# THE CIRCADIAN CLOCK OF THE *DROSOPHILA MELANOGASTER* EYE REGULATES MORNING ANTICIPATION AND IS SELECTIVELY LINKED TO THE CIRCADIAN NEURONAL NETWORK

by

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Dalhousie University is located in Mi'kma'ki, the ancestral and unceded territory of the Mi'kmaq. We are all Treaty people.

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# Abstract

How multiple circadian clocks within the circadian neuronal network (CNN) converge to regulate behaviour remains elusive. Circadian behaviour is an organism's response to environmental cues (e.g., day/night cycles), regulated by the circadian clock, a ~24-h transcription/translation negative feedback loop. Using *Drosophila melanogaster*, we aim to understand how the eye communicates to the CNN to regulate behaviour. Each hemisphere of the fly brain contains nine clusters of circadian neurons. Various aspects of circadian behaviour are regulated by the different neuronal clusters, suggesting that they must cooperate to regulate coherent behaviour. Given that light is the strongest environmental cue for circadian entrainment, and the visual system is an input point for light in flies and in mammals, we modified the circadian clock of the eye and measured clock oscillations in individual neuronal clusters to determine which brain clocks are linked to the eye clock. Our results demonstrate that the eye clock regulates morning anticipation behaviour and communicates to two neuronal clusters of the CNN. Our study suggests that the brain clocks use different neuronal pathways to communicate and regulate various aspects of behaviour.

# List of Abbreviations and Symbols Used

| | |
|---|---|
| aMe | Accessary medulla |
| *ckI* | casein kinase I (gene) |
| *ckII* | casein kinase II (gene) |
| CK2 | Casein kinase II (protein) |
| *clk* | clock (gene) |
| CLK | Clock (protein) |
| CNN | Circadian neuronal network |
| CRY | Cryptochrome (protein) |
| $cry^{01}/cry^{02}$ | cry null |
| *cryb* | crybaby |
| $cyc^{0}$ | cycle null |
| *cyc* | cycle (gene) |
| CYC | Cycle (protein) |
| DAM | Drosophila Activity Monitoring |
| *dbt* | doubletime (gene) |
| $dbt^{K38R}$ | doubletime K>R mutation |
| $dbt^{L}$ | doubletime long |
| $dbt^{s}$ | doubletime short |
| DBT | Doubletime (protein) |
| DD | Dark: dark |
| DN | Dorsal neuron |
| DN1a | Dorsal neuron 1a |

| | |
|---|---|
| DN1p | Dorsal neuron 1p |
| *flp* | flippase (gene) |
| FLP | Flippase (protein) |
| FRT | Flippase recombination target |
| GRASP | GFP Reconstitution Across Synaptic Partners |
| GWAS | Genome-Wide Association Studies |
| HB | Hofbauer-Buchner |
| JET | Jetlag (protein) |
| LABL | Locally Activatable Bioluminescence |
| LD | Light: dark (12-h: 12-h) |
| LL | Light: light |
| l-LNv | Large ventral lateral neuron |
| LNd | Dorsal lateral neuron |
| LNv | Ventral lateral neuron |
| LPN | Lateral posterior neuron |
| Luc | luciferase |
| NMO | Nemo (protein) |
| norpA | no receptor potential A |
| *pdf* | pigment-dispersing factor (gene) |
| $pdf^{01}$ | pdf null |
| PDF | Pigment dispersing factor (protein) |
| PDFR | Pigment dispersing factor receptor |
| pdf+ | pdf positive |

| | |
|---|---|
| *pdfr-* | pdfr negative |
| *pdfr-/-* | pdfr knockout |
| *per* | period (gene) |
| PER | Period (protein) |
| $per^{0}$ | per null |
| $per^{L}$ | per long |
| $per^{s}$ | per short |
| PP2A | Protein phosphatase 2A |
| Rh | Rhodopsin |
| scz | Schizophrenia |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| SGG | Shaggy (protein) |
| s-LNv | Small ventral lateral neuron |
| *Tik* | timekeeper |
| *tim* | timeless (gene) |
| TIM | Timeless (protein) |
| $tim^{0}$ | tim null |
| *ttx* | Tetrodotoxin |
| WT | Wild type |
| > | Driving expression |
| > + | Driving expression of wild-type gene |
| + > | Wild type expressing a gene |

# Chapter 1: Introduction

*1.1 Circadian rhythms*

Circadian rhythms are an organism's self-sustaining oscillations in behaviour and physiology in response to the earth's rotation. Various inputs such as the 24-hour day-night cycle (Aschoff et al. 1965), seasonal changes (Majercak et al. 1999, Nunes & Saunders 1999, Yellon & Goldman 1984, Nelson et al. 2000), temperature cycles (Yoshii et al. 2009, Buhr et al. 2010, Sweeney & Hastings 1960) as well as feeding (Dreyer et al. 2019, Stephan 2002) help synchronize circadian rhythms to the environment. However, even in constant conditions, meaning without environmental cues, rhythms persist (cf. Aschoff 1981). Circadian biology is present in most organisms including bacteria, algae, fungi, plants, insects and animals. The mechanisms regulating these rhythms are similar between the different species (Schibler 2006, Dunlap 1999, Rosbash 2009).

*1.2 The circadian clock*

Free-running rhythms are regulated by an organism's circadian clock (or biological clock). Free-running rhythms are defined by the rhythms that oscillate in the absence of environmental cues. The circadian clock is a transcriptional/translational negative feedback loop that oscillates with a period of ~24 h (Hardin et al. 1990). In *Drosophila melanogaster*, transcription factors Clock (CLK) (Allada et al. 1998, Darlington et al. 1998) and Cycle (CYC) (Rutila et al. 1998) form an activator complex. CLK and CYC activate the expression of hundreds of different genes, including *period (per)* and *timeless (tim)* (McDonald et al. 2001, Claridge-Chang et al. 2001, Abruzzi et al. 2011). Translation of the Period (PER) and Timeless (TIM) proteins occurs in the cytoplasm. PER and TIM accumulate during the night and form a repressor complex (Zeng et al. 1996, Gekakis et

al. 1995). The repressor complex, including other regulatory kinases, enters the nucleus to bind to the activator complex, inhibiting the activity of CLK and CYC (Menet et al. 2010, Yu et al. 2009). At the end of the ~24-h cycle, PER and TIM are degraded, which releases the activator complex and allows CLK and CYC to re-initiate transcription.

*1.3 Regulatory proteins of the clock*

Regulatory modifications occur throughout the ~24-h cycle of the circadian clock. Casein kinase II (CK2) and Shaggy (SGG) are a part of a phosphorylation cascade that regulates nuclear accumulation of the repressor complex. SGG phosphorylates TIM which then triggers phosphorylation by CK2 (Top et al. 2016, Lin et al. 2005, Allada & Meissner 2005, Meissner et al. 2008, Harms et al. 2004, Martinek et al. 2001). The PER protein is phosphorylated by a regulatory kinase known as Doubletime (DBT) to target PER for degradation (Kloss et al. 1998, Kloss et al. 2001, Cyran et al. 2005, Edery et al. 1994). Other kinases also contribute to the stabilization and degradation of PER. Nemo (NMO), a proline-directed kinase, physically associates with and helps regulate PER and CLK stability (Yu et al. 2011) and is required for the phosphorylation on PER as part of the DBT phosphorylation cascade (Chiu et al. 2011, Garbe et al. 2013). Protein phosphatase 2A (PP2A) plays a role in dephosphorylating PER, delaying the effects of DBT to assure appropriate timing of degradation. The degradation of TIM requires a deep-brain blue-light photoreceptor named cryptochrome (CRY) (Emery et al. 2000, Emery et al. 1998, Stanewsky et al. 1998). Once activated by light, CRY undergoes a conformational change which allows it to bind to TIM (Busza et al. 2004, Ozturk et al. 2011, Vaidya et al. 2013). This promotes ubiquitination of TIM by Jetlag (JET), a ubiquitin ligase. This ubiquitination

leads to the proteasomal degradation of TIM (Myers et al. 1996, Lee et al. 1996, Hunter-Ensor et al. 1996, Naidoo et al. 1999, Lin et al. 2001, Koh et al. 2006).

*1.4 Mutations of core circadian genes*

The core proteins of this transcription/translation negative feedback loop all have important roles and if these proteins are not properly expressed, clock function is affected which modifies behavioural rhythms in flies. Behaviour-modifying variants in the core genes exist. Many different variants of the *per* gene exist, including *per short* ($per^s$), an amino acid substitution at PER S589N (Konopka & Benzer 1971, Huang et al. 1995, Baylies et al. 1992, Colot et al. 1988). These variants have a shorter activity period in constant darkness (dark: dark, or DD) of ~19 h, compared to 23.6 h for WT flies (Kadener et al. 2008, Lin et al. 2002, Ko et al. 2007). Locomotion in wild type flies shows crepuscular, or bimodal rhythms, with an activity peak in the morning, a siesta in mid-day and another activity peak in the evening (Helfrich-Förster 2000). In *$per^s$ variants,* the evening peak of activity occurs earlier than normal in mutant flies in 12-h light: 12-h dark (light: dark, or LD) conditions. *per long* ($per^L$) is a missense mutation with an amino acid substitution at PER V243D (Curtin et al. 1995, Huang et al. 1995, Colot et al. 1988, Hardin et al. 1990). These variants have an activity period of ~29 h in DD compared to 23.6 h for WT flies (Lin et al. 2002, Rutila et al. 1996, Curtin et al.1995). In LD conditions, the evening peak occurs later than normal in mutant flies (Ko et al. 2007). There also exist different null variants that disrupt the clock entirely. *per null* ($per^0$) is a variant that causes arrhythmic activity by eliminating the clock (Konopka & Benzer 1971, Li et al. 2019). This variant introduces a stop codon in the PER sequence (Vosshall et al. 1994, Yu et al. 1987).

A variant of the *timeless* gene, *tim null* (*tim*$^{0}$), is a deletion of 64 base pairs (bp) of the *timeless* sequence (Myers et al. 1995), and this causes complete arrhythmicity in DD (Nishinokubi et al. 2003, Rothenfluh et al. 2000). *tim*RNAi is an RNAi-mediated silencing of *timeless* (Ni et al. 2011), which also causes arrhythmic behaviour in flies (Herrero et al. 2017). *cyc null* (*cyc*$^{0}$) is a non-sense mutation with a truncation at CYC K159term (Rutila et al. 1998, Park et al. 2000) and homozygotes have arrhythmic behaviour in DD (Rutila et al. 1998, Peng et al. 2003, Liu et al. 2015).

*1.5 Variants of regulatory genes*

The different regulatory proteins in the circadian clock can also modify behaviour if their genes are mutated. *Tik* is a *ckII* variant with two coding mutations within the presumptive ATP binding site (Allada et al. 2003). The two amino acid substitutions are at CK2 E165D and M161K (Lin et al. 2002, Bulat et al. 2014). Heterozygotes of this variant exhibit an activity period of 26.4 h, which is nearly 3 hours longer than WT (Lin et al. 2002, Allada et al. 2003). *dbt short* (*dbt*$^{s}$) is a variant of DBT which shortens the period of oscillation (Huang et al. 2014, Price et al. 1998). This variant consists of an amino acid replacement at DBT P47S (Kloss et al. 1998). *dbt*$^{s}$ heterozygotes exhibit activity rhythms with an average period of 21.8 h in DD, while homozoygotes produce an average period of 18 h (Price et al. 1998). *dbt long* (*dbt*$^{L}$) is an amino acid replacement at DBT M80I (Kloss et al. 1998) and lengthens the circadian period (Huang et al. 2014). Heterozygotes have an activity rhythm of 24.9 h in DD, whereas homozygotes have a period of 26.8 h (Price et al. 1998). A variant called *dbt*$^{K38R}$ is an amino acid substitution at DBT K38R. This variant eliminates kinase activity of DBT and disrupts phosphorylation and degradation of

PER and when overexpressing this mutant in all circadian cells, 62% of flies have an arrhythmic behavioural phenotype, and those that remained rhythmic had a ~33 h rhythm (Muskus et al. 2007).

