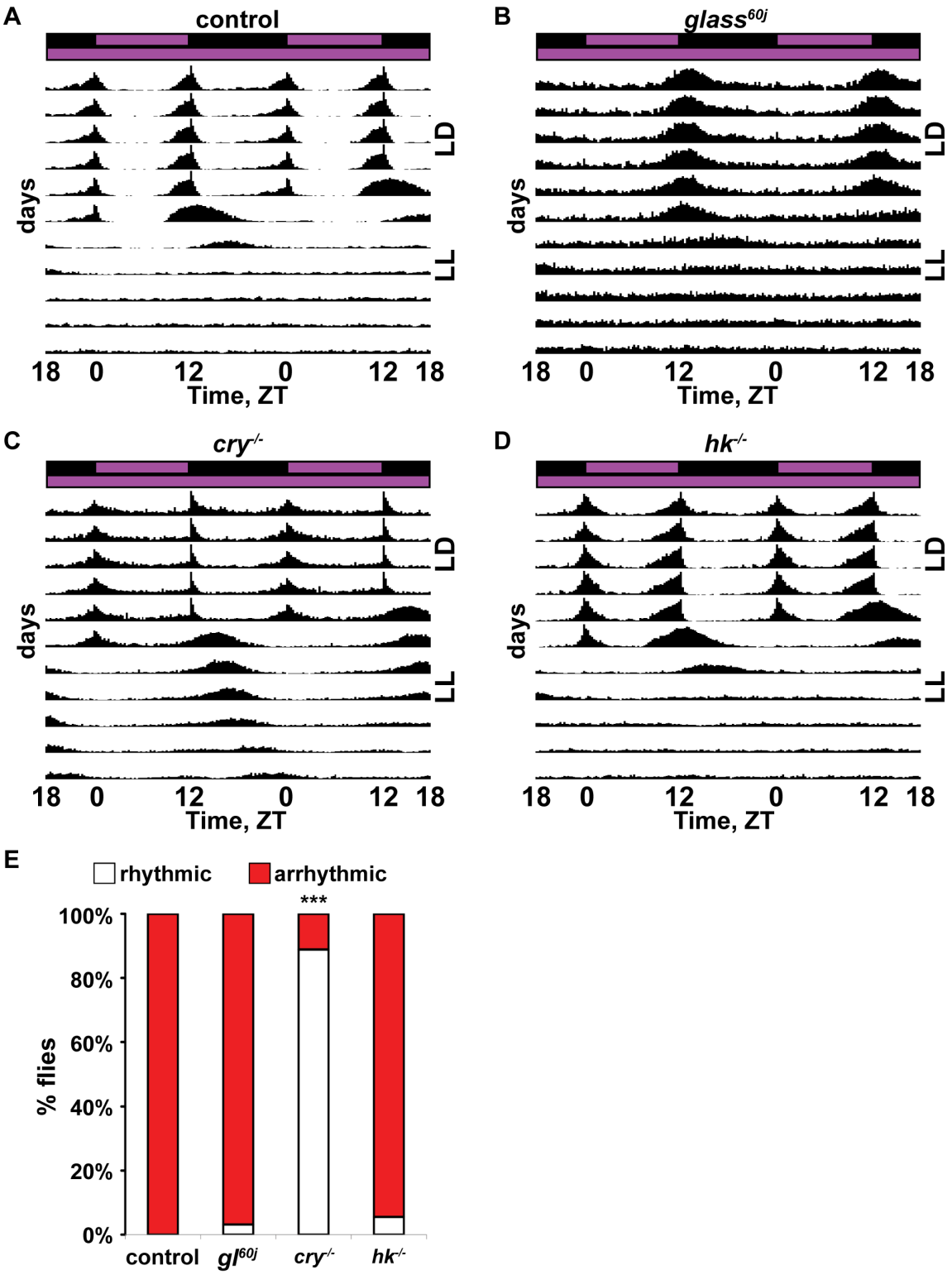

Figure 3–11 Constant UV light condition disrupts locomotor activity rhythm.
(A-C) Representative double plotted locomotor actogram in 5 days of standard 12h:12h UV (365 nm, 400 μW/cm^2) light: dark (LD) followed by 6 days of constant UV light condition (LL). **(A)** Control (w^{1118}; n= 47 flies) flies have normal entrainment in LD and becomes arrhythmic in LL.

(B) *glass*^{60j} (n= 87 flies) also becomes arrhythmic in LL. **(C)** *cry*$^{-/-}$ (n= 87 flies) on the other hand maintain rhythmicity in UV LL. **(D)** *hk*$^{-/-}$ (n= 90 flies) become arrhythmic in LL.
(E) Percentages of rhythmic and arrhythmic flies in LL. Data are represented as mean ± S.E.M. ***p < 0.001 vs. control.

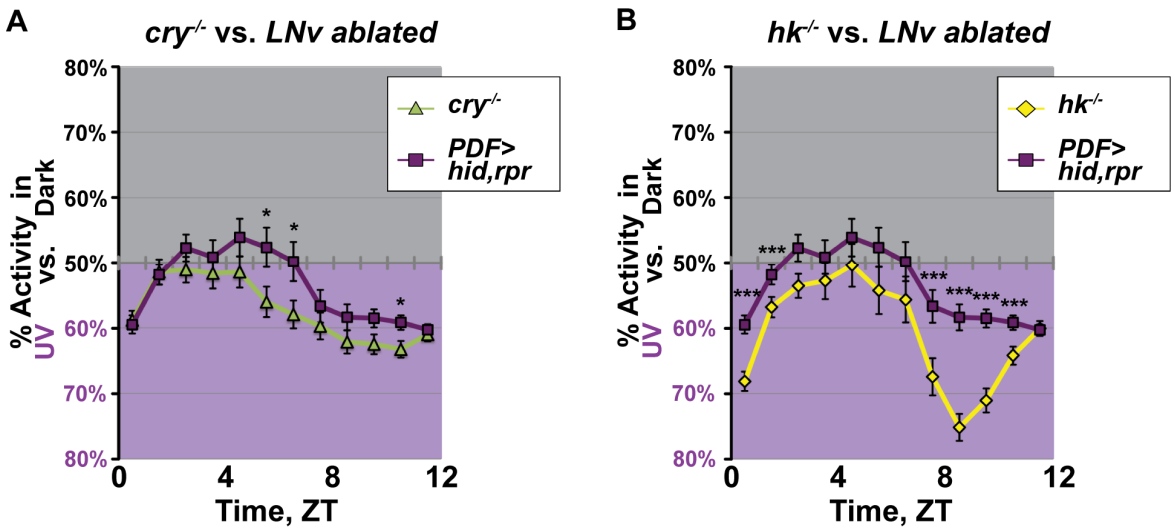

Figure 3–12 LNv -ablated or -silenced flies phenocopy the valence shift from UV light avoidance to attraction seen in *cry*$^{-/-}$ *and hk*$^{-/-}$ flies.
(A-B) UV avoidance behavior measured by preference for shaded environment vs. UV-exposed (365 nm, 400 μW/cm^2) calculated by percent of activity in each environment over total activity for each ZT. LNv ablated flies (*UAS-hid, rpr; pdf-GAL4-p12c*; n=76 flies) closely mimic the time-of-day dependent circadian modulation and valence of UV light avoidance behavior of **(A)** *cry*$^{-/-}$ (n=78, modified from Baik et al., 2017, *PNAS*) and **(B)** *hk*$^{-/-}$ (n=77, modified from Baik et al., 2017, *PNAS*) flies. Data are represented as mean ± S.E.M. *p < 0.05; ***p < 0.001.

3.3.6 LNv circadian neurons are necessary for normal UV avoidance behavior.

To test whether circadian lateral ventral neurons (LNv) are important for UV light-evoked avoidance behavior, I generated LNv ablated flies by transgenic *pdf-GAL4*-directed dual expression of the cell death genes *head involution defective* (*hid*) and *reaper*. Ablation of LNv neurons is confirmed by immunocytochemistry of whole adult fly brain as shown by the absence of PDF staining. PER staining shows specific absence of PER staining of LNv neurons, with the exception of the 5th small LNvs, which do not express PDF (Fig 3-13A (90)). PER also stains the lateral dorsal neurons (LNd) and dorsal neurons 1 and 3 (DN1, DN3) in both control and PDF-

expressing LNv ablated flies, demonstrating a PDF-positive LNv-specific circadian neuron ablation. I further confirmed the ablation of LNv neurons by behavioral analysis of disrupted afternoon and morning activity, phase advanced evening activity, and decreased anticipation (Fig 3-13 B-C, and Fig 3-14) (91-94)). The slight phase advance of evening activity in LNv ablated flies resembles the phase advance of evening activity seen in *pdf-null* flies (92). Further, LNv ablated flies show significantly higher midday activity/siesta disruption and lower average locomotor activity in both evening and morning relative to controls under 12h:12h UV LD (Fig 3-13B). Thus, the LNvs regulate midday behavior as well as evening and morning behavior (33, 91-94).

LNv ablated flies tested with the UV light choice assay show time-of-day dependent changes in avoidance/attraction (Fig 3-13 D, and Fig 3-14). Most striking, their UV light response shifts significantly from UV light avoidance to attraction compared to control flies (Fig 3-13D), which phenocopies the valence shift of UV light attraction seen in $cry^{-/-}$ and $hk^{-/-}$ flies (6). To test whether membrane excitability of LNvs is important for the timing and/or valence of UV light behavioral responses, I genetically attenuated membrane excitability of LNvs by expressing the *Drosophila* Open-Rectifier K^+ channel (dORK) specifically in LNv neurons using a *pdf-GAL4* driver (12, 95). Flies with electrically silenced-LNvs have light avoidance/attraction behavior that is qualitatively similar to that of LNv-ablated flies, as well as that of $cry^{-/-}$ and $hk^{-/-}$ flies (Fig 3-13 D-E, and Fig 3-14) as shown by the significant attenuation of UV-evoked avoidance that still peaks at midday (Fig 4 E). These results suggest that the LNv, like CRY and HK, contribute more to the regulation of the valence of the UV light response between avoidance and attraction than the time-of-day modulation of UV light avoidance/attraction. I conclude that

the LNv circadian neurons and their electrical activity modulate the valence of UV light choice response behavior in adult *Drosophila*.

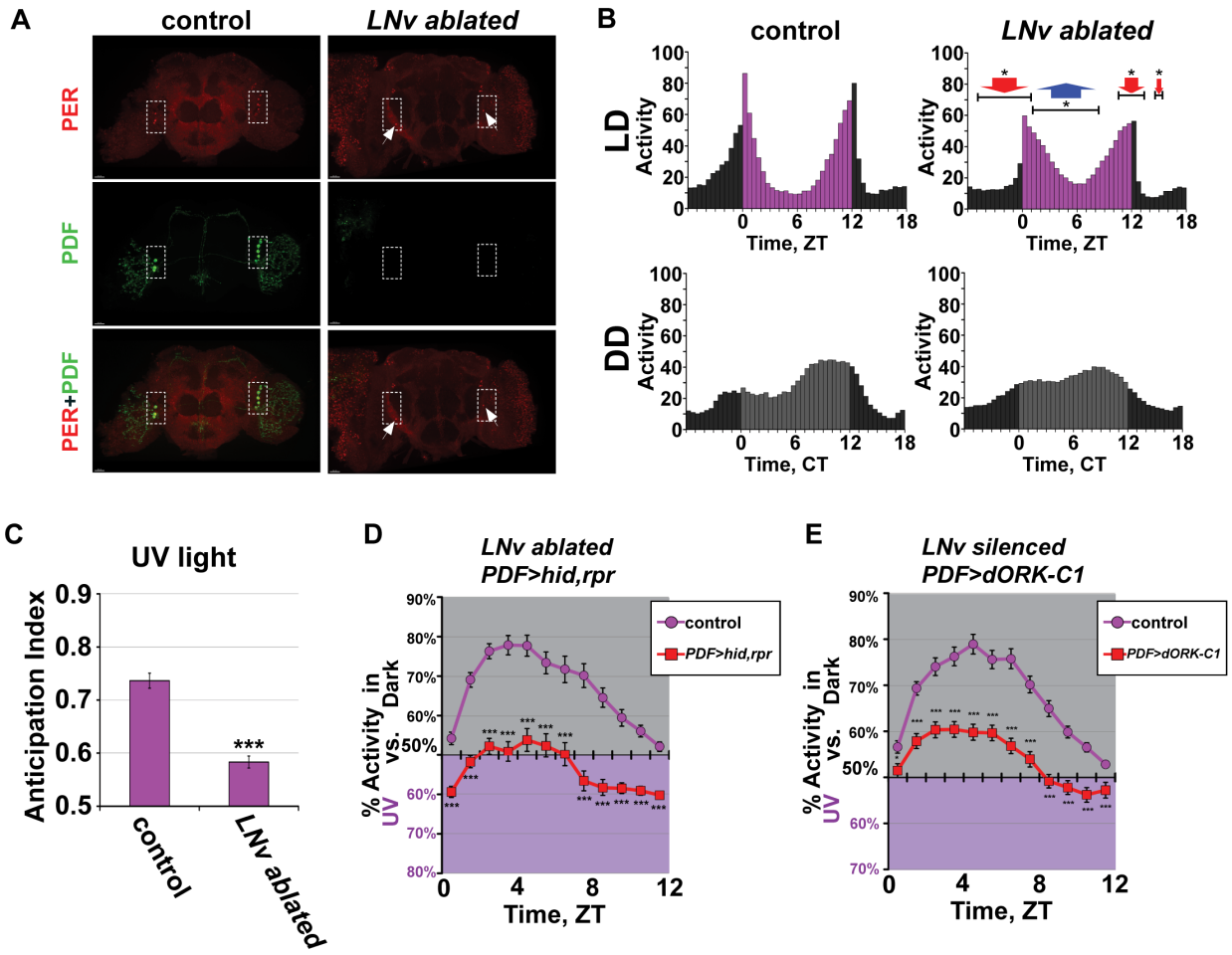

Figure 3–13 LNv circadian cells modulate the valence of UV light avoidance/attraction behavior but not timing.
(A) Control (*UAS-hid, rpr; +*; left panels) and LNv ablated (*UAS-hid, rpr; pdf-GAL4-p12c;* right panels) brains stained with anti-PER (red) and anti-PDF (green) shows successful ablation of PDF-positive LNv neurons (dashed white box) in the LNv ablated flies as evident by the lack of PDF staining, while PDF-negative 5th small-LNv is intact (arrow). Scale bar represents 30mm. **(B)** Average activity plot of control (w^{1118}; n= 96 flies) (left panels) and LNv ablated flies (*UAS-hid, rpr; pdf-GAL4-p12c;* n= 285 flies) (right panels) in 12h:12h UV (365 nm, 400 μW/cm^2) light: dark (LD) (top panels; 5 days) followed by constant darkness (DD) (bottom panels; 5 days). LNv ablated flies (right panels) have defective circadian activity profile in both UV LD and DD conditions (top panels, and bottom panels, respectively). Arrows represent significantly higher (blue arrow, *p<0.05) or significantly lower (red arrow, *p<0.05) average activity in LNv ablated flies compared to control in the represented bin(s) during LD. **(C)** Harrisingh morning anticipation index (ref. 45) for control (left) versus LNv ablated (*UAS-hid, rpr; pdf-GAL4-p12c;* right) during LD. LNv ablated flies have significantly lower morning anticipation compared to

control during UV (365 nm, 400 μW/cm^2) light LD (control, n=95 versus LNv ablated, n=280, ***p<0.001). **(D-E)** UV avoidance/attraction behavior measured by preference for shaded environment vs. UV-exposed (365 nm, 400 μW/cm^2) calculated by percent of activity in each environment over total activity for each ZT. Both **(D)** LNv ablated flies (*UAS-hid, rpr; pdf-GAL4-p12c*) and **(E)** LNv silenced flies (*w; pdf-GAL4; UAS-dORK-C1*) have significant defects in UV light avoidance behavior at all times of the day compared to control flies (n= 78 flies, control vs. n= 76 flies, LNv ablated; n= 110 flies, control vs. n= 92 flies, LNv silenced), but maintain time-of-day dependent pattern of modulation in avoidance behavior. Data are represented as mean ± S.E.M. *p < 0.05; ***p < 0.001 vs. control.

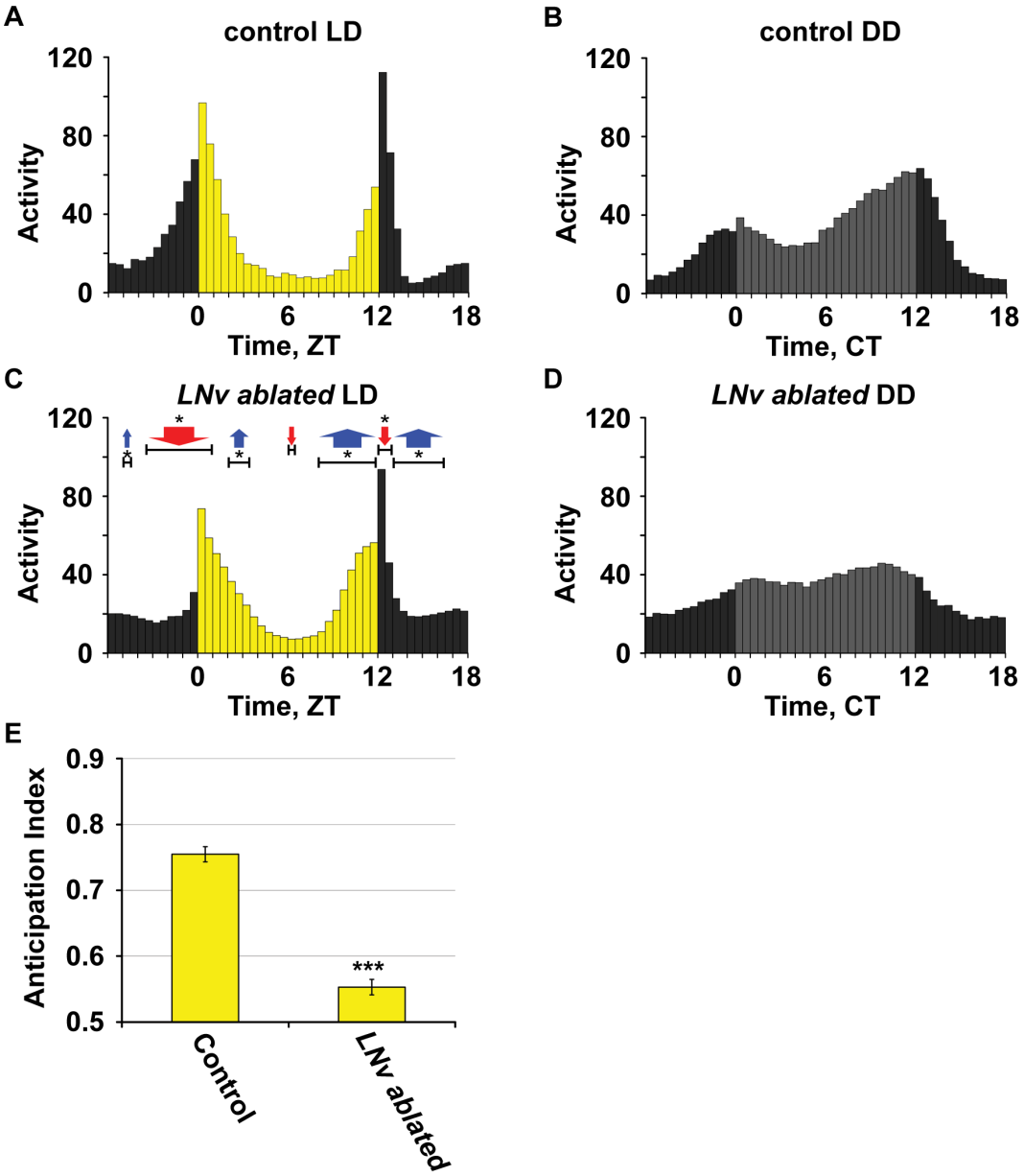

Figure 3–14 LNv ablated flies have defects in locomotor activity profile throughout the day.

(A-D) Average activity plot of control (n=96 flies) (top panels) and LNv ablated flies (*UAS-hid, rpr; pdf-GAL4-p12c;* n=256 flies) (bottom panels) in standard 12h:12h white light: dark (LD) (left panels; 5 days) followed by constant darkness (DD) (right panels; 5 days). Arrows represent significantly higher (blue arrow, *p<0.05) or significantly lower (red arrow, *p<0.05) average activity in LNv ablated flies compared to control in the represented bin(s) throughout the day during LD. Compared to **(A)** control flies (n=96 flies), **(C)** PDF+ (LNv) ablated flies (*UAS-hid, rpr; pdf-GAL4-p12c;* n=256 flies) show defective locomotor activity in LD. **(B, D)** Average activity plot in constant darkness (DD) (5 days) that followed LD. **(B)** Control and **(D)** LNv ablated flies both maintain rhythmicity in DD, but LNv ablated flies show defective locomotor activity in DD compared to control flies. **(E)** Harrisingh morning anticipation index for control (left) versus LNv ablated (*UAS-hid, rpr; pdf-GAL4-p12c;* right) during LD. LNv ablated flies have significantly lower morning anticipation compared to control during white light LD (control, n=64 versus LNv ablated, n=159, ***p<0.001). Data are represented as mean ± S.E.M. *p < 0.05; ***p < 0.001 vs. control.

3.3.7 The CRY/HK phototransduction pathway modulates the timing and valence of blue and orange light choice behavior.

Baseline FAD oxidized CRY exhibits a blue light excitation peak at 450 nm in addition to the UV light peak at 365 nm. CRY excitation ceases in the green light spectral range around 525 nm (7, 27, 29). Control flies tested for light choice in response to 450 nm blue light exhibit significant avoidance behavior that is qualitatively similar to UV light avoidance behavior (Fig 3-15 A-C). Mutant $cry^{-/-}$ null flies show a significant valence shift to blue light attraction that peaks towards the end of the day (Fig 3-15A). In contrast, $glass^{60j}$ mutant flies show mostly neutral behavior with very weak avoidance to blue light ($glass^{60j}$ flies express CRY) (Fig 3-15B). Control, $cry^{-/-}$, and $glass^{60j}$ flies tested for light choice in response to 595 nm orange light exhibit mostly neutral avoidance/attraction behavior with the exception of a few time points for $cry^{-/-}$, and $glass^{60j}$ flies which show very weak light avoidance / attraction to orange light (Fig 3-15C-D).

