The Role of the *Clock* Gene in Protection Against Neural and Retinal Degeneration

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Abstract

In order to survive, organisms evolved to adapt to environmental changes, so it is unsurprising that a biological system arose to deal with existence in a cycling 24-hour light and dark environment. Gene expression and protein activity are regulated by the circadian system in roughly twenty-four hour cycles to yield changes in behavior and activity of numerous biological systems. The circadian system is an important focus of research, because it influences critical processes, such as metabolism and sleep, and circadian rhythm disruption is observed in patients with a variety of diseases, including neurodegenerative diseases such as Alzheimer's and Parkinson's. *Clock* (*Clk*) is a primary circadian gene in both humans and *Drosophila melanogaster*, more commonly known as the fruit fly. In mutant flies lacking functional *Clk*, known as Clk^{Jrk}(*Jrk*) , one class of circadian neurons is absent. I show evidence that these neurons develop normally but degenerate later in *Jrk* mutants and can be rescued by *Clk* overexpression. *Jrk* mutants are also more susceptible to light-induced retinal degeneration. I hypothesize that normal circadian rhythms resulting from *Clk* expression protect neurons from daily, use-dependent damage. The underlying molecular mechanism of these results is still under investigation, but my data suggests that *Clk* does not function by inhibiting the apoptosis pathway. The results from this project will contribute to a greater understanding of the relationship between neurodegenerative diseases and circadian rhythm disruption. The project also has public health implications, because a large portion of the population, especially shift workers, have disrupted circadian rhythms that may lead to increased risk of disease.

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Introduction and Literature Survey

A. Circadian rhythms

A circadian rhythm is an essential, daily cycling behavior thought to prepare organisms for daily changes in their environment, such as light(Rosbash, 2009). Sleep, body temperature regulation, and metabolism are examples of processes regulated by the circadian system in humans. The molecular mechanisms of the circadian system are conserved between humans and numerous other organisms, so findings from research conducted in model organisms can give insight into the mechanisms of the human circadian system(Panda et al., 2002). This project focuses on the circadian system of *Drosophila melanogaster*, also known as the fruit fly.

Circadian rhythms are regulated by specific neurons that function as endogenous clocks, and these neurons communicate with each other and cells of other tissues to coordinate responses and activity. These neurons are sensitive to changes in external time cues, such as light and temperature, although they are capable of maintaining regular circadian rhythms in the absence of these stimuli, such as during complete darkness. In humans, the superchiasmatic nucleus (SCN) is the master clock, and it is sensitive to light stimulation received by the eyes as well as other input(Blau et al., 2007). In fruit flies, six subsets of neurons in the brain are the main circadian system controllers. They are the small ventral lateral neurons(sLNvs), the large ventral lateral neurons(lLNvs), the dorsal lateral neurons(LNds), and three groups of dorsal neurons(DNs)(Helfrich-Förster, 2003)(Figure 1). These neurons influence circadian behavior in *Drosophila.* Flies exhibit two distinct peaks in locomotor activity during one 24-hour cycle of light-dark, one peak occurs during the light to dark transition(morning) and the other peak occurs during the dark to light transition(evening)(Stoleru et al., 2004). The LNvs are responsible for regulation of morning activity and the LNds along with several other circadian neurons are

responsible for regulation of the evening peak in activity(Stoleru et al., 2004). The two circadian groups interact with each other but they can also function autonomously(Stoleru et al., 2004).

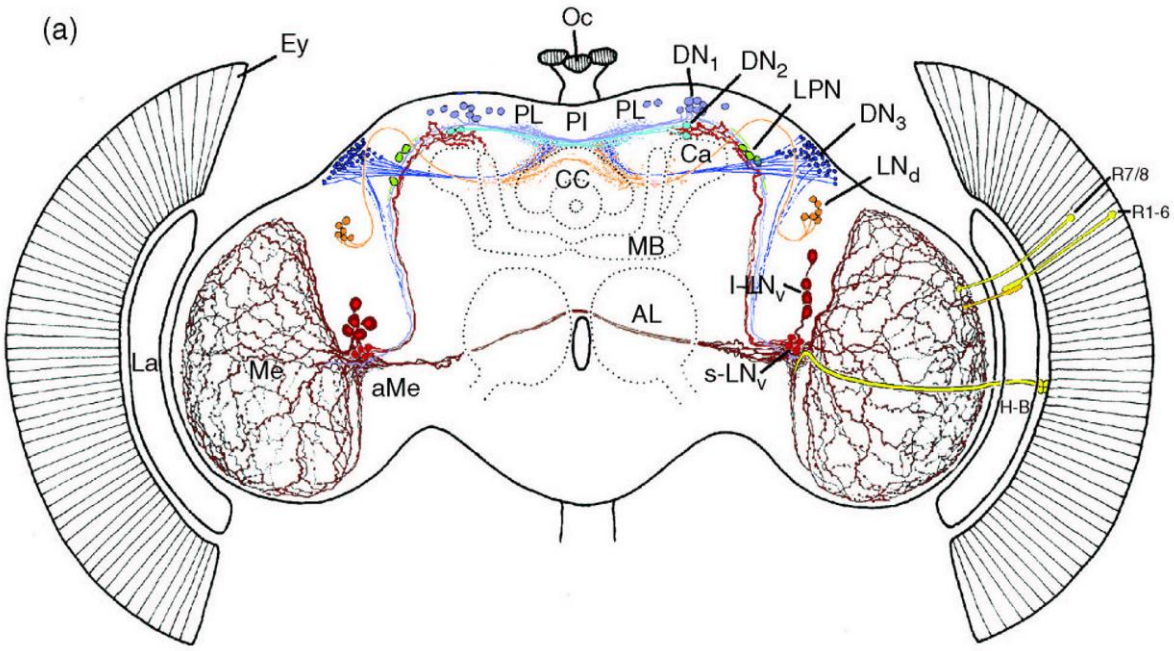


Figure 1. Anatomy of the circadian neurons in the brain of *Drosophila melanogaster*(Helfrich-Förster, 2003).

B. The molecular basis of the circadian system

The basis of the circadian system lies in the regulation of gene expression in individual cells(Benito et al., 2007). Specific portions of DNA called genes encode for the generation of proteins, which carry out various functions in the cell and body as a whole. Cells regulate the expression of genes by producing other proteins known as transcription factors. These transcription factors bind to the DNA at specific sites in front of genes and can inhibit or stimulate the transcription of the genes into RNA, which is then made into proteins.

In both mammals and fruit flies, the *Clock* gene is a major circadian system regulator(Figure 2). In flies, the transcription factors, CLOCK (CLK) and CYCLE (CYC),

combine and stimulate the transcription of the *period* (*per*) and *timeless* (*tim*) genes(Benito et al., 2007). *per* and *tim* encode for the proteins, PERIOD(PER) and TIMELESS(TIM). The two proteins bind together and enter the cell nucleus(Blau et al., 2007). Following entry, TIM begins to degrade and the liberated PER interacts with CLK and CYC to prevent them from activating gene expression(Blau et al., 2007). This halts the expression of *per* and *tim*, and the PER present in the nucleus degrades(Blau et al., 2007). The light-sensitive protein, CRYPTOCHROME (CRY), controls the rate of degradation of TIM and is one way by which the molecular clock synchronizes with light cycles(Blau et al., 2007). The expression of *Clk* is both necessary and sufficient to induce rhythms of gene expression that peak and fall at similar times each day(Kilman and Allada, 2009). These gene rhythms drive daily cycles of behavior. This is true even when *Clk* itself does not oscillate, though CLK's phosphorylation state normally does(Kilman and Allada, 2009). *Clk*'s prominent role in regulating the circadian system in both mammals and flies is one reason why *Clk* is an attractive research subject.

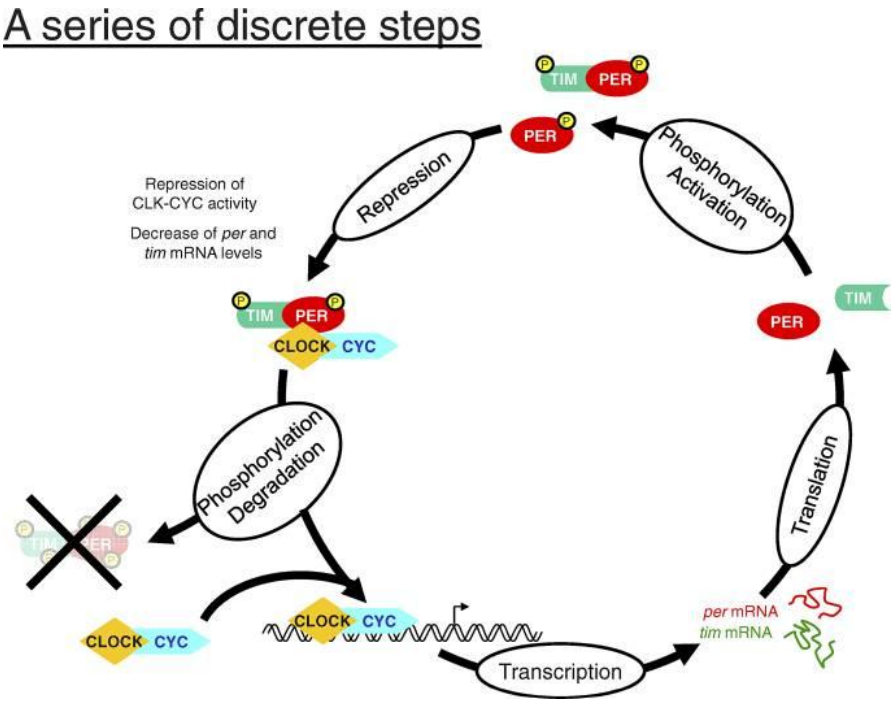


Figure 2. Representation of the molecular circadian clock in *Drosophila melanogaster*(Rosbash, 2009).

C. Disruption of the circadian system and disease

There is mounting evidence associating morbidity of numerous diseases with misregulation of the circadian system in humans, which is further supported by basic research investigating the harmful effects of disrupted circadian systems. The increased prevalence of artificial light has allowed humans to stay awake for longer periods, so it is important for researchers to understand how these changes in the circadian rhythms can affect the body and overall health(Santhi et al., 2011). The National Sleep Foundation claims that in the last century, Americans have decreased their total sleep time by nearly two hours(National Sleep Foundation, 2003). In 2009, surveys found that Americans were sleeping an average of 6.7 hours on weeknights and 7.1 on weekdays(National Sleep Foundation, 2009). Shift workers, who make up a large portion of the population, suffer from chronically disrupted circadian rhythms.

Circadian rhythm disruption is evidenced by abnormal or varying sleep-wake cycles. The categories of circadian rhythm and sleep disorders are separated into those that are voluntary or environment-based and those that are intrinsic(Sack et al., 2007). Voluntary circadian rhythm disorders, such as shift work disorder and jet lag disorder, are due to environmental impositions(Sack et al., 2007). Intrinsic disorders, such as delayed sleep phase disorder and restless leg syndrome, are caused by circadian system malfunctions(Sack et al., 2007). Short-term consequences of sleep deprivation and disrupted circadian rhythms include increased risk of injury or death, lower cognitive performance, and changes in metabolic hormones(Centers for Disease Control and Prevention, 2011). Long-term sleep loss leads to increased risk of obesity, diabetes, cancer, and possibly neurodegenerative diseases(Centers for Disease Control and Prevention, 2011). There is a connection between arrhythmicity and neuronal diseases, such as dementia and Alzheimer's(Reddy and O'Neill, 2010).

D. Disruption of the circadian system and neurodegeneration

Sleep disorders and irregular circadian behavior and neurodegenerative diseases, such as Alzheimer's and Huntington's, often occur concurrently(Wulff et al., 2010). Patients afflicted with neuronal diseases frequently exhibit arrhythmic behavior and have poor quality of sleep(Cardinali et al., 2010). Alzheimer's patients exhibit an abnormal circadian effect called sundowning, where there is a regular and daily increase in agitated or abnormal behavior in the late afternoon or evening(Cardinali et al., 2010). Alzheimer's, the most common neurological disorder associated with aging, is currently the fourth leading cause of death in the United States(Hung et al., 2010). The occurrence of irregular circadian behavior makes vigilance more difficult for caregivers and the irregular cycle is one reason why the elderly and people afflicted with neuronal diseases are institutionalized(Reddy and O'Neill, 2010).

The direction of causality between circadian rhythm disruptions and neurodegenerative diseases is currently inconclusive. In neurodegenerative diseases, there is extensive neuronal loss, and the loss of neurons in brain regions responsible for circadian regulation may cause the sleep disorders and disrupted circadian behavior(Jan et al., 2010). Neurodegeneration also leads to changes in release of neurotransmitters, which would have the potential to affect input to the central circadian pacemaker(Jan et al., 2010). There is evidence that sleep disorders exacerbate symptoms of neurodegenerative diseases and contribute to the progression of these diseases(Wulff et al., 2010). The relationship between circadian disorders and neurodegenerative diseases is supported by the reduction in neurodegenerative symptoms when sleep and circadian problems are treated. Therapeutic application of melatonin, an antioxidant substance with a role in sleep regulation, can reduce the severity of Alzheimer's and Parkinson's symptoms(Cardinali et al., 2010; Srinivasan et al., 2011). It is possible that there is a positive-feedback effect where

existing sleep disorders and disruption to the sleep-wake system promote development of neurological disorders that further worsen the sleep and circadian behavior due to extensive neuronal loss.