*1.6 Circadian neuronal clusters*

The circadian clock is present in every kind of tissue in the fly. By analysing reporter gene expression of *per* and PER immunolocalization, *per* is shown to express in the photoreceptor cells of the compound eye (R1-8), as well as the antennae, the proboscis, the ocelli, the oesophagus, the fat body, the brain glia and nine clusters of brain neurons (Siwicki et al. 1988, Plautz et al. 1997, Liu et al. 1988, Helfrich-Förster 2003, Nitabach & Taghert 2008). Circadian clock activity in the nine clusters of *per*-expressing neurons (more commonly known as the circadian neuronal clusters) is required for normal behaviour rhythms (Handler & Konopka 1979). In this earlier study, short period behavioural rhythms were restored in *per null* ($per^0$) flies by transplanting the brain of *per* short ($per^s$) mutants into the abdomen. The nine circadian neuronal clusters differ from one another by their size, morphology and their anatomical location. They are identified as small ventral lateral neurons (s-LNvs), large ventral lateral neurons (l-LNvs), dorsal lateral neurons (LNds), dorsal neurons (DN1as, DN1ps, DN2s, DN3s), the fifth small ventral lateral neuron ($5^{th}$ s-LNv) as well as the lateral posterior neurons (LPNs) (**Figure 1**) (Helfrich-Förster 2003, Ahmad et al. 2021).

*1.7 Behaviour regulation*

The circadian neuronal clusters regulate different aspects of circadian behaviour. The most common circadian behaviour studied in *Drosophila* is locomotion. Locomotion

in wild type flies shows crepuscular, or bimodal rhythms, with an activity peak in the morning, a siesta in mid-day and another activity peak in the evening (Helfrich-Förster 2000). These two peaks are referred to as morning anticipation (the fly's gradual increase in activity before sunrise, or "lights-on" during an experiment) and evening anticipation (activity before sunset, or "lights-off"). Cell specific ablation has revealed that the ventral lateral neurons (s-LNvs and l-LNvs) regulate morning anticipation and the dorsal lateral neurons (LNds) and fifth small ventral lateral neuron ($5^{th}$ s-LNv) regulate evening anticipation (Stoleru et al. 2004). Another study has revealed similar roles of the LNvs and LNds, by restoring PER in individual clusters in *per*$^0$ mutant flies (Grima et al. 2004). In the first study, the neurons were eliminated (and thus their circadian clock as well), whereas in the second: only the clock was eliminated and restored. The dorsal neuron 1s (DN1s) are downstream synaptic partners of the s-LNvs (Hyun et al. 2005, Mertens et al 2005. Shafer et al. 2008, Lear et al. 2005, Shafer et al. 2022). DN1s play a role in morning anticipation under light/dark (LD) conditions, in that they are sufficient to drive morning anticipation activity in LD cycles (Zhang, L. et al. 2010). They also seem to drive evening anticipation in certain temperature conditions (Zhang, Y. et al. 2010). These neurons also promote the fly's midday "siesta", which is their sleep during the day (Kunst et al. 2014, Liu et al. 2014, Guo et al. 2016).

*1.8 Connections between neuronal clusters*

The LNvs are known as the "master pacemaker neurons" and are thought to be at the top of a neuronal hierarchy, communicating to the other circadian neuronal clusters (more detail below). Synapses have been demonstrated between the LNvs and other

neurons. The s-LNvs extend to the DN1s and was determined through electron microscopy as well as GRASP (GFP Reconstitution Across Synaptic Partners) (Guo et al. 2016, Yasuyama & Meinertzhagen 2010). The hemibrain connectome is a large analysis of the fly brain showing potential connections between the clusters, and it suggests that the s-LNvs also form connections with the DN1ps, DN2s and LNds in the circadian neuronal network (CNN) (Kim et al. 2013, Scheffer et al. 2020). Additionally, there is communication between the neurons through neuropeptide release. The LNvs (s-LNvs and l-LNvs) produce and release a neuropeptide called pigment-dispersing factor (PDF). The intensity of PDF oscillates at the synaptic terminal of neurons (Park et al. 2000), and activates the PDF receptor (PDFR), a G-protein coupled receptor. Many of the circadian neuronal clusters express the PDFR: the DN1as, some DN1ps, DN2s, some DN3s, some LNds, the 5$^{th}$ s-LNv, and the s-LNvs themselves (Hyun et al. 2005, Mertens et al 2005. Shafer et al. 2008, Lear et al. 2005, Shafer et al. 2022). In summary, these are examples of the various connections between the circadian neurons, although it is not clear how these different connections converge to regulate behaviour.

*1.9 Master pacemaker neurons*

The LNvs are known as the master pacemaker neurons because they dominate behavioural rhythms in DD and constant temperature. Eliminating these neurons by driving a proapoptotic gene called *hid* causes locomotor behaviour to become arrhythmic in DD (Stoleru et al. 2004). Others have shown that when restoring PER only in the LNvs in a *per*$^{0}$ mutant fly, PER protein levels had sustained oscillations in the LNvs, unlike the other clusters (Grima et al. 2004). In models of the CNN, they are placed at the top of a neuronal

hierarchy because they are thought to synchronize the other circadian neuronal clusters in the fly brain through PDF signaling (Park et al. 2000, Stoleru et al. 2005). 70% of *pdf* null ($pdf^{01}$) flies gradually lose locomotor rhythmicity in DD, and by ablating the PDF-expressing neurons (LNvs) with the proapoptotic mutation *hid*, 83% of flies lose locomotor rhythmicity (Renn et al. 1999). In $pdf^{01}$ mutant flies, PER protein oscillations and nuclear localization are affected in the LNvs, in DD (Lin et al. 2004). In WT flies, peak levels of PER in the cytoplasm occur ~7 hours before subjective day. However, the mutant flies showed a dispersal of translocation, meaning at ~7 hours before subjective day, PER was localized in the nucleus as well as in the cytoplasm. Additionally, in $pdf^{01}$ mutant flies, the amplitude of *tim* mRNA oscillations is reduced in all clock neurons in DD (Peng et al. 2003). A more precise description of this hierarchical model has revealed that between the two LNv clusters, the s-LNvs appear to be the "true" master pacemaker neurons (Shafer & Taghert 2009). When knocking down PDF in the l-LNvs, locomotor behavioural rhythms remain unchanged, suggesting that PDF is not required in the l-LNvs for normal behaviour. On the other hand, when knocking down PDF in the s-LNvs, behaviour becomes arrhythmic in DD conditions, suggesting that the s-LNvs are responsible to maintain free-running rhythms (Shafer & Taghert 2009).

*1.10 Mechanistic differences between the neuronal clusters*

The underlying mechanism of the circadian clock has been presumed the same in each circadian neuronal cluster, although there are documented differences in expression of certain clock proteins (Top & Young 2018). For example, when looking at expression of an important regulatory kinase, Casein kinase II (CK2), it is only expressed in the two

LNv clusters, while there is no expression in the other clusters (Top et al. 2016). This brings up the question of how the CK2 mechanism is conducted in the clusters that do not express this kinase. As mentioned previously, one role of CK2 includes the phosphorylation of the timeless protein (TIM) and regulation of nuclear entry of the repressor complex. When looking at nuclear accumulation of the repressor complex in wild type TIM and mutant form of TIM, nuclear accumulation is affected differently in each neuronal cluster. For example, nuclear accumulation was delayed by 7-8 hours in the s-LNvs, while it appeared to function like wild type in the l-LNvs, and there was no accumulation at all in the DN3s. (Top et al. 2016). Interestingly, the l-LNvs contain CK2, suggesting that, despite the expression of this kinase in the l-LNvs, the CK2 mechanism for TIM nuclear entry is not utilized. On the other hand, the DN3s do not have CK2, but nuclear entry of TIM is affected. This suggests that the mutant form of TIM may disrupt another unknown mechanism that influences TIM nuclear entry and accumulation. This suggests that the circadian clocks are regulated differently within each neuronal cluster, given that mutations of circadian genes can be differently effective, or even deleterious, depending on the neuronal cluster in which they're expressed.

### *1.11 Input from the eye*

Light is the strongest environmental cue for circadian entrainment. In flies, light input communicates to the CNN through two pathways: the visual system, and through CRY, a deep-brain blue-light photoreceptor (Ogueta et al. 2018). In mammals, the visual system is their primary input point for light (Rea et al. 1998, Dibner et al. 2010). The mechanism through which CRY is activated by light and helps to synchronize the circadian

clocks in the fly brain is well established (Foley et al. 2020). The CRY protein directly communicates blue-light information to the circadian clock within the neuronal clusters and causes TIM degradation (Emery et al. 1998, Stanewsky et al. 1998, Emery et al. 2000). However, when knocking out *cry* (*cry*$^{01}$), flies are still able to entrain behavioural rhythms in LD conditions (Dolezelova et al. 2007), suggesting that the visual system plays an important role in communicating light information to the CNN to regulate behaviour (Schlichting 2020). In fact, the eye can act independently of CRY to communicate to the circadian clock neurons in the brain. *cry*$^{01}$ mutant flies can re-entrain their locomotor behaviour to a phase shift in LD cycle within a few days, however double mutant *cry*$^{01}$; *gmr*-hid flies (*gmr*-hid: eyeless flies) were unable to re-entrain their behaviour and remained arrhythmic (Yoshii et al. 2015). Light input is known to communicate to the CNN of the fly through retinal activation of rhodopsins (Helfrich-Förster et al. 2001). The fly eye is composed of three main structures: the compound eye, the Hofbauer-Buchner (HB) eyelet, and ocelli. The retina resides in the compound eye, and contains clusters of eight photoreceptor cells, named R1-8 (Hofbauer & Buchner 1989). Each photoreceptor cell expresses one or more rhodopsin(s), named Rh1-Rh7 (O'Tousa et al. 1985; Zuker et al. 1985, Fryxell and Meyerowitz 1987, Montell et al. 1987, Zuker et al. 1987, Feiler et al. 1992, Chou et al. 1996, Chou et al. 1999, Huber et al. 1997, Papatsenko et al. 1997, Salcedo et al. 1999). Once the rhodopsins are activated by light, this triggers a Gqα protein to activate *no receptor potential A* (*norpA*), a phospholipase C, that depolarizes the cell and triggers the release of histamine, influencing the entrainment of the CNN (Bloomquist et al. 1988, Montell 2012, Hardie & Juusola 2015). There has not yet been evidence of direct synaptic connections between the photoreceptor cells and the circadian clock neurons. R1-

6 can send projections from the retina into the lamina of the optic lobe, and the R7 and R8 cells have revealed a close proximity to the LNv dendrites, but no direct connection (Helfrich-Förster et al. 2002). This suggests there may be indirect synaptic connections, or non-synaptic signaling, from the photoreceptor cells to the circadian clock neurons. However, a separate structure within the fly eye, the HB eyelet (Hofbauer & Buchner 1989), has shown synaptic connections with circadian clock neurons. This light-sensing component of the fly eye is located between the retina and the lamina, and it communicates with the LNvs (Yasuyama & Meinertzhagen 1999, Malpel et al. 2002).

*1.12 Dominant circadian neurons in different environmental conditions*

The LNvs (more precisely: the s-LNvs) regulate behavioural rhythms in DD and are known as the master pacemaker neurons. However, in different environmental conditions, the LNvs do not dominate. In constant light (LL), there is evidence that it is the LNds that dominate behavioural rhythms (Murad et al. 2007, Picot et al. 2007). In LL, behaviour in flies becomes arrhythmic. However, with non-functional CRY ($cry^b$), flies regain behavioural rhythms. When expressing CRY in the LNvs of these $cry^b$ flies, locomotor behaviour remained rhythmic suggesting that the LNvs do not dominate behaviour in LL. On the other hand, when expressing CRY in the LNds, behaviour became arrhythmic, meaning that the LNds play a crucial role in regulating behavioural rhythms in LL. This suggests that depending on the light condition, different neuronal clusters can take over behavioural rhythms. Other neuronal clusters also tend to dominate under different environmental conditions. Temperature is an important environmental cue, and changes in temperature affect behaviour rhythms mainly through the DN1s (Yadlapalli et

al. 2018). Another recent study has revealed that the circadian neuronal clusters can fire independently from one another, in response to light (Li et al. 2018). This study first demonstrates that light-induced electrical responses of circadian neurons are driven by the phototransduction of the visual system, and not CRY: Light responses of the circadian neuronal clusters remained intact when knocking out CRY, whereas light response was eliminated in most neurons when expressing a mutation that eliminates phototransduction from the eye. Then, when measuring neuronal firing of each cluster via patch clamp recording, the evening neurons (LNds and 5$^{th}$ s-LNv) as well as the DN1as can fire in response to light, even after ablating the "master pacemaker neurons", the LNvs. Taking these many studies together, it is possible that the LNvs may not be at the top of a neuronal hierarchy. We believe that the CNN is not a linear network, but rather can be subdivided into multiple networks that respond to different cues and regulate specific behavioural outputs.