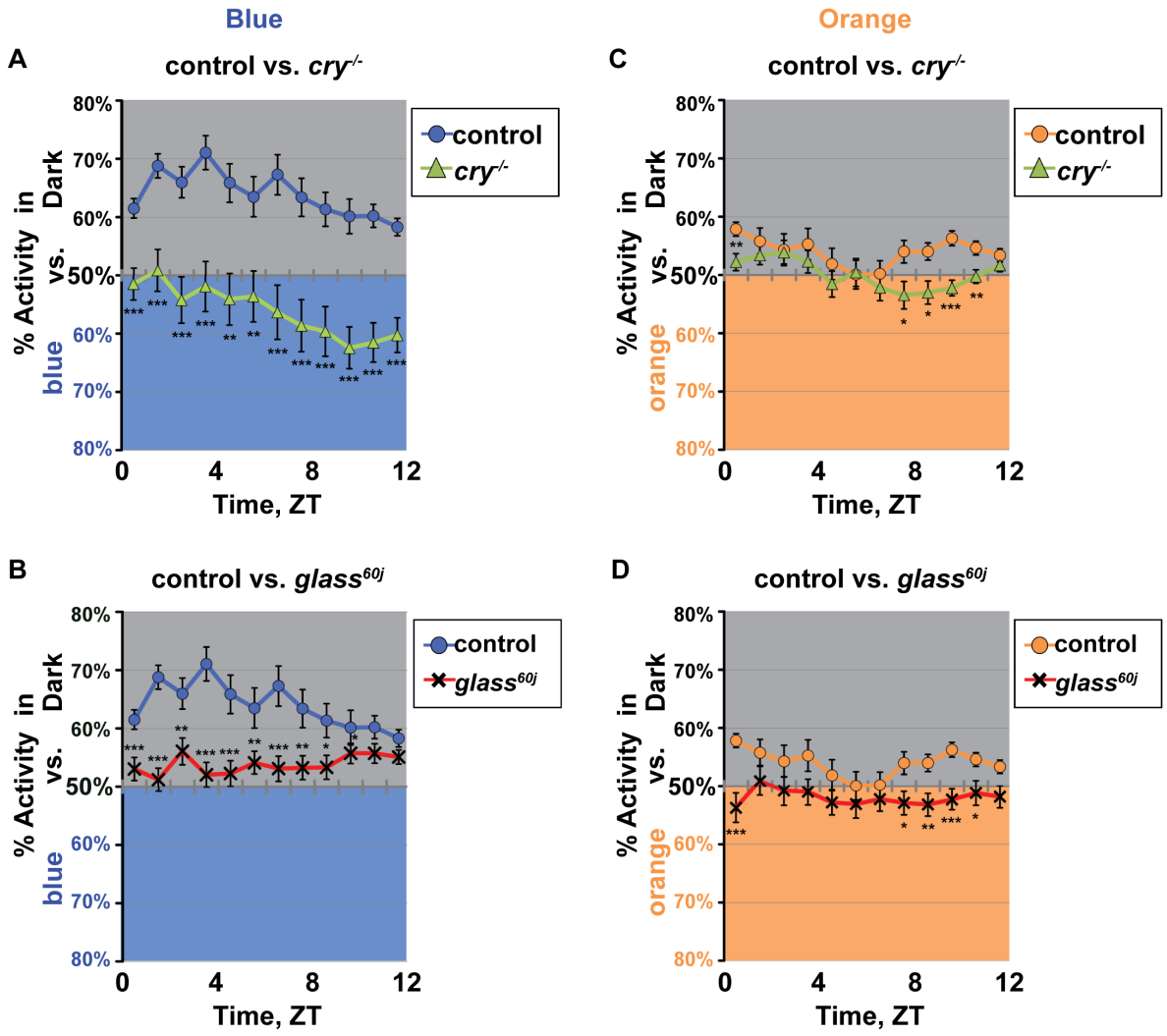

Figure 3–15 The CRY-mediated phototransduction modulates blue and orange light avoidance/ attraction behavior.

(A-B) Blue light avoidance behavior measured by preference by shaded environment vs. blue-light exposed environment (400 µW/cm^2, 460 nm) calculated by percent of activity in each environment over total activity for each ZT. **(A)** Control flies (w^{1118}; n= 75 flies) have significant avoidance to blue light with midday peak of avoidance. In contrast, null $cry^{-/-}$ flies (n=58 flies) show a significant valence shift from avoidance to attraction for blue light. **(B)** $glass^{60j}$ flies (n= 78 flies) avoid blue light at all times of the daytime, but have attenuated response relative to control. **(C-D)** Orange light avoidance behavior measured by preference by shaded environment vs. orange-light exposed environment (400 µW/cm^2, 595 nm) calculated by percent of activity in each environment over total activity for each ZT. **(C)** Control flies (n= 63 flies) and $cry^{-/-}$ flies (n= 64 flies) show overall neutral attraction/ avoidance responses to orange light, but $cry^{-/-}$ flies show small but significant valence shifts to attraction. **(D)** Similarly, $glass^{60j}$ flies (n= 62 flies) show overall neutral attraction / avoidance to orange light, but $glass^{60j}$ flies show small but significant valence shifts to attraction. Data are represented as mean ± S.E.M. *p < 0.05; **p < 0.01; ***p < 0.001 vs. control.

3.3.8 UV light-evoked attraction/avoidance behavioral response is intensity-dependent.

Previously reported avoidance to high intensity UV light was observed over 12 hr daytime at 1 hr resolution (Baik et al., 2018), while UV evoked positive phototaxis events occur within a few minutes (6). If UV light behavioral attraction/avoidance depends on the duration of UV exposure, 1-hr resolution binning could miss rapid positive phototaxis responses. To determine if flies exhibit rapid positive phototaxis responses at different intensities of UV light, I subjected adult flies to light choice assays collected and analyzed in 1 min bins. Wild-type control flies have little-to-no attraction to high intensity UV light (365 nm, 400 mW/cm^2) in the first few minutes of light exposure (Fig. 3-16). Mutant flies lacking all external opsin-based photoreceptors (*glass*^{60j} flies and *norpA*^{P24} flies) similarly lack clear positive phototaxis responses to high intensity UV light (Fig. 3-16 A, B). In contrast, mutant flies lacking blue/UV-specific CRY/HK-mediated (*cry*$^{-/-}$ and *hk*$^{-/-}$) or violet-peak Rh7-mediated (*rh7*^{1}) phototransduction show strong, fast kinetic attraction responses to high intensity UV light that slowly attenuate with time (Fig. 3-16 D-F, and Fig. 3-17 C).

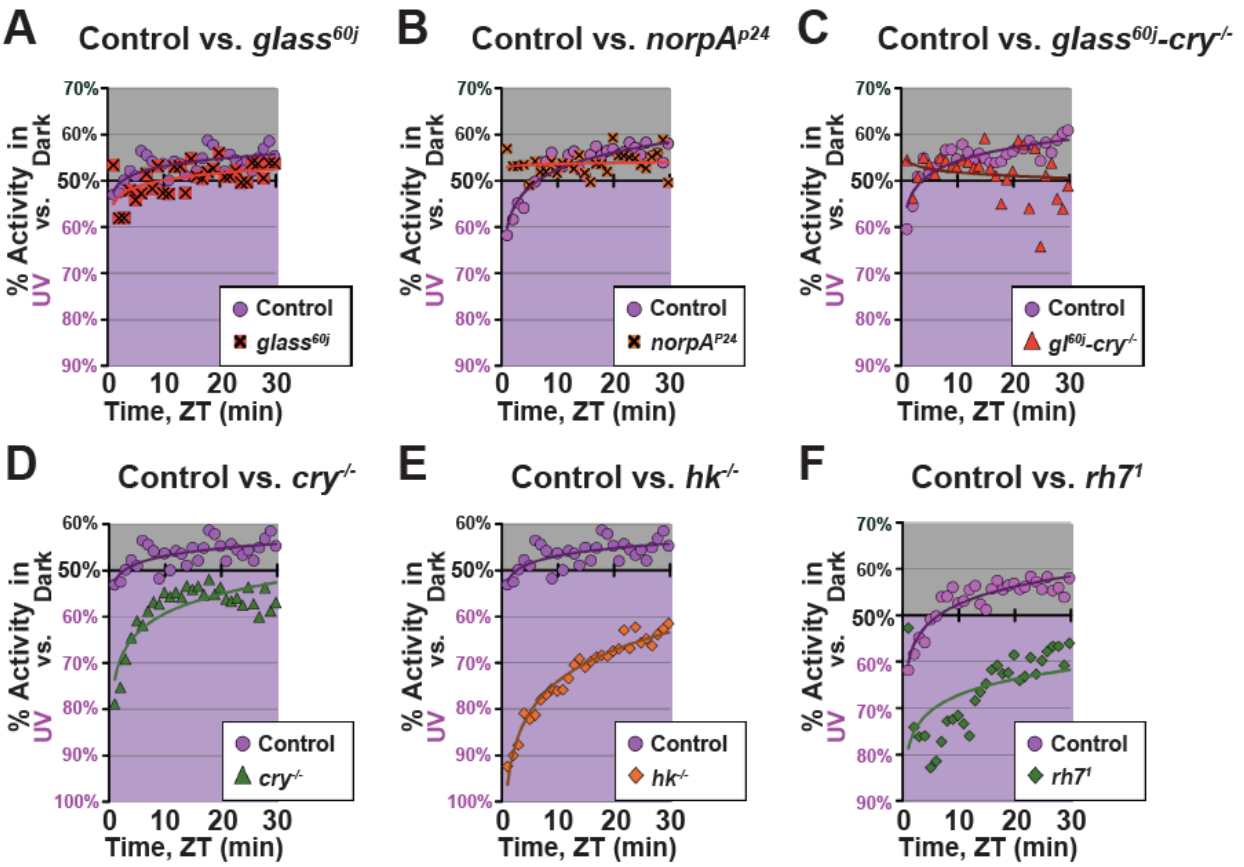

Figure 3–16 Internal neuronal photoreceptors are necessary for avoidance behavioral response to high intensity UV light.

UV attraction/avoidance behavior measured by preference for shaded environment vs. high intensity UV-exposed environment (365 nm, 400 μW/cm^2) during daytime of standard 12hr:12hr light:dark cycle. Preference is calculated by percent of activity in each environment over total activity for each time bin. **(A-F)** Preferences of wild-type control (w^{1118}) vs. mutant flies to high-intensity UV (365 nm, 400 μW/cm^2) in ZT 0-30 min shown in 1 min bin. Similar to wild-type flies that show little-to-no attraction in the first few minutes of UV light exposure, **(A)** $glass^{60j}$ (n=76 vs. control, n=76), **(B)** $norpA^{P24}$ (n=80 vs. control, n=79), and **(C)** $glass^{60j}$-$cry^{-/-}$ (n=55 vs. control, n=95) mutant flies lack clear positive phototaxis responses. In contrast, **(D)** $cry^{-/-}$ (n=78 vs. control, n=76), **(E)** $hk^{-/-}$ (n=77 vs. control, n=76), and **(F)** $rh7^1$ (n=42 vs. control, n=79) mutant flies show strong fast positive phototaxis responses to high intensity UV light.

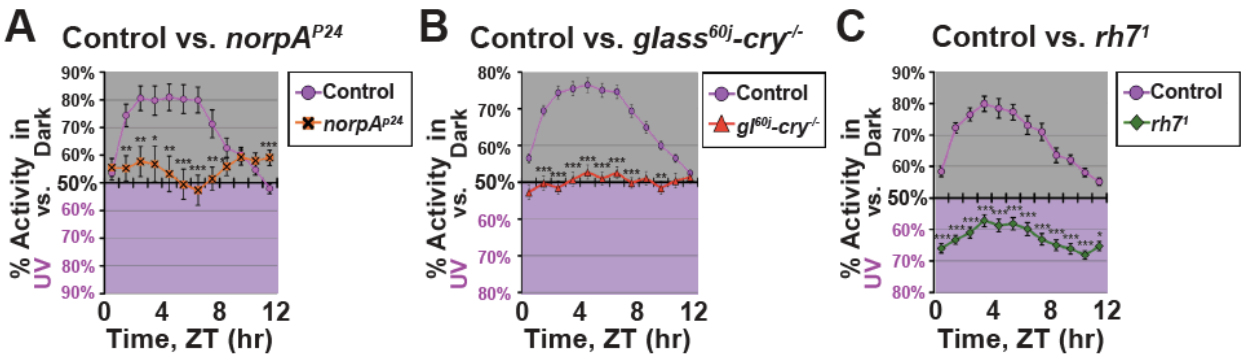

Figure 3–17 Both external photoreceptors and direct neuronal photoreceptors are necessary for UV light avoidance/attraction behavior.
(A-C) Preferences for high-intensity UV (400 μW/cm^2)–exposed vs. shaded environment during ZT 0-12 hr depicted in 1 hr bins for wild-type control (w^{1118}) vs. mutant flies. **(A)** $norpA^{P24}$ (n=80 vs. control, n=79) flies show slight avoidance to high intensity UV light. **(B)** $glass^{60j}$-$cry^{-/-}$ (n=55 vs. control, n=95) double mutant flies almost completely lack any preference for either shaded or UV-exposed environment. **(C)** $rh7^1$ (n=42 vs. control, n=79) mutant flies show attraction to high intensity UV light throughout the daytime.

To test if UV light intensity impacts the choice between positive phototaxis versus avoidance, I measured choice behavior under low intensity UV light (10 μW/cm^2). Wild-type control flies show strong attraction to dim UV light that slowly attenuates (Fig. 3-18). Thus attraction versus avoidance behavioral responses are UV light intensity-dependent. Mutant flies lacking CRY or HK also show attraction to low intensity UV light (Fig.2 3-18 B, C, F, G). Similarly, mutant flies lacking Rh7 show strong attraction to low intensity UV light (Fig. 2D, H). In contrast, mutant flies lacking all external opsin-based photoreceptors ($glass^{60j}$) are not attracted to low intensity UV light (Fig. 3-18A). Flies lacking CRY/HK- or Rh7-mediated phototransduction exhibit positive phototaxis, while flies lacking external opsin-based phototransduction exhibit very slight avoidance, regardless of the intensity of UV light intensity exposure. Over the 12-hr UV exposure during the daytime, *cry*-, *hk*- or *rh7*-null mutant flies still exhibit time-of-day dependence (Fig. 3-18 F-H), while external opsin-based photoreceptor mutant flies ($glass^{60j}$ and $norpA^{P24}$) show relative lack of circadian modulation in attraction/avoidance to UV light (Fig. 3-18E, and Fig. 3-17A). This suggests that CRY/HK and

Rh7-mediated phototransduction is important for deciphering the intensity of UV light, and thus signaling for avoidance in response to high intensity UV light. In contrast, external opsin-based phototransduction is necessary for evoking fast positive phototaxis response to low intensity UV light and time-of-day dependent modulation of attraction/avoidance. Double mutant flies lacking both opsin-based external photoreceptors and CRY-mediated phototransduction almost completely lack any preference for UV-exposed vs. shaded environment (Fig.3-16C, and Fig.3-17B).

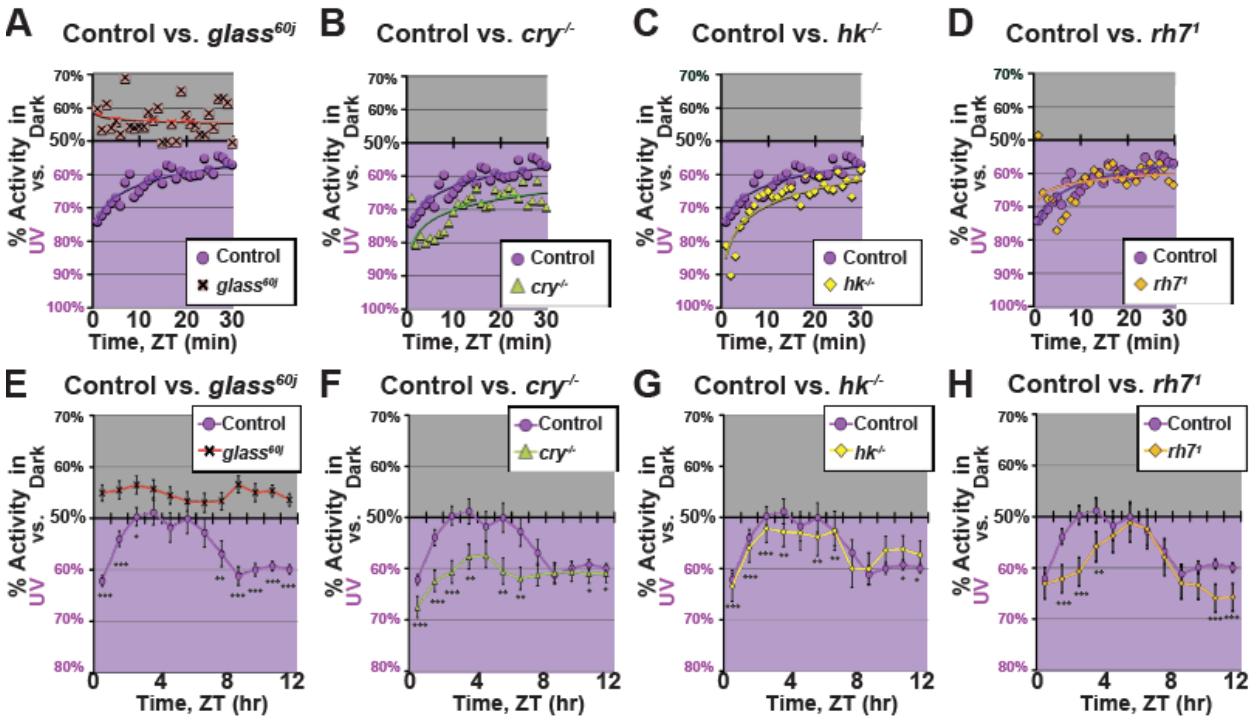

Figure 3–18 UV light-evoked attraction/avoidance behavioral response is intensity-dependent.

UV attraction/avoidance behavior measured by preference for shaded environment vs. low intensity UV-exposed environment (365 nm, 10 μW/cm^2) during daytime of standard 12hr:12hr light:dark cycle. Preference is calculated by percent of activity in each environment over total activity for each time bin. Preference wild-type control (w^{1118}; n=47) vs. mutant flies to low-intensity UV (10 μW/cm^2) in **(A-D)** ZT 0-30 min shown in 1 min bin and **(E-H)** in the daytime (ZT 0-12 hr) shown in 1 hr bin. Unlike wild-type control flies that show strong attraction to low intensity UV light, **(A)** $glass^{60j}$ flies (n=31) lack attraction to low intensity UV light in ZT 0-30min. **(B)** $cry^{-/-}$ (n=44) , **(C)** $hk^{-/-}$ (n=31), and **(D)** $rh7^{1}$ (n=105) mutant flies show strong attraction to low intensity UV light in ZT 0-30 min. **(E)** $glass^{60j}$ flies (n=31) sustain slight avoidance to low intensity UV light throughout ZT 0-12 hr. In contrast, wild-type control, **(F)**

cry$^{-/-}$ (n=44), **(G)** *hk*$^{-/-}$ (n=31), and **(H)** *rh7*^{1} (n=105) mutant flies exhibit attraction to low intensity UV light in ZT 0-12 hr shown in 1 hr bins.

3.3.9 High intensity UV light evokes avoidance in a clock-dependent and exposure duration-independent manner, while low intensity UV light evokes fast attraction response in an external opsin photoreceptor-dependent manner.

Behavioral attraction to low intensity UV light is observed most clearly in the first few minutes following light onset, followed by gradual decrease in the degree of attraction within 15 minutes. Under constant 12 hr UV light, time-of-day dependent changes in overall attraction/avoidance response behaviors are modulated by the circadian clock (9). Due to adaptation, it is difficult to determine the acute light responses using this chronic light choice assay. To determine acute light responses distributed throughout the day, I modified the light choice assay to administer acute 15 min pulses of UV light (365 nm, 400 μW/cm^2 for high intensity pulses or 10μW/cm^2 for low intensity pulses) throughout the 12 hr day on hourly intervals. The acute light response was measured in flies already entrained to 12:12 LD prior to experiment. The experiment was carried out under dark condition (DD) and light pulses were only administered during the subjective daytime to maintain prior circadian entrainment.

Using this acute repeated pulse light choice assay, wild-type control flies still exhibit robust avoidance to high intensity UV light in the early-mid daytime that shifts valence to light attraction towards the end of the day (Fig. 3-19A, and Fig. 3-21A). In contrast, circadian mutant flies, *timeless-null* (*tim*^{0}) and *clock-null* (*clk*^{OUT}) show strong acute attraction to high-intensity UV pulses that do not vary with time-of-day (Fig. 3-19E-F). This shows that avoidance behavior in response to acute high intensity UV light pulses is modulated by the circadian clock, rather than by the simple duration of UV light exposure. In contrast, wild-type control flies exhibit a fast positive phototaxis response to acute low-intensity UV light pulses throughout the entire day

that do not appear to be circadian modulated (Fig. 3-20A and Fig. 3-21A). Similarly, circadian mutants tim^{0} and clk^{OUT} also show rapid attraction to low-intensity UV light at all times of the day (Fig. 3-20E-F). This suggests the valance of UV light choice is gated by light intensity: avoidance is a chronic circadian clock-modulated response to high intensity UV light, while attraction is an acute circadian clock-independent behavioral response to low intensity UV light.

CRY-mediated electrical responses signal through an HK-dependent pathway that couples light activation to potassium channel modulation in a redox-dependent mechanism (4). Both mutant flies null for CRY/HK-mediated phototransduction show rapid acute attraction to both high- and low-intensity UV pulses that do not vary with time-of-day (Fig. 3-19 B, C, Fig. 3-20 B, C, and Fig. 3-21C, D), supporting earlier results showing that CRY codes for UV light avoidance responses (6, 9). In contrast, mutant flies lacking external opsin-based photoreceptors ($glass^{60j}$) lack clear preference (no clear attraction nor avoidance) throughout the day for both high- and low-intensity UV pulses (Fig. 3-19D, Fig.3-20D, and Fig. 3-21B). I conclude that high-intensity UV light-evoked avoidance signals via CRY/HK-mediated phototransduction mechanism is modulated by the circadian clock, and is independent of the duration of UV light exposure. In contrast, low-intensity UV light evokes attraction in a circadian clock-independent manner via external opsin-based photoreceptors. Thus, UV light intensity responses signal through different phototransduction channels in an intensity-dependent manner.

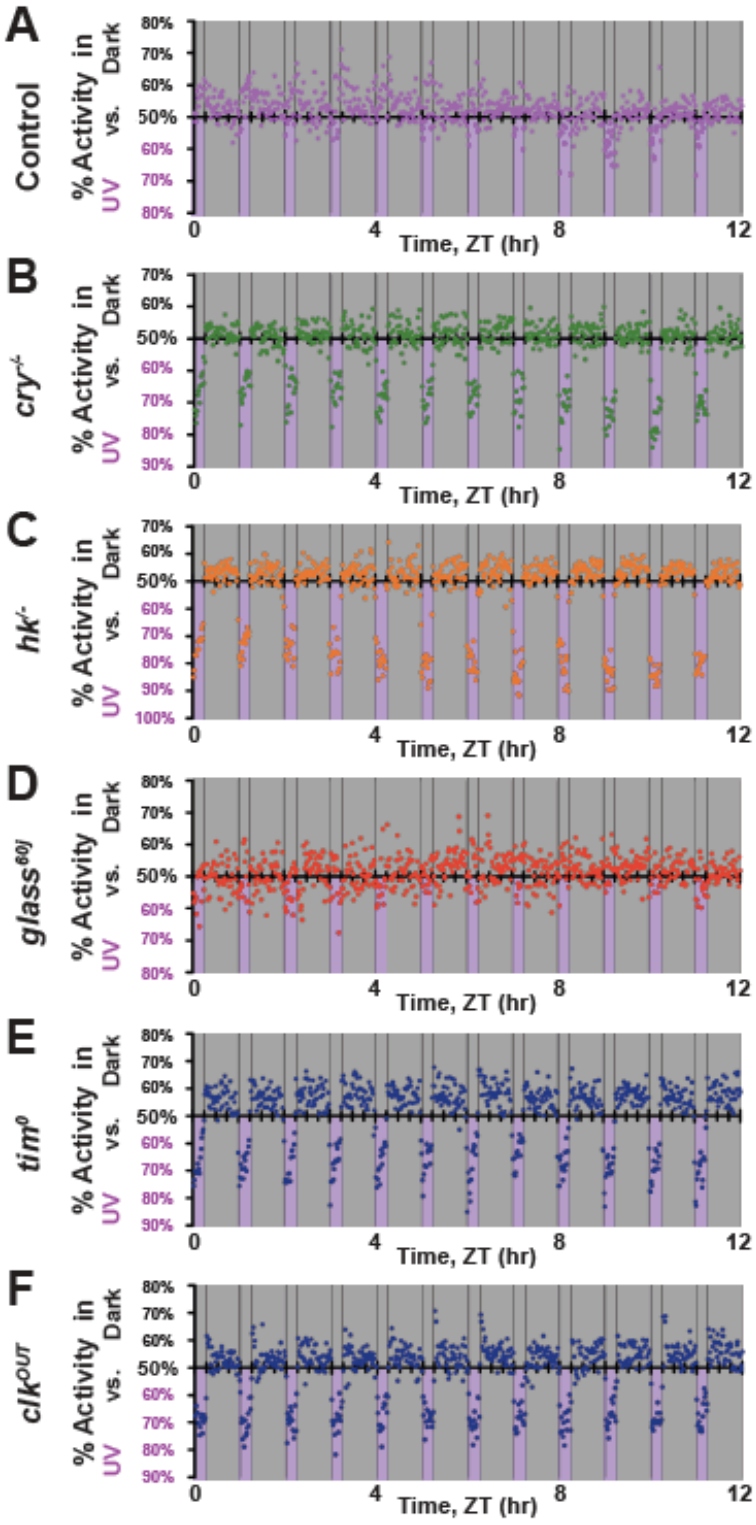

Figure 3–19 High intensity UV light evokes avoidance modulated by the circadian clock that is independent of duration of light exposure.