The sleep-wake cycle may contribute to the development of Alzheimer's disease by influencing the fluctuations of amyloid-β protein(Aβ) in the brain interstitial fluid(Kang et al., 2009). Aβ accumulation in the brain interstitial fluid(ISF) is a strong indicator of the onset of Alzheimer's disease(Kang et al., 2009). Fluctuations in Aβ are linked to the sleep-wake cycle in both mice and humans, and acute sleep deprivation causes an increase in the Aβ levels in mice that immediately decreased upon recovery sleep(Kang et al., 2009). When mice are chronically sleep deprived, there are greater amounts of Aβ plaques, a component of the pathology of Alzheimer's disease, in the sleep-deprived mice compared to the control mice(Kang et al., 2009). Orexin, a hormone that participates in control of wake and metabolism, is proposed to be involved in the control of Aβ fluctuations, because treatment of orexin increases ISF Aβ concentration and orexin receptor antagonists decrease ISF Aβ levels(Kang et al., 2009).

Components of the molecular circadian system may have roles in preventing the onset of neurodegenerative diseases. *per*, one of the central circadian genes that are conserved between species, may be involved in neuroprotection. *Drosophila per* null(per^{01}) mutants have accelerated aging and worsened neuron loss in a neurodegeneration-prone background(Krishnan et al., 2011). Researchers observed shortened average lifespan and accelerated neurodegeneration in double mutants of per^{01} and *sniffer* and in double mutants of per^{01} and *swiss cheese* (Krishnan et al., 2011). *sniffer* is a loss of function mutation that leads to oxidative stress-induced, age-related neuron degeneration and *swiss cheese* is a loss of function mutation that results in age-dependent lesions of the neuropil and neuron cell death through

apoptosis(Krishnan et al., 2011). The absence of *per* agonizes the propensity of the neuron degeneration in *sniffer* and *swiss cheese* mutants, which suggests that appropriate regulation of *per* can help protect cells against regulated cell death.

In *Jrk* mutants, I found a subset of circadian neurons disappeared after developing normally, which led to the hypothesis that functional *Clk* expression may have a role in protecting neurons from degeneration. Experiments focused on confirming the degeneration of the neurons, determining the mechanism of action for CLK, and subsequently, *Clk's* role in activity-dependent retinal degeneration as a test of the generality of *Clk*'s protective function.

E. Programmed cell death mechanisms

One possible mechanism by which *Clk* could deter degeneration is if it blocks the apoptosis pathway. Apoptosis is a well-known form of programmed cell death that is conserved between species. Apoptosis can be triggered by a variety of intracellular or extracellular signals. Intracellular signals from internal factors, such as excessive DNA damage, can activate the apoptosis mechanism. Hormones or other signals from nearby cells can also communicate to a cell to undergo apoptosis. For example, during metamorphoses in insects, a hormone called ecdysone induces changes throughout the pupa to drive the transformation from the larval state to an adult fly(Kirilly et al., 2011). It is known that there are massive alterations in organ structure and drastic neural remodeling during metamorphoses, and this transition is aided by apoptosis of select cells(Kirilly et al., 2011). The first step in the cell death process is the reception of an intracellular or extracellular apoptosis signal. Various events occur in preparation for the cell suicide: Ca^{2+} ions are released from the mitochondria and caspases are activated. Anti-caspase proteins, such as P35 and DIAP1, can inhibit apoptosis. The circadian system affects processes leading to programmed cell death, but the mechanism by which the circadian

system influences this pathway is still uncertain(Figure 3). For example, a mutation in *cry* causes certain tumor cells to be more responsive to signals stimulating a specific apoptotic pathway(Lee and Sancar, 2011).

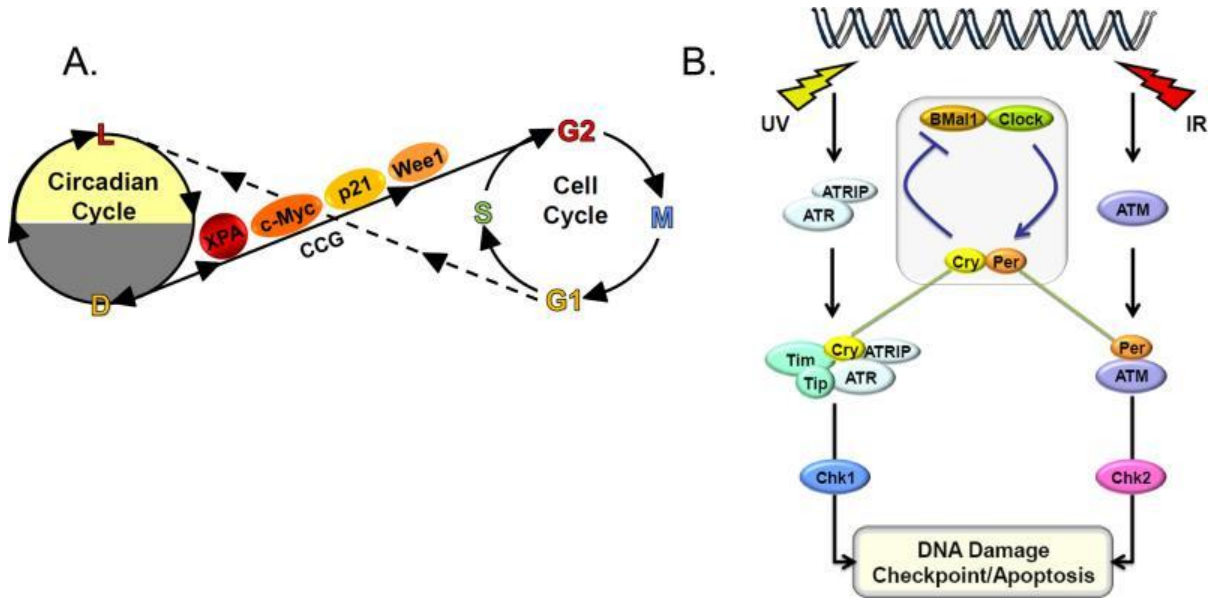


Figure 3. Two proposed ways in which the mammalian circadian system is connected to the DNA damage response and apoptosis in cells(Sancar et al., 2010).

Another possible programmed cell death mechanism that Clk may inhibit is death caused by prolonged overstimulation of neurons(Dong et al., 2009). This process of continued excitation leading to cell death is called excitotoxicity. Neuronal excitotoxicity may play a role in the onset of neurodegenerative diseases, such as Huntington's, Alzheimer's, and Parkinson's(Dong et al., 2009). When a neuron receives chronic overstimulation, the toxic levels of neurotransmitters and ions induce a large influx of Ca^{2+}, activating enzymes that lead the cell to undergo programmed cell death.

F. Disruption of the circadian system and retinal degeneration

Examining *Clk*'s function in protecting neurons led to additionally considering *Clk*'s role in the retina. Fruit flies exposed to constant light stimulation undergo retinal degeneration due to the inability of photoreceptors to turn off activity during light exposure(Dolph et al., 1993). If *Clk* inhibits excitoxicity of neurons in the brain, then it may also work in the retinas to protect retinal cells from overstimulation by light. The retina is constantly exposed to ultraviolet radiation in nature and is proposed to display circadian-dependent protection against damage and cell death.

A hypothesis of the origins of the circadian system is that it developed evolutionarily to help prepare and protect organisms from DNA damage caused by ultraviolet radiation(Rosbash, 2009). By cycling production of protective proteins, the cell can be safeguarded when necessary and save resources and energy when defense is not required(Rosbash, 2009). Ultraviolet radiation causes DNA damage in cells exposed to the sun and these damages can lead to DNA mutations, development of cancer, or cell death. A DNA repair mechanism in mice displays circadian cycling, so the probability of developing ultraviolet-induced skin cancer varies according to the cycling efficiency of the DNA excision repair system(Gaddameedhi et al., 2011). The susceptibility to light-induced retinal degeneration in diurnal rats is circadian-dependent(Organisciak et al., 2000). Rats that were exposed to light during the nighttime had significantly greater cell damage in the retinas than rats exposed to light during the daytime, which suggests that a circadian-regulated mechanism in the retina renders cells less susceptible to damage at certain time points(Organisciak et al., 2000). *Clk* controls the expression of numerous genes, so it is likely that a normal level of functional *Clk* expression may be critical for this protective effect.

Given the background information, this project aims to elucidate *Clk*'s role in protecting against neural degeneration and retinal degeneration in fruit flies by examining the anatomy of specific circadian neuron subsets in the brain and the anatomy of the retinal cells.

G. *Clk^{Jrk}* mutant as a model for elucidating *Clk* function

To determine the function of the *Clk* gene, the effects of the loss of *Clk* were examined. In fruit flies, a null *Clk* mutant has not been isolated, so the *Clk^{Jrk}(Jrk)* mutant is the most similar genotype to a *Clk* null. *Jrk* is a dominant negative mutation of *Clk*, meaning nonfunctional CLK protein is produced that lacks the ability to activate genes and inhibits the ability of normal CLK to function(Allada et al., 1998). *Jrk* mutants have negligible *Clk*-activated transcription.

In adult wild type flies, the lLNvs and all but one of the sLNvs produce pigment dispersing factor (PDF), a neuropeptide transmitter critical to circadian molecular rhythms and clock output(Helfrich-Förster, 1997;Blau and Young, 1999). These neurons display a characteristic anatomy that can be visualized by using fluorescent markers to detect, or stain, for the PDF protein, or by using genetic techniques to produce green fluorescent proteins(GFP) in only these cells. The sLNvs and lLNvs are nearly the only cells in the brain that express PDF, allowing detailed analysis of their structure with these methods.

The neuroanatomy of circadian pacemaker neurons in homozygote *Jrk* flies is significantly different from the wild type neuroanatomy. In wild type and *Jrk* heterozygote flies, both the large and small ventral lateral neurons(lLNvs and sLNvs) are visible when the brain is stained for PDF(Helfrich-Förster, 1997). There are four to five lLNvs and four sLNvs in each hemisphere of the brain(Figure 1)(Helfrich-Förster, 2003). The sLNvs also send axons towards the upper portion of the brain(Figure 1). In *Jrk* homozygotes however, the sLNvs and their axons are no longer visible using PDF staining and the lLNvs send aberrant projections upwards(Park

et al., 2000). In adult *Jrk* homozygotes, the sLNvs are not detectable with staining for PDF and from *in situ* hybridization(Park et al., 2000). The lLNvs also have altered neuron structure compared to those of wild type flies, sending aberrant projections upwards.

Jrk flies also have altered circadian behavior(Allada et al., 1998). Wild type flies show anticipation of light changes and morning and evening peaks of activity with depression of activity during the middle of the light period(Wheeler et al., 1993). *Jrk* homozygotes have no anticipatory behavior of light changes in 12 hour cycles of light and dark(LD) (Allada et al., 1998). When entrained wild type flies are placed in constant darkness(DD), the flies maintain their rhythmic behavior(Allada et al., 1998). *Jrk* homozygotes are arrhythmic in DD after entrainment(Allada et al., 1998).

Materials and Methods:

***Drosophila* stocks**

cry24G4;;Jrk, cry24G4, cry24G4;;GFPnlsJrk, cry24G4;;GFPnls, mGFP;cry13, mGFP;cry13Jrk, cry13G4, cry13Jrk, ClkG4, UGFPnls, UmGFP;Jrk, UmGFP, Jrk iso, Jrk sib ctrl, UKir2.1, UKir2.1Jrk, UP35, UP35;Jrk, tubG80^{ts}, tubG80^{ts}Jrk, tubG80^{ts};UClk, tubG80^{ts};UClkJrk, UClkJrk, UClk, UDiap1, UDiap1Jrk

Flies were raised at room temperature (~22°C) or at 25°C in 12 hour:12 hour light/dark conditions(LD) unless otherwise stated.

GAL4/UAS system

GAL4-UAS is a binary tissue-specific expression system in flies. One transgene carries the yeast GAL4 transcriptional activator driven by a fly tissue-specific promoter. Another transgene carries a gene of interest to be expressed under the control of yeast UAS (upstream activating sequence), the binding site for GAL4. Flies carrying both transgenes will express the gene of interest only in the spatiotemporal pattern of the chosen tissue-specific promoter that drives GAL4. Further temporal refinement of expression of specific genes was accomplished by using the temperature sensitive GAL80 in conjunction with the GAL4-UAS system. Flies were placed in 18°C and moved to 29°C at specific points in their life cycle depending on the experimental group. *Gal80* expression was under the control of the *tubulin* promoter. At 18°C, the GAL80 protein inhibits the GAL4-UAS system, blocking transgenic expression. At 29°C, GAL80 degrades, so the GAL4-UAS system was able to function and express the transgene.