*1.13 Circadian rhythms and mental health*

The underlying mechanisms of mental disorders remain elusive. Genome-Wide Association Studies (GWAS) have revealed many mutations associated with these disorders (Mathieson et al. 2012, Pardinas et al. 2018, Ormel et al. 2019, Jansen et al. 2019), however these mutations do not reveal any specific pathway causing mental disorders. Interestingly, there is a link between circadian rhythms and mental disorders (Walker et al. 2020), including seasonal affective disorder, depression, schizophrenia, bipolar disorder, and other brain disorders such as neurodegenerative disease (Albrecht 2013, Videnovic et al. 2014, Lamont et al. 2007, Logan & McClung 2019, Jones & Benca

2015, Walker et al. 2020). At the molecular level, mutations in circadian genes that force a phase change with daily environmental cues are linked to the development of mental disorders and sleep disorders in humans (Baresic et al. 2020, Escudero & Johnstone 2014, Mullins et al. 2019). Importantly, the circadian neuronal clusters respond differently to mutations (Johnstone et al. 2022). Mutations that alter circadian clocks can have a severe effect in one neuronal cluster and no effect in another cluster (Top et al. 2016, Top & Young 2018), which means clock function can change in different parts of the brain. This suggests that other behaviour genes (i.e., genes implicated in mental/behavioural disorders) may also function differently (in different regions) in response to mutations. Therefore, we believe that a specific gene/mutation is not the cause of mental disorders, but rather the loss of synchrony between circadian clocks, leading to mutations and eventually changes in behaviour.

### *1.14 Central hypothesis*

We believe that the various circadian clocks in the brain, as well as the clocks in the peripheral tissues, must synchronize with each other to regulate a coherent behavioural output. We hypothesize that a loss of synchrony between the circadian neuronal clusters in *Drosophila* contributes to behavioural changes. We therefore want to determine how the circadian clocks communicate with each other. To do that, we must know how the circadian clocks of the brain respond to an upstream clock. Given that the photoreceptor cells contain a circadian clock (Siwicki et al. 1988, Plautz et al. 1997, Liu et al. 1988, Helfrich-Förster, C. 2003), we want to discover potential connections between the circadian clocks of the eye and the brain. Therefore, we aim to determine which brain clocks are responsive to

these eye clocks. This is possible because of a novel technique that we recently created, which allows the direct observation of circadian clock oscillations in individual neuronal clusters, in real time and *in vivo* (Johnstone et al. 2022). The field has mainly focused on behaviour studies in attempt to discover connections between the clocks in the brain, however this method only measures behavioural output and does not measure the circadian clocks directly. Others have been able to measure changes in PER/TIM levels with RNAseq or immunofluorescence, however these experiments consume a lot of time and materials. Additionally, these methods cannot measure clock oscillations *in vivo* or at different time-points. The technique used in our study measures the activity of the *period* promoter in specific clusters, and how these levels are affected when modifying the clocks in the eye. Given that the circadian neuronal clusters in the fly brain regulate specific aspects of circadian behaviour, we believe that there are specific neuronal networks dedicated to a certain aspect/type of behaviour, and different communication pathways are necessary to regulate these various aspects of behaviour.

# Chapter 2: Methods

*2.1 Fly strains*

The following fly strains were used in our experiments: Iso31 (Bloomington stock number: 5905), **Gal4/UAS strains**: Rh1-Gal4 (Bloomington: 8688) Rh5-Gal4 (Bloomington: 66671), Rh6-Gal4 (Bloomington: 66672), pdf-Gal4 (Bloomington: 6899), VT020611-Gal4 (Sekiguchi et al. 2020), VT027163-Gal4 (Sekiguchi et al. 2020), UAS-$dbt^{K38R}$ (Muskus et al. 2007), UAS-*Tik* (Bloomington: 24624), UAS-*tim*RNAi (Bloomington: 40864), UAS-FLP2 (Nern et al. 2011), UAS-*Kir2.1* (Bloomington: 6595), UAS-*hid* (Bloomington: 65403). **LexA/LexAop strains**: R15C11-LexA (Bloomington: 52492), R67D09-LexA (Bloomington: 53596), R77H08-LexA (Bloomington: 54392), R18H11-LexA (Bloomington: 52535), pdf-LexA (Bloomington: 84429), LexAop-FLPL (Bloomington: 55819, 90892). Flies were kept on a standard yeast, agar, molasses and cornmeal medium at room temperature (18-25°C).

*2.2 Locomotion assays*

Locomotor activity of individuals was measured using the Drosophila Activity Monitor system (DAM). Flies were entrained at 25°C in a light-dark (LD) cycle (12-h:12-h) for three days prior to the beginning of the locomotion assay. Individual flies were placed into a glass cuvette containing food and secured with a cotton plug. A monitor carries 32 cuvettes (32 individual flies), and therefore and up to 32 replicates were measured for each genotype. Infrared beams located in the monitor measured the fly's activity as they traversed the cuvette. Locomotion was measured for two days in a light-dark cycle (12-h:12-h), followed by seven days in constant darkness at 25°C. Analysis of behavioural rhythms was performed using DAMFileScan113X, measuring monitor counts in 30-min

bins. Behavioural periodicity was measured in ImageJ plug-in ActogramJ using Lomb-Scargle analysis. Morning anticipation analysis was measured in Microsoft Excel. Data points from the last ~2.5 h before the change from "lights-off" to "lights-on" (at 21-23.5 h) were divided by the morning peak of activity at the time of light change (24 h). The mean of the activity for each fly spanning 21-23.5 h were used to generate the mean +/- SD for each genotype. Statistical analysis completed with Student T test. Graphs were created using GraphPad Prism.

*2.3 Luminescence assays*

Luminescence of flies was measured using the LumiCycle 32 Color (Actimetrics). The LumiCycle 32 was adapted for the use of *Drosophila* by using custom made 35 mm dishes, designed together by our lab and Actimetrics. Each dish contained standard fly food with the addition of D-luciferin potassium salt (Cayman Chemicals or Gold Biotechnology) to a final concentration of 15 mM. Flies were entrained at 25°C in a light-dark (LD) cycle (12-h: 12-h) three days prior to the beginning of the luminescence assay. Flies were placed in their respective dishes and covered with a coverslip, in the dark (using red light). 15 flies were placed in each dish, corresponding to one replicate. 3-4 replicates were used for each genotype measured. Throughout the entirety of the experiment, flies were kept at 25ºC in constant darkness. Luminescence from each dish was recorded in 4-min intervals on a standard Windows-operated PC using software by Actimetrics, for 9 days.

*2.4 Luminescence analysis*

Luminescence analysis has been described previously (Johnstone et al. 2022). Lumicycle Analysis software normalized the exponential delay of luminescence using a polynomial curve fit, with no smoothing. Data was exported into .cvs files for analysis. A custom python code was used to organize the data into 30-min bins and quantify period, peaks and troughs, and oscillations of the luminescence signal through the course of 9 days. A sinusoidal curve was fitted to each day +/- half a day. Overlapping values of the peaks and troughs of the oscillations were averaged for x- and y- coordinates before being plotted +/- standard deviation. The period of oscillations was analyzed with a custom python code, using a Morlet wavelet fit. The bottom 25% of best fits were omitted from analysis. 15 data points were measured every hour (15x4 min in an hour). The zero-hour mark represented 10:00 am (subjective dawn). The period of shortest wave match was 16 hours, and the period of longest wave match was 72 hours. The increment of wavelet fit was 1. The highest confidence interval values were used to plot period across time. Statistical analysis completed by Student T test. Graphs were created using GraphPad Prism.

# Chapter 3: Results

*3.1 Eye clock regulates morning anticipation*

We know that circadian rhythms are entrained by environmental cues, and the strongest cue is the 24-hour day/night cycle. One pathway in which light input is communicated to the CNN is through the retina. However, there is a lack of understanding of how the circadian clocks in the eye are linked to the brain clocks, and if/how the eye clock helps to regulate behaviour. Before measuring the connection between the eye clock and brain clocks, we first needed to test if the eye clock plays a role in regulating locomotion. We expressed three different transgenes into the photoreceptor cells of the retina using the common Gal4/UAS binary expression system (**Figure 2A**) (Brand & Perrimon 1993). GAL4/UAS was first engineered in yeast *Saccharomyces Cerevisiae* (Webster et al. 1988) and was soon after used in *Drosophila melanogaster* (Brand & Perrimon 1993). *Drosophila* do not have the Gal4 and UAS sequences in their genome, therefore making this system exploitable for the expression of specific genes of interest in a variety of cells and tissues in the fly. We used Rh1-Gal4 to drive these transgenes in the fly eye. Rh1, or Rhodopsin 1, is expressed in six of the eight photoreceptor cells of the compound eye (R1-6) (**Table 1**) (Montell 2012), which is the reason why we chose this driver. Other known rhodopsins are each expressed in either one or two photoreceptor cells (Fryxell & Meyerowitz, 1987, Montell et al. 1987, Zuker et al. 1987, Feiler et al. 1992, Chou et al. 1996, Chou et al. 1999, Huber et al. 1997, Papatsenko et al. 1997, Salcedo et al. 1999), making Rh1-Gal4 the best driver to target the majority of the photoreceptors. We were interested in modifying the clock of the eye and measuring locomotion, to discover potential roles of the eye in influencing the CNN and regulating behaviour. We overexpressed $dbt^{K38R}$ (**Figure 3A**), a variant of *dbt* [encodes for Casein Kinase I (CKI)],

which interferes with PER degradation. In order to alter a different step of the clock, we expressed *Timekeeper* (*Tik*) (**Figure 3B**), a mutant form of casein kinase II (CK2), which prevents the stabilization of TIM and nuclear entry, interfering with TIM degradation. These two kinase variants alter the function of the clock; however, we were also interested in looking at the effect on locomotion when eliminating the clock. We therefore expressed *tim*RNAi (**Figure 3C**), which silences transcription of the *timeless* repressor gene, eliminating the clock entirely. All three mutant flies show a noticeable effect on morning anticipation in LD (light: dark, days 1-2) (**Figure 3D**). The flies expressing the transgenes in the eye do not anticipate the day and are startled by the light, unlike the WT flies who display morning anticipation behaviour. Even in constant darkness (DD), when the endogenous clock is functioning with no influence of light, all the mutant flies exhibit a delay in their morning activity (day 3-9) (**Figure 3A-C**). Additionally, there is an observable decrease in the evening peak of activity (the transition from day to night, or "lights-off" during the experiment) in LD in $dbt^{K38R}$ flies (**Figure 3A**). A low level of the evening peak is observed through the first ~3-4 days of DD in the $dbt^{K38R}$ flies as well as the *Tik* flies (**Figure 3A-B**). In *tim*RNAi flies, there is no noticeable decrease in the evening peak at any time during the experiment (**Figure 3C**). Evening anticipation is only reduced when overexpressing the kinase variants, $dbt^{K38R}$ and *Tik*, but not when eliminating the clock entirely with *tim*RNAi. The reason for this is unclear, however this may be due to the way the clock is affected. For example, when the clock function is altered, these modified signals from the eye affect evening anticipation. On the other hand, when eliminating the clock entirely, there are no signals from the eye, leaving evening activity unaffected. The period of activity rhythms was not affected in any of the variants in DD