UV attraction/avoidance behavior measured by preference for shaded environment vs. high intensity UV-exposed environment (365 nm, 400 μW/cm^2) during acute 15 min pulses of light

throughout the 12 hr day on hourly intervals. Preference is calculated by percent of activity in each environment over total activity for each 1min bin. **(A)** Wild-type control (w^{1118}; n=61) flies show avoidance to high intensity UV light in the early-mid daytime. In contrast, **(B)** $cry^{-/-}$ (n=63) and **(C)** $hk^{-/-}$ (n=61) mutant flies show attraction to high-intensity 15 min UV light exposures at all time-of-day. **(D)** $glass^{60j}$ (n=58) mutant flies lack clear preference at all times of the daytime. **(E)** $timeless^{0}$ (n=100) and **(F)** $clock^{OUT}$ (n=102) circadian mutant flies show attraction to high-intensity 15 min UV light exposures regardless of time-of-day.

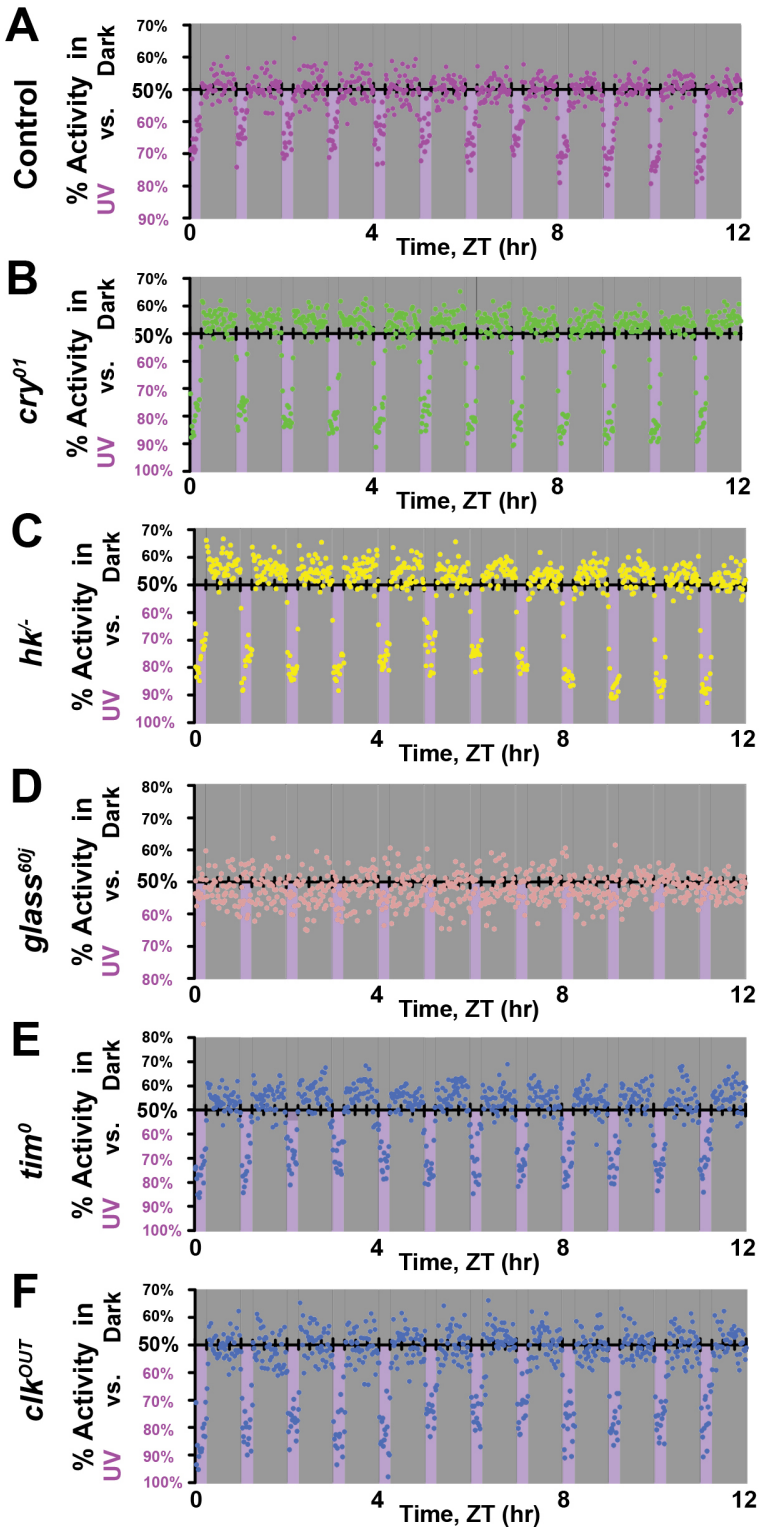

Figure 3–20 External opsin-based photoreceptors modulate attraction behavior in response to low intensity UV light.

UV attraction/avoidance behavior measured by preference for shaded environment vs. low intensity UV-exposed environment (365 nm, 10 μW/cm^2) during acute 15 min pulses of light

throughout the 12 hr day on hourly intervals. Preference is calculated by percent of activity in each environment over total activity for each 1 min bin. **(A)** Wild-type control (w^{1118}; n=61), **(B)** $cry^{-/-}$ (n=60) and **(C)** $hk^{-/-}$ (n=62) flies show attraction to low-intensity 15 min UV light exposures at all time-of-day. In contrast, **(D)** $glass^{60j}$ (n=62) mutant flies lack attraction. **(E)** $timeless^{0}$ (n=44) and **(F)** $clock^{OUT}$ (n=40) circadian mutant flies show attraction to high-intensity 15 min UV light exposures regardless of time-of-day.

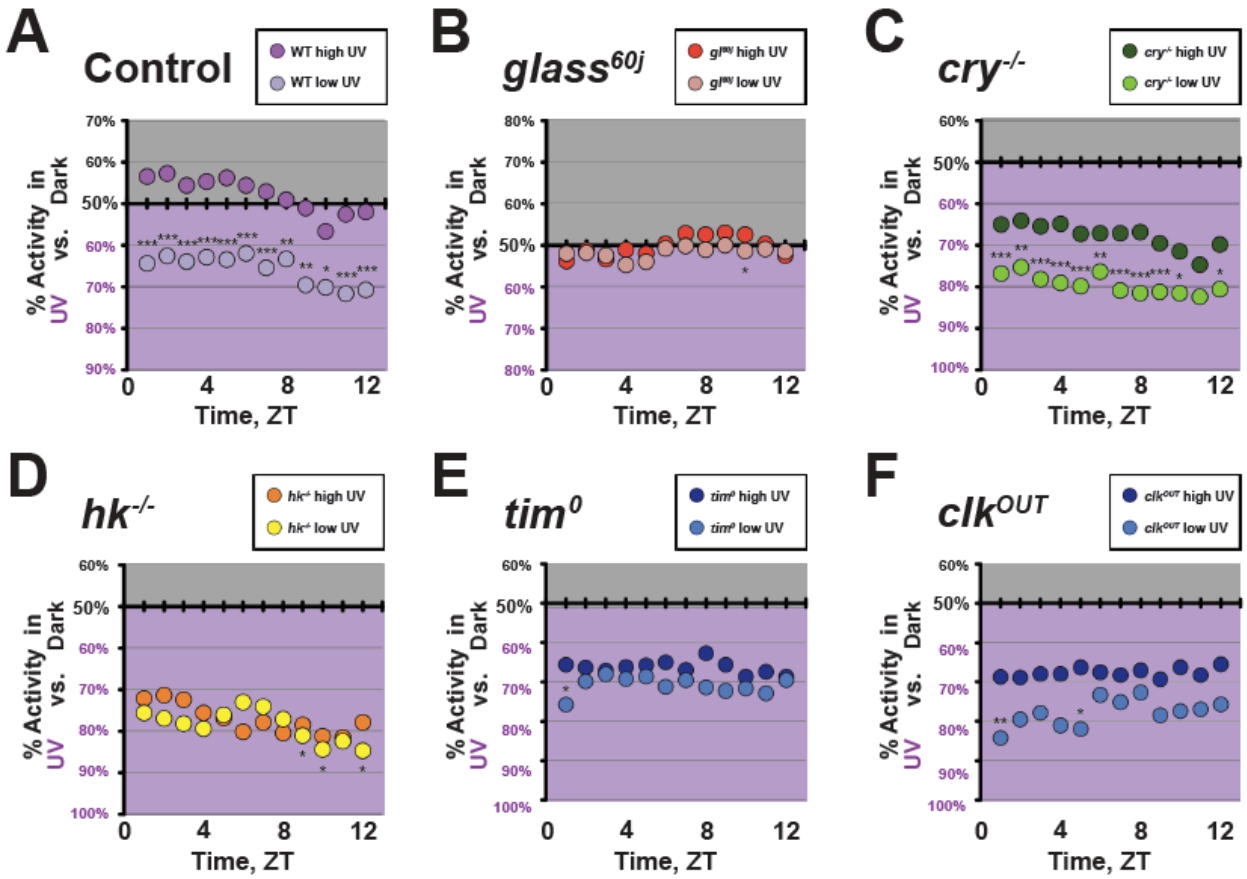

Figure 3–21 UV light-evoked attraction/avoidance behavioral response is intensity-dependent.
(A-D) Average preference for shaded environment vs. high- (365 nm, 400 µW/cm^2) or low- (10 µW/cm^2) intensity UV-exposed environment during the 15 min UV light pulses for **(A)** wild-type control (w^{1118}; high intensity n=61 vs. low intensity n=61), **(B)** $glass^{60j}$ (high intensity n=58 vs. low intensity n=62), **(C)** $cry^{-/-}$ (high intensity n=63 vs. low intensity n=60), and **(D)** $hk^{-/-}$ (high intensity n=61 vs. low intensity n=62). **(E)** $timeless^{0}$ (high intensity n=100 vs. low intensity n=44), and **(F)** clk^{OUT} (high intensity n=109 vs. low intensity n=40).

3.3.10 External and internal phototransduction mechanisms modulate distinct time-of-day dependent spatial preference of light environment.

To resolve behavioral responses to UV light at high spatial resolution, I used multibeam locomotor activity monitors (with infrared beams spaced every 3 mm, 17 beams on each light exposed versus shaded side with a total of 34 beams per fly) (Fig. 3-22). Wild-type control flies strongly prefer shaded locations at the end of tubes that are the most distant from the UV exposed light area. This behavior is subject to circadian-modulated time-of-day driven preferences with UV avoidance behavior that peaks during midday (Fig. 3-23). Wild-type control flies venture out of the shade to UV-exposed areas at the beginning (ZT0-1) and the end (ZT10-12) of the day, even though UV light intensity does not vary throughout the 12-hr day (Fig. 3-23A). During midday (ZT1-10), control flies strongly prefer to be at the edge furthest away from the UV-exposed environment, close to the food. However, food location does not dictate this behavioral preference for location in wild-type flies. The time at which wild-type flies prefer the furthest edge away from the UV-exposed environment, coincides with the trough of circadian feeding rhythm (78), and the other end of the tube on the UV exposed side is not a strongly preferred location at any time of day.

In marked contrast to wild-type flies, *cry-null* flies strongly prefer the edge closest to the food in the UV exposed area and avoid the mid-region between both the shaded- and the UV-light exposed environments (Fig. 3-23B). Similarly, *rh7-null* flies strongly prefer the UV exposed area, although not as close to the food, and avoid the mid-region between both the shaded- and the UV-light exposed environments at all times of the day (Fig. 3-23D). Clk^{OUT} circadian mutant flies prefer the UV-light exposed environment regardless of the time-of-day, and prefer the edge closest to the food in the UV exposed area, similar to *cry-null* flies. However, unlike *cry-* or *rh7-null* flies, clk^{OUT} mutants do not avoid the mid-region between the shaded- and

the UV-light exposed environments (Fig. 3-23E). *Glass*^{60j} mutant flies, lacking all external opsin-based photoreceptors, prefer the shaded environment at all times of the day and lack clear time-of-day dependent modulation. Unlike control flies, *glass*^{60j} flies prefer the mid-region within the shaded environment at all times of the day rather than the edge furthest away from the UV light (Fig. 3-23C). In spite of this subtle difference with control flies, *glass*^{60j} flies, which completely lack image-forming vision, are still capable of discerning UV light and show almost no locomotion in the UV light-exposed area, likely by CRY-mediated light sensing.

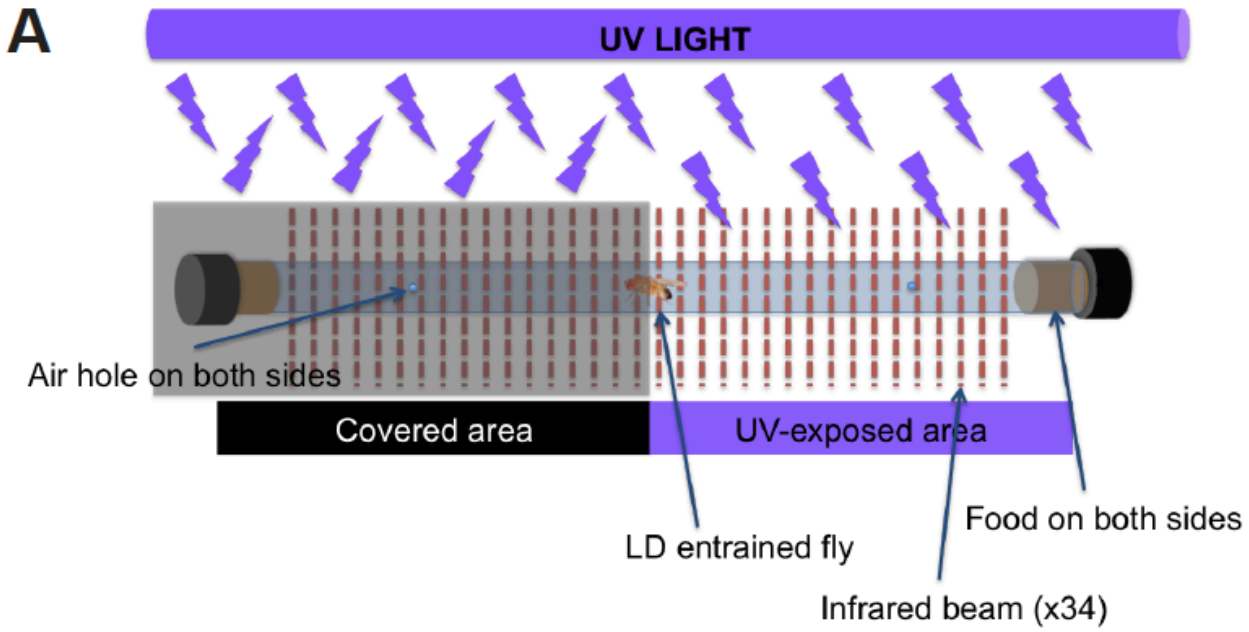

Figure 3–22 High spatiotemporal resolution of preference and activity using multibeam monitors.
(A) Multibeam locomotor activity monitors with infrared beams spaced every 3 mm. Each fly has 17 beams on light exposed side, and another 17 beams on shaded side, with a total of 34 beams per fly. Air hole and food were placed equally on both sides.

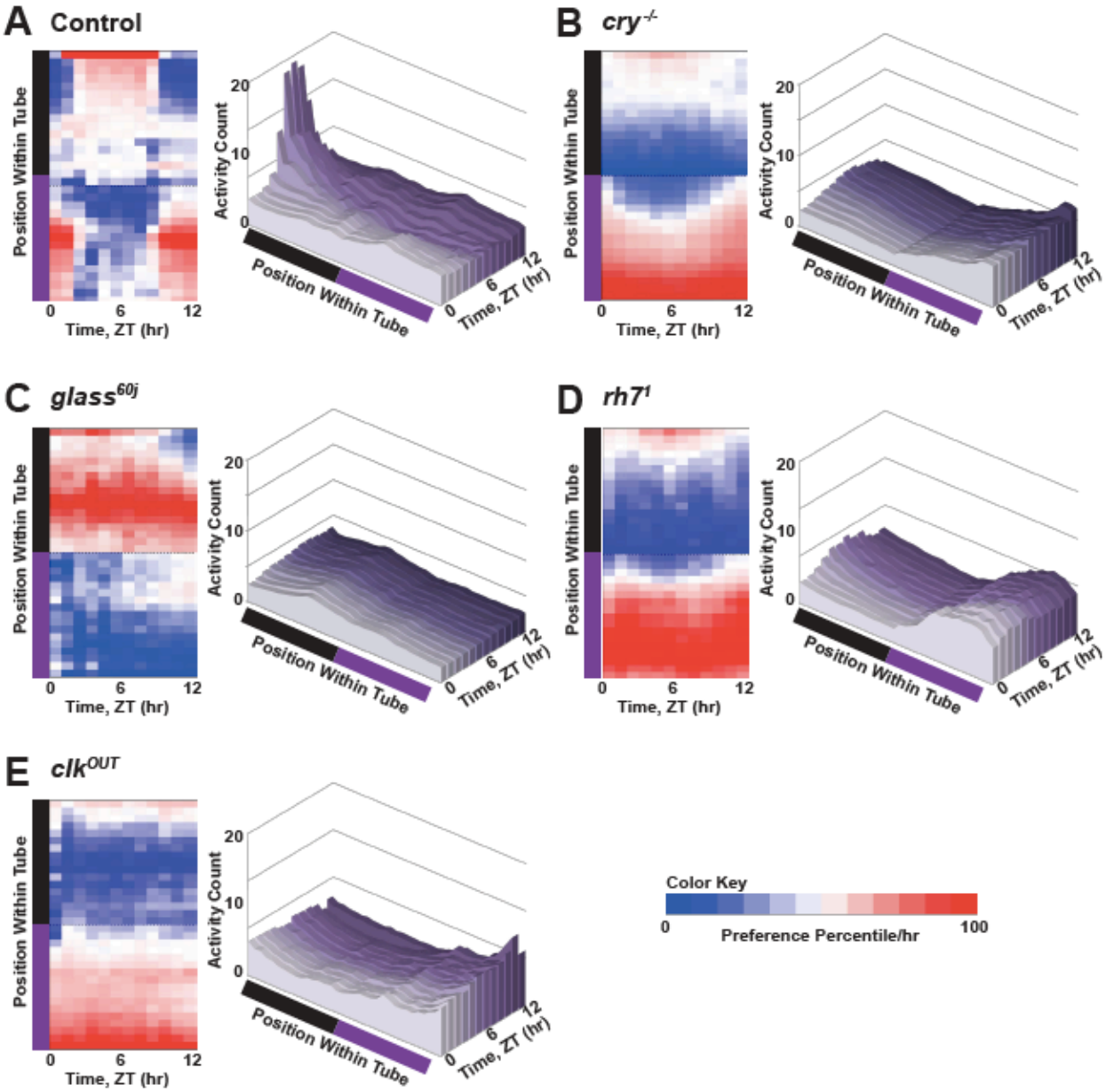

Figure 3–23 Avoidance/attraction behavior has distinct time-of-day dependent spatial preference.

(A-C) Time-of-day dependent spatial preference of avoidance/attraction behavior in response to high-intensity UV (365 nm, 400 μW/cm^2) vs. shade was monitored with high spatial and temporal resolutions using multibeam locomotor activity monitors. Heat map of preference for each 1hr bin (left) and activity count at each position for every 1hr bin (right) are shown. **(A)** Wild-type control (*w*^{1118}; n=144) flies prefer the mid-area of UV-exposed environment during the early morning and hours before simulated dusk. But during the midday prefer the edge of the shaded environment, furthest away from the UV-environment and close to the food. In contrast, **(B)** *cry*$^{-/-}$ (n=61) flies strongly prefer area furthest out in UV-exposed environment at all times of the day. **(C)** *glass*^{60j} (n=64) flies prefer the mid-area within the shaded environment, regardless of the time of day. **(D)** *rh7*^{1} (n=46) flies strongly prefer the outer edge of UV-exposed

environment at all times of the day. **(E)** clk^{OUT} (n=48) flies prefer the area furthest out in UV-exposed environment close to the food, at all times of the day.

3.4 Discussion

The ability to discern the changes in intensity, spectral content, timing and exposure length of light provides valuable environmental information crucial to an organism's well being and survival. UV light avoidance behavior has been demonstrated in foraging larva and egg-laying activity in females (54, 55). I demonstrate that the CRY/HK signaling pathway mediates UV light avoidance behavior in adult male *Drosophila*. During peak UV light intensity (mid-day in most natural environments), flies (especially males) tend to take a "siesta" rest and thus avoid heat exposure and desiccation. UV light avoidance behavior is highest during the mid-day (Fig. 4), despite unvarying UV intensity for our experimental conditions during the daytime (ZT0-12). This suggests that CRY/HK-mediated UV light avoidance behavior may be under circadian control comparable to larval avoidance behavior shown to be dependent on opsin-based photoreceptors and subsets of circadian pacemaker neurons and circadian genes (80, 81).

CRY dually mediates attraction and avoidance behaviors to UV light depending on UV light intensity. The differences in the CRY-mediated behavioral response to varying intensities of UV light poses the interesting question of whether CRY may be important not only for the acute sensory detection of the light, but also for modulating more complex aspects of behavior such as executive choice. CRY-mediated behavioral responses likely depend on spectral composition, intensity, and duration of light exposure, as well as integration with other sensory cues, most notably temperature (96-100). CRY-mediated electrophysiological light responses vary monotonically depending on UV light intensity. Thus the cell-autonomous neuronal CRY light sensor codes for graded responses to UV light intensity rather than gated on/off responses.

Opsin-based light sensing is clearly critical for behavioral light responses. The gl^{60j} mutant exhibits the developmental loss of all external opsin-based photoreceptors, HB eyelet, and the DN1p subset of circadian neurons (21, 84, 85). The DN1s have been implicated in light evoked morning arousal activity (101, 102). HB eyelet cells project into the accessory medulla and to the LNvs (65, 68). The $norpA^{P24}$ mutant disrupts opsin-based phototransduction in eyes without disrupting phototransduction in the HB eyelet or development of the DN1p circadian neurons, thus the gl^{60j} and $norpA^{P24}$ mutants are not functionally equivalent (21, 86, 87). In contrast to the dramatic loss of arousal response to UV light in gl^{60j} mutants, $norpA^{P24}$ mutants show UV light arousal responses that closely resemble that of controls (Fig. 2E), suggesting the DN1ps and/or the HB eyelet may also contribute to the UV light arousal response. CRY's contribution is functionally distinct from that of opsins as shown by both electrophysiological and behavioral results. In conclusion, CRY is a major modulator of a wide range of fly behavioral responses to UV light.