Brain dissections

Brains were dissected in PBS(phosphate buffer saline) using forceps and a light microscope. They were placed into fixative(4% formaldehyde with methanol in PBS) within 10 minutes after

dissection. Brains were fixed for approximately 30 minutes for adults and 20 minutes for larval and pupal brains. Larval and pupal stages were determined according to the aging system of Bainbridge and Bownes, 1981. The brains were washed 5 to 6 times in PBT(0.3% TritonX100 in PBS). Then, the brains were incubated for one or two nights in a primary antibody solution (10% goat normal serum in PBS with primary antibody) at 18°C or for at least four hours at room temperature. For PDF staining, the primary antibody concentration was 0.125% by volume of mouse anti-PDF (DSHB). For PER staining, the primary antibody concentration was 0.125% by volume(Siwicki et al., 1988). The brains were washed in PBT 5 to 6 times after primary antibody staining. If the brains were left in primary antibody for two nights, they were placed in PBT for about 24 hours before washing. After washing, the brains were incubated in a secondary antibody solution (10% goat normal serum in PBS with secondary antibody). The secondary antibody concentrations were 0.083% by volume. The secondary antibodies used were Alexa Fluor 594 donkey anti-mouse, Alexa Fluor 594 donkey anti-rabbit, and Cy5 donkey anti-mouse. After another PBT washing cycle, the brains were washed using PBS 4 to 5 times. They were then mounted on slides using 80% glycerol in PBS. The brains were imaged using a laser scanning confocal microscope. ImageJ and Adobe Photoshop were used to edit images.

Retinal dissections

The head was separated into two halves using the forceps with brain tissue still connected to the retinas. The heads were placed into a fixative solution(4% formaldehyde without methanol in PBS) within 10 minutes of initial dissection. The heads were fixed for approximately half an hour to 45 minutes before removal of the brain tissue, the exoskeleton surrounding the eyes, and the lamina. The eyes were left in fixative for an additional half an hour, so the total fixing time was about an hour to an hour and a half. The retinas were washed in PBT 4 to 5 times and then

PBS 4 to 5 times. They were placed in a phalloidin solution(0.2% of a 0.1mg/ml phalloidin stock in PBS)overnight in an 18°C incubator. The retinas were washed in PBT 4 to 5 times and then PBS 4 to 5 times. The retinas were mounted onto a glass slide using 80% glycerol in PBS with the exoskeleton side facing downwards. The retinas were imaged using a laser scanning confocal microscope.

Activity eductions

Fly activity data was collected using the *Drosophila* Activity Monitoring(DAM) System and data was processed using DAMFile Scan(Pfeiffenberger et al., 2010a). The computer programs ClockLab and Counting Macro were used to analyze the data(Pfeiffenberger et al., 2010b).

Results:

A. *Clk* overexpression in *Jrk* homozygotes

I confirmed previously published findings of the neuroanatomy of *Jrk* homozygotes using flies from a uniform, or isogenic, genetic background(Figure 4A, B).

Clk was overexpressed in *Jrk* homozygotes by using *cry24GAL4* to drive *UASClk*(*UClk*). *Clk* overexpression was performed to confirm that the loss of sLNvs is due to loss of functional CLK and that *Clk* expression is necessary for visualization of the sLNvs in adult brains. The morphology of the *cry24/+;;Jrk/UClkJrk* brain is markedly different from that of a *Jrk* homozygote brain. *cry24/+;;Jrk/UClkJrk* exhibit the same sLNv morphology as wild type brains (Figure 4C).

The *cry24* promoter is expressed in the PDF-positive neurons and drives *Clk* expression strongly enough to successfully rescue the phenotype. The rescue of the wild type phenotype in *Jrk* homozygotes when functional *Clk* expression is induced are evidence that the lack of functional CLK is responsible for the loss of the sLNv PDF-staining and altered projections of the lLNVs. The introduction of CLK is able to reverse the mutant phenotype by rescuing the sLNvs.

The locomotor activities of the flies were also examined. As expected, the *cry24/+;;Jrk/Jrk* and *UClkJrk/Jrk* flies showed activity characteristic of *Jrk* homozygotes, such as lack of anticipation and nocturnal activity(Figure 5). When *cry24* is used to overexpress *Clk* in *Jrk* homozygotes, behavior is partially rescued. The anticipation of lights off is restored and flies are less nocturnal, but anticipation of lights on and other characteristics of wild type behavior still remain absent(Figure 5). The average period of rescued flies was 23.650±0.183 hours while the average period of wild type flies was 24.591±0.113 hours(Table 1). The rescued flies were

moderately rhythmic in DD (P-S = 20.783±5.939)(Table 1). The partial rescue of behavior despite the complete rescue in neuroanatomy suggests that gene oscillations, also dependent on *Clk*, may remain aberrant.

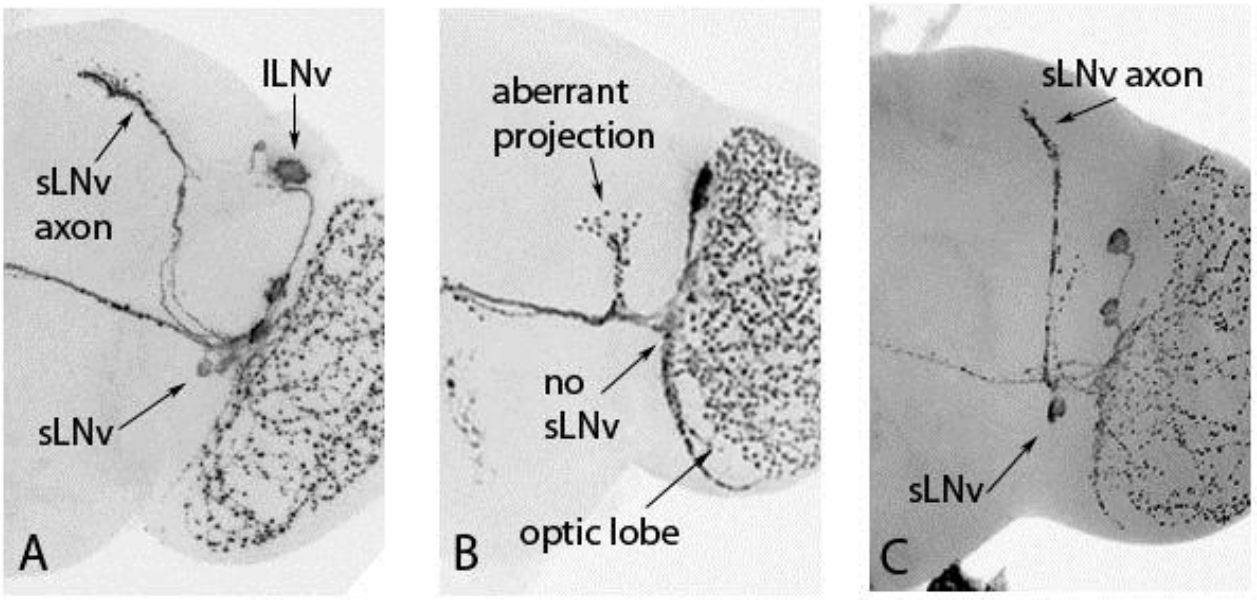


Figure 4. *Clk* overexpression rescues PDF-stained sLNv morphology. **A.** Wild type (*Jrk sibling control*) brains. sLNv somata and axons are visible as indicated by arrows. **B.** *Jrk* homozygote brains. No sLNvs and lLNvs show aberrant projections. **C.** *cry24/+;;Jrk/UClkJrk* adult brain. sLNv morphology is rescued when *Clk* is overexpressed.

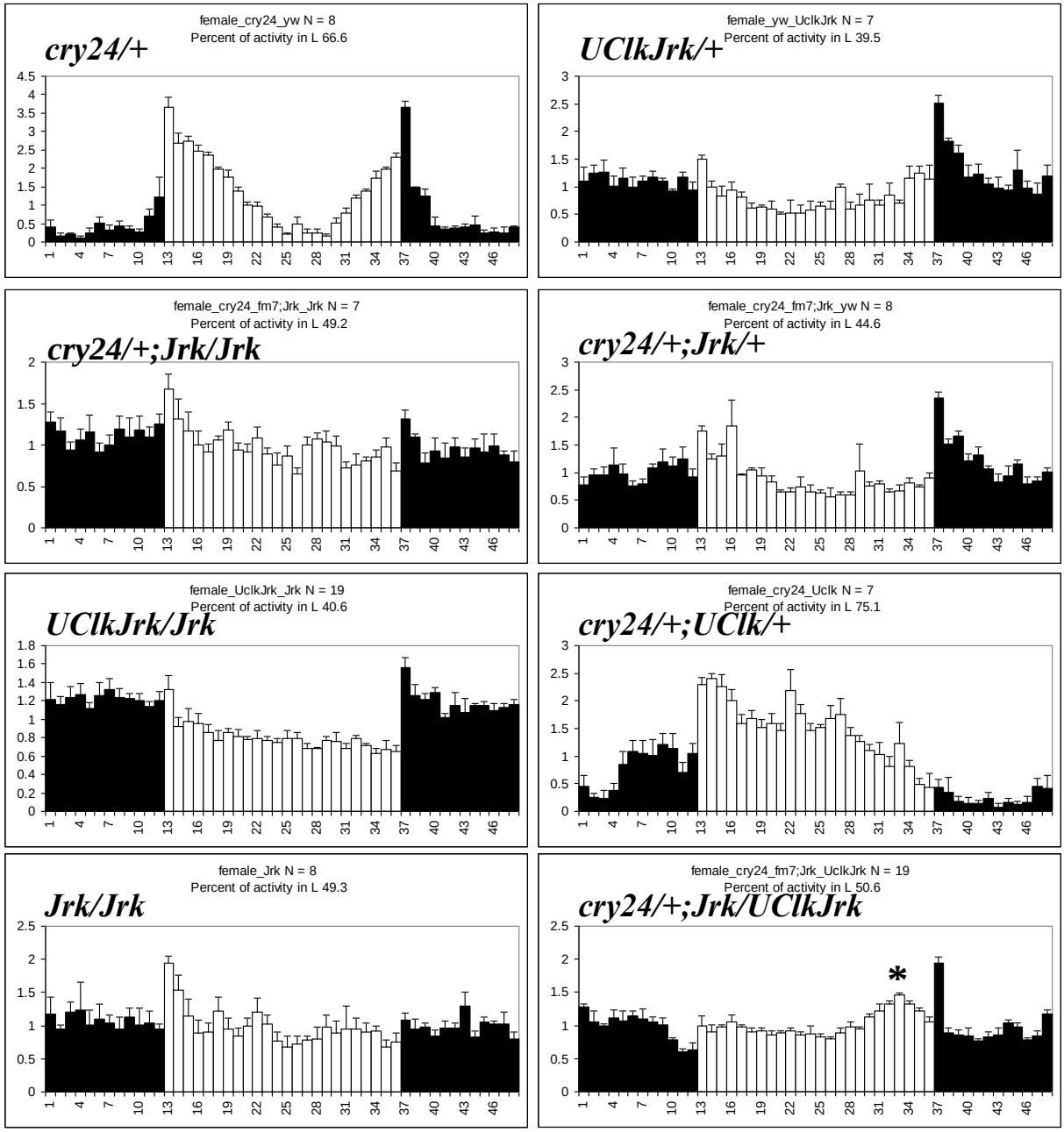


Figure 5. Activity eductions of experimental and control flies.
cry24/+;;Jrk/UClkJrk flies show rescue of anticipation of lights off (*).

Parental Cross	Average Period (hr)	SEM	Average (P-S)	SEM	N
cry24;Jrk x Jrk	N/A	N/A	0.376	±0.834	21
UClkJrk x Jrk	N/A	N/A	0.226	±0.226	18
cry24 x yw	24.591	±0.113	60.817	±14.673	15
cry24;Jrk x UClkJrk	23.650	±0.183	20.783	±5.939	24
cry24 x UClk	23.250	±0.299	13.971	±3.689	17
yw x UClkJrk	17.500	±17.500	2.361	±1.501	14
Jrk	N/A	N/A	0.359	±0.332	14
cry24;Jrk x yw	N/A	N/A	0.564	0.500	16

Table 1. DD behavior for *cry24*-driven *Clk* rescue. (P-S) ≥10 indicates that the fly exhibited rhythmic behavior and larger numbers indicate stronger rhythmicity.