(**Figure 3E**). These data suggest that the circadian clock of the eye regulates morning anticipation behaviour and modifying the eye clock with a dominant negative kinase (*dbt*$^{K38R}$ and *Tik*) affects evening activity.

| Driver | Target |
|---|---|
| pdf-Gal4 | s-LNvs and l-LNvs |
| Rh1-Gal4 | Photoreceptor cells R1-6 |
| Rh5-Gal4 | Photoreceptor cell R7 |
| Rh6-Gal4 | Photoreceptor cell R8 and HB eyelet |
| VT027163-Gal4 | s-LNvs |
| VT026011-Gal4 | LNds and 5$^{th}$ s-LNv |
| Mai179-Gal4; pdf-Gal80 | Three of six LNds and 5$^{th}$ s-LNv |
| R15C11-LexA | Two of four LNds |
| R67D09-LexA | l-LNvs |
| R77H08-LexA | DN3s |
| R18H11-LexA | DN1s |
| pdf-LexA | s-LNvs and l-LNvs |

**Table 1: Gal4 and LexA drivers.** Driver lines used in this study and their target cells.

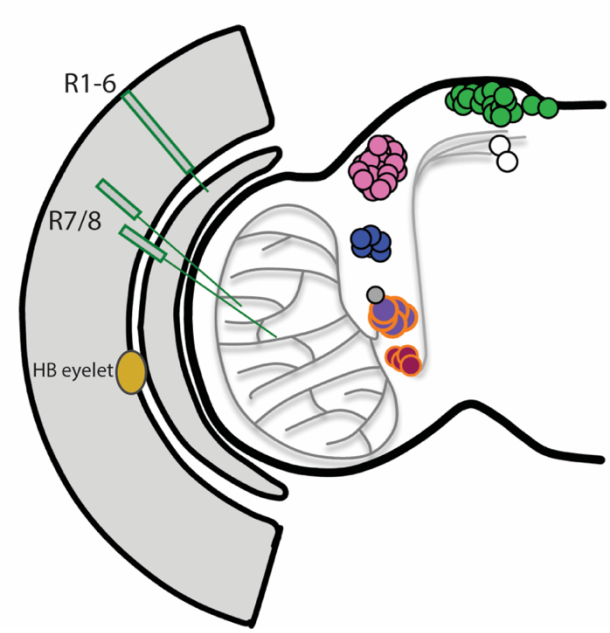



**Figure 1: Schematic of the circadian centers of the fly brain.** Adapted from Top & Young 2018. One hemisphere of the fly brain represented with compound eye (containing photoreceptor cells R1-8), HB eyelet (yellow ellipse) and circadian neuronal clusters: **Green**: DN1s, **pink**: DN3s, **blue**: LNds, **purple/ orange**: l-LNvs, **maroon/ orange**: s-LNvs.

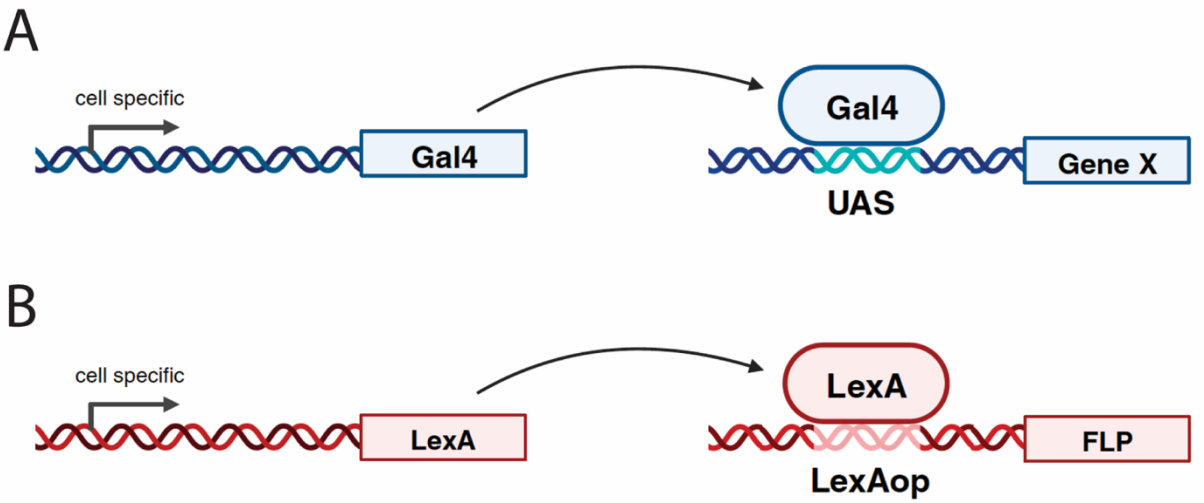



**Figure 2: GAL4/UAS and LexA/LexAop systems.** Two common binary expression systems used in *Drosophila* for cell-specific expression of genes of interest. Created with BioRender. **(A)** GAL4/UAS: A promoter of interest is fused to the *gal4* gene, expressing GAL4 in a cell-specific manner. GAL4, a transcriptional activator, binds to the upstream activating sequence (UAS) of a gene of interest, thus activating their transcription. **(B)** LexA/LexAop: A promoter of interest is fused to the *LexA* gene, expressing LexA in individual circadian neuronal clusters. LexA, a transcriptional activator, binds to the upstream activating sequence (UAS) of the *flp* gene, thus expressing FLP and activating LABL (LABL: Appendix A).

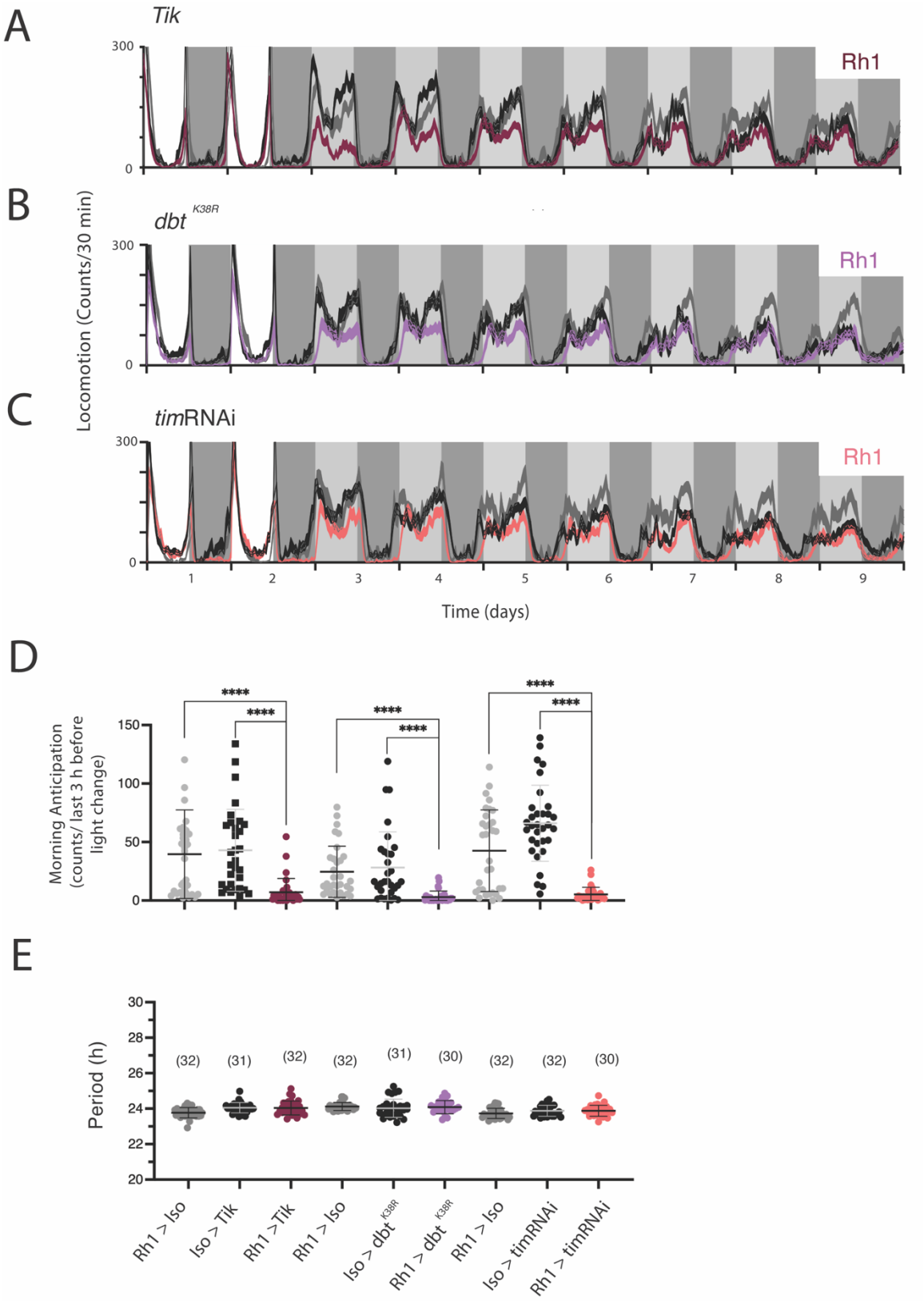

A
Tik
300
0
Rh1
B
dbt K38R
300
0
Rh1
C
timRNAi
300
0
Rh1
Locomotion (Counts/30 min)
1
2
3
4
5
6
7
8
9
Time (days)
D
Morning Anticipation (counts/ last 3 h before light change)
150
100
50
0
****
****
****
****
****
****
E
Period (h)
30
28
26
24
22
20
(32)
(31)
(32)
(32)
(31)
(30)
(32)
(32)
(30)
Rh1 > Iso
Iso > Tik
Rh1 > Tik
Rh1 > Iso
Iso > dbt K38R
Rh1 > dbt K38R
Rh1 > Iso
Iso > timRNAi
Rh1 > timRNAi

**Figure 3: Locomotor activity in flies expressing circadian transgenes in the eye.**

Activity rhythms of flies were assessed when expressing circadian transgenes in the eye using the Gal4 driver Rhodopsin 1 (Rh1-Gal4). **(A)** Rh1-Gal4 > UAS-*Tik* (maroon). **(B)** Rh1-Gal4 > UAS-$dbt^{K38R}$ (purple). **(C)** Rh1-Gal4 > UAS-*tim*RNAi (salmon). All grey curves represent controls using only the Gal4 drivers [Rh1-Gal4 > + (wild-type)]. All black curves represent controls using only the transgene [+ (wild-type) > UAS-*Tik* or UAS-$dbt^{K38R}$ or UAS-*tim*RNAi]. Locomotion counts are averaged into 30-min bins, and the mean of 30-32 flies presented, +/- SEM (thickness of curve). n= 30-32. White and dark grey backgrounds (days 1-2) represent day and night. Light grey and dark grey backgrounds (days 3-9) represent subjective day and subjective night, respectively. **(D)** Morning anticipation analysis (3 hours before change from "lights-off" to "lights-on") +/- standard deviation (SD). **(E)** Period analysis of the activity rhythms +/- SD.

*3.2 Eye clock communicates to the clock of the LNd and l-LNvs*

Once we determined that the eye clock plays a role in morning anticipation (and evening activity), we sought to determine which brain clocks are responsive to the eye clock. We simultaneously altered the eye clock with circadian gene variants using the GAL4/UAS system (**Figure 2A**), and directly measured circadian clock oscillations in distinct neuronal clusters. To measure these circadian clock oscillations in individual clusters, we used a technique called LABL (**Appendix A**) (Johnstone et al. 2022). LABL is a genetically encoded reporter that allows the measurement of transcriptional oscillations of *period* in target neurons in real time and *in vivo*. We expressed the same three transgenes (Figure 1) to alter the circadian clock of the eye with Rh1-Gal4, and LABL was simultaneously activated in individual neuronal clusters using the LexA/LexAop system (**Figure 2B**). Simultaneous modification of the eye and activation of LABL in neuronal clusters required the use of two distinct systems, which is why we used both Gal4/UAS and LexA/LexAop. When modifying the eye clock with *Tik,* the luminescence oscillations of the LNd neurons (**Figure 4A, blue**) and LNv neurons (**Figure 4A, purple and orange**) show little to no effect compared to WT (**Figure 4A, black curves**). The other neuronal clusters measured (DN1s and DN3s) were not affected (**Figure 4A, pink and green**).