The circadian clock and circadian circuit neurons modulate both the valence of UV light avoidance/attraction and the timing of rhythmic behavioral responses to high intensity UV light. CRY/HK- and opsin-mediated external and internal photoreceptors all contribute to circadian clock entrainment to light (11, 21) and modulate complex short wavelength light avoidance/attraction behavior. The disrupted valence is particularly clear and prominent in the circadian-disrupting LL light condition without the circadian "filtering" on time-of-day dependent modulation for all genotypes including wild-type. In LL, both $cry^{-/-}$ and $hk^{-/-}$ flies display a steady attraction to UV light exposed environment at all times of day. Together, these results strongly suggest that both the circadian clock and the CRY/HK signaling pathway code for UV light avoidance. Mutant $hk^{-/-}$ flies exhibit strong behavioral attraction to UV light

exposed environments, even more so than *cry*$^{-/-}$ flies for both LD and LL light choice assays. HK is a functional redox sensor (73, 74, 103). It is likely that very high intensity UV light generates redox signals even in the absence of CRY (83, 104). Surprisingly, all circadian mutants tested show degrees of valence shift from avoidance to attraction to the UV light-exposed environment and that this can be rescued by genetic clock restoration.

In addition to rhythmic UV light avoidance, circadian regulated behavioral preference for temperature further refines a complex and adaptive behavioral output in *Drosophila* (105, 106). Certain *Drosophila* sensory responses, including temperature preference, olfactory response, and gustatory response, are under circadian control (106-108). An afternoon peak (A-peak) of locomotor activity appears under semi-natural conditions with increased temperature or light intensities and likely facilitates escape from harmful environments to shade to avoid desiccation (99, 109). Midday avoidance is coordinated between multiple sensory modalities, as temperature preference is dependent on light environment (110). Timing of peak midday UV avoidance coincides with siesta rest in adult flies, which prefer to sleep in dark environments (79). In the absence of environmental choice between UV light exposure and shade, LNv ablated flies exhibit disrupted midday siesta rest with significantly higher locomotor activity along with dampened evening and morning activity relative to controls under 12h:12h UV LD (Fig 4B). CRY also mediates similar valence control over blue light responses. I propose that electrical signaling by LNv neurons and UV light sensing by CRY/HK are crucial in regulating the valence of the UV light response between avoidance and attraction to coordinate the fundamental escape from light (58). This provides both core circadian clock components and the CRY/HK as coordinating elements for Pittendrigh's idea of "escape from light" that the circadian systems were evolved to effectively escape the harmful effects of short wavelength light (58).

Insect behavior, including circadian rhythmic behavior, is subject to a multitude of sensory inputs. The combination of these sensory cues in natural environments is sufficiently powerful to mask major clock mutations, which yield severe behavioral impairments in less complex laboratory conditions (109). I demonstrate that flies complex spatiotemporal behavioral light responses integrate multiple photic inputs to navigate their light environment. Low intensity UV evokes attraction, which has fast kinetics and diminishes rather fast, confirming its importance as a survival mechanism for escape behavior. Here I show that this fast attraction behavior is induced by external opsin-based photoreceptors at all times of the day. High intensity UV evokes avoidance via internal/direct neuronal photoreceptors, CRY and Rh7. In addition to intensity of light, circadian time and light spectra are important for avoidance/attraction signaling (9). In contrast to fast kinetics of short wavelength light attraction, CRY- and Rh7-evoked avoidance is slowly modulated by the circadian clock, changing over hours span during the daytime (6, 9).

External and internal photoreceptors all contribute to light entrainment of circadian locomotor activity rhythm (11, 19-21, 32, 60, 64-72). Both internal photoreceptors, CRY and Rh7, are expressed in circadian pacemaker neurons in the *Drosophila* brain, including lateral ventral neurons (LNv) (1, 2, 11). Double knockout mutant flies lacking both external and internal photoreceptors, $glass^{60j}$-$cry^{-/-}$, almost completely lack any preference of light environment, and thus do not display neither avoidance nor attraction to UV light. This suggests CRY- and Rh7-phototransduction signals converge to mediate avoidance/attraction behavior. I demonstrate that in addition to circadian entrainment by light, organisms can behaviorally control how much light input is received by the circadian system under different light conditions. I did not test optimally

preferred intensities of light, but flies clearly do not prefer high intensity short wavelength light, especially in the midday (6, 9, 111).

Here I implemented multibeam activity monitors to acquire high temporal and spatial resolution of UV-evoked avoidance/attraction behavior. Interestingly, both control and $glass^{60j}$ flies both show minimal behavioral preference for the shaded food-containing edge towards end of day, even though it coincides with the time of peak in feeding rhythm (78). In contrast, *cry-null* and clk^{OUT} mutant flies show strong positional preference for the UV-exposed area close to food at all times of day with overall less time-of-day modulated positional preference as compared to wild-type controls. This suggests that observed spatial preference in the far edge in midday is not due to the presence of food. I show that there are multiple levels of sensory processing even for a single type of input such as light. I conclude that multiple phototransduction mechanisms modulate a complex behavioral output depending on its spectra, intensity, and time/duration of exposure.

At the organismal level, integration of multiple types of sensory inputs, including light and temperature, are crucial to synchronize to the changing environment. Both light and temperature cycles shape complex behavioral output, including locomotor activity and sleep in *Drosophila* (110, 112-119). Multisensory entrainment of the circadian clock occurs by integrating different sensory cues, including light inputs via CRY and thermal inputs from trpA1 (99, 114, 120, 121). Rhythmic preference of both light and temperature environment further reinforces an intricate and complex behavioral output (Figs.1-5; (6, 106, 110, 122, 123).

In conclusion, complex spatiotemporal behavioral responses to UV light are mediated through multiple phototransduction pathways. Acute high intensity UV light exposure (minutes) in wild type flies evokes a transient phototaxis attractive response, which is mediated by opsin-

based phototransduction in the eyes. With longer high intensity UV light exposure (tens of minutes), avoidance is the dominant response, which is mediated by the CRY/HK and Rh7 pathways. Both rapid/acute and long term/chronic CRY/HK mediated behavioral responses vary by time of day, while external photoreceptor/opsin mediated responses do not appear to vary by time of day. Acute low intensity UV light exposure (minutes) in wild type flies evokes a longer lasting transient phototaxis attractive response that resolves to mostly neutral responses. Spatial responses to UV light are strongly influenced by both neuronal cell autonomous phototransduction (CRY/HK) and external opsin-based photoreceptor phototransduction, indicating integrated contributions from both major UV sensing photosystems.

Reprinted with permission from:

Baik LS, Fogle KJ, Roberts L, Galschoidt AM, Chevez JA, Recinos Y, & Holmes TC (2017) CRYPTOCHROME mediates behavioral executive choice in response to UV light. *Proceedings of the National Academy of Sciences of the United States of America* 114(4):776-781.

Baik LS, Recinos Y, Chevez JA, & Holmes TC (2018) Circadian modulation of light-evoked avoidance/attraction behavior in Drosophila. *PloS one* 13(8):e0201927.

Chapter 4 : Circadian basis of time-of-day activity and light-evoked attraction/avoidance in day- vs. night-biting mosquitoes

4.1 Introduction

Mosquitoes present a worldwide threat by spreading human diseases, including Zika, Chikungunya, West Nile, Yellow Fever, and Dengue Fever viruses. Insects display a wide range of short wavelength light modulated behaviors, including attraction/avoidance (4, 6, 9, 124-127). It has been long assumed that insect responses to ultraviolet (UV) light are mediated by UV-sensitive opsins expressed in eyes and other external photoreceptors. However, various insects, including mosquitoes and flies often express other non-opsin photoreceptors including the blue-light sensitive flavoprotein CRYPTOCHROME (CRY). *In vitro* analysis of purified *Drosophila* and mosquito CRY proteins show spectral absorbance peaks at 450nm (blue) and 365nm (UV-A) (7, 27). *Drosophila* CRY mediates a wide range of behavioral responses to blue and UV light, including attraction and avoidance (6, 9).

The circadian clock further refines the light-mediated behaviors according to daily environmental changes (6, 9). Time-of-day dependent changes in ultraviolet (UV) light-evoked attraction/avoidance behavior of *Drosophila melanogaster* is modulated by the circadian clock (9). Different mosquito species have evolved distinct circadian timing of behaviors according to their temporal/ecological niches, which likely minimize inter-species competition. Some mosquito species are diurnal (i.e., *Aedes aegypti*) while others are nocturnal (i.e., *Anopheles coluzzii*). The timing of behavioral niche is also sex dependent within and between different mosquito species. For an example, two closely related nocturnal mosquito species *Anopheles coluzzii* and *Anopheles gambiae* show temporal separation in timing of male swarming (128). Numerous mosquito behaviors change with the time-of-day, including flight activity, mating, oviposition, and biting (128-139). Considering the magnitude of their impact on health and

ecology, relatively little is known about the species basis of diurnality/nocturnality and behavioral timing in mosquitoes. Here, I measured the time-of-day light-evoked attraction/avoidance behavior in mosquitoes. Further, I map circadian neuronal circuit species differences between day- and night-biting mosquitoes and within these circuits investigate the timing of rhythmic circadian protein cycling in diurnal and nocturnal mosquito brains. Lastly, I investigate the role of circadian clock in timing of attraction/avoidance behavioral response to light.

4.2 Materials and Methods

4.2.1 Immunocytochemistry

All mosquitoes were reared in standard 12:12 light:dark (LD) schedule in 27°C, and 80% humidity in large cages, with access to 10% sucrose diet. Brains were dissected in 1X PBS, fixed in 4% paraformaldehyde (PFA) for 30min, washed 3X 10min in PBS-Triton-X 1%, incubated in blocking buffer (10% Horse Serum-PBS-Triton-X 0.5%) at room temperature before incubation with mouse anti-PDF C7, monoclonal (1:10,000) and rabbit anti-PER, polyclonal (1:1,000) antibodies overnight in 4^{o}C. Brains were washed 3X 10min in PBS-Triton-X 0.5% then incubated in goat α-mouse-Alexa- (1:500) and goat α-rabbit-Alexa-594 (1:500) secondary antibodies in blocking buffer overnight in 4^{o}C. Brains were washed 5X 15min in PBS-Triton-X 0.5% before mounting in Vectashield mounting media (Vector Laboratories). Microscopy was performed using Zeiss LSM700 confocal microscope and analyzed using Imaris software (Bitplane).

4.2.2 Light Choice Behavior Assay

All mosquitoes were reared in standard 12:12 light:dark (LD) schedule in 27°C, and 80% humidity in large cages, with access to 10% sucrose diet. Mosquitoes were entrained to LD schedule for minimum of 3 days prior to testing. Individual mosquitoes were each placed into 25mm diameter x 125mm length pyrex glass tubes (Trikinetics) plugged with plugs on either side, which was soaked with 10% sucrose providing a food source, while simultaneously allowing airflow. Tubes containing individual mosquitoes were placed in humidity-, temperature-, and light-controlled incubator and allowed to acclimate for a full day. One half of the tubes were covered with infrared (IR) filters (LEE Filters 4 x 4" Infrared (87C) Polyester Filter), providing the mosquitoes with a choice of a shaded environment (IR filtered) versus light-exposed (not covered with IR filter) during the 12 hours of light. Philips TL-D Blacklight ultraviolet light (UV) source with narrow peak wavelength of 365 nm and intensity of 400 $\mu W/cm^2$ was used for UV light. Blue (450 nm, Supernight) and red (630 nm, Supernight) LED strips set around 400 $\mu W/cm^2$ was used as blue and red light sources. Additionally, IR LED strips (Infrared 850 nm 3528 LED Strip Light, 78/m, 8mm wide, by the 5m Reel) placed on aluminum heat sink was placed under the entire setup. With each light source, same LD schedule as the LD entrainment schedule prior to experiment was continued to minimize any disturbance to the circadian time. For constant light (LL) light choice assay, the UV light was constantly left on, instead of LD. Webcam (Microsoft Q2F-00013 USB 2.0 LifeCam) took pictures at 5min intervals for 3-5 days of experiment. Each mosquito's preference in the UV-exposed versus shaded side of the tube was analyzed by the ImageJ program. Preference was averaged for each time point per mosquito and statistical measurements were analyzed by t-test using Microsoft Excel and Sigma Plot.

4.3 Results

4.3.1 Diurnal and Nocturnal mosquitoes display specie- and sex-specific avoidance/attraction behavior to UV light.

I measured the light environment preference behaviors of both diurnal (*Aedes aegypti*) and nocturnal (*Anopheles coluzzii*) mosquito species throughout the 24 hr day. All mosquitoes were entrained to a standard 12hr:12hr white light:dark (LD) schedule prior to testing. Young adult (3-5 days post-eclosion) mosquitoes were then presented with a choice of light versus shaded environments during the subjective daytime (ZT 0-12). Preference for each mosquito was measured every 5 mins, as being present in either the light-exposed or in the shaded-environment, and quantified as % of preference (i.e., % of time present in each environment) for each time point averaged over 3-5 days of experiment duration.

Mosquitoes show robust modulation in their degree of avoidance versus attraction behavior depending on time-of-day, despite the constant intensity of light presented. We observe striking differences between nocturnal versus diurnal species of mosquitoes in their light-evoked attraction/avoidance behavior. Diurnal *Aedes aegypti* (*Ae. aegypti*) females are behaviorally attracted to UV light during the day (Fig. 4-1 A, I). In contrast, nocturnal *Anopheles coluzzii* (*An. coluzzii)* females strongly avoid UV light during most of the daytime (Fig. 1B, I). Both *Ae. aegypti and An. coluzzii* females attenuate their UV light avoidance behavior in the afternoon, shifting to greater attraction during the approach of dusk even though there are no changes in light intensity until lights off (Fig. 4-1 A-B, E, G). This temporal "anticipatory" afternoon behavior starts much earlier for diurnal *Ae. aegypti* females, which increase their attraction behavior beginning around mid-afternoon (ZT6) (Fig. 4-1 A, E). In contrast, nocturnal *An.*

coluzzii, females show sharp decreases in UV light avoidance starting before dusk (ZT11). Precisely at dusk, they enter the previously UV illuminated area, rapidly reaching 60% preference for this area 10 minutes after dusk and peak 70% preference one hour after dusk (Fig. 4-1 B).

Females mosquitoes require a blood meal before laying eggs, thus we considered the possibility of sex differences in between females and male for their avoidance/attraction behavioral responses to UV light of both diurnal and nocturnal mosquito species. Similar to the respective female counterparts, diurnal *Ae. aegypti* males are attracted to UV light during the late subjective daytime, but to a significantly lesser extent than females, which are attracted to UV light throughout the entire day (Fig. 4-1 C, I). Nocturnal *An. coluzzii* males strongly avoid UV light, similar to *An. coluzzii* females (Fig. 4-1 D, I).

Both species show sex-specific differences in timing of "anticipation" of increase in attraction/decrease in avoidance while temporally approaching dusk. *Ae. aegypti* male attraction peaks much earlier (ZT10) than that of females (ZT 12) (Fig. 4-1 A, C, E, F). Similarly as dusk approaches, *An. coluzzii* males initiate decreases in avoidance much earlier than females during their transition to attraction in the previously UV light illuminated area (Fig. 4-1 B, D, G, H). We observe sex-dependent differences even after the UV light is turned off, simulating the subjective nighttime (ZT 12-24). For both species, females prefer the previously light-exposed environment throughout the night. This nighttime preference for previously UV-light exposed environment is significantly higher in females, compared to males (Fig. 4-1 A-D, J).

In summary, I show that mosquitoes have diurnal/nocturnal species-specific and sex-dependent differences throughout the valence range UV light-evoked attraction/avoidance behaviors during their systematic changes at different times of the day. Diurnal *Ae. aegypti*

shows stronger attraction to UV light during the daytime. In contrast, nocturnal *An. coluzzii* strongly avoids UV light during the daytime. Both species of mosquitoes display increase in attraction/decrease in avoidance towards simulated dusk, with earlier anticipation in males than females for both species.

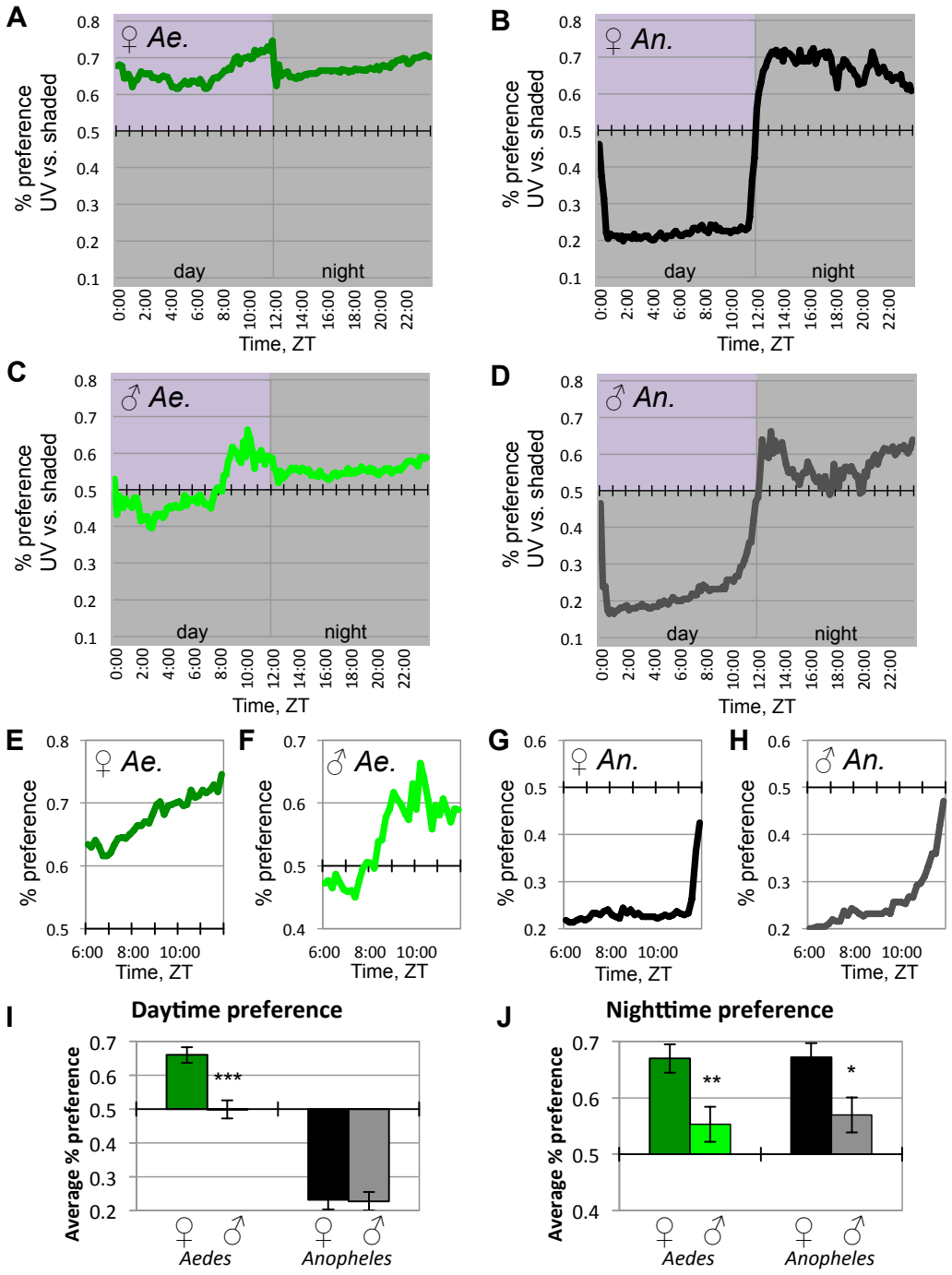

Figure 4–1 UV light-evoked attraction/avoidance responses in diurnal and nocturnal mosquitoes are specie- and sex-dependent.

(A-D) Attraction/avoidance behavior to UV light, measured by % of preference in UV-exposed versus shaded environment throughout 12hr:12hr UV light:dark for female *Ae. aegypti* **(A)**, female *An. coluzzii* **(B)**, male *Ae. aegypti* **(C)**, male *An. coluzzii* **(D)**. **(E-H)** Attraction/avoidance behavior to UV light, measured by % of preference in UV-exposed versus shaded-environment during ZT6-12 for female *Ae. aegypti* **(E)**, male *Ae. aegypti* **(F)**, female *An. coluzzii* **(G)**, male *An. coluzzii* **(H)**. **(I-J)** Average attraction/avoidance behavioral preference to UV light-exposed

versus shaded-environment for daytime **(I)** and nighttime **(J)** in *Ae. aegypti* and *An. coluzzii* female and male mosquitoes.

4.3.2 Mosquito light environmental preference is wavelength-dependent.

While attraction/avoidance behavior in response to light is common among insects, color spectral specificity of attraction/avoidance behavior varies between different insect species (6, 9, 127, 140-145). Another diptera fruit flies, *Drosophila melanogaster*, avoid short wavelength light, but not long wavelength light. *Drosophila* avoidance of UV light peaks in the midday during their low locomotor activity "siesta" (6, 9). To determine the spectral dependence of mosquito attraction/avoidance behavioral light responses, we tested their environmental preferences for visible short wavelength blue light and visible long wavelength red light for comparison with their responses to UV light.

Diurnal mosquito, *Ae. aegypti* females are attracted to both blue and red light during the daytime, very similar to their behavioral attraction to UV light (Fig. 4- 1A, Fig. 4-2 A, C). *Ae. aegypti* females are equally attracted to all light wavelengths tested (Fig. 4-2 E). Nocturnal *An. coluzzii* females, which strongly avoid UV light (Fig. 4-1 B), also avoid blue light during the day (Fig. 4-2 B). Their magnitude of blue light avoidance is significantly lower than their UV light avoidance (Fig. 4-2 E). In contrast to their striking short wavelength avoidance during the day, female *An. coluzzii* are slightly attracted to long wavelength red light (Fig. 4-2 D, E). Females of both species prefer dark nighttime environments with prior UV light-exposure (Fig. 4-1 J, Fig. 4-2 F). This nighttime preference for prior UV-light exposed environments in both mosquito species is significantly higher than their nighttime preference for areas with prior blue- or red-light exposure, for which they are weakly attracted to (Fig. 4-2 F). Thus, attraction/avoidance

behavioral responses are wavelength-dependent and show striking differences in both overall profile and anticipation of dusk between nocturnal and diurnal species.