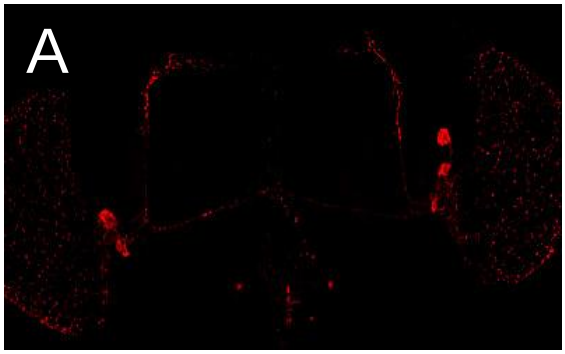
B. Temperature-dependent induction of *Clk* overexpression

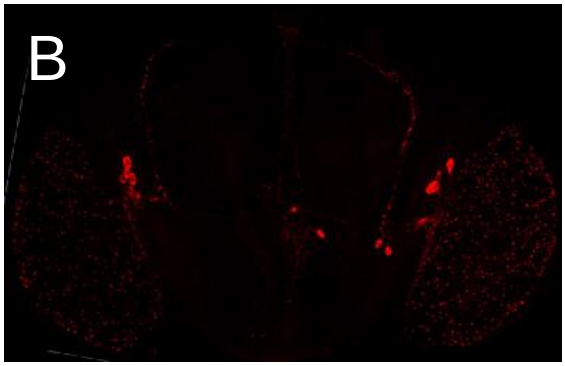
A temperature-sensitive repressor of GAL4 was used to induce *Clk* overexpression during different stages of development in *Jrk* homozygotes in order to determine the critical period when *Clk* expression can rescue sLNv anatomy. The *tubG80^{ts}* driver was used to inhibit GAL4-UAS system at 18°C and the GAL4-UAS system was disinhibited at 29°C. Initiating induction of *Clk* overexpression during the larval or early pupal stage results in rescue of the sLNvs(Table 2, Figure 6C, D). When *Clk* overexpression is first induced during adulthood however, the phenotype cannot be rescued(Table 2, Figure 6E). Functional CLK is necessary during the early developmental stages for proper maintenance of PDF-stained sLNvs.

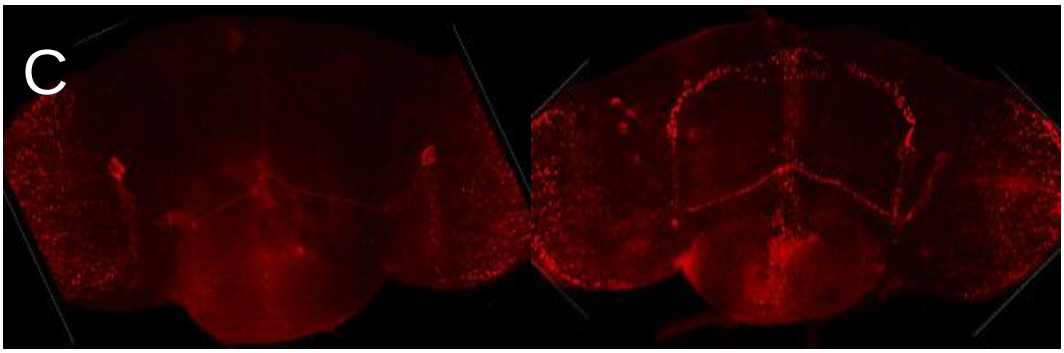
18°C to 29°C	***cry24/+;+/tubG80^{ts};Jrk/UClkJrk***	***cry24/+;;Jrk/Jrk***	***cry24/+;+/tubG80^{ts}***
L1	wild type(n=8)	*Jrk* homozygote(n=6)	wild type(n=9)
L2	wild type(n=5)	*Jrk* homozygote(n=9)	wild type(n=6)
early L3	wild type(n=6)	*Jrk* homozygote(n=6)	N/A(n=0)
late L3	wild type(n=3), *Jrk* homozygote(n=1)	*Jrk* homozygote(n=4)	wild type(n=4)
pupal	*Jrk* homozygote(n=5), partial rescue(n=4)	*Jrk* homozygote(n=11)	wild type(n=12)
adult	*Jrk* homozygote(n=2)	*Jrk* homozygote(n=1)	wild type(n=15)

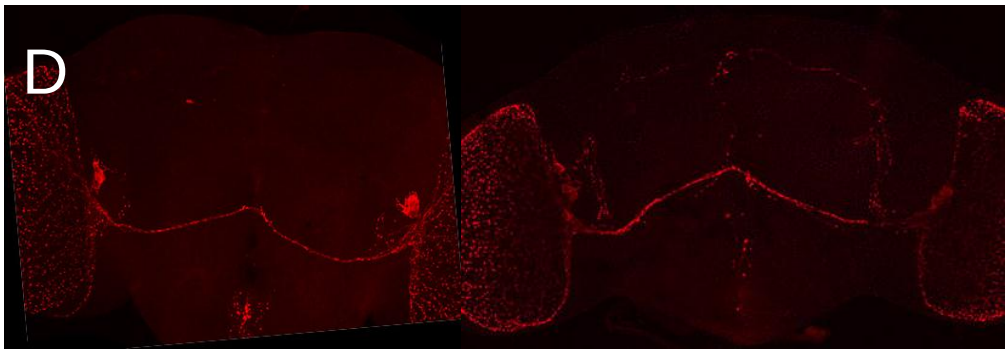
Table 2. The critical period of functional *Clk* expression for rescue of the PDF-stained sLNvs is during the late L3 to pupal stages. Flies were moved from 18°C to 29°C at various developmental stages.

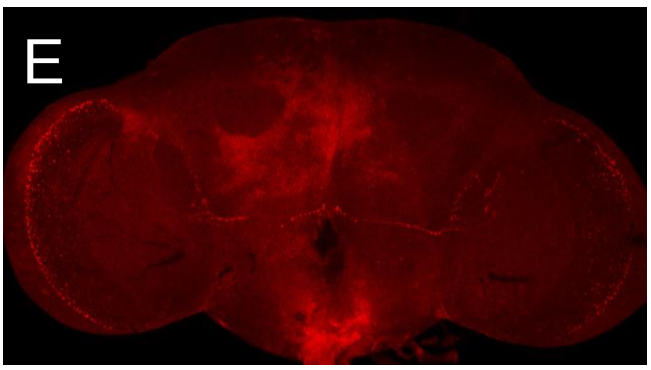
Figure 6. The critical period of functional *Clk* expression for rescue of the PDF-stained sLNvs is during the late L3 to pupal stages. **A.** *cry24/+;+/tubG80^{ts};Jrk/UClkJrk* adult brain when temperature-shifted at L1. **B.** *cry24/+;+/tubG80^{ts};Jrk/UClkJrk* adult brain when temperature-shifted at early L3. **C.** *cry24/+;+/tubG80^{ts};Jrk/UClkJrk* adult brains when temperature-shifted at late L3. Left brain displays *Jrk* homozygote phenotype and right brain displays wild type phenotype. **D.** *cry24/+;+/tubG80^{ts};Jrk/UClkJrk* adult brains when temperature-shifted as pupae. Left brain displays *Jrk* homozygote phenotype and right brain displays wild type phenotype. **E.** *cry24/+;+/tubG80^{ts};Jrk/UClkJrk* adult brains when temperature-shifted 1 day post-eclosion.











C. Visualization of sLNvs during various stages of development

There are three possible explanations for the disappearance of the PDF-stained sLNvs in adult *Jrk* homozygote brains. First, CLK may be necessary during embryogenesis to allow development of the sLNvs. The sLNvs may never develop in *Jrk* homozygotes. Second, the sLNvs may develop properly in *Jrk* homozygote embryonic stages but degenerate when functional *Clk* is not expressed in pupal life. A third possible explanation is that the sLNvs are still present in adult brains, but they do not produce PDF and the PDF staining is unable to detect the sLNvs.

It is possible that although the sLNvs develop normally, they are not detected by commonly used markers, because these markers are dependent on *Clk* expression. Transgenic expression of *GFP* was used to resolve this uncertainty. I tested many different drivers to find one that would continue to produce GFP in *Jrk* flies, allowing me to track these cells in the absence of canonical clock markers (PER, TIM). *cry24* was a strong driver of membrane GFP expression(mGFP) in the PDF-positive neurons(data not shown). It was also shown in the previous experiments that the *cry24* driver is able to fully rescue anatomy and partially rescue behavior. I first used *cry24* to drive mGFP and examined the sLNvs in different stages of development. These data show that the sLNv are present in *Jrk* larvae and develop relatively normally(Figure 7A, B). Two sLNvs are labeled with nuclear GFP(GFPnls), and the sLNvs form synaptic connections with two other neurons, the DN_2s, which is consistent with previously published data(Helfrich-Förster, 2003). At pupal stages however, *cry24* expression becomes too broad to definitively identify the sLNvs(data not shown). In addition, further rescue attempts with an activity-inhibiting transgene resulted in lethality(discussed in section IV.F), limiting the utility of *cry24* for mechanistic studies.

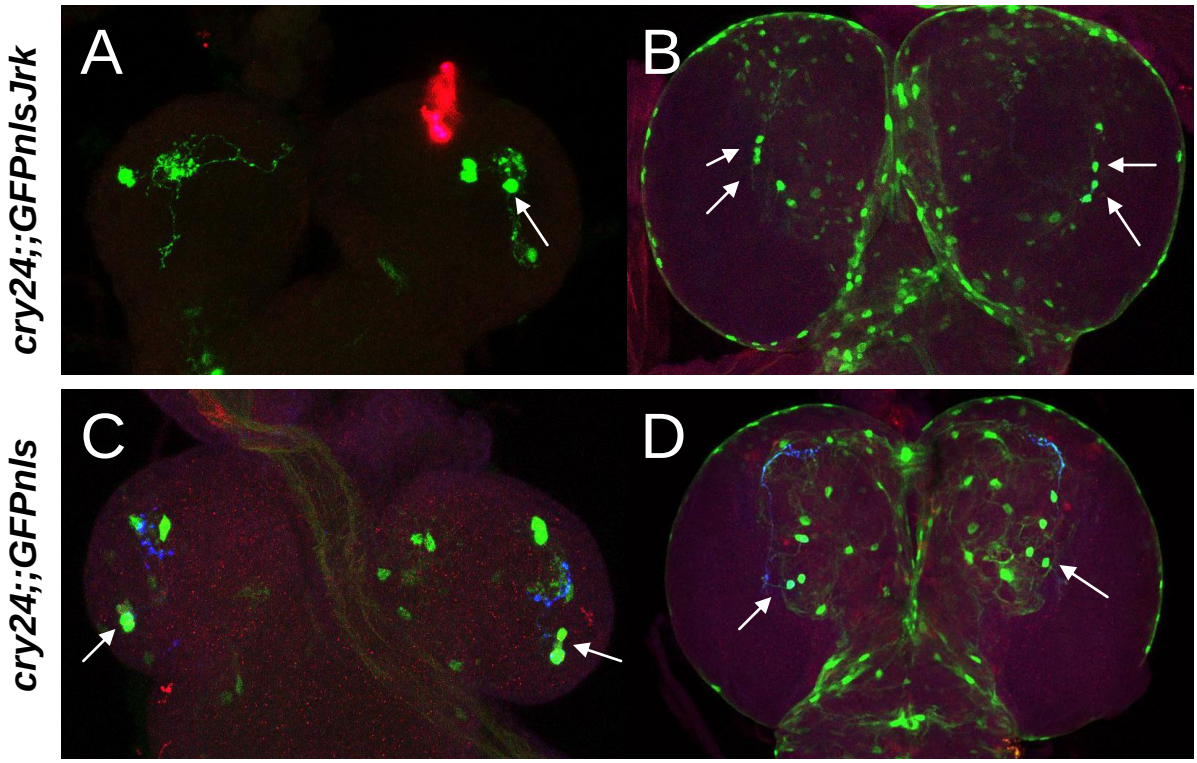


Figure 7. sLNvs are present in *Jrk* homozygotes during L1 and L3. GFPnls(green) and PDF(blue). **A.** *cry24;;GFPnlsJrk* homozygote L1 brain. sLNvs indicated by arrows. **B.** *cry24;;GFPnlsJrk* homozygote L3 brain. sLNvs indicated by arrows. **C.** *cry24;;GFPnls* homozygote L1 brain. sLNvs indicated by arrows. **D.** *cry24;;GFPnls* homozygote L3 brain. sLNvs indicated by arrows.

D. *cry13* expression

The *Clk* overexpression experiment was repeated using *cry13* instead of *cry24. cry13* expression should also be retained in *Jrk* flies, but its expression is weaker and less broad, which would allow us to distinguish the sLNv from other GFP-expressing cells. First, GFP expression was examined at different developmental time points. The sLNvs are present during the larval stages and pupal stages P1 to P4 in *mGFP/+;Jrk/cry13Jrk* flies, although there is no PDF staining of the sLNvs(Figure 8.1C-F). The sLNvs do not show GFP expression in *mGFP/+;+/cry13* larval and early pupal brains, but they are labeled by PDF staining(Figure 8.1A, B). In *Jrk* mutants, *cry* is upregulated, so the presence of GFP expression in the *Jrk* homozygote mutants but not in the

control is consistent with increased *cry* expression in *Jrk* homozygotes(Glossop et al., 2003). The sLNvs are not visible using GFP during the P6 to P7 stages and during adulthood in *mGFP/+;Jrk/cry13Jrk*(Figure 8.2F, H). The lLNvs do not appear until the late pupal stages and they are visible using PDF staining(Figure 8.2D, H). The *cry13*-dependent expression of GFP in late larval stages of *Jrk* flies suggested that this driver would be useful in dissecting the molecular mechanisms of sLNv loss in *Jrk*.