When altering the eye clock with $dbt^{K38R}$, the luminescence oscillations in the LNd neurons were lengthened during days ~4-6 (**Figure 5A, blue**). When measuring the period of oscillation, we observed a period of ~25.5-26 h (**Figure 5B, blue**), compared to WT flies with a period of ~24 h (**Figure 5B, grey**). Interestingly, the oscillations of the LNds do seem to revert to a normal rhythm at day ~7 (see discussion). The oscillations in the LNvs showed a very slight phase advancement of the luminescence oscillation (**Figure 5A,**

**purple and orange**), while the DN3 and DN1 oscillations were not affected (**Figure 5A, pink and green**).

When eliminating the eye clock using *tim*RNAi, the oscillations of the LNds were affected, showing arrhythmic luminescence signal during days ~3-5 (**Figure 6A, blue**). The oscillations in the l-LNvs were also noticeably arrhythmic at days ~3-5 (**Figure 6A, purple**). When measuring both pdf+ neurons (s-LNvs and l-LNvs), oscillations remained like wild-type (**Figure 6A, orange**). The DN3s and DN1s also remained unchanged (**Figure 6A, pink and green**). Because of inaccurate measurements of the peaks and troughs of the LNds and l-LNv oscillations during days ~3-5 (**Figure 6A, blue and purple dots**), we excluded these data points. In addition, the arrhythmic luminescence signals in the LNds and l-LNvs (**Figure 6C**) caused inaccurate measurements of the period, revealing ~40-60-h rhythms (**Figure 6B, blue and purple**). Interestingly, the oscillations of the LNds and l-LNvs revert to a normal rhythm at around day ~6, similar to the results with $dbt^{K38R}$ (**Figure 5**) (see discussion). These data suggest that the LNd clock is largely responsive to the eye clock, while the l-LNv clock is responsive only when eliminating the clock of the eye entirely. When measuring luminescence in both s-LNv and l-LNv clusters (orange), oscillations remained like wild type, which could mean that between the two LNv clusters, only the l-LNv clock is responsive to the eye while the s-LNvs are not. A lack of an s-LNv specific LexA driver prohibits us from confirming this suggestion. For the remainder of this study, we focused on the $dbt^{K38R}$ and *tim*RNAi transgenes, given that their effect on the LNd and l-LNv oscillations were more prominent than with *Tik*.

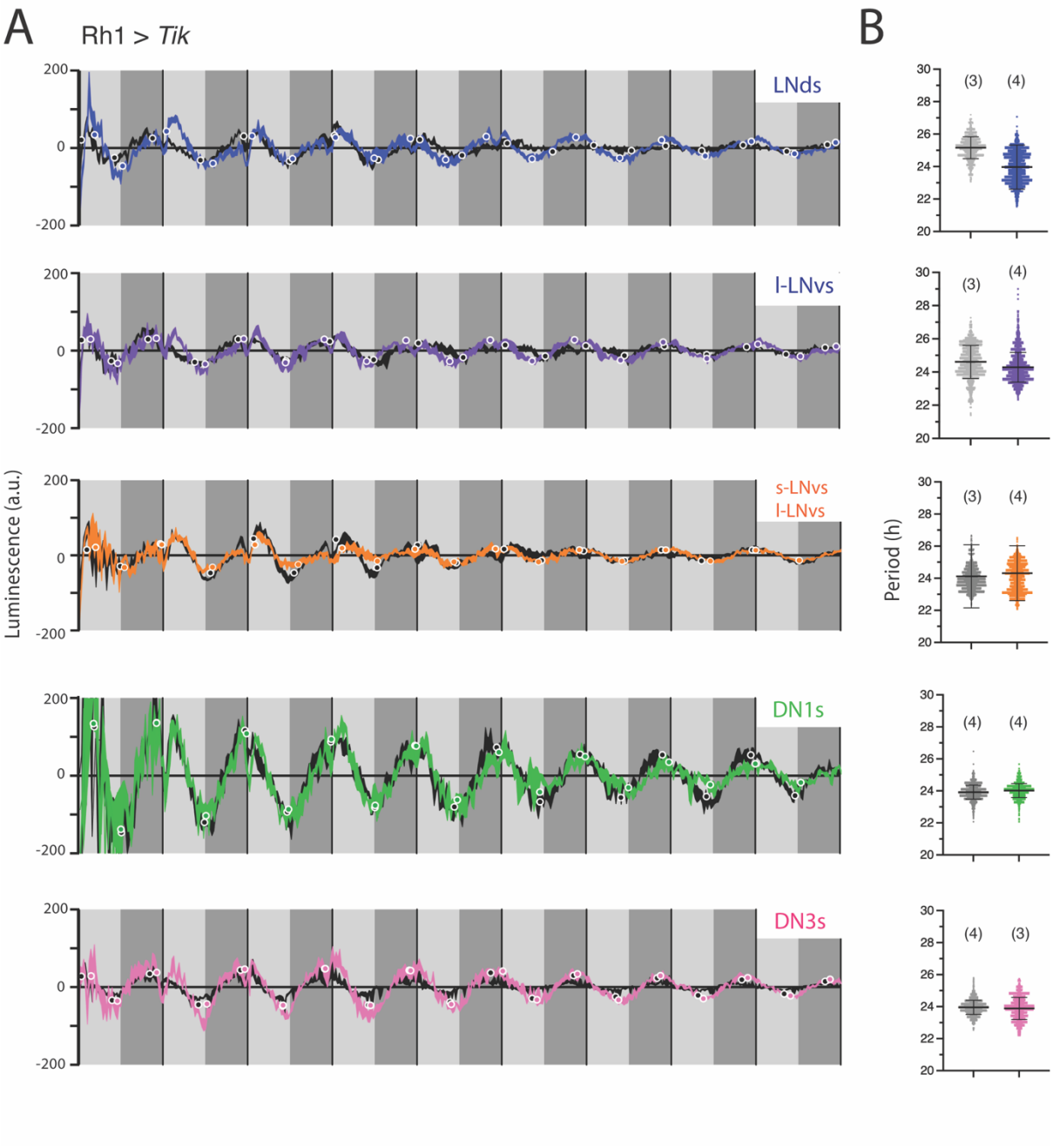

A
Rh1 > Tik
LNds
l-LNvs
s-LNvs
l-LNvs
DN1s
DN3s
Luminescence (a.u.)
200
0
-200
B
Period (h)
30
28
26
24
22
20
(3)
(4)

**Figure 4: Clock oscillation response of neuronal clusters with eye clock expressing *Tik***

**(A)** Oscillation response of distinct neuronal clusters when expressing UAS-*Tik* with Gal4 driver Rhodopsin 1 (Rh1-Gal4). LABL was activated in distinct neuronal clusters by expressing LexAop-FLPL with specific LexA drivers: R15C11-LexA (LNds, blue), R67D09-LexA (l-LNvs, purple), pdf-LexA (s-LNvs and l-LNvs, orange), R18H11-LexA (DN1s, green), R77H08-LexA (DN3s, pink). All black curves represent controls lacking UAS-*Tik*. Luminescence signal from flies is normalized to exponential decay of signal, the values are averaged into 30 min bins, and the mean of the three-four replicates presented, +/- SEM (thickness of curve). n= 3-4. Each replicate contains 15 flies. Peaks and troughs are represented by dots, and are the mean of three-four replicates, +/- SEM. Light grey and dark grey backgrounds represent subjective day and subjective night, respectively. Vertical solid black lines divide days. **(B)** Period analysis of oscillation rhythms +/- standard deviation (SD).

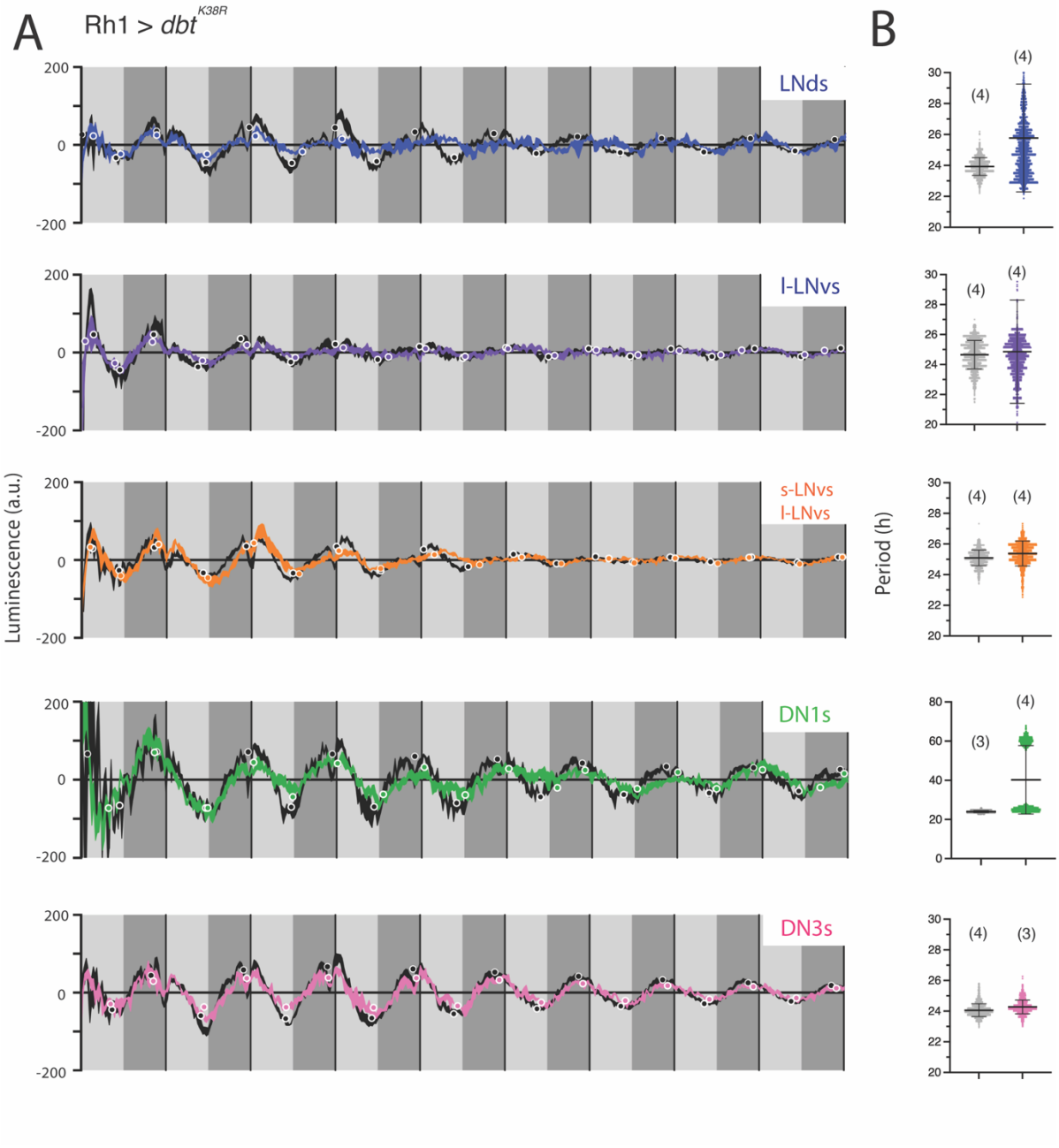

A
Rh1 > dbt K38R
LNds
l-LNvs
s-LNvs
l-LNvs
DN1s
DN3s
Luminescence (a.u.)
200
0
-200
B
Period (h)
(4)
(3)

**Figure 5: Clock oscillation response of neuronal clusters with eye clock expressing *dbt$^{K38R}$***

**(A)** Oscillation response of distinct neuronal clusters when expressing UAS-*dbt$^{K38R}$* with Gal4 driver Rhodopsin 1 (Rh1-Gal4). LABL was activated in distinct neuronal clusters by expressing LexAop-FLPL with specific LexA drivers: R15C11-LexA (LNds, blue), R67D09-LexA (l-LNvs, purple), pdf-LexA (both LNvs, orange), R18H11-LexA (DN1s, green), R77H08-LexA (DN3s, pink). All black curves represent controls lacking UAS-*dbt$^{K38R}$*. Luminescence signal from flies is normalized to exponential decay of signal, the values are averaged into 30 min bins, and the mean of the three-four replicates presented, +/- SEM (thickness of curve). n= 3-4. Each replicate contains 15 flies. Peaks and troughs are represented by dots, and are the mean of three-four replicates, +/- SEM. Light grey and dark grey backgrounds represent subjective day and subjective night, respectively. Vertical solid black lines divide days. (**B)** Period analysis of oscillation rhythms +/- standard deviation (SD).