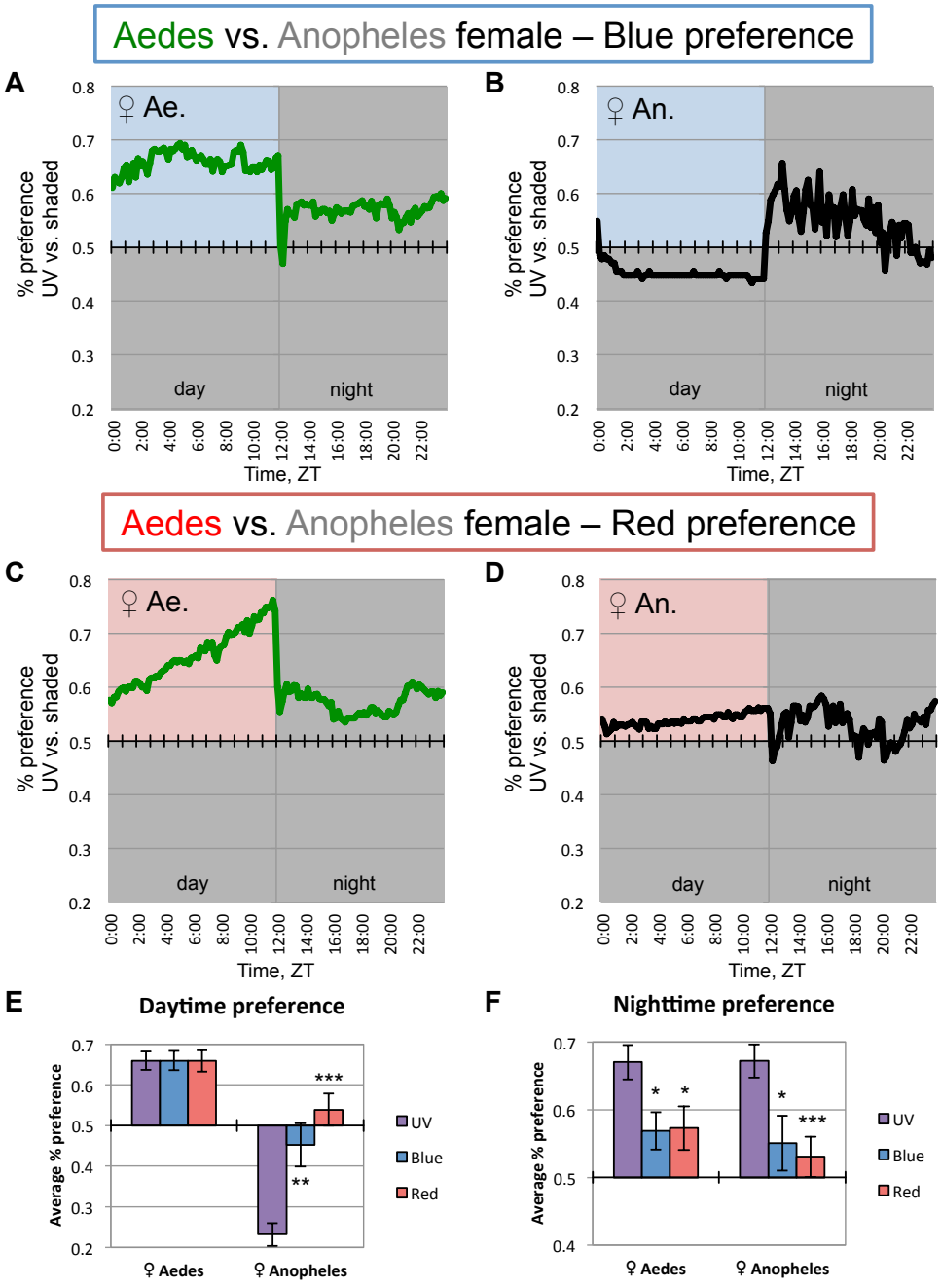

Figure 4–2 Mosquito light-evoked attraction/avoidance behavior is wavelength-specific. **(A-B)** Attraction/avoidance behavior to blue light (450 nm LED, 400 µW/cm^2), measured by % preference in blue light-exposed versus shaded environment throughout 12hr:12hr blue light:dark for female *Ae. aegypti* **(A)**, female *An. coluzzii* **(B)**. **(C-D)** Attraction/avoidance behavior to red

light (620 nm LED, 400 μW/cm^2), measured by % preference in red light-exposed versus shaded environment throughout 12hr:12hr red light:dark for female *Ae. aegypti* **(C)**, female *An. coluzzii* **(D)**. **(E-F)** Average attraction/avoidance behavioral preference to light-exposed versus shaded-environment for daytime **(E)** and nighttime **(F)** in *Ae. aegypti* and *An. coluzzii* female mosquitoes.

4.3.3 PER and PDF staining reveals location of circadian neuronal circuit in diurnal and nocturnal mosquitoes.

Mosquito attraction/avoidance behavior to light varies systematically with time-of-day, suggesting that this behavior is under circadian regulation. To determine whether the circadian clock and circuit modulates attraction/avoidance to light, we first sought to anatomically map the circadian neuronal network in the central brain of female mosquitoes. Insect circadian neurons are defined by the cyclic expression of PERIOD (PER), a highly conserved core circadian gene/protein that drives rhythmic oscillations of physiology and behavior. In many insects and other invertebrates, Pigment Dispersing Factor (PDF) is a neuropeptide co-expressed with PER in certain subsets of circadian neurons (146). PDF appears to be important for circadian phase between PER cycling oscillators (75, 147-149). In *Drosophila melanogaster*, PDF is co-expressed with PER light sensitive CRY in the small- and large-lateral ventral neurons (LNvs) (1, 2, 5, 14, 92). PDF-expressing LNvs in *Drosophila* modulate circadian- and light-mediated behaviors such as circadian locomotion, arousal, and light-evoked attraction/avoidance behaviors (6, 9, 32-35). Here we map the circadian neuronal circuits of *Ae. aegypti* and *An. coluzzii* female adult brains using anti-PER and anti-PDF immunocytochemistry staining.

In both *Ae. aegypti* and *An. coluzzii* female adult brains, PER and PDF proteins are co-expressed in similar neuroanatomical pattern the lateral ventral area, and are anatomically similar to *Drosophila* melanogaster and some other insects (1, 2). These PDF$^+$ and PER$^+$ neurons can be further distinguished by size and location as large-lateral ventral neurons (l-LNvs) and small-

lateral ventral neurons (s-LNvs) (Figs. 4-3 and 4-4). Furthermore, *Ae. aegypti* and *An. coluzzii* LNvs have large neuronal arbors in the optic lobes and clearly visible dorsal projections as shared features with the well characterized *Drosophila* melanogaster neurons (92, 150) (Figs. 4-4 and 4-5). In *Drosophila*, s-LNvs dorsal projections make synaptic connections to DNs (151-154) while the l-LNv arbors in the optic lobe appear to be dendrites based on labeling with the dendritic marker DenMark (155). Similar to *Drosophila*, neurites from PDF^+ LNv neurons project dorsally towards the DNs in *Ae. aegypti* (Fig. 4-3 A and 4-5). In *An. coluzzii* brains, PDF^+ LNv neurites project dorsally towards to the DNs and then extend medially towards the PER^+ PI neurons (Fig. 4-3 B and 4-5).

Female *Ae. aegypti* brains have approximately 8-9 PDF^+ l-LNvs and 9-10 PDF^+s-LNvs, while female *An. coluzzii* brains have approximately 10 PDF^+ l-LNvs and 9-10 PDF^+ s-LNvs per hemisphere (Figs. 4-3 and 4-4, and Table 1). Both species of mosquitoes have larger number of LNvs compared to *Drosophila melanogaster*, which has 5-6 l-LNvs and 4-5 PDF^+ s-LNvs, but otherwise their neuroanatomical features are highly similar to *Drosophila melanogaster* and other insects (156-161). In the lateral ventral region amongst the LNv, there are PER^+/PDF^- neurons, again, consistent with a PER^+/PDF^- "5^{th} s-LNv" neuron seen in flies. I find approximately 3 PDF^- putative l-LNvs and 6 PDF^- putative s-LNvs in female *Ae. aegypti*, and approximately 3-4 PDF^- putative l-LNvs and 4-6 PDF^- putative s-LNvs in female *An. coluzzii* in each side of the brain (Figs. 4-3, 4-4 and Table 1). The neuroanatomical location of PER^+ neurons show species specific similarities and differences for cell groups between diurnal *Ae. aegypti* and nocturnal *An. coluzzii* outside of the lateral ventral area. Similarly located neuronal groups include PER^+/PDF^- 3-4 dorsal neurons (DNs) in *Ae. aegypti* and *An. coluzzii* (Figs. 4-3, 4-4 and Table 1). Differentially located neuronal groups include approximately 5 PER^+ neurons

in the medial-anterior region of *Ae. aegypti* female brains, which we call medial-anterior neurons (m-ANs) here (Figs. 4-3, 4-4 and Table 1). Based on location and size, another differentially located neuronal group include approximately 7 PER^{+}/PDF^{-} neurons, which resemble the pars intercerebralis (PI) in *An. Coluzzii*. In *Drosophila melanogaster*, PI neurons are physiologically circadian rhythmic although by way of other clock neuron input (78, 151). These PI-like PER^{+}/PDF^{-} neurons are not detected in *Ae. aegypti* (Figs. 4-3, 4-4 and Table 1). In summary, I observe both shared and distinct anatomical features of circadian neuronal circuit of diurnal versus nocturnal mosquito species.

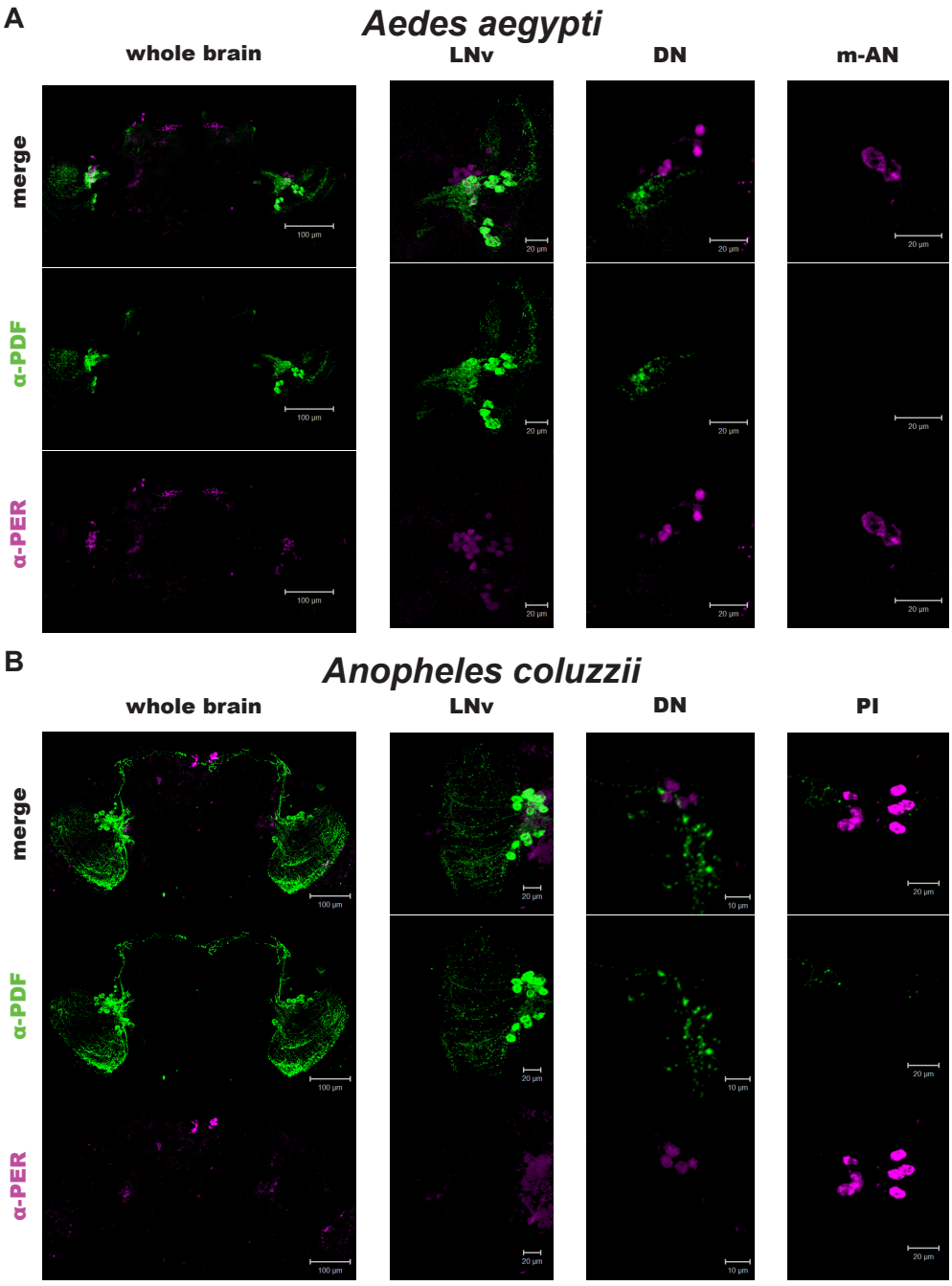

Figure 4–3 Circadian neuronal circuit of diurnal and nocturnal mosquito brains.
(A-B) Representative confocal images of adult female *Ae. aegypti* **(A)** and *An. coluzzii* **(B)** mosquito brains immunocytochemistry stained with anti–PER (magenta) and anti–PDF (green) antibodies.

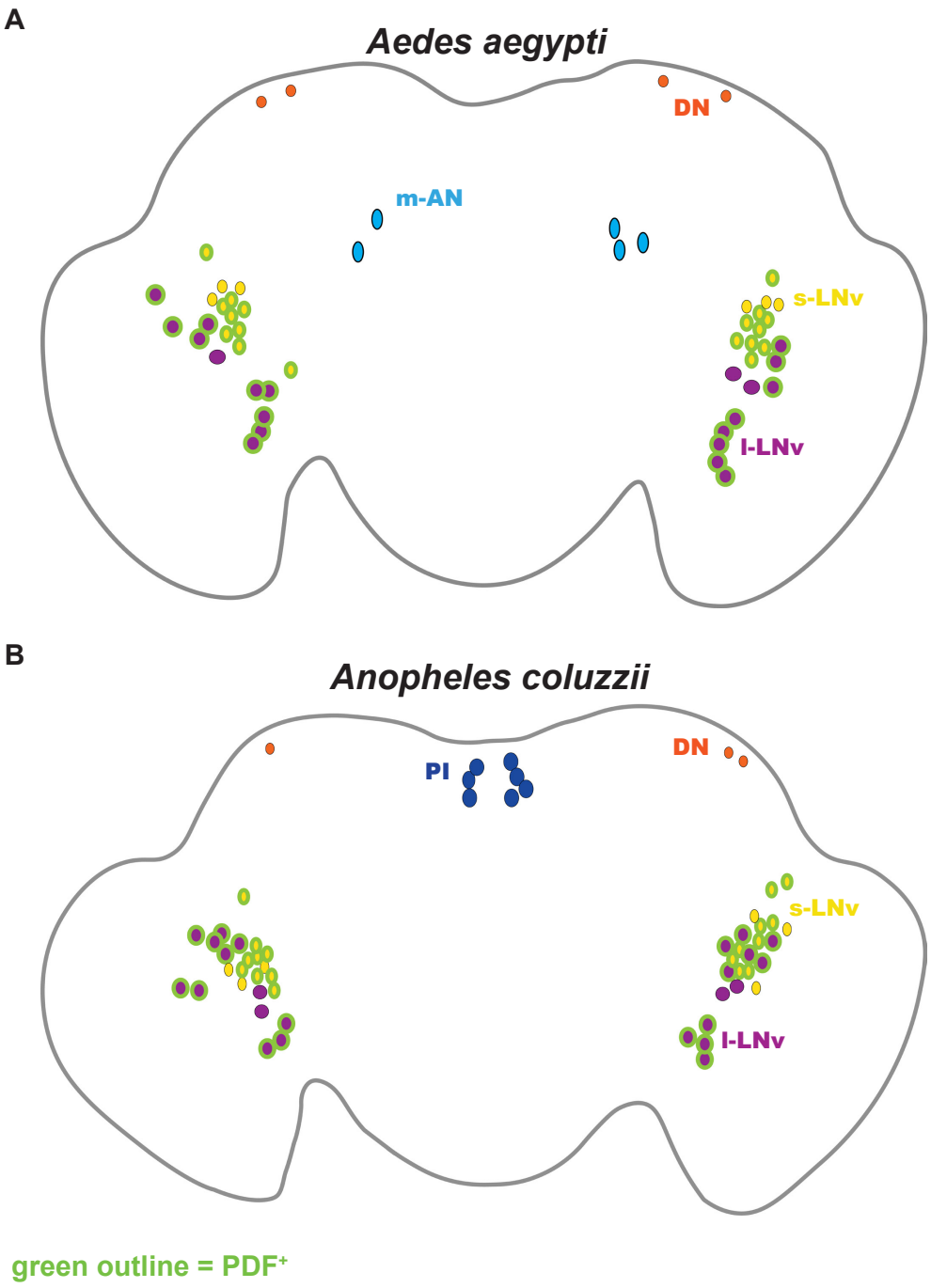

Figure 4–4 Schematic representation of *Aedes aegypti* and *Anopheles coluzzii* circadian neurons.
(A-B) Illustration of representative adult female *Ae. aegypti* **(A)** and *An. coluzzii* **(B)** mosquito central brain and its neurons expressing PER and/or PDF, all overlaid over anti-PDF stained confocal image.

Table 1. Mean number of circadian neurons ±SEM (n) in female mosquito brain

	Avg. number of neurons per hemisphere				*Avg. number of neurons per brain*		
	PDF^+ l-LNv	PDF^+ s-LNv	PDF^- l-LNv	PDF^- s-LNv	DNs	PI Neurons	m-ANs
Aedes aegypti	8.6 ± 0.4 (n= 18)	9.3 ± 0.5 (n= 18)	3.1 ± 0.4 (n= 19)	5.8 ± 1.1 (n= 19)	4.1 ± 0.3 (n= 30)	-	4.8 ± 0.4 (n= 31)
Anopheles coluzzii	10 ± 0.5 (n= 20)	9.8 ± 0.5 (n= 21)	3.5 ± 0.7 (n= 8)	5.2 ± 0.8 (n= 8)	3.3 ± 0.4 (n= 26)	7.3 ± 0.1 (n= 22)	-

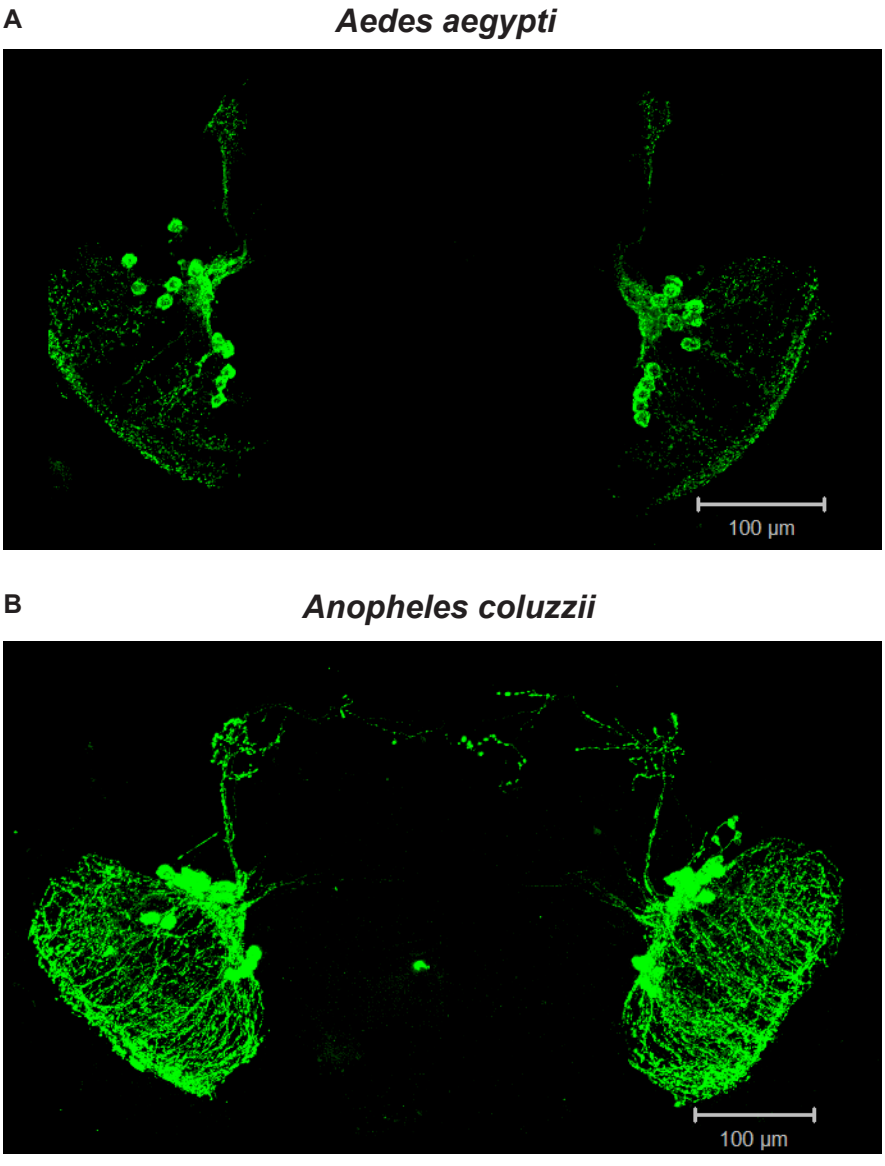

Figure 4–5 PDF neuronal circuit and projections of mosquito brains.

(A-B) Representative confocal images of adult female *Ae. aegypti* **(A)** and *An. coluzzii* **(B)** mosquito brains immunocytochemistry stained with–PDF (green) antibody.

4.3.4 PER oscillation exhibits opposing phase peaks between diurnal and nocturnal mosquitoes.

We measured PER protein oscillating levels throughout the 24hr day using anti-PER immunocytochemistry staining at 6hr intervals in the brains of standard 12hr: 12hr light:dark (LD) entrained *Ae. aegypti* and *An. coluzzii* female mosquitoes. Mosquito brains were co-stained with PDF to allow sub-group identification of PDF^+ versus PDF^- LNvs. We observe robust PER rhythm in both *Ae. aegypti* and *An. coluzzii* circadian neurons, in a neuronal subgroup-specific manner. Most notably, PER cycling in PDF^+ LNvs oscillate in opposite phases between diurnal *Ae. aegypti* versus nocturnal *An. coluzzii* brains (Fig. 4-6). PER protein levels peak in late night/early day in PDF^+ s-LNv and l-LNv of the diurnal mosquito *Ae. aegypti* (Fig. 4-6 A, C, D). In contrast, PER protein levels peak in late day/early night in PDF^+ s-LNv and l-LNv of the nocturnal mosquito *An. coluzzii* (Fig. 4-6 B, E, F). PER protein levels peak in early daytime in PER^+ DNs of both diurnal *Ae. aegypti* and nocturnal *An. coluzzii* (Fig. 4-7 and 4-9). Similarly, PER protein levels peak during the daytime in *Ae. aegypti* specific m-ANs (Fig. 4-8). In *An. coluzzii* PI-like neurons, PER protein levels peak in the early day (Fig. 4-10). In summary, diurnal and nocturnal mosquitoes have distinct circadian molecular signatures in the brain, the most striking feature is the early day PER peak in diurnal mosquito *Ae. aegypti* versus the early evening PER peak in nocturnal *An. coluzzii* in PDF^+ LNv neurons. These results suggest a mechanism for diurnal and nocturnality dictated at the neuronal/circuit level.