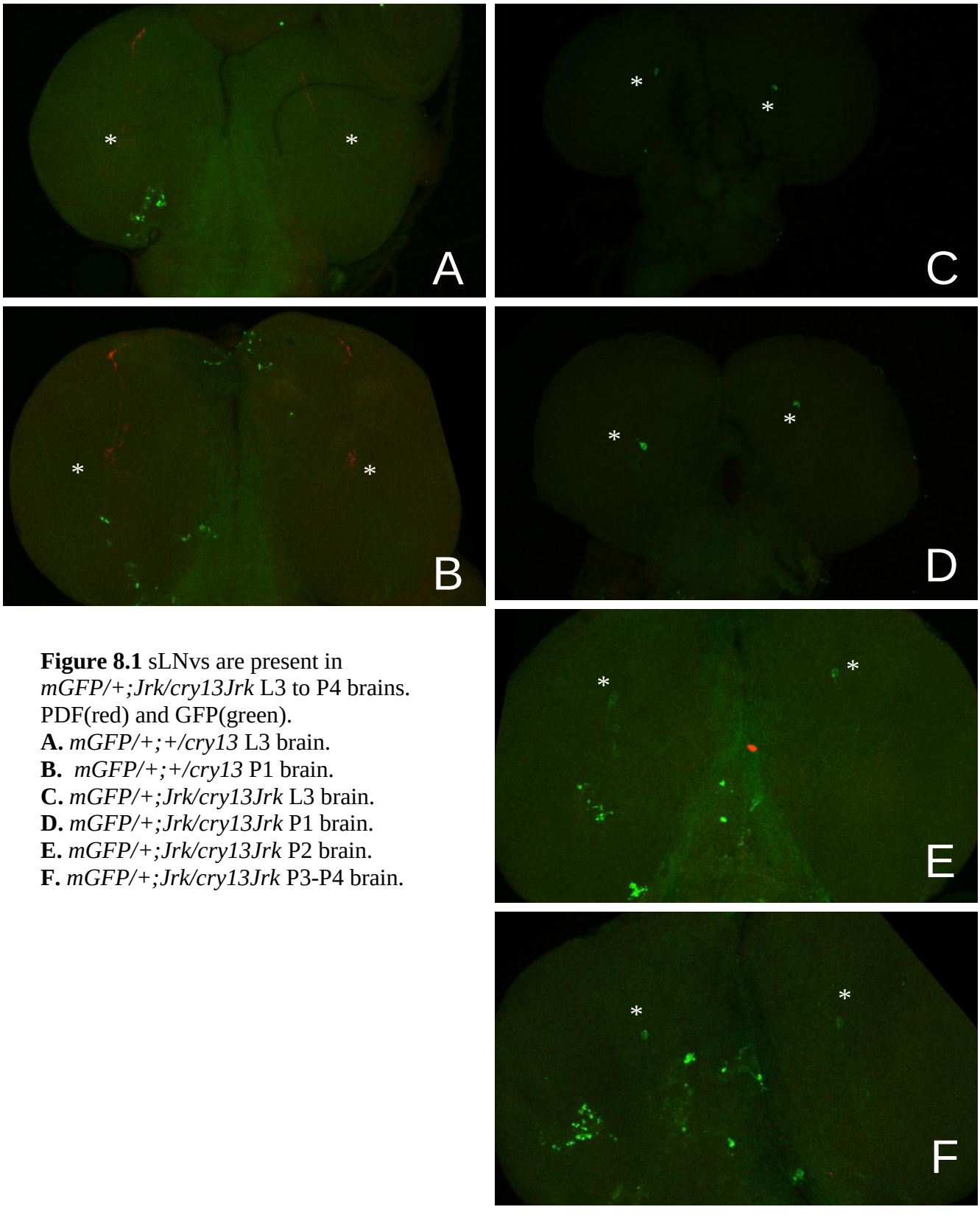


Figure 8.1 sLNvs are present in *mGFP/+;Jrk/cry13Jrk* L3 to P4 brains. PDF(red) and GFP(green).
A. *mGFP/+;+/cry13* L3 brain.
B. *mGFP/+;+/cry13* P1 brain.
C. *mGFP/+;Jrk/cry13Jrk* L3 brain.
D. *mGFP/+;Jrk/cry13Jrk* P1 brain.
E. *mGFP/+;Jrk/cry13Jrk* P2 brain.
F. *mGFP/+;Jrk/cry13Jrk* P3-P4 brain.

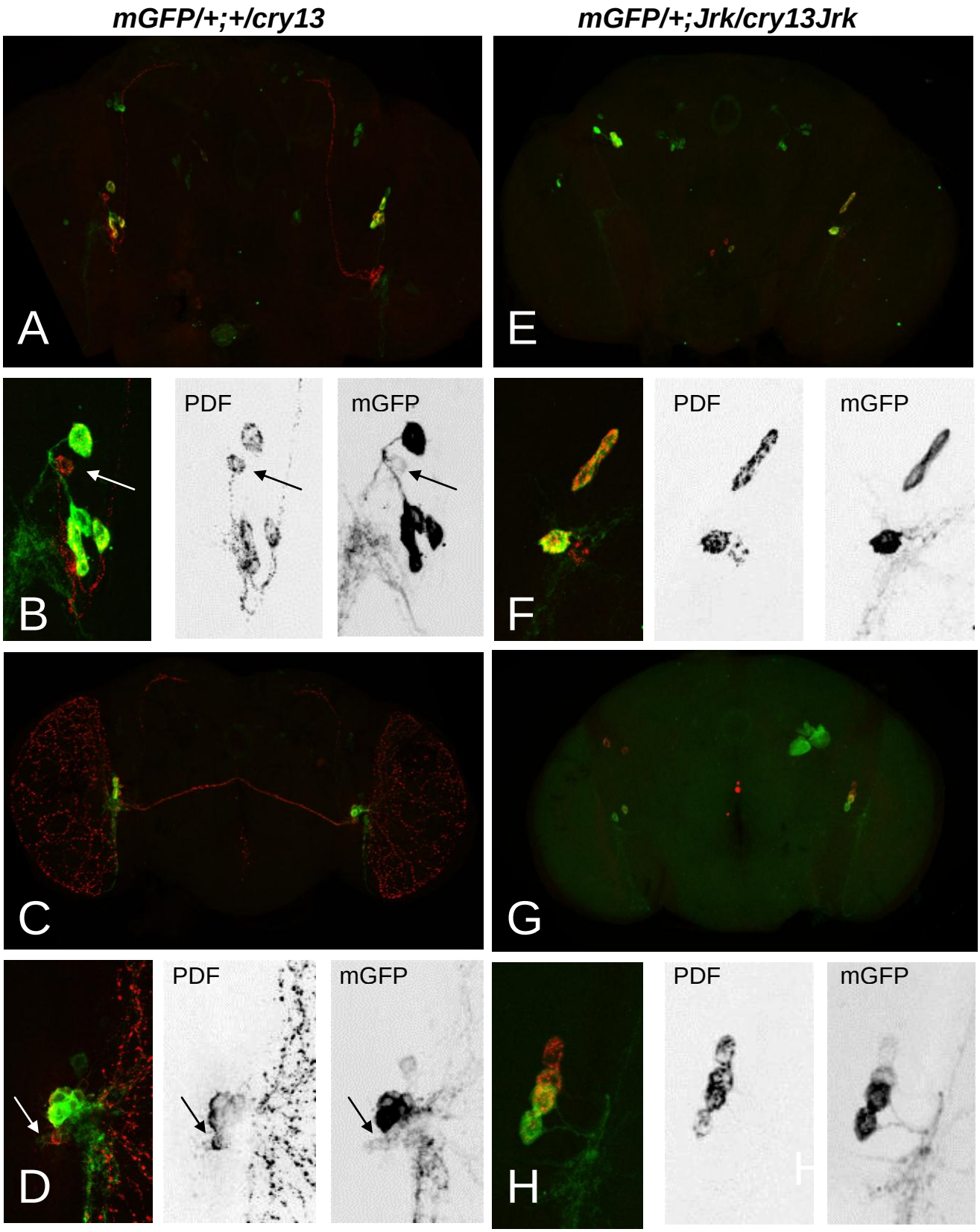


Figure 8.2. sLNvs are not present in P6-P7 or adult *Jrk* brains. PDF(red) and GFP(green). **A.** *mGFP/+;+/cry13* P6-P7 brain. **B.** *mGFP/+;+/cry13* P6-P7 LNvs. sLNvs are indicated by arrows. **C.** *mGFP/+;+/cry13* adult brain. **D.** *mGFP/+;+/cry13* adult LNvs. sLNvs are indicated by arrows. **E.** *mGFP/+;Jrk/cry13Jrk* P6-P7 brain. **F.** *mGFP/+;Jrk/cry13Jrk* P6-P7 LNvs. **G.** *mGFP/+;Jrk/cry13Jrk* adult brain. **H.** *mGFP/+;Jrk/cry13Jrk* adult LNvs.

E. *P35* and *Diap1* overexpression in *Jrk* homozygotes

P35 and DIAP1 are anti-apoptosis proteins, so neuron-specific expression of *P35* and *Diap1* transgenes should inhibit death of sLNvs if they undergo apoptosis. *P35* expression driven by *cry24* results in broad GFPnls expression in the sLNv region. This renders the experiment uninterpretable because identification of the sLNvs relies on position and morphology in *Jrk* flies. The *cry13* driver was then used, because although it does not express as strongly as *cry24*, it has a more restricted expression pattern and a *cry13* line with *mGFP* was readily available. The change from GFPnls to mGFP also aided in reducing the pan-neuronal presence of GFP seen with the *cry24*-driven GFPnls. There is no rescue of the sLNv morphology in *mGFP/P35;cry13Jrk/Jrk*(Figure 9G, H).

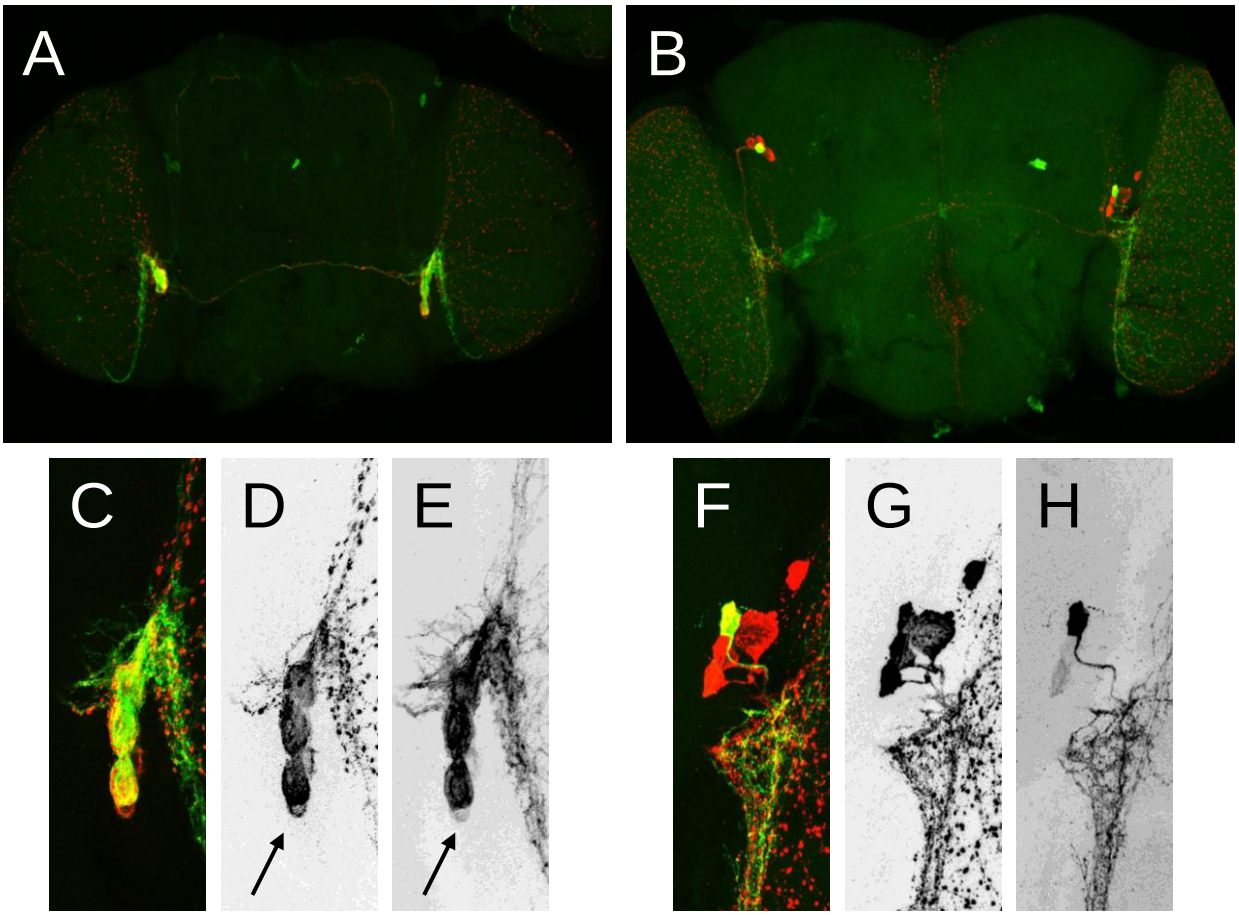


Figure 9. *P35* expression does not rescue the sLNvs in *Jrk* homozygotes. PDF(red) and mGFP(green). **A.** *mGFP/P35; cry13/+* brain. **B.** *mGFP/P35;cry13Jrk/Jrk* brain. **C.** *mGFP/P35; cry13/+* LNvs. **D.** PDF staining of *mGFP/P35; cry13/+* brain. Arrow indicates a sLNv. **E.** mGFP labeling of *mGFP/P35; cry13/+* brain LNvs. Arrow indicates sLNv. **F.** *mGFP/P35;cry13Jrk/Jrk* LNvs. **G.** PDF staining of *mGFP/P35;cry13Jrk/Jrk* LNvs. **H.** mGFP labeling of *mGFP/P35;cry13Jrk/Jrk* LNvs. No sLNvs are present.