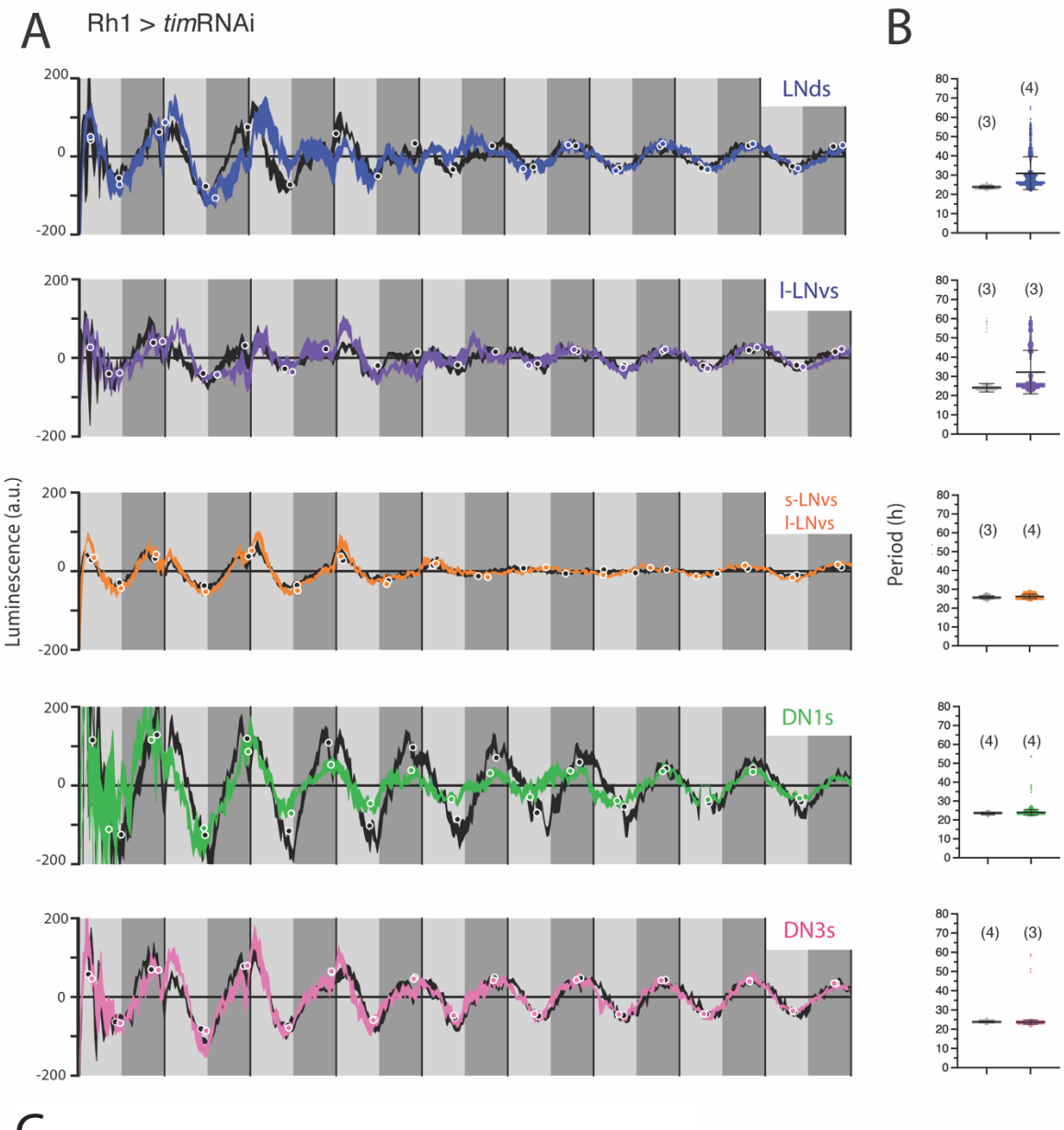

A
Rh1 > timRNAi
LNds
l-LNvs
s-LNvs
l-LNvs
DN1s
DN3s
Luminescence (a.u.)
B
Period (h)
(3)
(4)
C

**Figure 6: Clock oscillation response of neuronal clusters with eye clock expressing *tim*RNAi**

**(A)** Oscillation response of distinct neuronal clusters when expressing UAS-*tim*RNAi with driver Rhodopsin 1 (Rh1-Gal4). LABL was activated in distinct neuronal clusters by expressing LexAop-FLPL with specific LexA drivers: R15C11-LexA (LNds, blue), R67D09-LexA (l-LNvs, purple), pdf-LexA (both LNvs, orange), R18H11-LexA (DN1s, green), R77H08-LexA (DN3s, pink). All black curves represent controls lacking UAS-*tim*RNAi. Luminescence signal from flies is normalized to exponential decay of signal, the values are averaged into 30 min bins, and the mean of the three-four replicates presented, +/- SEM (thickness of curve). n= 3-4. Each replicate contains 15 flies. Peaks and troughs are represented by dots, and are the mean of three-four replicates, +/- SEM. Light grey and dark grey backgrounds represent subjective day and subjective night, respectively. Vertical solid black lines divide days. **(B)** Period analysis of oscillation rhythms +/- standard deviation (SD). **(C)** Luminescence signal plotted in 30-min bins. Upper panel represents one single replicate from LNd oscillation response (**blue curve in A**). Lower panel represent one single replicate from l-LNv oscillation response (**purple curve in A**).

*3.3 LNv clock does not communicate to LNd clock*

Earlier studies suggest that the LNvs are the master pacemaker neurons, and they are claimed to regulate morning anticipation (Stoleru et al. 2004, Grima et al. 2004). We have determined that the eye clock also regulates morning anticipation (**Figure 3**). We've also revealed that the LNd and l-LNv clocks are linked to the eye clock (**Figure 5-6**). Therefore, we were curious if these two neuronal clusters (LNds and l-LNvs) are a part of the same network receiving input from the eye clock to regulate morning anticipation, or different networks that respond to the eye clock separately. We wanted to test whether the LNv clock transmits information to the LNd clock. To test this, we used the GAL4/UAS system to alter the circadian clock of both LNvs (pdf-Gal4, **Table 1**), and simultaneously measured the luminescence oscillations of the LNds using LABL (**Appendix A**) (Johnstone et al. 2022). The oscillations in the LNds remained unchanged after we expressed $dbt^{K38R}$ in the LNv clocks (**Figure 7, red**). This result suggests that when altering the circadian clock of the LNvs, the LNd clock does not respond. However, we used a driver specific to both s-LNvs and l-LNvs in this experiment (pdf-Gal4). Given that we demonstrated a link between the eye clock and the l-LNv clock (**Figure 6, purple**), there is a possibility that we could observe a more noticeable effect when inducing l-LNv clock-specific changes. We believe if circadian clock regulation of the LNvs does not influence the LNd clock, there is a possibility that LNv neuronal activity does, while bypassing the circadian clock.

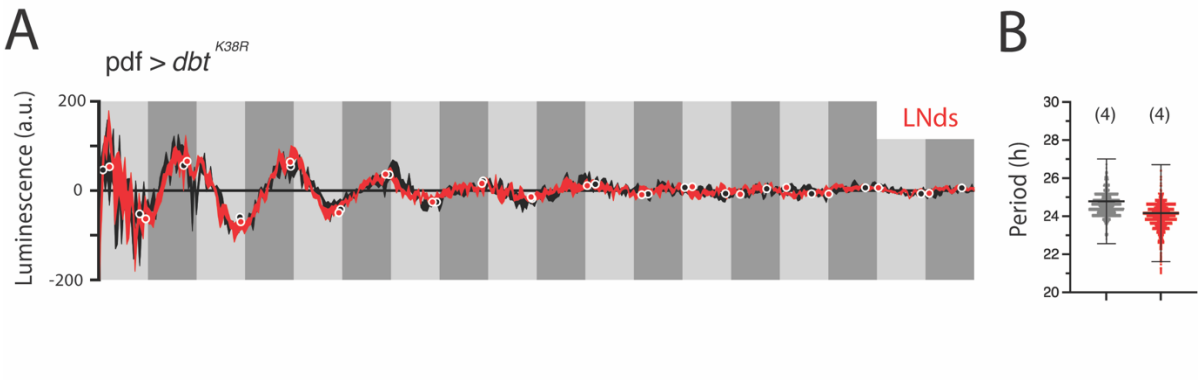



**Figure 7: LNd clock oscillation response to LNv clock expressing *dbt*$^{K38R}$**

**(A)** Oscillation response of the LNd clock when expressing clock mutant UAS-*dbt*$^{K38R}$ in the LNvs with pdf-Gal4. LABL was activated in the LNds by expressing LexAop-FLPL with driver R15C11-LexA (red curve). Black curve represents control lacking UAS-*dbt*$^{K38R}$. Luminescence signal from flies is normalized to exponential decay of signal, the values are averaged into 30 min bins, and the mean of the four replicates presented, +/- SEM (thickness of curve). n= 4. Each replicate contains 15 flies. Peaks and troughs are represented by dots, and are the mean of three-four replicates, +/- SEM. Light grey and dark grey backgrounds represent subjective day and subjective night, respectively. Vertical solid black lines divide days. **(B)** Period analysis of oscillation rhythms +/- SD.

*3.4 s-LNv clock regulates morning anticipation, LNd clock regulates evening anticipation*

Our earlier results suggest that input from the eye clock plays a role in regulating morning anticipation (and evening anticipation) (**Figure 1**). Earlier studies claim that the LNvs regulate morning anticipation and the LNds regulate evening anticipation (Stoleru et al. 2004, Grima et al. 2004, Stoleru et al. 2005, Guo et al. 2014). Given that the eye clock and the brain clocks have similar roles in how they regulate behaviour, we believe these clocks are connected. We therefore aimed to confirm the earlier studies to better understand how the LNvs and LNds collaborate with the eye clock to regulate behaviour. First, we altered the circadian clock of the LNvs and LNds with $dbt^{K38R}$ (**Figure 8A**). When expressing $dbt^{K38R}$ in the s-LNv clock, activity rhythms were lengthened to ~25-25.5 h (**Figure 8A, maroon**) and the flies remained fully rhythmic. Our data supports earlier reports that the $dbt^{K38R}$ mutant lengthens activity rhythms (Muskus et al. 2007), but in the 2007 study, $dbt^{K38R}$ produced 62% arrhythmic flies, and those who remained rhythmic had a 33-h behavioural period. However, these behavioural results were observed when expressing $dbt^{K38R}$ in all circadian tissues (tim-Gal4: *tim*-expressing tissues), which we believe explains this difference. When targeting both s-LNv and l-LNv clusters with $dbt^{K38R}$, this resulted in 50% arrhythmic behaviour in DD (**Figure 8A, orange**). The remaining rhythmic flies did not observe any change in periodicity, remaining at ~24 h, which contradicts the earlier study (Muskus et al. 2007). This may be explained by the same reasoning with our results targeting the s-LNvs just above (**Figure 8A, maroon**), meaning the effects are dependent on the number of neurons/tissues in which $dbt^{K38R}$ is expressed. There is also a possibility that our pdf-Gal4 driver lacks enough strength to lengthen the behavioural period. When expressing the same mutant in the LNd neurons,