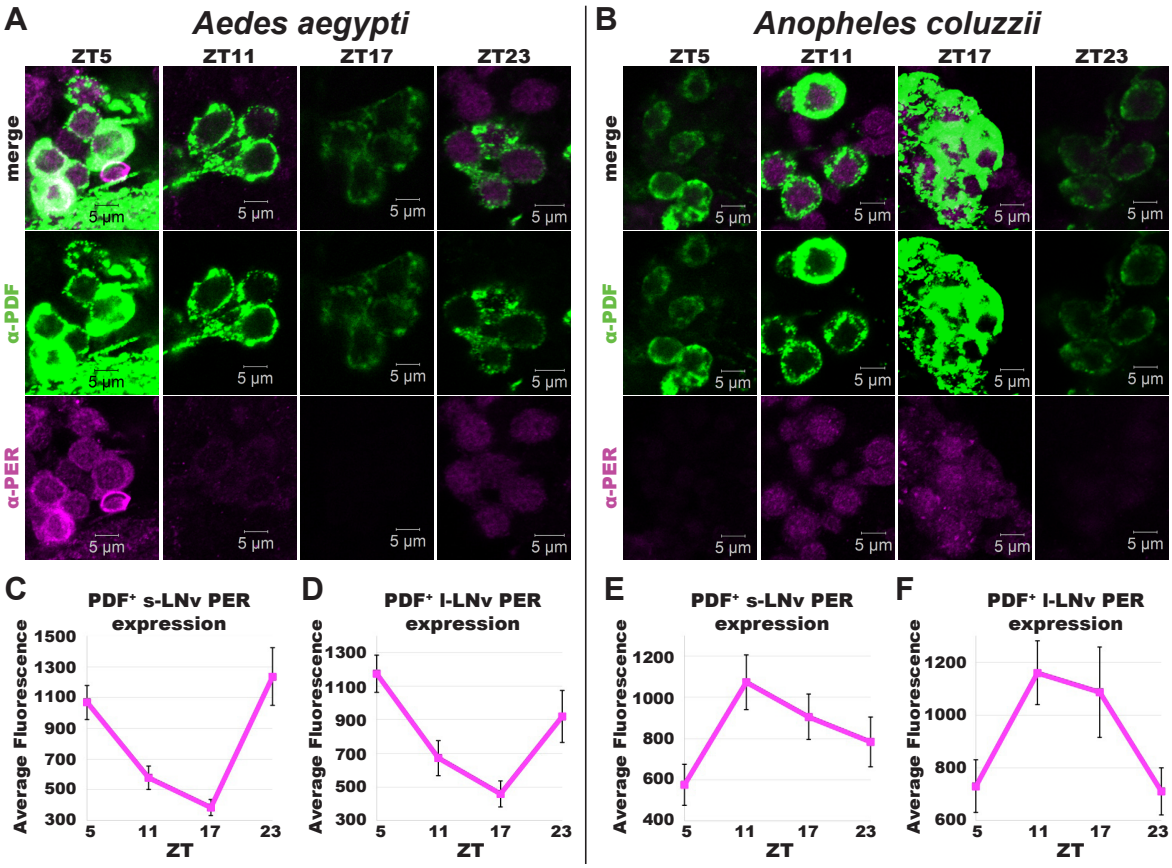

Figure 4–6 Diurnal versus nocturnal mosquito PER expression in PDF+ LNv neurons oscillate in anti-phasic manner

(A-B) Representative confocal images of adult female *Ae. aegypti* **(A)** and *An. coluzzii* **(B)** mosquito lateral ventral neurons (LNv) immunocytochemistry stained with α –PER (magenta) and α –PDF (green) antibodies at ZTs 5, 11, 17, and 23. **(C-F)** PERIOD expression levels at each ZT for *Ae. aegypti* s-LNv **(C)** and l-LNv **(D)**, and *An. colluzzii* s-LNv **(E)** and l-LNv **(F)**.

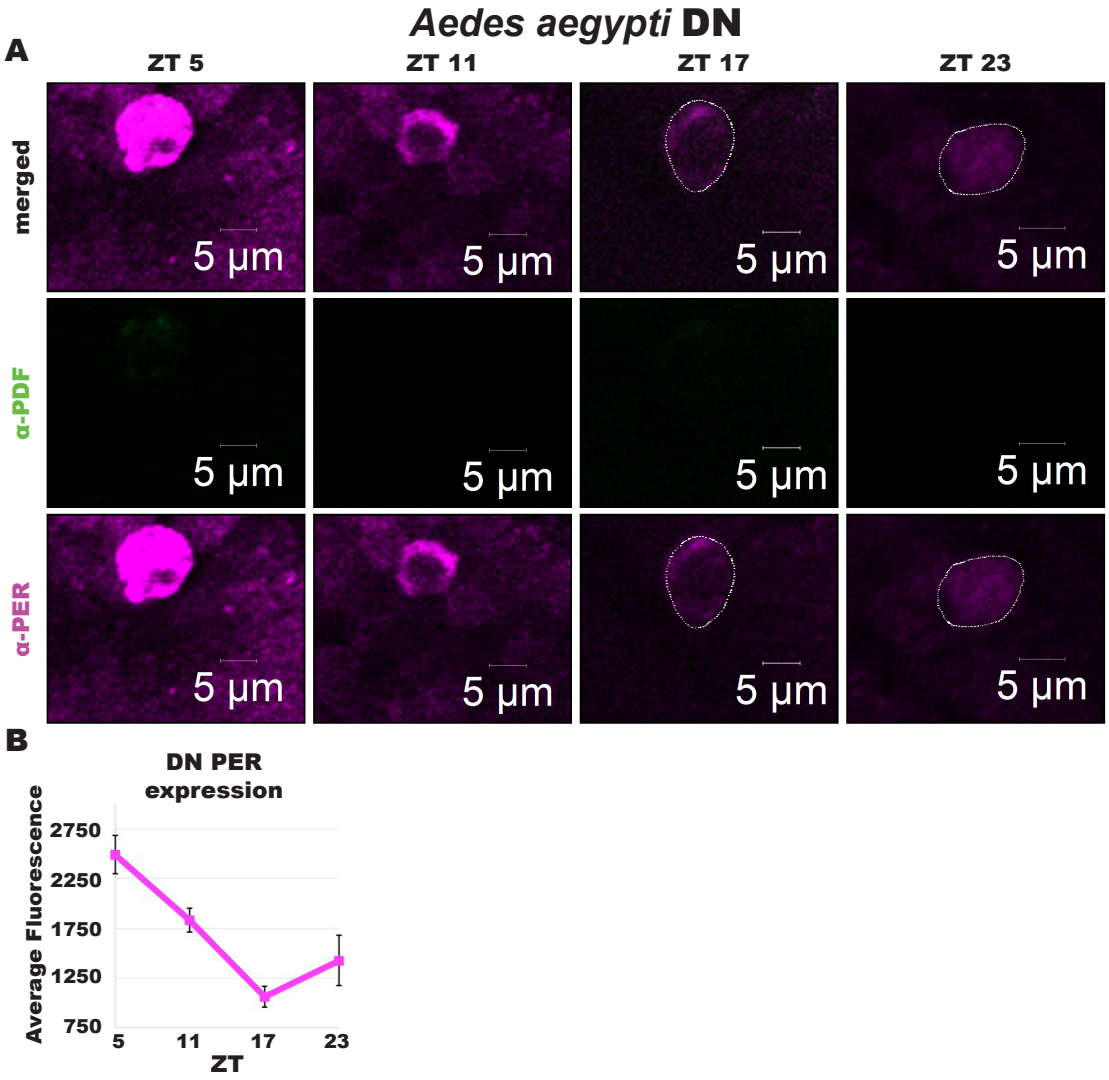

Figure 4–7 Oscillatory PER expression of *Aedes aegypti* DNs.
(A) Representative confocal images of adult female *Ae. aegypti* mosquito dorsal neurons (DNs) immunocytochemistry stained with α–PER (magenta) and α–PDF (green) antibodies at ZTs 5, 11, 17, and 23. **(B)** PERIOD expression levels at each ZT for *Ae. aegypti* DNs (ZT 5, n=27; ZT 11, n=17; ZT 17, n=6, ZT 23, n=7). Data are represented as mean ± S.E.M.

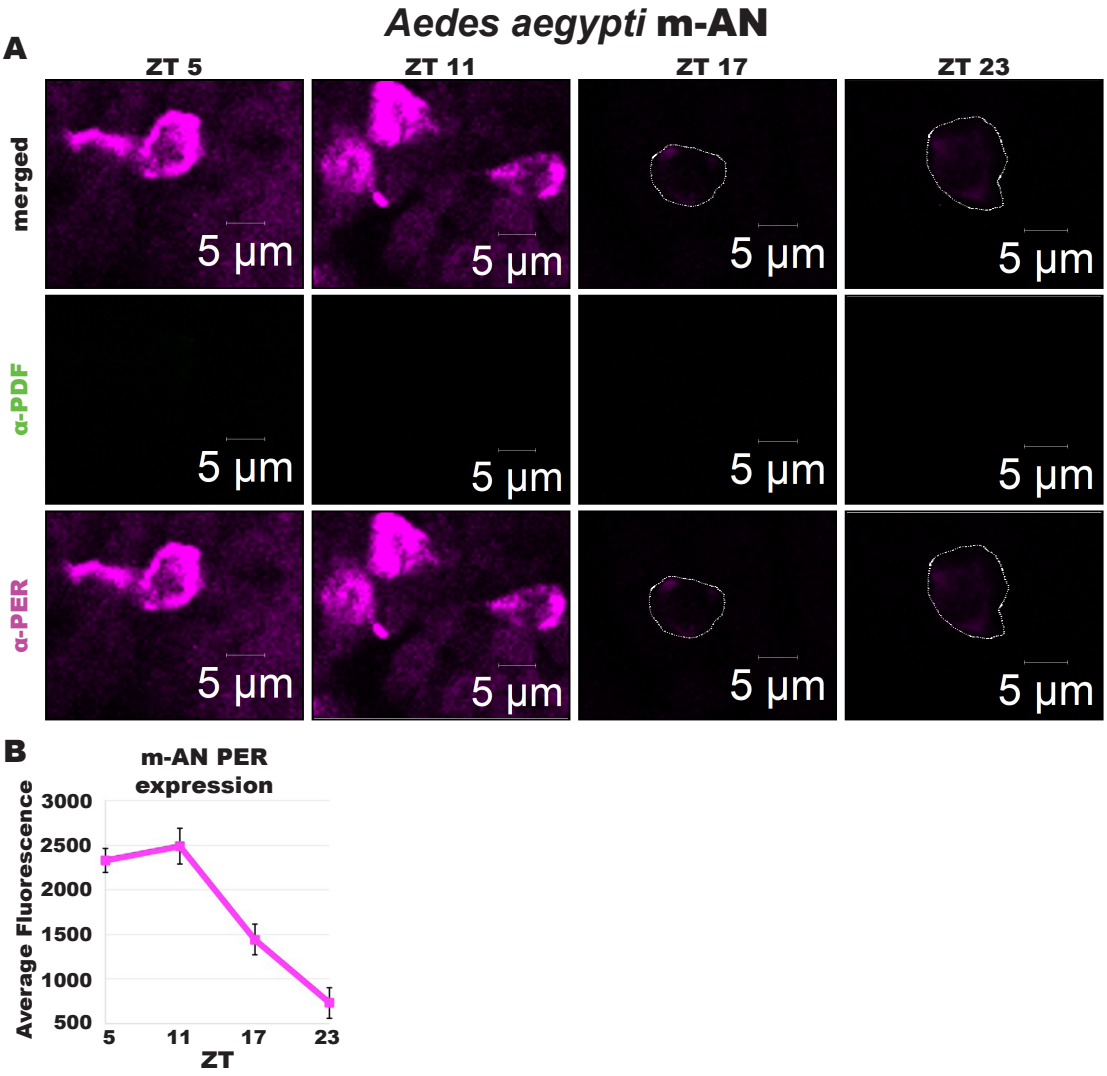

Figure 4–8 Oscillatory PER expression of *Aedes aegypti* m-ANs.
(A) Representative confocal images of adult female *Ae. aegypti* mosquito mid-anterior neurons (m-ANs) immunocytochemistry stained with α–PER (magenta) and α–PDF (green) antibodies at ZTs 5, 11, 17, and 23. **(B)** PERIOD expression levels at each ZT for *Ae. aegypti* m-ANs (ZT 5, n=27; ZT 11, n=17; ZT 17, n=6, ZT 23, n=7). Data are represented as mean ± S.E.M.

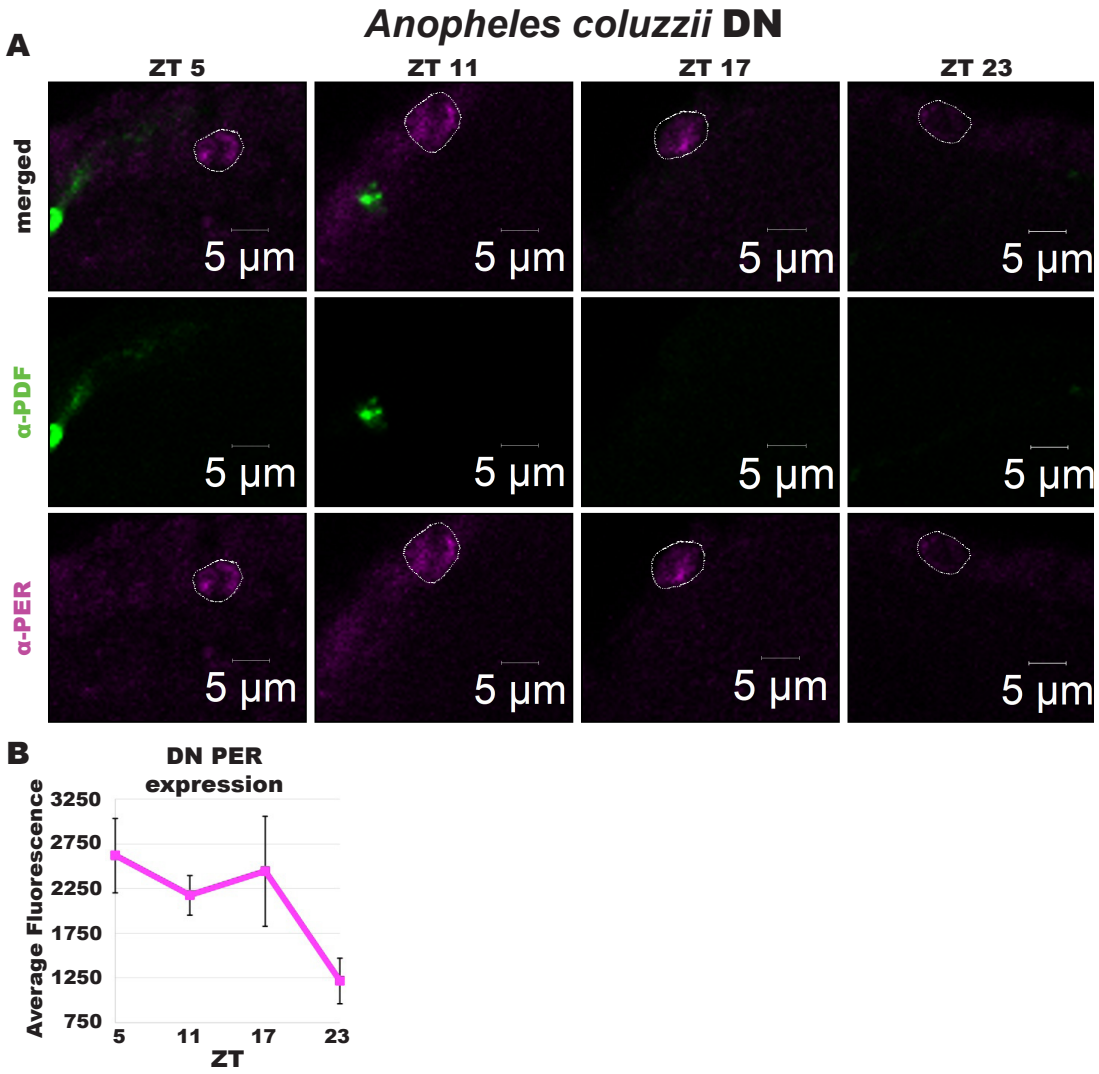

Figure 4–9 Oscillatory PER expression of *Anopheles coluzzii* DNs.
(A) Representative confocal images of adult female *An. coluzzii* mosquito dorsal neurons (DNs) immunocytochemistry stained with α–PER (magenta) and α–PDF (green) antibodies at ZTs 5, 11, 17, and 23. **(B)** PERIOD expression levels at each ZT for *An. colluzzii* DNs (ZT 5, n=13; ZT 11, n=31; ZT 17, n=9, ZT 23, n=8)**.** Data are represented as mean ± S.E.M.

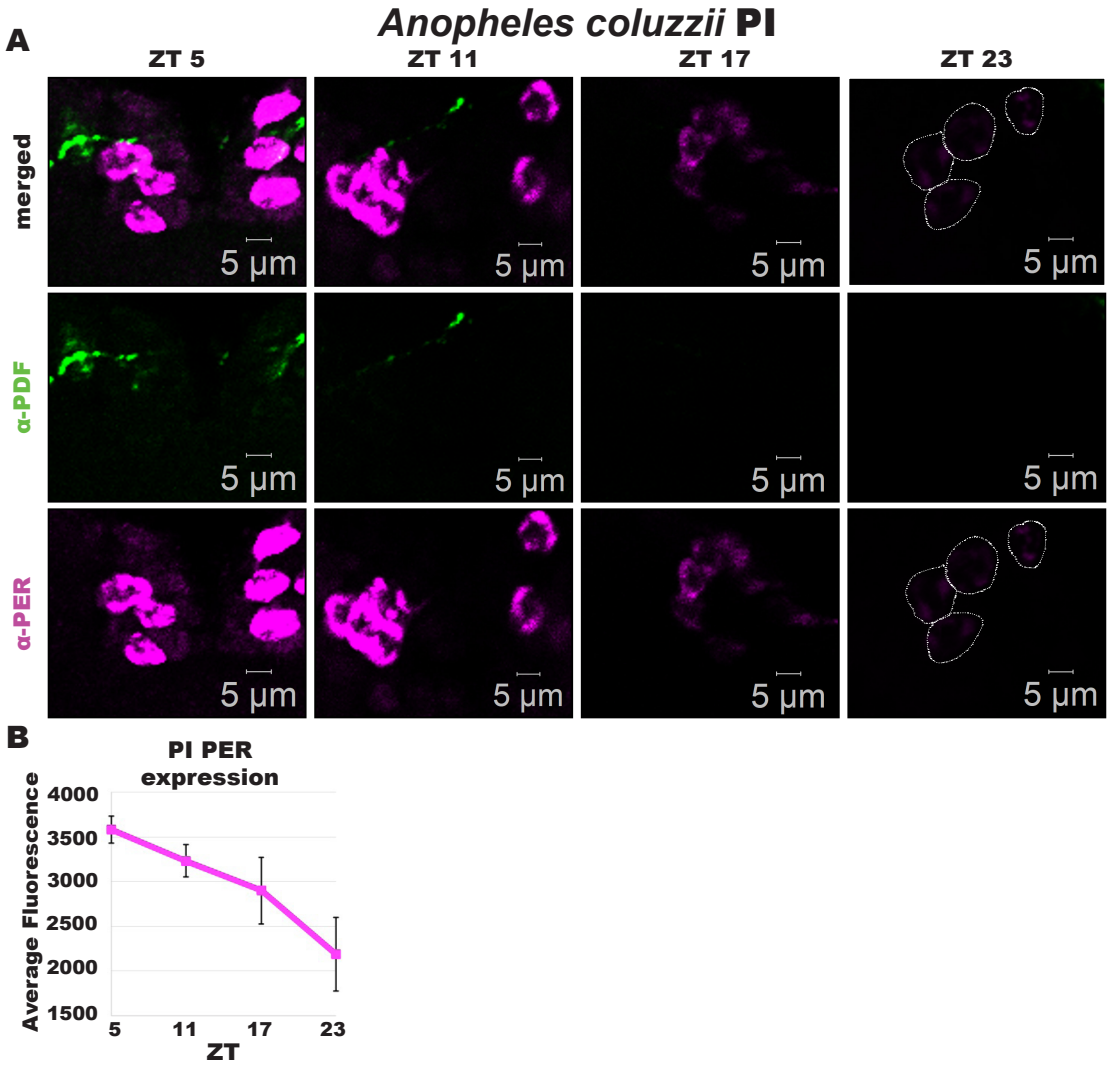

Figure 4–10 Oscillatory PER expression of *Anopheles coluzzii* PI neurons.
(A) Representative confocal images of adult female *An. coluzzii* mosquito pars intercerebralis (PI) neurons immunocytochemistry stained with α–PER (magenta) and α–PDF (green) antibodies at ZTs 5, 11, 17, and 23. **(B)** PERIOD expression levels at each ZT for *An. coluzzii* DNs (ZT 5, n=13; ZT 11, n=31; ZT 17, n=9, ZT 23, n=8)**.** Data are represented as mean ± S.E.M.

4.3.5 Constant light exposure disrupts circadian protein expression and clock-modulated avoidance/attraction behavioral responses to UV light.

Constant light conditions disrupt circadian clock gene expression and rhythmic behaviors in many animals (46, 162, 163). In mosquitoes, constant light condition results in arrhythmic

flight activity (164-166). To examine the functional linkage between circadian clock disruption and attraction/avoidance behavioral responses to UV light, *Ae. aegypti* and *An. coluzzii* female mosquitoes were exposed to constant UV light exposure (UV LL) for 3-5 days. Using anti-PER immunocytochemistry, we measured PER protein levels corresponding to species specific peak times in female mosquito brains following 3-5 days of UV LL. PER protein levels show severely reduced expression in mosquito brains following UV LL compared to LD in both *Ae. aegypti* and *An. coluzzii* (Fig. 4-11 A-D). In many brains, PER protein levels in LNvs could not be quantified because there was no visible PER staining following UV LL (not shown). Thus for LNvs, PER protein levels in PDF^{+} neurons were quantified.

To determine whether functional ablation of the circadian clock modulates UV light-evoked attraction/avoidance behavior in mosquitoes, we measured the behavioral preference for UV-exposed versus shaded light environments under UV LL condition. Under control LD conditions, one of the most striking time-of-day dependent phenotypes seen is the dusk-anticipatory increase in attraction/decrease in avoidance (Fig. 4-1). In contrast, under the experimental UV LL condition, both *Ae. aegypti* and *An. coluzzii* mosquitoes lack clear time-of-day dependent changes in attraction/avoidance behavior, including the dusk-anticipation (Fig. 4-11 E-H). *Ae. aegypti* females are attracted to UV light regardless of time-of-day (Fig. 4-11 E, G). *An. coluzzii* females lack subjective day versus night differences in avoidance/attraction, and overall lack any clear preferences for either UV-exposed or shaded environments under UV LL condition (Fig. 4-11 F, H). My results show that clock ablating constant UV light exposure disrupts circadian function in both diurnal and nocturnal mosquito species, and that the circadian clock modulates the timing of UV-evoked attraction/avoidance behavior of both diurnal and nocturnal mosquitoes.