To verify *P35* expression in the *P35;Jrk* line, the presence of tritocerebral cells in older adults was used as a marker of the anti-apoptotic activity of *P35*. Previously published data indicates that PDF-positive tritocerebral cells degenerate a few days after eclosion(Helfrich-Förster, 1997). When *pdf* homozygote, *pdf/P35*, and *pdf/P35;+/Jrk* brains were examined however, the tritocerebral cells were still present in all groups 5 to 8 days after eclosion(Figure 10). Previous laboratory methods may have used ineffective methods of detecting the PDF-positive tritocerebral cells, so the tritocerebral cells appeared to degenerate although they are still

present. Alternatively, this phenotype may occur in some genetic backgrounds and not others. The rescue of PDF-positive tritocerebral cells is not a reliable marker of *P35* expression, so there is currently no positive control confirming that *P35* is expressed at sufficient levels to prevent apoptosis.

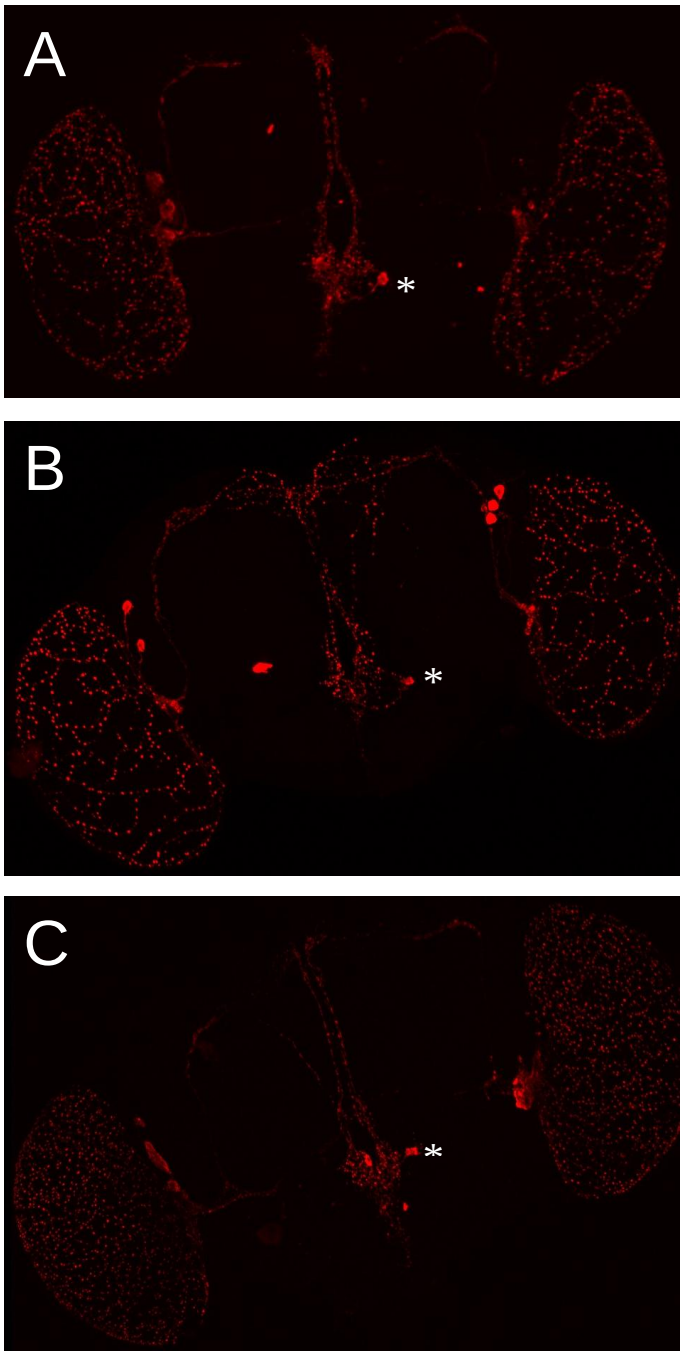


Figure 10. No degeneration of PDF-stained tritocerebral cells(*) after 5 to 8 days post-eclosion. **A.** *pdf/UP35* brain(7 to 8 days post-eclosion). **B.** *pdf/UP35;+/Jrk* brain(5 to 8 days post-eclosion). **C.** *pdf* homozygote brain(7 days post-eclosion).

When *Diap1* is expressed in the sLNvs using *cry13*, there is no rescue of the sLNvs. The neuroanatomy of *mGFP/+;cry13Jrk/Diap1Jrk* is characteristic of *Jrk* homozygotes and no sLNvs are present(Figure 11E, F). The results from the *P35* and *Diap1* experiments suggested that the sLNvs do not undergo apoptosis. The sLNvs may degenerate by a different programmed cell death mechanism, so the possibility of excitotoxicity-induced neurodegeneration was explored.

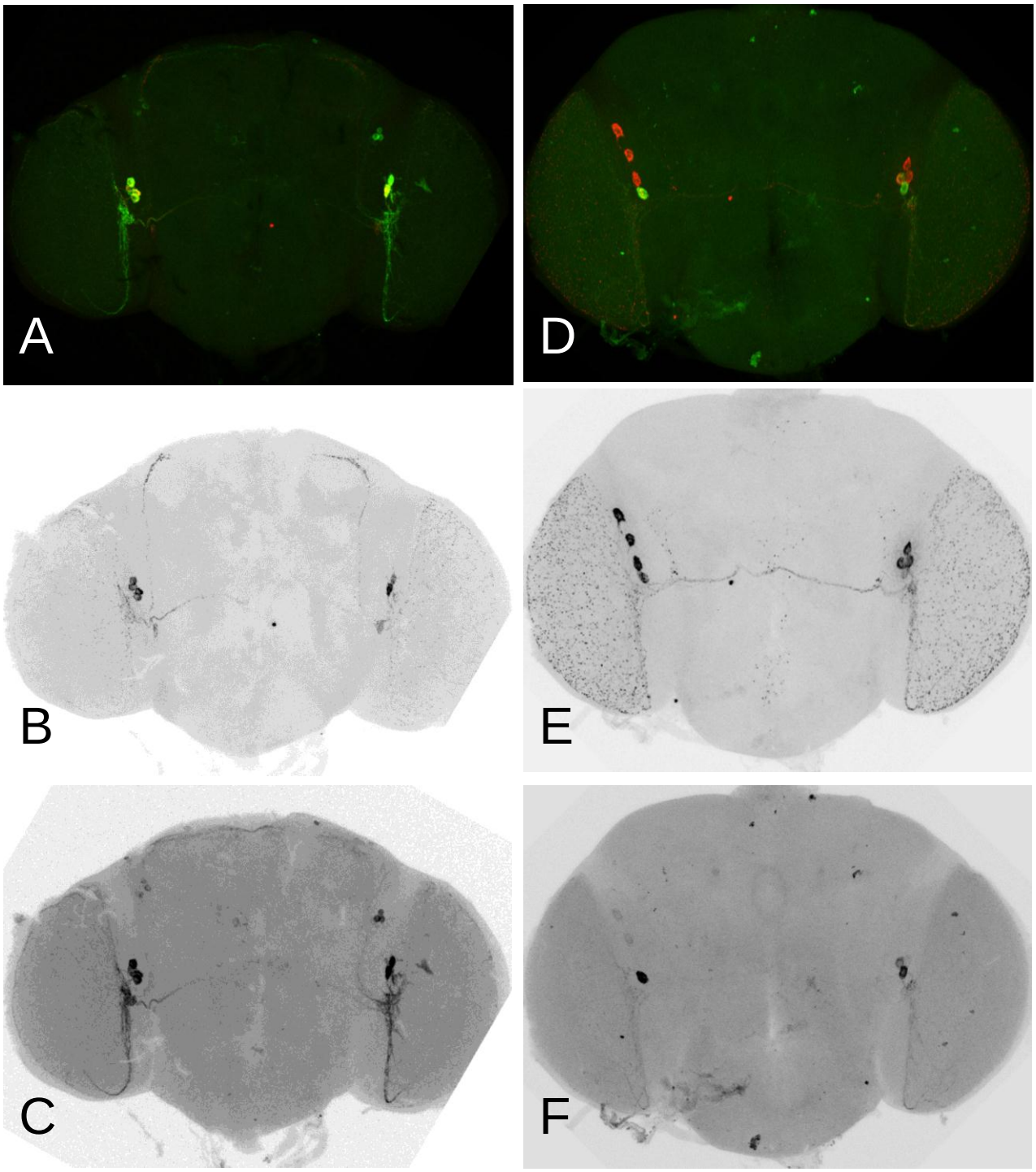


Figure 11. No rescue of sLNvs in *mGFP/+;cry13Jrk/Diap1Jrk*. PDF(red) and mGFP(green). **A.** *mGFP/+;cry13/Diap1* brain. **B.** PDF staining of *mGFP/+;cry13/UDiap1* brain. **C.** mGFP labeling of *mGFP/+;cry13/Diap1* brain. **D.** *mGFP/+;cry13Jrk/Diap1Jrk* brain. **E.** PDF staining of *mGFP/+;cry13Jrk/Diap1Jrk* brain. **F.** mGFP labeling of *mGFP/+;cry13Jrk/Diap1Jrk*.

F. *Kir* overexpression in *Jrk* homozygotes

Transgenic expression of the inward-rectifier K^+ channel(*Kir*) causes silencing of neurons(Nitabach et al., 2002). *Kir* expression provides a molecular method of selectively inhibiting activity in neurons. In normal, rhythmic flies, the circadian clock drives 24-hour oscillations of resting membrane potential and neural activity in clock neurons(Cao and Nitabach, 2008). Unpublished work from the Allada lab also indicates that DN_1 pacemaker neurons are depolarized in *Jrk*(M. Flourakis, personal communication). If the sLNvs in *Jrk* homozygotes degenerate due to excitotoxicity, then *Kir*-induced silencing of sLNvs will protect the neurons from cell death.

Kir overexpression driven by *cry13* in *Jrk* homozygotes does not rescue the sLNv morphology. The sLNvs are not detectable using PDF staining and mGFP labeling(Figure 12E, F). When *cry24* was used to verify the results, it was discovered that *cry24/+;;Kir/+* and *cry24/+;;KirJrk/Jrk* genotypes are lethal. *4-1G4* is another driver that was used, but *4-1/+;Jrk/KirJrk* is also lethal.

Because many of the results with the *cry13* transgene were unexpected, I tested whether *cry13*-driven expression of Clk could rescue the sLNv. There was no rescue of *Jrk* homozygote sLNv morphology, which did not agree with the results obtained using *cry24*. *cry13* is more weakly and narrowly expressed than *cry24*, so it is concluded that *cry13* is not strongly expressed enough or expressed early enough to drive sufficient *Clk* overexpression for rescue of *Jrk* homozygotes. The data from experiments using *cry13* above to test the role of apoptosis and activity dependence are therefore inconclusive, because these transgenes may also have been inadequately expressed. Further actions are being taken to confirm the results of the *cry13*-driven experiments and will be elaborated upon in the Discussion section.