there is a noticeable decrease in the peak of evening activity throughout the entirety of the experiment (**Figure 8A, blue**). This suggests that the LNd clock regulates evening activity and the LNvs are crucial in regulating activity rhythms in DD, confirming earlier studies (Stoleru et al. 2004, Grima et al. 2004). When eliminating the clock entirely in the s-LNvs by silencing transcription of the *timeless* gene with *tim*RNAi (**Figure 8C, maroon**), morning anticipation was eliminated in LD, and 57.1% of flies became arrhythmic in DD. When expressing *tim*RNAi in both s-LNv and l-LNv clusters, there is a decrease in the amplitude of activity rhythms, and a severe reduction of the morning peak of activity in DD (**Figure 8C, orange**). In LD, we expected a loss of morning anticipation due to our results with the s-LNvs (**Figure 8A, maroon**) revealing an effect on morning anticipation in the s-LNvs. Earlier studies also claim that the LNvs regulate morning anticipation (Stoleru et al. 2004, Grima et al. 2004). However, behaviour remained like WT in LD. We believe this may be due to differences in strength between the drivers specific to the s-LNvs (VT027163-Gal4, **Table 1**) and both LNv clusters (pdf-Gal4, **Table 1**). On the other hand, there is also a possibility that the addition of the l-LNvs in the pdf-Gal4 driver is changing behaviour. Taking together these results, this suggests that the s-LNv clock regulates morning anticipation. Expressing *tim*RNAi in the LNds did not reveal an effect on activity rhythms (**Figure 8C, blue**), contradicting previous studies demonstrating that eliminating the clock in the LNds reduces evening anticipation (Stoleru et al. 2004, Grima et al. 2004), as well as previous data from our lab using different Gal4 drivers that target the LNds. For example, we have tested the effects on locomotion when expressing UAS-*tim*RNAi with Mai179-Gal4; pdf-Gal80, which targets three of the six LNds (**Table 1**), and this revealed a loss of evening anticipation in LD (**Appendix B**). This leads us to believe

that the driver used in this study (VT020611-Gal4, **Table 1**) may have off-target effects which is affecting our results. A different driver specific to the LNds will be important to confirm a role of the LNd clock in regulating evening anticipation.

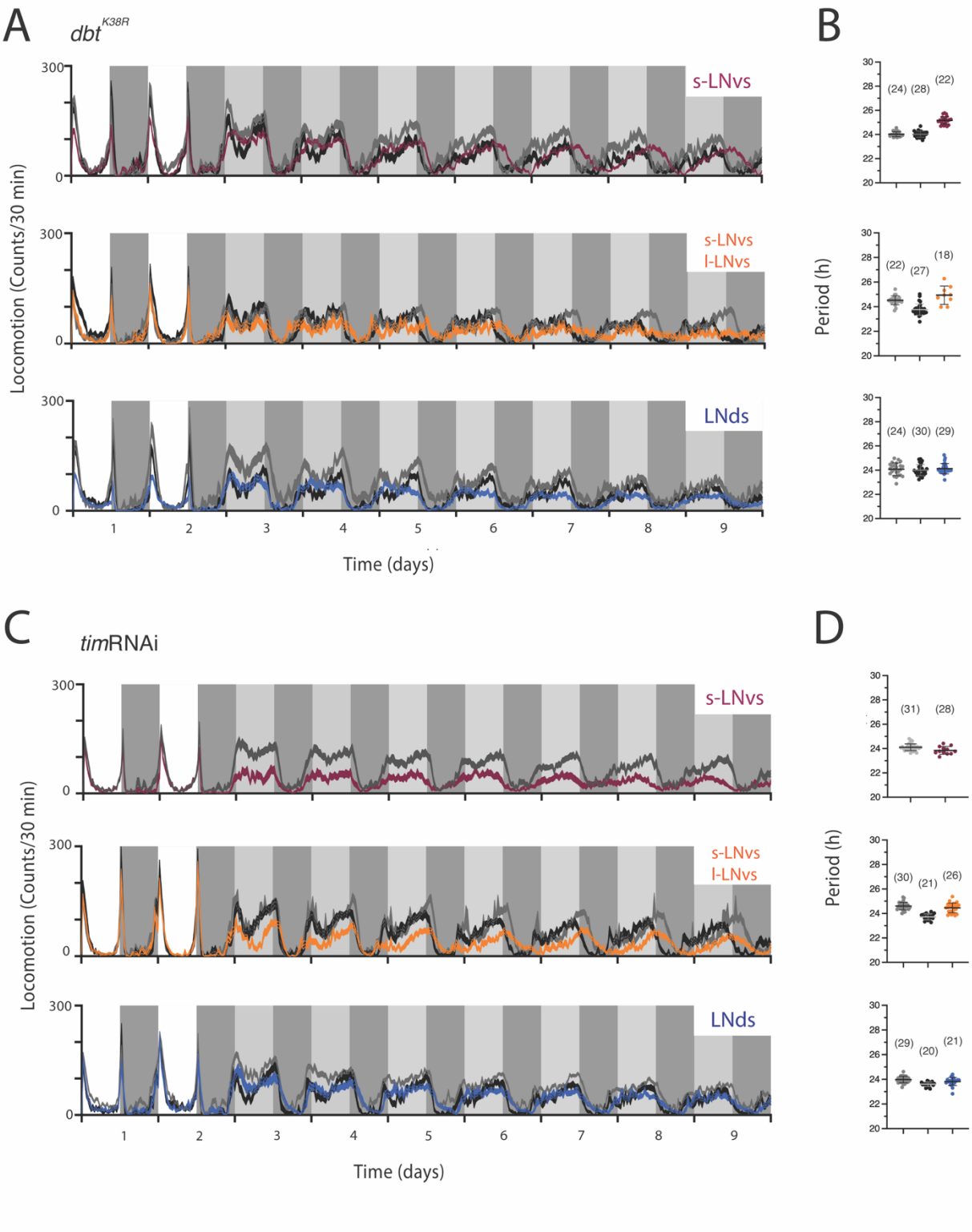

A
dbt K38R
300
0
s-LNvs
s-LNvs
l-LNvs
LNds
Locomotion (Counts/30 min)
1
2
3
4
5
6
7
8
9
Time (days)
B
Period (h)
(24) (28) (22)
(22) (27) (18)
(24) (30) (29)
C
timRNAi
D
(31) (28)
(30) (21) (26)
(29) (20) (21)

**Figure 8: Locomotor activity in flies expressing circadian transgenes in specific neuronal clusters**

Activity rhythms of flies were assessed when expressing circadian transgenes in the LNvs and LNds. (**A)** Clock transgene UAS-*dbt*$^{K38R}$ expressed in the s-LNvs (VT027163-Gal4, maroon), both s-LNv and l-LNv clusters (pdf-Gal4, orange) and LNds (VT020611-Gal4, blue). (**B)** Period analysis of activity rhythms +/- SD. (**C).** Clock transgene UAS-*tim*RNAi expressed in the s-LNvs (VT027163-Gal4, maroon), both s-LNv and l-LNv clusters (pdf-Gal4, orange) and LNds and 5$^{th}$ s-LNv (VT020611-Gal4, blue). (**D)** Period analysis of the activity rhythms +/- SD. White and dark grey backgrounds (days 1-2) represent day and night. Light grey and dark grey backgrounds (days 3-9) represent subjective day and subjective night, respectively. All grey curves represent controls using only the Gal4 drivers (VT027163-Gal4 or VT020611-Gal4 or pdf-Gal4 > +). All black curves represent controls using only the transgene (+ > UAS-*dbt*$^{K38R}$ or UAS-*tim*RNAi). Locomotion counts are averaged into 30 min bins, and the mean of 18-31 flies presented, +/- SEM (thickness of curve). n= 18-31.

*3.5 l-LNv firing regulates morning anticipation*

Next, we wanted to determine if neuronal activity of the LNvs and LNds play a role in regulating activity rhythms, specifically morning anticipation. Our earlier results revealed that the circadian clock of the LNvs may not be instructing the LNd clock (**Figure 7**). We therefore hypothesized that the LNvs could communicate (without using their circadian clock) to the LNds clock through neuronal firing. We decided to measure locomotion when expressing the mutation *Kir2.1* (a potassium channel) in these neurons which inhibits neuronal firing (**Figure 9A, B**). Inhibiting firing in the s-LNvs did not affect activity in LD, but severely affected activity rhythms once switching to DD, resulting in 98% arrhythmic activity (**Figure 9A, maroon**). Period analysis was not possible in the s-LNvs due to the arrhythmic behaviour (**Figure 9B**). Inhibiting neuronal firing in both s-LNv and l-LNv clusters revealed 100% arrhythmic activity in DD (**Figure 9A, orange**). These two results confirm the dominant role of the LNvs in regulating activity in DD (Stoleru et al. 2004, Grima et al. 2004). Additionally with pdf-Gal4, in LD conditions, morning anticipation was reduced compared to one of our control genotypes (+ > UAS-*Kir2.1*). Our second control (pdf-Gal4 > +) revealed locomotion rhythms with very low amplitude which leads us to believe that the results from this control are not comparable to our other genotypes. These results suggest that neuronal firing of the LNvs is important to maintain activity rhythms in DD, but more specifically the l-LNvs, given that inhibition of neuronal firing in the s-LNvs alone did not affect morning anticipation in LD (**Figure 9A, maroon**). When expressing the same mutant in the LNd neurons, activity rhythms were not affected (**Figure 9A, blue**). We were also interested in observing the effects of ablating the cells with a mutation called *hid*. Ablating the cells eliminates all function of the cell,

including circadian clock function and neuronal activity simultaneously. When expressing this mutation in the s-LNvs, the flies exhibited normal activity rhythms in LD, but were 75% arrhythmic in DD (**Figure 9C, maroon**). This is in agreement with our conclusions with *Kir2.1*, and previous studies (Shafer & Taghert, 2009), that the s-LNvs regulate behaviour in DD conditions. When expressing *hid* in both LNv neurons, this resulted in 64.3% arrhythmic behaviour when switching to DD (**Figure 9C, orange**). Earlier studies show that when ablating the pdf+ neurons with *hid*, morning anticipation is lost in LD (Stoleru et al. 2004) and we were expecting similar results, however behaviour remained like WT in LD. This contradicts the earlier studies, given that our locomotion results with the pdf-Gal4 driver did not reveal an effect on morning anticipation in LD. This could be explained by the strength of our pdf-Gal4 driver just like our results expressing circadian transgenes (**Figure 8A, C**). In fact, when comparing our locomotion data with *hid*, the driver specific to the s-LNvs (VT027163-Gal4) expressing *hid* resulted in 75% arrhythmicity, while the pdf-Gal4 driver expressing *hid* resulted in only 64.3%, suggesting that the pdf-Gal4 driver is weaker than our s-LNv driver. However, we also cannot rule out the possibility that the l-LNvs could be functioning differently than the s-LNvs and changing the phenotype of the fly, like our results with *tim*RNAi (**Figure 8C**). These results show that neuronal firing from the s-LNvs is important for regulating activity rhythms in constant darkness (DD), but it may not be important in LD. Our results with the driver targeting both s-LNv and l-LNv clusters (pdf-Gal4) do reveal that neuronal firing from the LNvs regulate morning anticipation in LD (**Figure 9A, orange**). This suggests that l-LNv firing may play an additional role in regulating morning anticipation in LD. Since the LNds are the end point of the circuit in which we are exploring, we wanted to test the

consequences of ablating these neurons as well. We attempted to express *hid* in the LNds, although the data is unavailable because it did not result in any progeny. This further supports our suggestion when expressing *tim*RNAi (**Figure 8**), that the Gal4 driver for the LNds may have non-specific targets and expressing *hid* may be ablating non-specific cells that are important for the development/survival of the fly. Investigation of the expression of this driver will be necessary to confirm this suggestion.