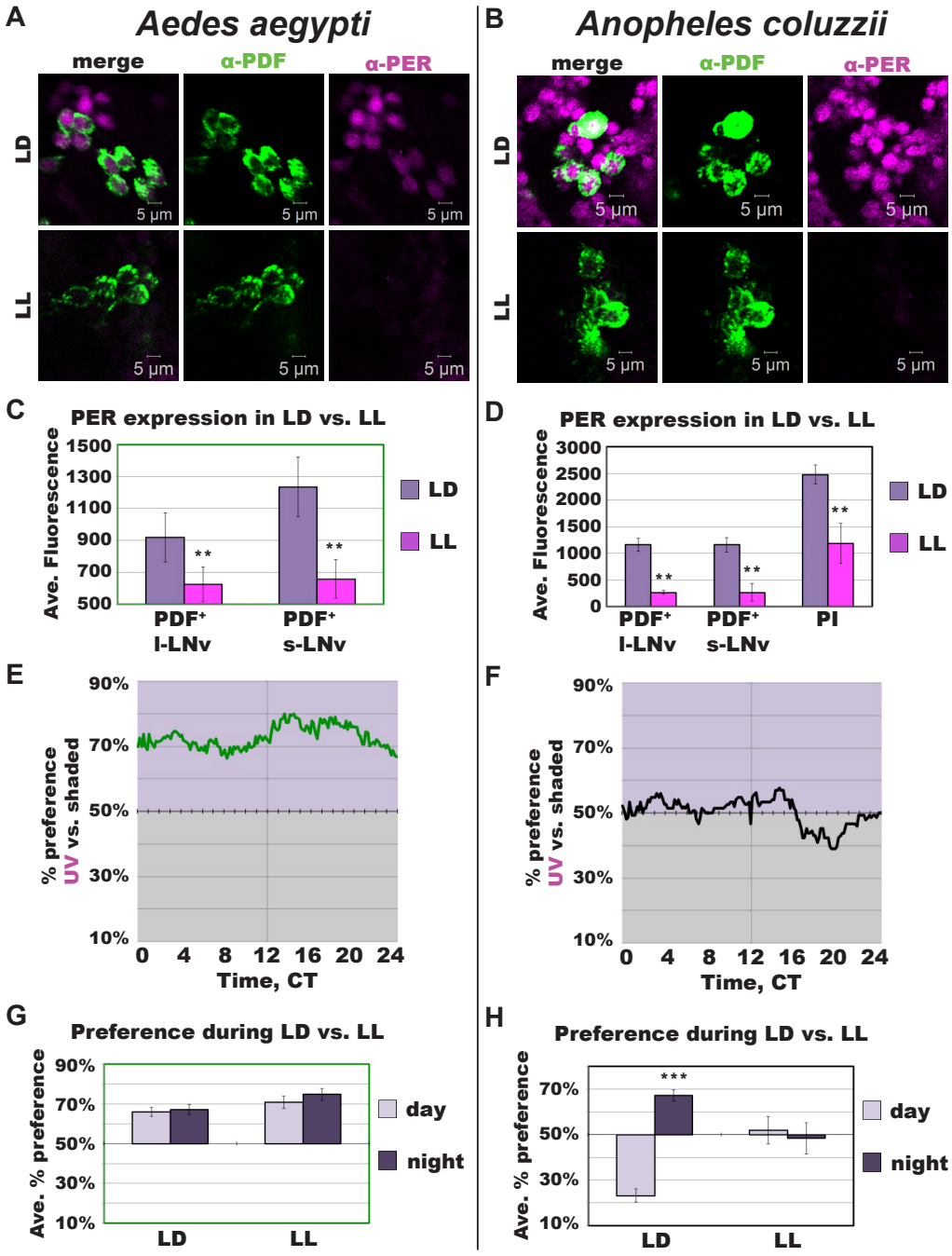

Figure 4–11 Constant UV light exposure disrupts circadian protein expression and clock modulation of attraction/avoidance behavioral responses to UV light in mosquitoes.
(A-B) Representative confocal images of anti–PER (magenta) and anti–PDF (green) immunocytochemistry stained adult female mosquito brains under LD or following UV LL exposure for *Ae. aegypti* **(A)** and *An. coluzzii* **(B)**. **(C-D)** Average fluorescence intensity of circadian neurons under LD versus LL conditions for *Ae. aegypti* ZT/CT23 **(C)** and *An. coluzzii* ZT/CT11 **(D)**. **(E-F)** Attraction/avoidance behavior to UV light, measured by % of preference in UV-exposed versus shaded during UV LL for female *Ae. aegypti* **(E)**, female *An. coluzzii* **(F)**. **(G-H)** Average attraction/avoidance behavioral preference to light-exposed versus shaded-

environment during subjective daytime versus nighttime under LD or LL conditions for *Ae. aegypti* **(G)** and *An. coluzzii* **(H)** female mosquitoes.

4.4 Discussion

Using our novel assay to measure light environmental preference in mosquitoes, I show that mosquito attraction/avoidance behavior to light is specie-, sex-, spectra-, and time-of-day-dependent. Diurnal versus nocturnal mosquitoes have opposite attraction/avoidance behavioral valence for short wavelength light. Daytime-active mosquitoes, *Ae. aegypti*, are attracted to a wide range of light spectra during the daytime. In contrast nighttime-active mosquitoes *An. coluzzii* are strongly photophobic in response to short wavelength light. Both species exhibit anticipatory behavior of decreased avoidance and increased attraction during the temporal approach to dusk. This correlates with the ecological timing of increased flight activity and host seeking behaviors (164, 165, 167, 168). Interestingly, males of both diurnal and nocturnal species show earlier anticipatory behavior of increased UV attraction, compared to females. Both *Ae. aegypti* and *An. coluzzii* males exhibit earlier flight activity onset towards dusk, compared females (130, 132). Male mosquitoes form 'swarms' in anticipation of female mosquitoes flying through to mate. The timing of male swarming is species specific. This dictates a temporal niche for mating of different mosquito species, whereby male mosquitoes evolved pre-dusk earlier anticipation to optimize their chance of mating (128). Our previous work shows that light-evoked attraction/avoidance behavior is mediated by both opsin- and non-opsin based photoreceptors in *Drosophila* (6, 9). Further investigation is needed to determine whether if different phototransduction mechanisms are involved in mosquito light preference behavior.

I show the location and oscillation of core circadian protein at neuronal subgroup level in diurnal and nocturnal mosquitoes. Intriguingly, I find core circadian protein expressions oscillate

in anti-phasic manner between diurnal and nocturnal mosquitoes, which could mechanistically underlie diurnal versus nocturnal behaviors. Conceptual support for this finding is that for diurnal versus nocturnal mammals, the circadian clock in non-suprachiasmatic nuclei neurons and periphery tissues cycle in opposite phases between diurnal versus nocturnal rodents and primates (169-175). It is not known currently what factors contribute to differential phase timing of circadian protein cycling among different tissue and cellular types. My detailed characterization of light-evoked attraction/avoidance behavior in mosquitoes shows timing features that suggest that these processes are under circadian regulation as I find in *Drosophila melanogaster* (6, 9). Experimental verification for this is shown by temporal disruption of light-evoked attraction/avoidance behavior by environmentally shutting down the circadian clock with constant light (LL). A wide range of behaviors in mosquito and other insects are temporally modulated, including mating, seeking a blood-meal, biting, oviposition, flight activity, and sleep (128, 133, 164, 165, 176-183). Light treatments that lead to the loss of circadian function also disrupts biting, flight activity, and oviposition behaviors (164-166, 168, 177, 178). In summary, I demonstrated a circadian basis of time-of-day dependent activity and light-induced attraction/avoidance behaviors in diurnal and nocturnal mosquitoes.

Chapter 5 : Conclusion and Final Remarks

Insects display a wide range of physiological and behavioral responses to light, with strong spectral sensitivity for short wavelength light. It had been long assumed that external photoreceptors are solely responsible for phototransduction in insects. The recent discovery of other phototransduction pathways, such as direct neuronal photoreceptors CRY and Rh7 in the brain has propelled my thesis research. Long-term goal of my thesis was to establish an integrative framework that addresses how light activates classical and non-classical photoreceptors to modulate complex insect physiology and behaviors. I carried out detailed investigation of redox mechanism underlying CRY/HK-modulated phototransduction. I examined how multiple light inputs differentially influence circadian and light-evoked behaviors in *Drosophila* at the molecular, physiological, neuronal, and genetic levels. In mosquitoes, I identified the molecular mechanism underlying diurnal versus nocturnal light-evoked behaviors.

Chapter 2 of this thesis investigated the structural and biophysical basis of CRY-mediated phototransduction mechanism. The crystal structure of *Drosophila* CRY revealed a series of tryptophan residues near its "active site" where the FAD cofactor binds. In a closely related protein, (6-4) photolyase that shares many structural similarities, the tryptophan residues near FAD binding site has been shown to be critical in its light-induced DNA repair activities (184). These tryptophan residues function as an electron transfer chain upon photoactivation of FAD. Furthermore, I previously demonstrated that CRY redox couples with HK for its phototransduction mechanism. There had been a controversy as to whether if these tryptophan residues function electron transfer chain to modulate photoactivation of CRY. Here I tested for the importance of the tryptophan residues *in vivo*, using transgenic mutant analysis paired with highly sensitive evoked potential electrophysiology and behavioral analysis. I was able to

demonstrate that the tryptophan residue closest to FAD binding site of CRY mediates electron transfer to evoked rapid neuronal response to short wavelength light. Furthermore, I showed that slow circadian photoentrainment is mediated by conformational change of CRY's flexible CTT. By testing for function of CRY under a much more native environment paired with electrophysiological and behavioral measures, I was able to resolve the controversial results others previously observed *in vitro*. Interestingly, other types of CRY such as mammalian CRYs exist without the FAD cofactor. These mammalian-type CRYs play a role in circadian molecular function, but are not light sensitive. My results also explain how CRY can modulate the circadian timing, independent of the series tryptophan residues functioning in light-activated electron transfer. Results outlined in Chapter 2 gives insights into the biophysical properties of evolutionarily ancient phototransduction mechanism, as well as the basic molecular mechanism of the circadian clock mediated by CRY.

In Chapter 3 of this thesis, I investigated the complexity of short wavelength light-evoked behaviors in *Drosophila*, with emphasis on the attraction/avoidance behavior. Previously, adult *Drosophila* was assumed to be simply attracted to light. Here I conducted a comprehensive study of light-evoked attraction/avoidance behavior with respect to spectra, intensity, time-of-day, duration of exposure, phototransduction pathways, circadian genes, neuronal circuit, and spatial information. I developed several behavioral assays monitoring *Drosophila* attraction/avoidance behavior at high spatial and temporal resolutions. I found that flies can exhibit both attraction and avoidance behaviors depending on several external and internal factors. External factors include the spectra, intensity, and duration of light, while internal factors include circadian timing and different phototransduction pathways involved at the molecular and neuronal circuitry levels. I demonstrated that flies integrate multiple photic inputs to navigate their light

environment in a complex manner. Results found in Chapter 3 also established basis for testing light-evoked responses of mosquitoes, outlined in Chapter 4.

In Chapter 4 of this thesis, I examined the molecular basis of diurnality and nocturnality and behavioral timing in mosquitoes. I described the circadian neuronal circuit and rhythmic expression of core circadian protein and neuropeptide in diurnal and nocturnal mosquito brains. I developed a novel assay measuring mosquito light environmental preferences. I found that mosquito attraction/avoidance behavior to light is specie-, sex-, spectra-, and time-of-day-dependent. Lastly, I show that the circadian clock modulates the timing and valence of light-evoked attraction/avoidance behavioral responses in mosquitoes. This is the first comparative analysis of neuronal circuit and expression of circadian genes in diurnal and nocturnal mosquito species. Anti-phasic circadian protein expression and distinct circadian anatomical circuit suggest the fundamental basis underlying diurnality versus nocturnality.

To test my hypothesis that short wavelength light modulates a wide range of insect behaviors, I developed and employed several assays and reagents, including molecular, genetic, physiological, and behavioral approaches. I demonstrated the biophysical mechanism underlying CRY-mediated phototransduction. My work in *Drosophila* demonstrates the complexity of insect behavior, which can be manipulated using different spectra, intensity, and timing of light exposure. I discovered striking differences between diurnal and nocturnal mosquito species in their circadian neuronal circuit. I demonstrated specie-, circadian-, and spectra-specific modulation of light-evoked attraction/avoidance behaviors in mosquitoes. Several assays I developed here can be further adapted to test other mosquito and insect species. My work shows that light is a powerful cue that drives complex insect behavior.

REFERENCES

1. Yoshii T, Todo T, Wulbeck C, Stanewsky R, & Helfrich-Forster C (2008) Cryptochrome is present in the compound eyes and a subset of Drosophila's clock neurons. *The Journal of comparative neurology* 508(6):952-966.
2. Benito J, Houl JH, Roman GW, & Hardin PE (2008) The blue-light photoreceptor CRYPTOCHROME is expressed in a subset of circadian oscillator neurons in the Drosophila CNS. *Journal of biological rhythms* 23(4):296-307.
3. Sheeba V*, et al.* (2008) Pigment dispersing factor-dependent and -independent circadian locomotor behavioral rhythms. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 28(1):217-227.
4. Fogle KJ*, et al.* (2015) CRYPTOCHROME-mediated phototransduction by modulation of the potassium ion channel beta-subunit redox sensor. *Proceedings of the National Academy of Sciences of the United States of America* 112(7):2245-2250.
5. Fogle KJ, Parson KG, Dahm NA, & Holmes TC (2011) CRYPTOCHROME is a blue-light sensor that regulates neuronal firing rate. *Science (New York, N.Y.)* 331(6023):1409-1413.
6. Baik LS*, et al.* (2017) CRYPTOCHROME mediates behavioral executive choice in response to UV light. *Proceedings of the National Academy of Sciences of the United States of America* 114(4):776-781.
7. Hoang N*, et al.* (2008) Human and Drosophila cryptochromes are light activated by flavin photoreduction in living cells. *PLoS biology* 6(7):e160.
8. Ozturk N, Song SH, Selby CP, & Sancar A (2008) Animal type 1 cryptochromes. Analysis of the redox state of the flavin cofactor by site-directed mutagenesis. *The Journal of biological chemistry* 283(6):3256-3263.
9. Baik LS, Recinos Y, Chevez JA, & Holmes TC (2018) Circadian modulation of light-evoked avoidance/attraction behavior in Drosophila. *PloS one* 13(8):e0201927.
10. LaDeau SL, Kilpatrick AM, & Marra PP (2007) West Nile virus emergence and large-scale declines of North American bird populations. *Nature* 447(7145):710-713.
11. Ni JD, Baik LS, Holmes TC, & Montell C (2017) A rhodopsin in the brain functions in circadian photoentrainment in Drosophila. *Nature* 545(7654):340-344.
12. Nitabach MN, Blau J, & Holmes TC (2002) Electrical silencing of Drosophila pacemaker neurons stops the free-running circadian clock. *Cell* 109(4):485-495.
13. Nitabach MN*, et al.* (2006) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 26(2):479-489.
14. Sheeba V, Gu H, Sharma VK, O'Dowd DK, & Holmes TC (2007) Circadian- and light-dependent regulation of resting membrane potential and spontaneous action potential firing of *Drosophila* circadian pacemaker neurons. *Journal of neurophysiology* 99(2):976-988.
15. Cao G & Nitabach MN (2008) Circadian control of membrane excitability in Drosophila melanogaster lateral ventral clock neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 28(25):6493-6501.
16. Flourakis M*, et al.* (2015) A Conserved Bicycle Model for Circadian Clock Control of Membrane Excitability. *Cell* 162(4):836-848.
17. Michel S, Geusz ME, Zaritsky JJ, & Block GD (1993) Circadian rhythm in membrane conductance expressed in isolated neurons. *Science (New York, N.Y.)* 259(5092):239-241.
18. Belle MD, Diekman CO, Forger DB, & Piggins HD (2009) Daily electrical silencing in the mammalian circadian clock. *Science (New York, N.Y.)* 326(5950):281-284.
19. Emery P, So WV, Kaneko M, Hall JC, & Rosbash M (1998) CRY, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell* 95(5):669-679.
20. Stanewsky R*, et al.* (1998) The cryb mutation identifies cryptochrome as a circadian photoreceptor in Drosophila. *Cell* 95(5):681-692.
21. Helfrich-Forster C, Winter C, Hofbauer A, Hall JC, & Stanewsky R (2001) The circadian clock of fruit flies is blind after elimination of all known photoreceptors. *Neuron* 30(1):249-261.
22. Rosato E*, et al.* (2001) Light-dependent interaction between Drosophila CRY and the clock protein PER mediated by the carboxy terminus of CRY. *Current biology : CB* 11(12):909-917.

23. Lin FJ, Song W, Meyer-Bernstein E, Naidoo N, & Sehgal A (2001) Photic signaling by cryptochrome in the Drosophila circadian system. *Molecular and cellular biology* 21(21):7287-7294.
24. Busza A, Emery-Le M, Rosbash M, & Emery P (2004) Roles of the two Drosophila CRYPTOCHROME structural domains in circadian photoreception. *Science (New York, N.Y.)* 304(5676):1503-1506.
25. Peschel N, Chen KF, Szabo G, & Stanewsky R (2009) Light-dependent interactions between the Drosophila circadian clock factors cryptochrome, jetlag, and timeless. *Current biology : CB* 19(3):241-247.
26. Liu B, Liu H, Zhong D, & Lin C (2010) Searching for a photocycle of the cryptochrome photoreceptors. *Current opinion in plant biology* 13(5):578-586.
27. Bouly JP*, et al.* (2007) Cryptochrome blue light photoreceptors are activated through interconversion of flavin redox states. *The Journal of biological chemistry* 282(13):9383-9391.
28. Partch CL & Sancar A (2005) Photochemistry and photobiology of cryptochrome blue-light photopigments: the search for a photocycle. *Photochemistry and photobiology* 81(6):1291-1304.
29. Berndt A*, et al.* (2007) A novel photoreaction mechanism for the circadian blue light photoreceptor Drosophila cryptochrome. *The Journal of biological chemistry* 282(17):13011-13021.
30. Schleicher E*, et al.* (2007) Electron nuclear double resonance differentiates complementary roles for active site histidines in (6-4) photolyase. *The Journal of biological chemistry* 282(7):4738-4747.
31. Xu L*, et al.* (2008) Active site of Escherichia coli DNA photolyase: Asn378 is crucial both for stabilizing the neutral flavin radical cofactor and for DNA repair. *Biochemistry* 47(33):8736-8743.
32. Sheeba V*, et al.* (2008) Large ventral lateral neurons modulate arousal and sleep in Drosophila. *Current biology : CB* 18(20):1537-1545.
33. Parisky KM*, et al.* (2008) PDF cells are a GABA-responsive wake-promoting component of the Drosophila sleep circuit. *Neuron* 60(4):672-682.
34. Shang Y, Griffith LC, & Rosbash M (2008) Light-arousal and circadian photoreception circuits intersect at the large PDF cells of the Drosophila brain. *Proceedings of the National Academy of Sciences of the United States of America* 105(50):19587-19594.
35. Kumar S, Chen D, & Sehgal A (2012) Dopamine acts through Cryptochrome to promote acute arousal in Drosophila. *Genes & development* 26(11):1224-1234.
36. Ceriani MF*, et al.* (1999) Light-dependent sequestration of TIMELESS by CRYPTOCHROME. *Science (New York, N.Y.)* 285(5427):553-556.
37. Zoltowski BD*, et al.* (2011) Structure of full-length Drosophila cryptochrome. *Nature* 480(7377):396-399.
38. Lukacs A, Eker AP, Byrdin M, Brettel K, & Vos MH (2008) Electron hopping through the 15 A triple tryptophan molecular wire in DNA photolyase occurs within 30 ps. *Journal of the American Chemical Society* 130(44):14394-14395.
39. Pfeiffer BD*, et al.* (2010) Refinement of tools for targeted gene expression in Drosophila. *Genetics* 186(2):735-755.
40. Li MT*, et al.* (2018) Hub-organized parallel circuits of central circadian pacemaker neurons for visual photoentrainment in Drosophila. *Nature communications* 9(1):4247.
41. Levy C*, et al.* (2013) Updated structure of Drosophila cryptochrome. *Nature* 495(7441):E3-4.
42. Lin C, Top D, Manahan CC, Young MW, & Crane BR (2018) Circadian clock activity of cryptochrome relies on tryptophan-mediated photoreduction. *Proceedings of the National Academy of Sciences of the United States of America* 115(15):3822-3827.
43. Ozturk N, Selby CP, Annayev Y, Zhong D, & Sancar A (2011) Reaction mechanism of Drosophila cryptochrome. *Proceedings of the National Academy of Sciences of the United States of America* 108(2):516-521.
44. Vaidya AT*, et al.* (2013) Flavin reduction activates Drosophila cryptochrome. *Proceedings of the National Academy of Sciences of the United States of America* 110(51):20455-20460.
45. Konopka RJ & Benzer S (1971) Clock mutants of Drosophila melanogaster. *Proceedings of the National Academy of Sciences of the United States of America* 68(9):2112-2116.
46. Daan S & Pittendrigh CS (1976) A functional analysis of circadian pacemakers in nocturnal rodents. *Journal of comparative physiology. A, Sensory, neural, and behavioral physiology* 106(3):267-290.
47. Emery P, Stanewsky R, Hall JC, & Rosbash M (2000) A unique circadian-rhythm photoreceptor. *Nature* 404(6777):456-457.
48. Dolezelova E, Dolezel D, & Hall JC (2007) Rhythm defects caused by newly engineered null mutations in Drosophila's cryptochrome gene. *Genetics* 177(1):329-345.
49. Garbe DS*, et al.* (2015) Context-specific comparison of sleep acquisition systems in Drosophila. *Biology open* 4(11):1558-1568.

50. Miller GV, Hansen KN, & Stark WS (1981) Phototaxis in Drosophila: R1–6 input and interaction among ocellar and compound eye receptors. *Journal of Insect Physiology* 27(11):813-819.
51. Heisenberg M & Buchner E (1977) The role of retinula cell types in visual behavior ofDrosophila melanogaster. *Journal of comparative physiology* 117(2):127-162.
52. Yamaguchi S, Desplan C, & Heisenberg M (2010) Contribution of photoreceptor subtypes to spectral wavelength preference in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America* 107(12):5634-5639.
53. Gao S*, et al.* (2008) The neural substrate of spectral preference in Drosophila. *Neuron* 60(2):328-342.
54. Xiang Y*, et al.* (2010) Light-avoidance-mediating photoreceptors tile the Drosophila larval body wall. *Nature* 468(7326):921-926.
55. Zhu EY, Guntur AR, He R, Stern U, & Yang CH (2014) Egg-laying demand induces aversion of UV light in Drosophila females. *Current biology : CB* 24(23):2797-2804.
56. Sakai T, Isono K, Tomaru M, Fukatami A, & Oguma Y (2002) Light wavelength dependency of mating activity in the Drosophila melanogaster species subgroup. *Genes & genetic systems* 77(3):187-195.
57. Majercak J, Sidote D, Hardin PE, & Edery I (1999) How a circadian clock adapts to seasonal decreases in temperature and day length. *Neuron* 24(1):219-230.
58. Pittendrigh C (1965) On the mechanism of the entrainment of a circadian rhythm by light cycles. *Circadian Clocks*:277-297.
59. Heisenberg M & Buchner EJJocp (1977) The role of retinula cell types in visual behavior ofDrosophila melanogaster. 117(2):127-162.
60. Saint-Charles A*, et al.* (2016) Four of the six Drosophila rhodopsin-expressing photoreceptors can mediate circadian entrainment in low light. *The Journal of comparative neurology* 524(14):2828-2844.
61. Chou WH*, et al.* (1996) Identification of a novel Drosophila opsin reveals specific patterning of the R7 and R8 photoreceptor cells. *Neuron* 17(6):1101-1115.
62. Feiler R*, et al.* (1992) Ectopic expression of ultraviolet-rhodopsins in the blue photoreceptor cells of Drosophila: visual physiology and photochemistry of transgenic animals. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 12(10):3862-3868.
63. Salcedo E*, et al.* (1999) Blue- and green-absorbing visual pigments of Drosophila: ectopic expression and physiological characterization of the R8 photoreceptor cell-specific Rh5 and Rh6 rhodopsins. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 19(24):10716-10726.
64. Veleri S, Rieger D, Helfrich-Forster C, & Stanewsky R (2007) Hofbauer-Buchner eyelet affects circadian photosensitivity and coordinates TIM and PER expression in Drosophila clock neurons. *Journal of biological rhythms* 22(1):29-42.
65. Helfrich-Forster C*, et al.* (2002) The extraretinal eyelet of Drosophila: development, ultrastructure, and putative circadian function. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 22(21):9255-9266.
66. Kistenpfennig C, Hirsh J, Yoshii T, & Helfrich-Forster C (2012) Phase-shifting the fruit fly clock without cryptochrome. *Journal of biological rhythms* 27(2):117-125.
67. Klarsfeld A*, et al.* (2004) Novel features of cryptochrome-mediated photoreception in the brain circadian clock of Drosophila. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 24(6):1468-1477.
68. Malpel S, Klarsfeld A, & Rouyer F (2002) Larval optic nerve and adult extra-retinal photoreceptors sequentially associate with clock neurons during Drosophila brain development. *Development (Cambridge, England)* 129(6):1443-1453.
69. Rieger D, Stanewsky R, & Helfrich-Forster C (2003) Cryptochrome, compound eyes, Hofbauer-Buchner eyelets, and ocelli play different roles in the entrainment and masking pathway of the locomotor activity rhythm in the fruit fly Drosophila melanogaster. *Journal of biological rhythms* 18(5):377-391.
70. Schlichting M, Grebler R, Menegazzi P, & Helfrich-Forster C (2015) Twilight dominates over moonlight in adjusting Drosophila's activity pattern. *Journal of biological rhythms* 30(2):117-128.
71. Schlichting M, Grebler R, Peschel N, Yoshii T, & Helfrich-Forster C (2014) Moonlight detection by Drosophila's endogenous clock depends on multiple photopigments in the compound eyes. *Journal of biological rhythms* 29(2):75-86.
72. Schlichting M*, et al.* (2016) A Neural Network Underlying Circadian Entrainment and Photoperiodic Adjustment of Sleep and Activity in Drosophila. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 36(35):9084-9096.