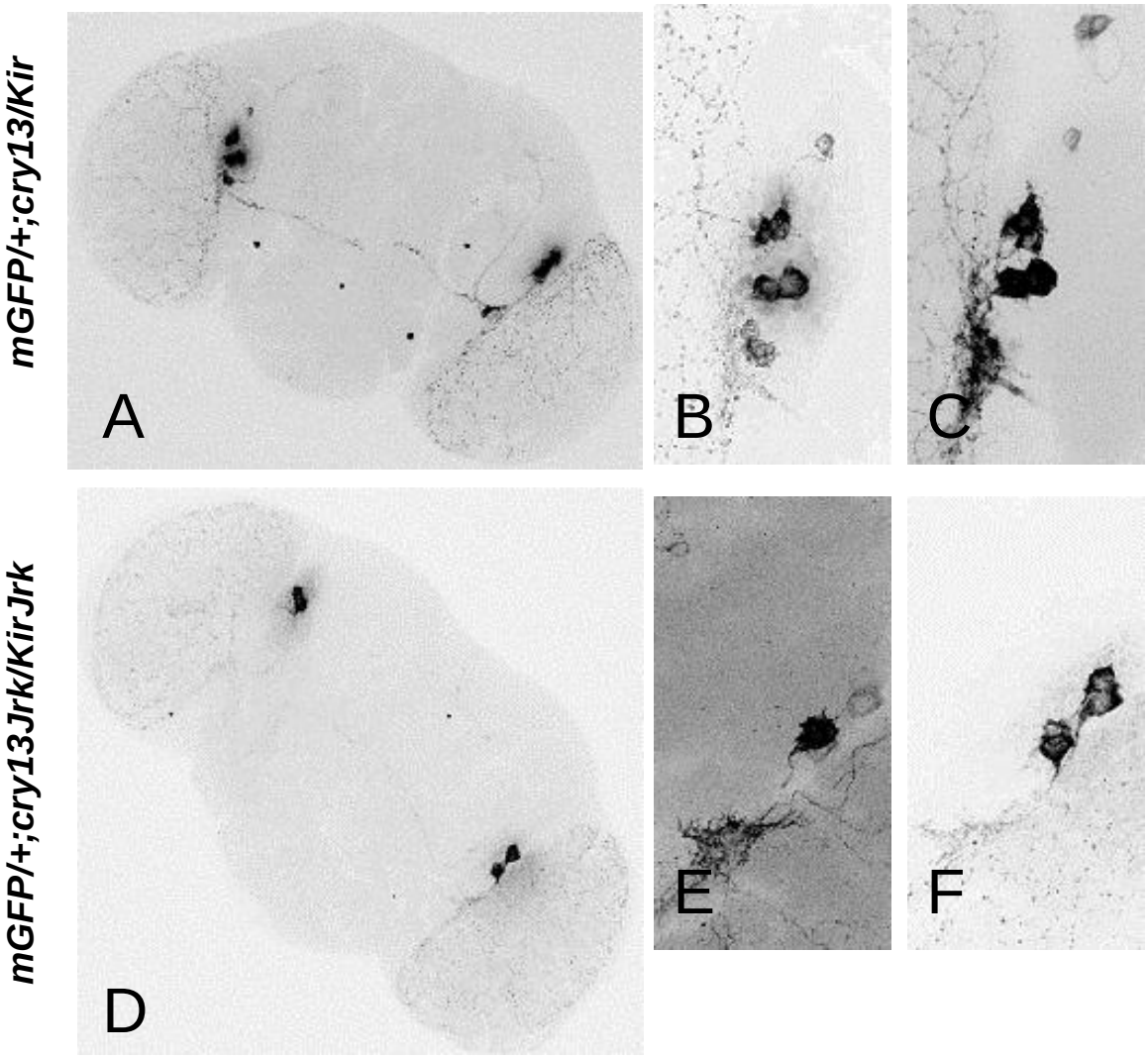


Figure 12. No rescue of sLNvs with *Kir* expression in *Jrk homozygote* adult brains dissected 4 to 6 days post-eclosion. **A.** PDF staining of *mGFP/+;cry13/Kir* brain. **B.** PDF staining of *mGFP/+;cry13/Kir* LNvs**. C.** mGFP labeling of *mGFP/+;cry13/Kir* LNvs. **D.** PDF staining of *mGFP/+;cry13Jrk/KirJrk* brain. **E.** PDF staining of *mGFP/+;cry13Jrk/KirJrk* LNvs. **F.** mGFP labeling of *mGFP/+;cry13Jrk/KirJrk* LNvs

G. Exposure of *Jrk* mutants to LL and DD conditions

I used another approach to test for a role of *Clk* in activity-dependent apoptosis. CRY-positive neurons, such as the LNvs, are sensitive to light entrainment, so they must receive light-input that modifies their activity(Miyasako et al., 2007). To see if the sLNvs would undergo light-dependent degeneration, adult flies were exposed to constant light(LL). For parallel experiments to reduce light-dependent activation of the sLNvs, flies were raised from embryos in constant dark (DD).

In *Jrk* heterozygote(*cry24/+;;GFPnlsJrk/+*) and wild type(*cry24/+;;GFPnls/+*) flies raised in constant light (LL) at 29°C for approximately one month, the neuroanatomy has significantly altered from those of wild type flies raised in LD. The flies were raised at 29°C to exacerbate the LL effects and accelerate neurodegeneration. *Jrk* heterozygotes display wild type neuroanatomy when raised in LD. When experimental and control flies were raised in LL, PDF-stained LNvs disappear or fewer are present(Figure 13F, H). PDF expression in axons transversing the optic lobe has decreased(Figure 13C, D). There are fewer LNvs in *Jrk* heterozygote brains compared to wild type brains, which suggests that flies with reduced levels of CLK are more susceptible to light-induced degeneration of the sLNvs. Broad GFPnls expression makes it difficult to draw conclusions from GFP labeling, so *cry13*-driven mGFP expression in flies raised at 25°C in LL is being investigated.

I also examined *Jrk* homozygote flies raised in DD to test whether reduction in sLNv activation would rescue the sLNvs. The sLNvs are not rescued in *Jrk* homozygotes(Figure 14G, H).

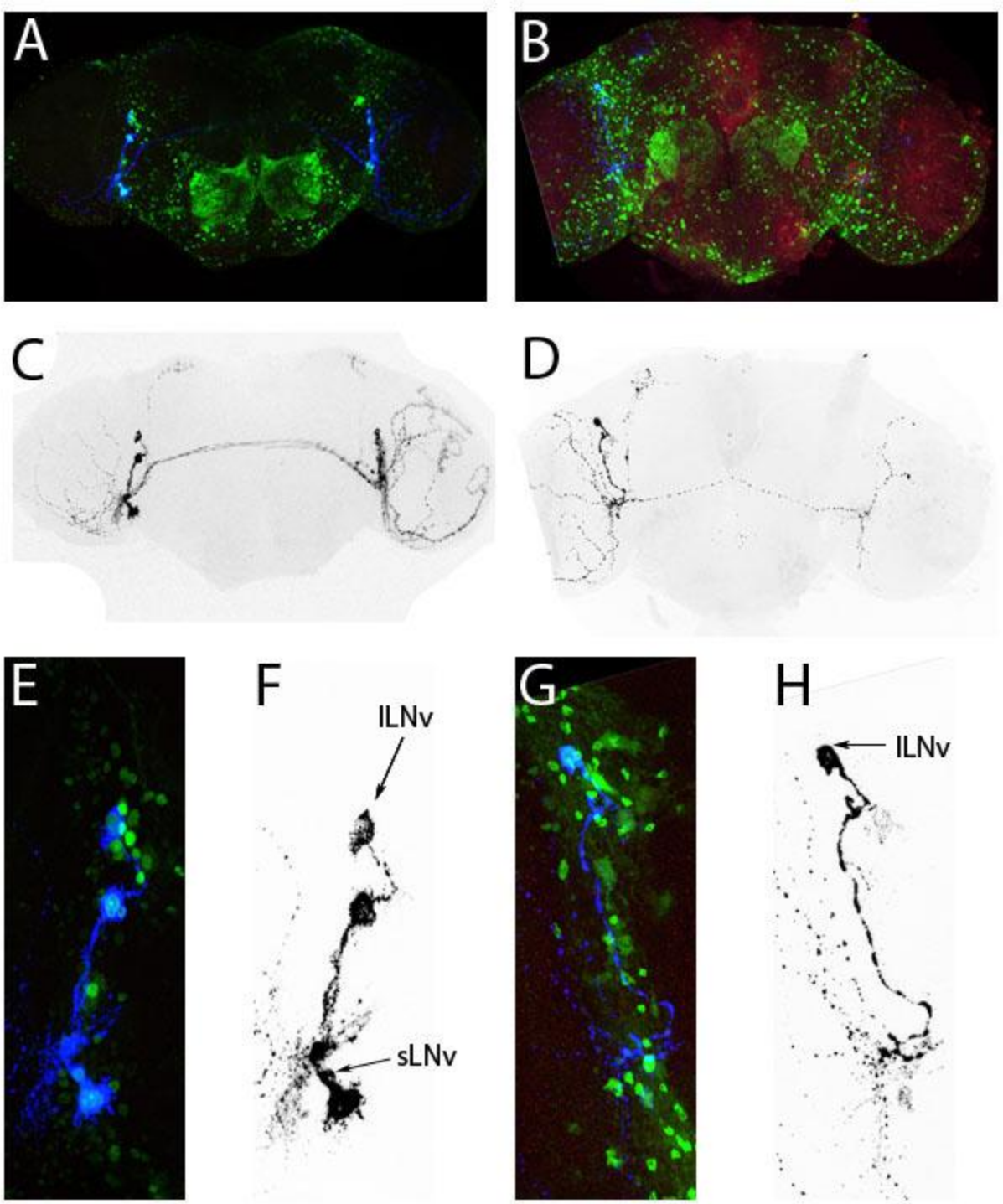


Figure 13. LNvs of *Jrk* heterozygotes are more susceptible to light-induced excitotoxicity than LNvs of wild type flies. PDF(blue) and GFPnls(green). **A.** *cry24/+;;GFPnls/+* brain. **B.** *cry24/+;;GFPnlsJrk/+* brain. **C.** PDF staining in *cry24/+;;GFPnls/+* brain. **D.** PDF staining of *cry24/+;;GFPnlsJrk/+* brain. **E.** c*ry24/+;;GFPnls/+* LNvs. **F.** PDF expression *cry24/+;;GFPnls/+* LNvs. **G.** *cry24/+;;GFPnlsJrk/+* LNvs. **H.** PDF staining of *cry24/+;;GFPnlsJrk/+* LNvs. No sLNvs visible.

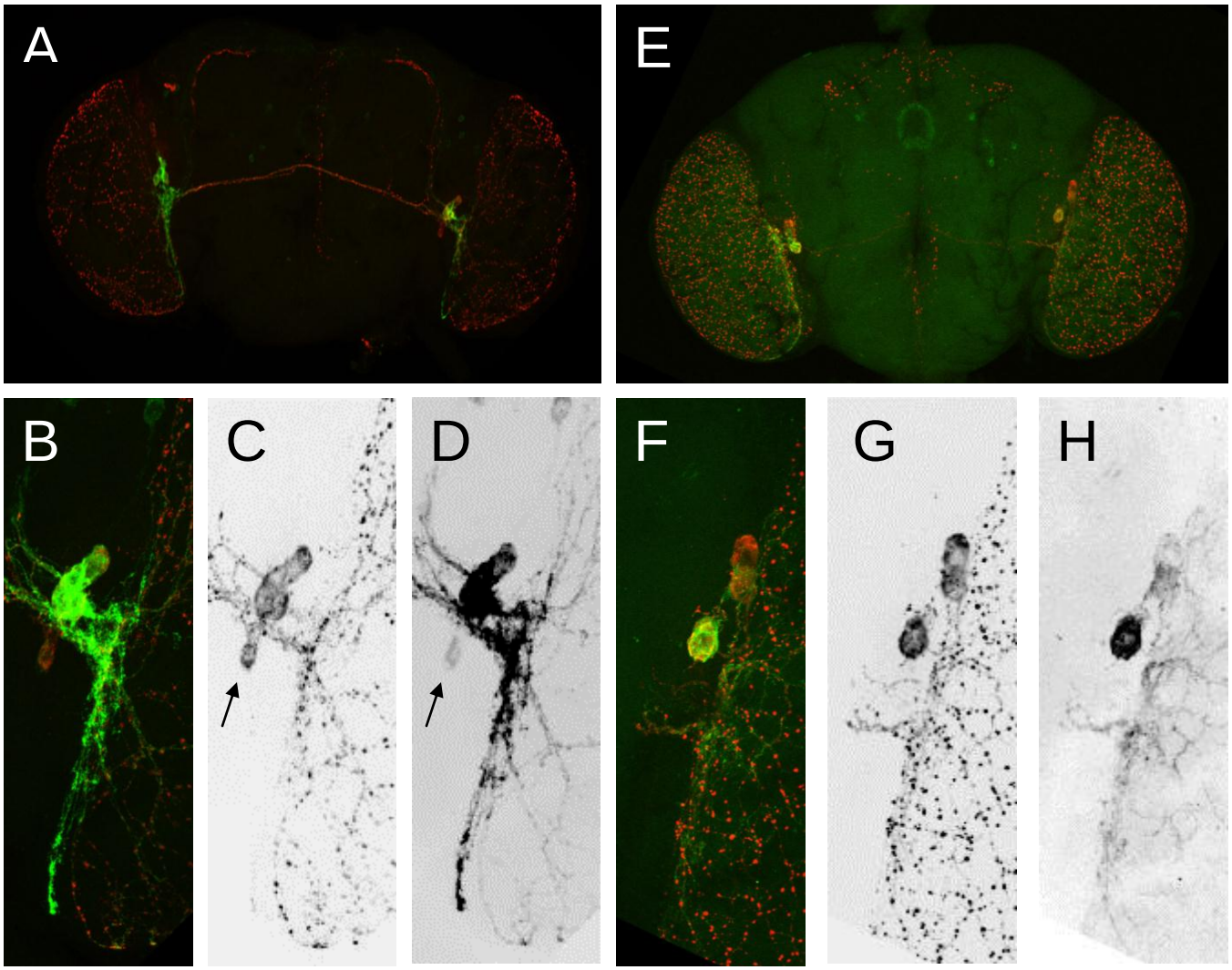


Figure 14. Raising *mGFP/mGFP;Jrk/cry13Jrk* flies in DD does not rescue the sLNvs. **A.** *mGFP/mGFP; cry13/+* brain in LD. **B.** *mGFP/mGFP;cry13/+* LNvs in LD. **C.** PDF staining of *mGFP/mGFP;cry13/+* LNvs in LD. Arrow indicates sLNvs. **D.** mGFP labeling of *mGFP/mGFP;cry13/+* LNvs in LD. Arrow indicates sLNvs. **E.** *mGFP/mGFP;Jrk/cry13Jrk* brain. **F.** *mGFP/mGFP;Jrk/cry13Jrk* LNvs in DD. **G.** PDF staining of *mGFP/mGFP;Jrk/cry13Jrk* LNvs in DD. **H.** mGFP labeling of *mGFP/mGFP;Jrk/cry13Jrk* LNvs in DD.

H. Visualization of *Jrk* heterozygote retinas in LL

An additional system for exploring *Clk*'s influence on excitotoxicity of cells is the retina. Retinal cells modulate their activity based on changes in light exposure.

The *Drosophila melanogaster* retina is composed of an array of distinct units of eight cells, called rhabdomeres. In a properly formed retina, seven of the eight cells can be clearly visualized using whole mount staining and microscopy(Figure 15, 0 days LL). During LL, the retinal cells exhibit constant activity and firing(Dolph et al., 1993). The chronically high levels of activity cause degeneration of the retinal cells due to excitoxicity(Dolph et al., 1993). When wild type(*Jrk sib ctrl)* flies and *Jrk iso* flies have not been exposed to LL conditions (0 days LL), there is little difference between the retinal structure(Figure 15). The rhabdomeres are located in the regular array, indicating healthy and intact retinal cells. In just two days under the LL conditions, the retinal cells begin to exhibit degeneration(Figure 15). The cells are less distinct and in some areas have lost the rigid array structure. The *Jrk* heterozygote retinas exhibit greater progress of degeneration than the control retinas(Figure 15B). At five days under LL conditions, the retinas in both genotypes have completely degenerated(Figure 15). The rhabdomeres are no longer distinguishable and the actin structures of the cells have become irregular and degenerated. The LL conditions are responsible for the degeneration of the retinal cells. When the flies under LL for five days are compared with age-matched flies who were raised in LD for five days, the LL fly retinas show degeneration while the LD flies show little change from flies in LL for zero days(Figure 15). The LD retinas are still healthy and properly formed. This experiment was independently repeated by a colleague and the results were consistent with my work, showing an increase in susceptibility to light-induced degeneration in *Jrk* heterozygotes (F. Xu, personal communication).

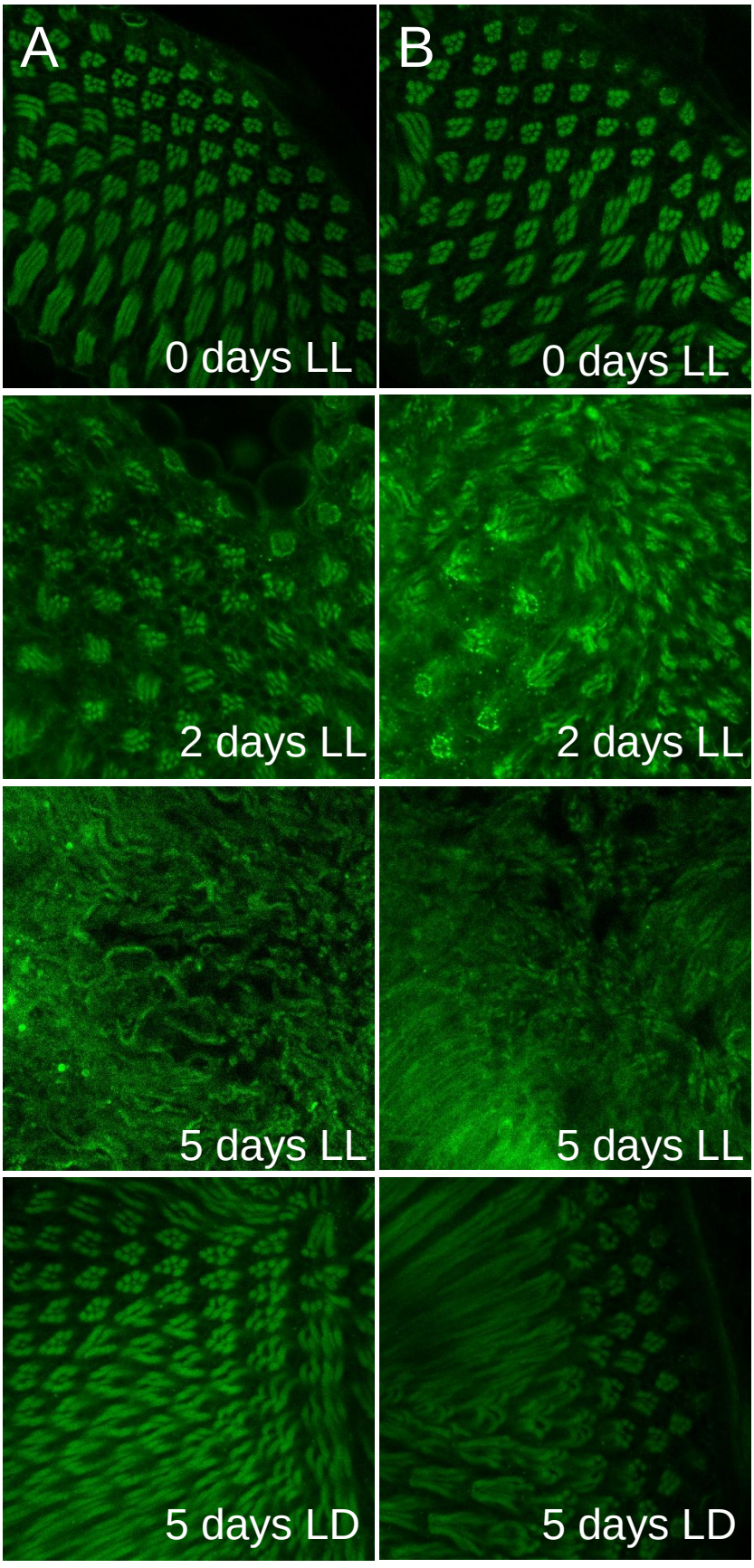


Figure 15. *Jrk* heterozygote retinal cells are more susceptible to light-induced degeneration. **A.** *Jrk sibling control* retinas. **B.** *Jrk/+* retinas.

Discussion

Overall mechanism of *Clk* function

Taken together, these data suggest *Clk* may have a function in neuroprotection. The sLNvs are appropriately formed during early development. At a critical point during the mid-pupal stage, functional *Clk* expression is necessary to protect the neurons from degenerating. Induction of *Clk* expression after this critical period will not rescue the *Jrk* homozygote neuroanatomy because the sLNvs have already degenerated or they have progressed too far in the degeneration process. The neurons may undergo programmed cell death using a novel mechanism and not through a caspase-dependent pathway. The data here regarding cell death mechanisms are not conclusive however, due to possibly insufficient driver expression. The trigger or signal for degeneration in the sLNvs may be excessive levels of neurotransmitters or possibly a specific hormone released during the massive remodeling that occurs during the pupal stage.

Further confirmation of sLNv degeneration

As an alternative explanation for the inability to visualize the sLNvs using GFP expression, *cry* promoter may be inactivated or expression reduced in adults so absence of GFP expression in the sLNvs is due to insufficient activation by *cry* and not due to degeneration of the sLNvs. To address this issue, a "memory" experiment will be performed. An *actin^CD2^Gal4;UFlp;Jrk* fly will be produced to cross to a *Gal4-GFP-Jrk* containing fly. The GAL4 driver will only induce expression of *UFlp* in specific cells and the FLP will cause GFP expression to be driven by the *actin* promoter. This will allow GFP expression to be permanently expressed by the ubiquitous *actin* promoter, independent of *cry* expression in adults, but spatially restricted to cells that expressed Gal4, for instance *cry24*-specific cells. Currently, the fly lines necessary for this experiment are being constructed using genetic crosses.

Another method of confirming the degeneration of the sLNvs is dissecting pupae at specific time intervals and imaging brains to visualize the degeneration of the sLNvs. The dissections will be focused on flies in the P5 to P7 stage because the sLNvs are still present at P5 but gone by P6 or P7 in the *mGFP/+;Jrk/cry13Jrk* flies. Visual confirmation of the degeneration process of the sLNvs will be significant evidence supporting the hypothesis of the neuroprotective role of *Clk* expression. Propidium iodide staining is a commonly used technique that distinguishes apoptotic or necrotic cells from normal cells(Riccardi and Nicoletti, 2006). DNA in the nuclei is cleaved during apoptosis and necrosis. The propidium iodide binds to DNA, and the appearance of the nuclei in apoptotic and necrotic cells differs from those in normal cells due to the extensive DNA cleavage(Riccardi and Nicoletti, 2006). The propidium iodide staining can confirm the degradation of the sLNvs when the time interval of degeneration is established.

Modifications of *P35, Kir,* and *Diap1* experiments

An immediate experiment that can be conducted to answer the question of the significance of the *Diap1* results is performing DIAP1 staining in *mGFP/+;cry13/Diap1* and *mGFP/+;cry13Jrk/Diap1Jrk* brains. If the *cry13* promoter is driving *Diap1* expression, then DIAP1 will be detected in the sLNvs of larvae during staining. The staining protocol for DIAP1 is being developed and results of this experiment will be obtained by the end of the academic year.

An initial experiment to verify the results of the *P35* experiment is construction of the *P35*;*cry13Jrk* line. The expression of two copies of *cry13* may be able to drive sufficient *P35* expression. Additionally, a method for verifying the *P35* expression in the *P35;Jrk* line is still being developed. It would be ideal to identify specific neurons that develop normally and then

degenerate at a later time point. These neurons would need to be easily detectable with available markers. Rescue of these neurons using *P35;Jrk* would verify the function of the *P35* transgene. Another approach that will be performed is switching to the *cry39* driver, a promoter that is more strongly expressed than *cry13* although its expression is still weaker than *cry24.* Reverting to usage of *cry24* is not ideal because construction of a *cry24;mGFP;Jrk* line will result in a fly with transgenes on three chromosomes, which is difficult to produce and will not be a healthy line. It is more efficient to examine *cry39*'s suitability before resorting to *cry24*. *cry39*-driven *Clk* overexpression to rescue the *Jrk* homozygote neuroanatomy will be performed first in order to ascertain whether *cry39* has sufficient expression in the sLNvs. In case *cry39* is found to be a suitable driver, the *mGFPcry39* and *mGFPcry39;Jrk* lines are being constructed and will be used.

Further explorations of mechanisms of *Clk* function

The light-induced degeneration of the sLNvs experiment is currently being repeated under 25°C to confirm the previously obtained results and to establish the time course of the neuron degeneration. If neurons with reduced levels of CLK are more susceptible to excitotoxicity, it is expected that the neurons of *Jrk* heterozygote flies will begin to degenerate before neurons of wild type flies.

Ecdysone is a likely candidate for a cell death signal. It is a hormone that is released during metamorphosis that triggers massive neural remodeling in the pupae in preparation for adulthood. *Drosophila* neurons have been identified that under go caspase-dependent and ecdysone-induced cell death. The Corazonin-positive group of peptidergic neurons in the *Drosophila* larval ventral nerve cord degenerate during the pupal stage(Choi et al., 2006). The

cells can be rescued with dominant negative expression of a specific ecdysone receptor and they are also rescued by expression of *P35*(Choi et al., 2006).

Significance and implications of results

The proposed molecular role of *Clk* in *Drosophila* is consistent with data obtained from previously published research. This project suggests that *Clk* is important during developmental periods for protection of specific neurons in the brain. Chronic sleep loss during critical developmental periods in children can lead to cognitive impairment and impaired brain development from widespread neuronal loss(Jan et al., 2010). Sleep in adolescent mice aids with synaptic pruning and contributes to homeostasis of synaptic connections in the brain(Maret et al., 2011). Wake favors the development of cortical spines and results in a net increase in cortical spines while sleep favors the decrease of cortical spines in the adolescent mice(Maret et al., 2011). This suggests that disruption of normal sleep-wake cycles in human adolescents may also interfere with the normal fluctuations between neuron cortical spine growth and retraction during a vulnerable period of brain development. Chronic insults to the sleep-wake system during childhood and adolescence in humans may have irreversible and long-term consequences on the developing brain. When the circadian system is disrupted, such as in *Jrk* mutants or in humans with irregular sleep-wake cycles, neurons may be less protected against daily use-dependent insults or less protected from the effects of normal cell pruning processes. Therefore, it would be important to develop public health policies that encourage regular circadian behavior and instigate changes in employment hours to mitigate potential negative effects arising from disrupted circadian rhythms.

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