73. Pan Y, Weng J, Cao Y, Bhosle RC, & Zhou M (2008) Functional coupling between the Kv1.1 channel and aldoketoreductase Kvbeta1. *The Journal of biological chemistry* 283(13):8634-8642.
74. Weng J, Cao Y, Moss N, & Zhou M (2006) Modulation of voltage-dependent Shaker family potassium channels by an aldo-keto reductase. *The Journal of biological chemistry* 281(22):15194-15200.
75. Roberts L*, et al.* (2015) Light evokes rapid circadian network oscillator desynchrony followed by gradual phase retuning of synchrony. *Current biology : CB* 25(7):858-867.
76. Roberts L, Leise TL, Welsh DK, & Holmes TC (2016) Functional Contributions of Strong and Weak Cellular Oscillators to Synchrony and Light-shifted Phase Dynamics. *Journal of biological rhythms* 31(4):337-351.
77. Kaneko M, Helfrich-Forster C, & Hall JC (1997) Spatial and temporal expression of the period and timeless genes in the developing nervous system of Drosophila: newly identified pacemaker candidates and novel features of clock gene product cycling. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 17(17):6745-6760.
78. Barber AF, Erion R, Holmes TC, & Sehgal A (2016) Circadian and feeding cues integrate to drive rhythms of physiology in Drosophila insulin-producing cells. *Genes & development* 30(23):2596-2606.
79. Garbe DS*, et al.* (2015) Context-specific comparison of sleep acquisition systems in Drosophila. *Biology Open* 4(11):1558-1568.
80. Keene AC*, et al.* (2011) Distinct visual pathways mediate Drosophila larval light avoidance and circadian clock entrainment. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 31(17):6527-6534.
81. Kane EA*, et al.* (2013) Sensorimotor structure of Drosophila larva phototaxis. *Proceedings of the National Academy of Sciences of the United States of America* 110(40):E3868-3877.
82. Harrisingh MC, Wu Y, Lnenicka GA, & Nitabach MN (2007) Intracellular Ca2+ regulates free-running circadian clock oscillation in vivo. *J of Neurosci* 27(46):12489-12499.
83. Guntur AR*, et al.* (2015) Drosophila TRPA1 isoforms detect UV light via photochemical production of H2O2. *Proceedings of the National Academy of Sciences of the United States of America* 112(42):E5753-5761.
84. Shafer OT, Helfrich-Forster C, Renn SC, & Taghert PH (2006) Reevaluation of Drosophila melanogaster's neuronal circadian pacemakers reveals new neuronal classes. *The Journal of comparative neurology* 498(2):180-193.
85. Moses K, Ellis MC, & Rubin GM (1989) The glass gene encodes a zinc-finger protein required by Drosophila photoreceptor cells. *Nature* 340(6234):531-536.
86. Zhu L, McKay RR, & Shortridge RD (1993) Tissue-specific expression of phospholipase C encoded by the norpA gene of Drosophila melanogaster. *The Journal of biological chemistry* 268(21):15994-16001.
87. McKay RR*, et al.* (1995) Phospholipase C rescues visual defect in norpA mutant of Drosophila melanogaster. *The Journal of biological chemistry* 270(22):13271-13276.
88. Daan S & Pittendrigh CS (1976) A functional analysis of circadian pacemakers in nocturnal rodents. *J of Comp Physiol* 106(3):267-290.
89. Konopka RJ, Pittendrigh C, & Orr D (1989) Reciprocal behaviour associated with altered homeostasis and photosensitivity of Drosophila clock mutants. *Journal of neurogenetics* 6(1):1-10.
90. Rieger D, Shafer OT, Tomioka K, & Helfrich-Förster C (2006) Functional analysis of circadian pacemaker neurons in Drosophila melanogaster. *Journal of Neuroscience* 26(9):2531-2543.
91. Sheeba V, Fogle KJ, & Holmes TC (2010) Persistence of morning anticipation behavior and high amplitude morning startle response following functional loss of small ventral lateral neurons in Drosophila. *PloS one* 5(7):e11628.
92. Renn SC, Park JH, Rosbash M, Hall JC, & Taghert PH (1999) A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. *Cell* 99(7):791-802.
93. Grima B, Chélot E, Xia R, & Rouyer F (2004) Morning and evening peaks of activity rely on different clock neurons of the Drosophila brain. *Nature* 431(7010):869-873.
94. Stoleru D, Peng Y, Agosto J, & Rosbash M (2004) Coupled oscillators control morning and evening locomotor behaviour of Drosophila. *Nature* 431(7010):862-868.
95. Nitabach MN, Sheeba V, Vera DA, Blau J, & Holmes TC (2005) Membrane electrical excitability is necessary for the free-running larval Drosophila circadian clock. *Journal of neurobiology* 62(1):1-13.
96. Hamada FN*, et al.* (2008) An internal thermal sensor controlling temperature preference in Drosophila. *Nature* 454(7201):217-220.

97. Zhong L*, et al.* (2012) Thermosensory and nonthermosensory isoforms of Drosophila melanogaster TRPA1 reveal heat-sensor domains of a thermoTRP Channel. *Cell reports* 1(1):43-55.
98. Das A, Holmes TC, & Sheeba V (2016) dTRPA1 in Non-circadian Neurons Modulates Temperature-Dependent Rhythmic Activity in Drosophila melanogaster. *Journal of biological rhythms*.
99. Das A, Holmes TC, & Sheeba V (2015) dTRPA1 Modulates Afternoon Peak of Activity of Fruit Flies Drosophila melanogaster. *PloS one* 10(7):e0134213.
100. Gentile C, Sehadova H, Simoni A, Chen C, & Stanewsky R (2013) Cryptochrome antagonizes synchronization of Drosophila's circadian clock to temperature cycles. *Current biology : CB* 23(3):185-195.
101. Zhang L*, et al.* (2010) DN1(p) circadian neurons coordinate acute light and PDF inputs to produce robust daily behavior in Drosophila. *Current biology : CB* 20(7):591-599.
102. Guo F*, et al.* (2016) Circadian neuron feedback controls the Drosophila sleep--activity profile. *Nature* 536(7616):292-297.
103. Barski OA, Tipparaju SM, & Bhatnagar A (2008) The aldo-keto reductase superfamily and its role in drug metabolism and detoxification. *Drug metabolism reviews* 40(4):553-624.
104. Fork RL (1971) Laser stimulation of nerve cells in Aplysia. *Science (New York, N.Y.)* 171(3974):907-908.
105. Stevenson R (1985) The relative importance of behavioral and physiological adjustments controlling body temperature in terrestrial ectotherms. *The American Naturalist* 126(3):362-386.
106. Kaneko H*, et al.* (2012) Circadian rhythm of temperature preference and its neural control in Drosophila. *Current biology : CB* 22(19):1851-1857.
107. Krishnan B, Dryer SE, & Hardin PE (1999) Circadian rhythms in olfactory responses of Drosophila melanogaster. *Nature* 400(6742):375-378.
108. Chatterjee A, Tanoue S, Houl JH, & Hardin PE (2010) Regulation of gustatory physiology and appetitive behavior by the Drosophila circadian clock. *Current biology : CB* 20(4):300-309.
109. Vanin S*, et al.* (2012) Unexpected features of Drosophila circadian behavioural rhythms under natural conditions. *Nature* 484(7394):371-375.
110. Head LM*, et al.* (2015) The influence of light on temperature preference in Drosophila. *Current biology : CB* 25(8):1063-1068.
111. Rieger D*, et al.* (2007) The fruit fly Drosophila melanogaster favors dim light and times its activity peaks to early dawn and late dusk. *Journal of biological rhythms* 22(5):387-399.
112. Chen C, Xu M, Anantaprakorn Y, Rosing M, & Stanewsky R (2018) nocte Is Required for Integrating Light and Temperature Inputs in Circadian Clock Neurons of Drosophila. *Current biology : CB* 28(10):1595-1605.e1593.
113. Harper REF, Dayan P, Albert JT, & Stanewsky R (2016) Sensory Conflict Disrupts Activity of the Drosophila Circadian Network. *Cell reports* 17(7):1711-1718.
114. Harper REF, Ogueta M, Dayan P, Stanewsky R, & Albert JT (2017) Light Dominates Peripheral Circadian Oscillations in Drosophila melanogaster During Sensory Conflict. *Journal of biological rhythms* 32(5):423-432.
115. Lamaze A*, et al.* (2017) Regulation of sleep plasticity by a thermo-sensitive circuit in Drosophila. *Scientific reports* 7:40304.
116. Parisky KM, Agosto Rivera JL, Donelson NC, Kotecha S, & Griffith LC (2016) Reorganization of Sleep by Temperature in Drosophila Requires Light, the Homeostat, and the Circadian Clock. *Current biology : CB* 26(7):882-892.
117. Yadlapalli S*, et al.* (2018) Circadian clock neurons constantly monitor environmental temperature to set sleep timing. *Nature* 555(7694):98-102.
118. Yoshii T, Vanin S, Costa R, & Helfrich-Forster C (2009) Synergic entrainment of Drosophila's circadian clock by light and temperature. *Journal of biological rhythms* 24(6):452-464.
119. Currie J, Goda T, & Wijnen H (2009) Selective entrainment of the Drosophila circadian clock to daily gradients in environmental temperature. *BMC biology* 7:49.
120. Das A, Holmes TC, & Sheeba V (2016) dTRPA1 in Non-circadian Neurons Modulates Temperature-dependent Rhythmic Activity in Drosophila melanogaster. *Journal of biological rhythms* 31(3):272-288.
121. Green EW*, et al.* (2015) Drosophila circadian rhythms in seminatural environments: Summer afternoon component is not an artifact and requires TrpA1 channels. *Proceedings of the National Academy of Sciences of the United States of America* 112(28):8702-8707.
122. Tang X*, et al.* (2017) The role of PDF neurons in setting the preferred temperature before dawn in Drosophila. *eLife* 6.

123. Stevenson RD (1985) The Relative Importance of Behavioral and Physiological Adjustments Controlling Body Temperature in Terrestrial Ectotherms. 126(3):362-386.
124. Farnworth B, Innes J, Kelly C, Littler R, & Waas JR (2018) Photons and foraging: Artificial light at night generates avoidance behaviour in male, but not female, New Zealand weta. *Environmental pollution (Barking, Essex : 1987)* 236:82-90.
125. Knop E*, et al.* (2017) Artificial light at night as a new threat to pollination. *Nature* 548(7666):206-209.
126. Altermatt F & Ebert D (2016) Reduced flight-to-light behaviour of moth populations exposed to long-term urban light pollution. *Biology letters* 12(4).
127. Tokushima Y*, et al.* (2016) Broadband Photoreceptors Are Involved in Violet Light Preference in the Parasitoid Fly Exorista Japonica. *PloS one* 11(8):e0160441.
128. Sawadogo SP*, et al.* (2013) Differences in timing of mating swarms in sympatric populations of Anopheles coluzzii and Anopheles gambiae s.s. (formerly An. gambiae M and S molecular forms) in Burkina Faso, West Africa. *Parasit Vectors* 6:275.
129. Rund SS, Gentile JE, & Duffield GE (2013) Extensive circadian and light regulation of the transcriptome in the malaria mosquito Anopheles gambiae. *BMC genomics* 14:218.
130. Rund SS, Lee SJ, Bush BR, & Duffield GE (2012) Strain- and sex-specific differences in daily flight activity and the circadian clock of Anopheles gambiae mosquitoes. *Journal of insect physiology* 58(12):1609-1619.
131. Fritz ML*, et al.* (2008) Ovipositional periodicity of caged Anopheles gambiae individuals. *Journal of circadian rhythms* 6:2.
132. Farnesi LC, Barbosa CS, Araripe LO, & Bruno RV (2018) The influence of a light and dark cycle on the egg laying activity of Aedes aegypti (Linnaeus, 1762) (Diptera: Culicidae). *Memorias do Instituto Oswaldo Cruz* 113(4):e170362.
133. Sumba LA*, et al.* (2004) Daily oviposition patterns of the African malaria mosquito Anopheles gambiae Giles (Diptera: Culicidae) on different types of aqueous substrates. *Journal of circadian rhythms* 2(1):6.
134. Das S & Dimopoulos G (2008) Molecular analysis of photic inhibition of blood-feeding in Anopheles gambiae. *BMC physiology* 8:23.
135. Jones MD, Hill M, & Hope AM (1967) The circadian flight activity of the mosquito Anopheles Gambiae: phase setting by the light regime. *The Journal of experimental biology* 47(3):503-511.
136. Taylor B & Jones MD (1969) The circadian rhythm of flight activity in the mosquito Aedes aegypti (L.). The phase-setting effects of light-on and light-off. *The Journal of experimental biology* 51(1):59-70.
137. Gentile C, Rivas GB, Meireles-Filho AC, Lima JB, & Peixoto AA (2009) Circadian expression of clock genes in two mosquito disease vectors: cry2 is different. *Journal of biological rhythms* 24(6):444-451.
138. Bhatt RM & Kohli VK (1996) Biting rhythms of some anophelines in central Gujarat. *Indian journal of malariology* 33(4):180-190.
139. Jaal Z & Macdonald WW (1993) The ecology of anopheline mosquitos in northwest coastal Malaysia: host preferences and biting-cycles. *The Southeast Asian journal of tropical medicine and public health* 24(3):530-535.
140. Coombe P (1982) Visual behaviour of the greenhouse whitefly, Trialeurodes vaporariorum. *J Phys Ent* 7(3):243-251.
141. Kishi M, Wakakuwa M, Kansako M, Inuma T, & Arikawa K (2014) Action Spectrum of Phototactic Behavior and Compound Eye Spectral Sensitivity in the Yellow Tea Thrips, Scirtothrips dorsalis Hood (Thysanoptera: Thripidae). *Japanese J Applied Ent Zoology* 58(1).
142. Otani Y, Wakakuwa M, & Arikawa K (2014) Relationship between Action Spectrum and Spectral Sensitivity of Compound Eyes Relating Phototactic Behavior of the Western Flower Thrips, Frankliniella occidentalis. *Japanese J Applied Ent Zoology* 58(3).
143. Green C & Cosens D (1983) Spectral responses of the tsetse fly, Glossina morsitans morsitans. *J Insect Physiol* 29(10):795-800.
144. van Langevelde F, Ettema JA, Donners M, WallisDeVries MF, & Groenendijk D (2011) Effect of spectral composition of artificial light on the attraction of moths. *Biological Conservation* 144(9):2274-2281.
145. Balamurali G, Nicholls E, Somanathan H, & de Ibarra NH (2018) A comparative analysis of colour preferences in temperate and tropical social bees. *The Science of Nature* 105(1-2):8.
146. Helfrich-Forster C, Nitabach MN, & Holmes TC (2011) Insect circadian clock outputs. *Essays in biochemistry* 49(1):87-101.
147. Helfrich-Forster C (2009) Neuropeptide PDF plays multiple roles in the circadian clock of Drosophila melanogaster. *Sleep Biological Rhythms* 7(3):130-143.

148. Sabado V, Vienne L, Nunes JM, Rosbash M, & Nagoshi E (2017) Fluorescence circadian imaging reveals a PDF-dependent transcriptional regulation of the Drosophila molecular clock. *J Sci Rep* 7:41560.
149. Mertens I*, et al.* (2005) PDF receptor signaling in Drosophila contributes to both circadian and geotactic behaviors. *Neuron* 48(2):213-219.
150. Helfrich-Forster C (2003) The neuroarchitecture of the circadian clock in the brain of Drosophila melanogaster. *Microscopy research and technique* 62(2):94-102.
151. Cavanaugh DJ*, et al.* (2014) Identification of a circadian output circuit for rest: activity rhythms in Drosophila. *Cell* 157(3):689-701.
152. Seluzicki A*, et al.* (2014) Dual PDF signaling pathways reset clocks via TIMELESS and acutely excite target neurons to control circadian behavior. *PLoS biology* 12(3):e1001810.
153. Tang X*, et al.* (2017) The role of PDF neurons in setting the preferred temperature before dawn in Drosophila. *eLife* 6:e23206.
154. Gorostiza EA, Depetris-Chauvin A, Frenkel L, Pírez N, & Ceriani MF (2014) Circadian pacemaker neurons change synaptic contacts across the day. *Current biology : CB* 24(18):2161-2167.
155. Nicolai LJ*, et al.* (2010) Genetically encoded dendritic marker sheds light on neuronal connectivity in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America* 107(47):20553-20558.
156. Beer K*, et al.* (2018) Pigment-Dispersing Factor-expressing neurons convey circadian information in the honey bee brain. *Open Biology* 8(1):170224.
157. Helfrich-Förster C (2005) Organization of endogenous clocks in insects. (Portland Press Limited).
158. Kobelková A, Závodská R, Sauman I, Bazalová O, & Dolezel D (2015) Expression of clock genes period and timeless in the central nervous system of the Mediterranean flour moth, Ephestia kuehniella. *Journal of biological rhythms* 30(2):104-116.
159. Sauman I*, et al.* (2005) Connecting the navigational clock to sun compass input in monarch butterfly brain. *Neuron* 46(3):457-467.
160. Sauman I & Reppert SM (1996) Circadian clock neurons in the silkmoth Antheraea pernyi: novel mechanisms of period protein regulation. *Neuron* 17(5):889-900.
161. Wise S*, et al.* (2002) Neuroanatomical studies of period gene expression in the hawkmoth, Manduca sexta. *The Journal of comparative neurology* 447(4):366-380.
162. Konopka RJ, Pittendrigh C, & Orr D (1989) Reciprocal behaviour associated with altered homeostasis and photosensitivity of Drosophila clock mutants. *Journal of neurogenetics* 6(1):1-10.
163. Froy O, Gotter AL, Casselman AL, & Reppert SM (2003) Illuminating the circadian clock in monarch butterfly migration. *Science (New York, N.Y.)* 300(5623):1303-1305.
164. Taylor B & Jones M (1969) The circadian rhythm of flight activity in the mosquito Aedes aegypti (L.): the phase-setting effects of light-on and light-off. *J Exp Biol* 51(1):59-70.
165. Jones M, Hill M, & Hope A (1967) The Circadian Flight Activity of the Mosquito, Anopheles Gambiae: Phase Setting by the Light Regime. *J Exp Biol* 47(3):503-511.
166. Nayar J & Sauerman D (1971) The effect of light regimes on the circadian rhythm of flight activity in the mosquito Aedes taeniorhynchus. *J Exp Biol* 54(3):745-756.
167. Jones M, Cubbin C, & Marsh D (1972) The circadian rhythm of flight activity of the mosquito Anopheles gambiae: The light-response rhythm. *J Exp Biol* 57(2):337-346.
168. Sheppard AD*, et al.* (2017) Light manipulation of mosquito behaviour: acute and sustained photic suppression of biting activity in the Anopheles gambiae malaria mosquito. *Parasites & Vectors* 10(1):255.
169. Mure LS*, et al.* (2018) Diurnal transcriptome atlas of a primate across major neural and peripheral tissues. *Science (New York, N.Y.)* 359(6381):eaao0318.
170. Ramanathan C, Nunez A, & Smale L (2008) Daily rhythms in PER1 within and beyond the suprachiasmatic nucleus of female grass rats (Arvicanthis niloticus). *Neuroscience* 156(1):48-58.
171. Ramanathan C, Stowie A, Smale L, & Nunez AA (2010) Phase preference for the display of activity is associated with the phase of extra-suprachiasmatic nucleus oscillators within and between species. *Neuroscience* 170(3):758-772.
172. Lambert CM, Machida KK, Smale L, Nunez AA, & Weaver DR (2005) Analysis of the prokineticin 2 system in a diurnal rodent, the unstriped Nile grass rat (Arvicanthis niloticus). *Journal of biological rhythms* 20(3):206-218.
173. Lemos DR, Downs JL, & Urbanski HF (2006) Twenty-four-hour rhythmic gene expression in the rhesus macaque adrenal gland. *Mol Endocr* 20(5):1164-1176.

174. Murphy B*, et al.* (2015) Evidence of a molecular clock in the ovine ovary and the influence of photoperiod. *Theriogenology* 84(2):208-216.
175. Valenzuela F*, et al.* (2008) Clock gene expression in adult primate suprachiasmatic nuclei and adrenal: is the adrenal a peripheral clock responsive to melatonin? *Endocrinology* 149(4):1454-1461.
176. Rund SS, Lee SJ, Bush BR, & Duffield GE (2012) Strain-and sex-specific differences in daily flight activity and the circadian clock of Anopheles gambiae mosquitoes. *J Insect Physiol* 58(12):1609-1619.
177. Farnesi LC, Barbosa CS, Araripe LO, & Bruno RV (2018) The influence of a light and dark cycle on the egg laying activity of Aedes aegypti (Linnaeus, 1762)(Diptera: Culicidae). *Mem Inst Oswaldo Cruz* 113(4).
178. Das S & Dimopoulos G (2008) Molecular analysis of photic inhibition of blood-feeding in Anopheles gambiae. *BMC Physiology* 8(1):23.
179. Gentile C, Rivas GB, Meireles-Filho AC, Lima JB, & Peixoto AA (2009) Circadian expression of clock genes in two mosquito disease vectors: cry2 is different. *Journal of biological rhythms* 24(6):444-451.
180. Bhatt R & Kohli V (1996) Biting rhythms of some anophelines in central Gujarat. *Indian J of Malariology* 33(4):180-190.
181. Jaal Z & Macdonald W (1993) The ecology of anopheline mosquitos in northwest coastal Malaysia: host preferences and biting-cycles. *The Southeast Asian journal of tropical medicine public health* 24(3):530-535.
182. Fritz ML*, et al.* (2008) Ovipositional periodicity of caged Anopheles gambiae individuals. *J Circadian Rhythms* 6(1):2.
183. Rund SS*, et al.* (2013) Daily rhythms in antennal protein and olfactory sensitivity in the malaria mosquito Anopheles gambiae. *Scientific reports* 3:2494.
184. Li J*, et al.* (2010) Dynamics and mechanism of repair of ultraviolet-induced (6-4) photoproduct by photolyase. *Nature* 466(7308):887-890.