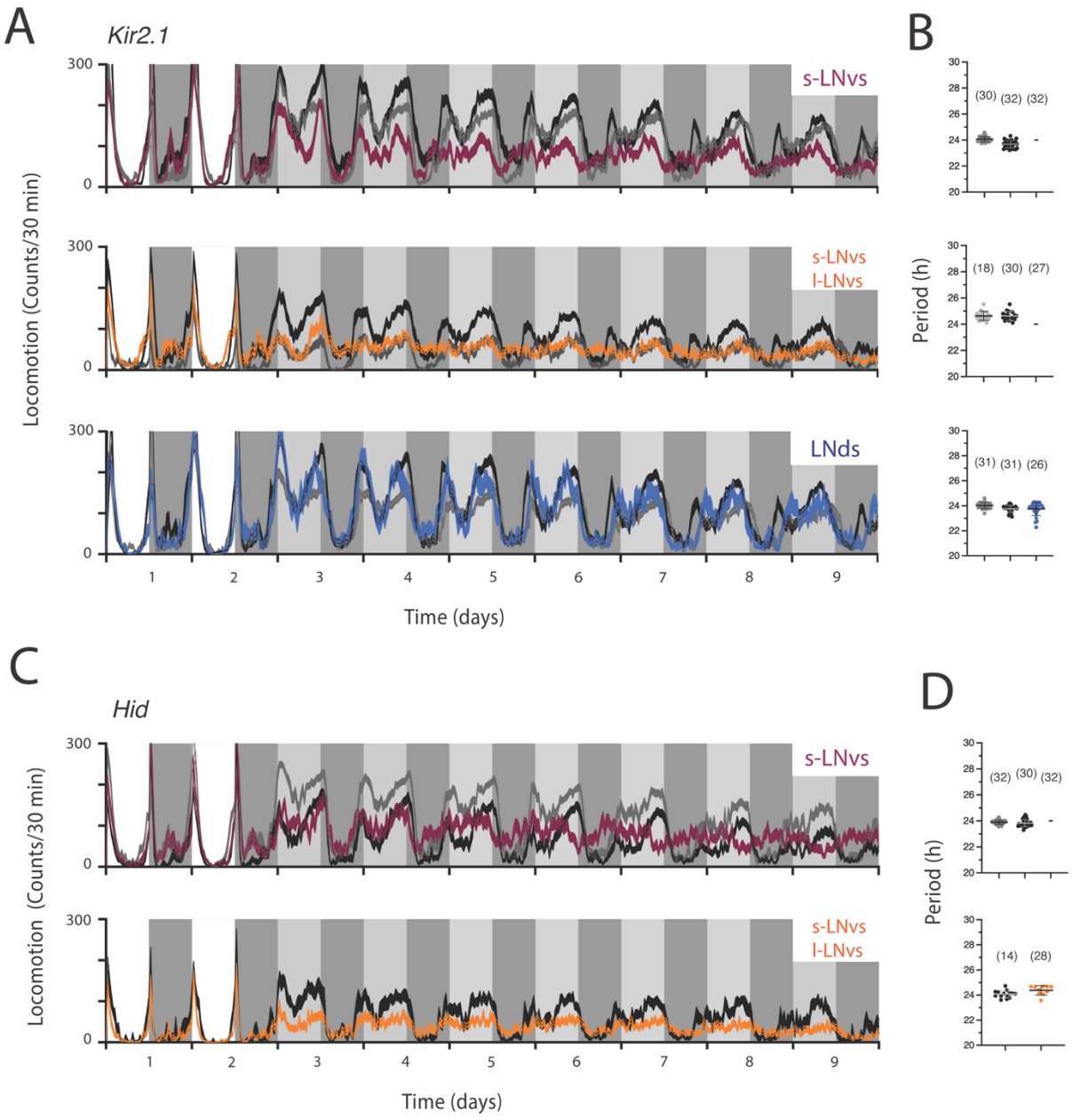

A
Kir2.1
s-LNvs
s-LNvs
l-LNvs
LNds
Locomotion (Counts/30 min)
Time (days)
B
Period (h)
(30) (32) (32)
(18) (30) (27)
(31) (31) (26)
C
Hid
s-LNvs
s-LNvs
l-LNvs
Locomotion (Counts/30 min)
Time (days)
D
Period (h)
(32) (30) (32)
(14) (28)

**Figure 9: Locomotor activity in flies expressing *Kir2.1* and *hid* in specific neuronal clusters**

Activity rhythms of flies were assessed when expressing mutants in the LNvs and LNds. (**A)** UAS-*Kir2.1* expressed in the s-LNvs (VT027163-Gal4, maroon), both s-LNv and l-LNv clusters (pdf-Gal4, orange) and LNds (VT020611-Gal4, blue). (**B)** Period analysis of activity rhythms +/- SD. (**C)** UAS-*hid* expressed in the s-LNvs (VT027163-Gal4, maroon) and both s-LNv and l-LNv clusters (pdf-Gal4, orange). (**D)** Period analysis of the activity rhythms +/- SD. White and dark grey backgrounds (days 1-2) represent day and night. Light grey and dark grey backgrounds (days 3-9) represent subjective day and subjective night, respectively. All grey curves represent controls using only the Gal4 drivers (VT027163-Gal4 or VT020611-Gal4 or pdf-Gal4 > +). All black curves represent controls using only the transgene (+ > UAS-*Kir2.1* or UAS-*hid*). Locomotion counts are averaged into 30 min bins, and the mean of 14-32 flies presented, +/- SEM (thickness of curve). n= 14-32.

*3.6 Neuronal firing from retinal cells regulates morning anticipation*

Given our results that demonstrate a role of the retinal clocks in regulating morning anticipation (and evening anticipation) (**Figure 3**), we next wanted to test if neuronal firing from these retinal cells regulates activity rhythms. We were interested in measuring neuronal activity of the retinal cells because we discovered that neuronal activity may be important in the l-LNvs (**Figure 9A, orange**), and we wanted to see if a similar mechanism was also important in retinal cells. We expressed the *Kir2.1* mutant in the R1-6 photoreceptor cells of the eye, inhibiting neuronal firing, and this led to a loss in morning anticipation in LD (**Figure 10, teal**). This result suggests that neuronal activity from the retinal cells is critical in maintaining morning anticipation. We additionally expressed *hid* in the same retinal cells, although activity was not affected (**Figure 10, pink**). This may be due to a dominant negative phenotype of the eye, meaning the inhibition of neuronal firing alters the input to the neuronal pathway necessary for regulating morning anticipation, however when killing the retinal cells completely, the brain clocks can still function autonomously to regulate behaviour. It is also a possibility that *hid* was not successful in killing these photoreceptor cells. We are confident that the transgene can be successfully expressed since we see a behavioural effect when expressing in the s-LNvs (**Figure 9C, orange**). However, we have not confirmed that the transgene was successfully expressed in the eye. This can be validated using expression of a fluorescent reporter such as GFP to report both Rh1-Gal4 activity and a loss of fluorescence due to *hid* function. We conclude from these data that neuronal firing from the photoreceptor cells R1-6 could contribute to the regulation of morning anticipation.

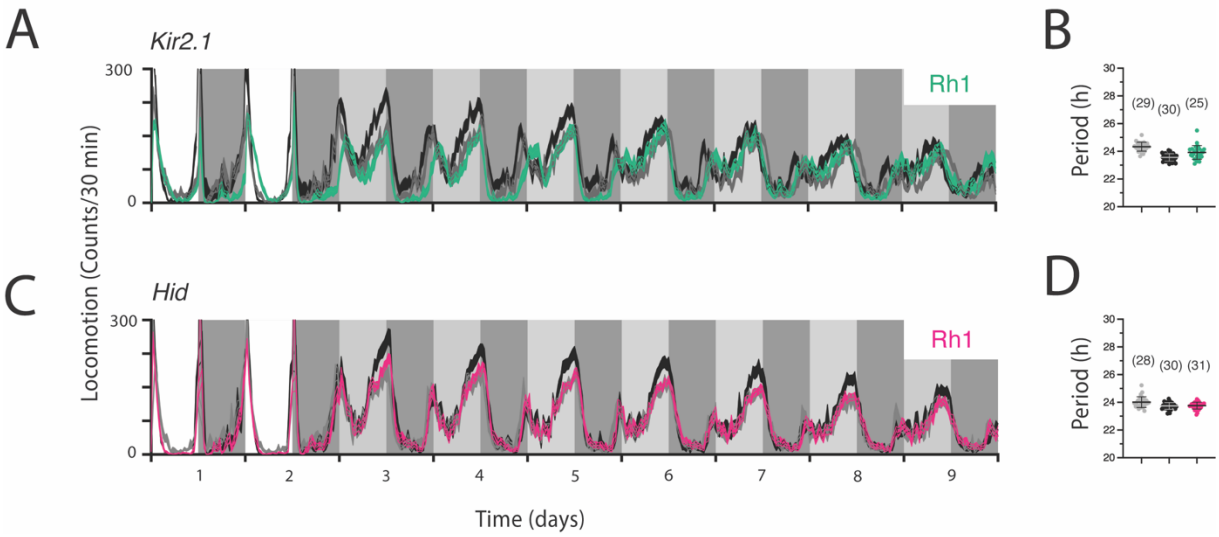



**Figure 10: Locomotor activity in flies expressing *Kir2.1* and *hid* in retinal cells R1-6**

Activity rhythms of flies were assessed when expressing mutants in photoreceptor cells R1-6 of the retina. (**A**) Rh1-Gal4 > UAS-*Kir2.1* (**B**) Period analysis of activity rhythms +/- SD. (**C**) Rh1-Gal4 > UAS-*hid*. (**D**) Period analysis of the activity rhythms +/- SD. White and dark grey backgrounds (days 1-2) represent day and night. Light grey and dark grey backgrounds (days 3-9) represent subjective day and subjective night, respectively. All grey curves represent controls using only the Gal4 drivers (Rh1-Gal4 > +). All black curves represent controls using only the transgene (+ > UAS-*Kir2.1* or UAS-*hid*). Locomotion counts are averaged into 30 min bins, and the mean of 25-31 flies presented, +/- SEM (thickness of curve). n= 25-31.

*3.7 R7 and R8 photoreceptor cells (and HB eyelet) regulate evening activity*

The driver used in this study (Rh1-Gal4) targets six of the eight photoreceptor cells of the compound eye (R1-6), giving us a lot of information on how these retinal cells contribute to the regulation of behaviour. However, the R7 and R8 photoreceptor cells have also revealed important roles in behavioural synchronization of the circadian clocks in the brain (Ogueta, M. et al. 2018, Ni et al. 2017, Senthilan et al. 2019). We were therefore curious to see if these retinal cells had similar effects as the R1-6 cells. To determine if the R7 and R8 photoreceptor cells and their clocks also regulate activity rhythms, we expressed various mutants in these cells using Rh5-Gal4 (**Table 1**), which targets the R7 cells, and Rh6-Gal4 (**Table 1**), which targets the R8 cells as well as the HB eyelet (**Figure 11**). When expressing $dbt^{K38R}$ with both drivers, there is a decrease in the peak of evening activity in DD (**Figure 11A, yellow and green**). This suggests that the circadian clock of the R7-8 retinal cells, as well as the HB eyelet, contributes to regulating evening activity. Silencing neuronal firing by expressing *Kir2.1* in the R7 cells did not affect the activity of the flies (**Figure 11C, yellow**), and with Rh6-Gal4, there is a slight decrease in the peak of evening activity in DD (**Figure 11C, green**). This suggests that neuronal firing of the R8 retinal cells helps to regulate evening activity. However, given that Rh6-Gal4 also targets the HB eyelet, the results obtained may not be specific to the role of the R8 retinal cells. Additionally, the HB eyelet does show a prominent role in communicating to the the CNN (Helfrich-Förster 2020). We have yet to distinguish between these retinal cells and the HB eyelet, and this will require further investigation. Finally, when ablating the cells by expressing *hid* in the R7 cells, there is a decrease in the peak of evening activity (**Figure 11E, yellow**), but in the R8 cells and HB eyelet, there is no effect (**Figure 11E, green**).

Similar to the results obtained for R1-6, it is possible that *hid* was not successfully expressed in these retinal cells, and this will be necessary to test to ensure that our results are valid. The period of activity rhythms was not affected in any of the experiments (**Figure 11B, D, F**). These data suggest that the clock in the R7-8 cells (and HB eyelet), as well as neuronal firing from R8 cells and HB eyelet, contribute to regulating evening activity.

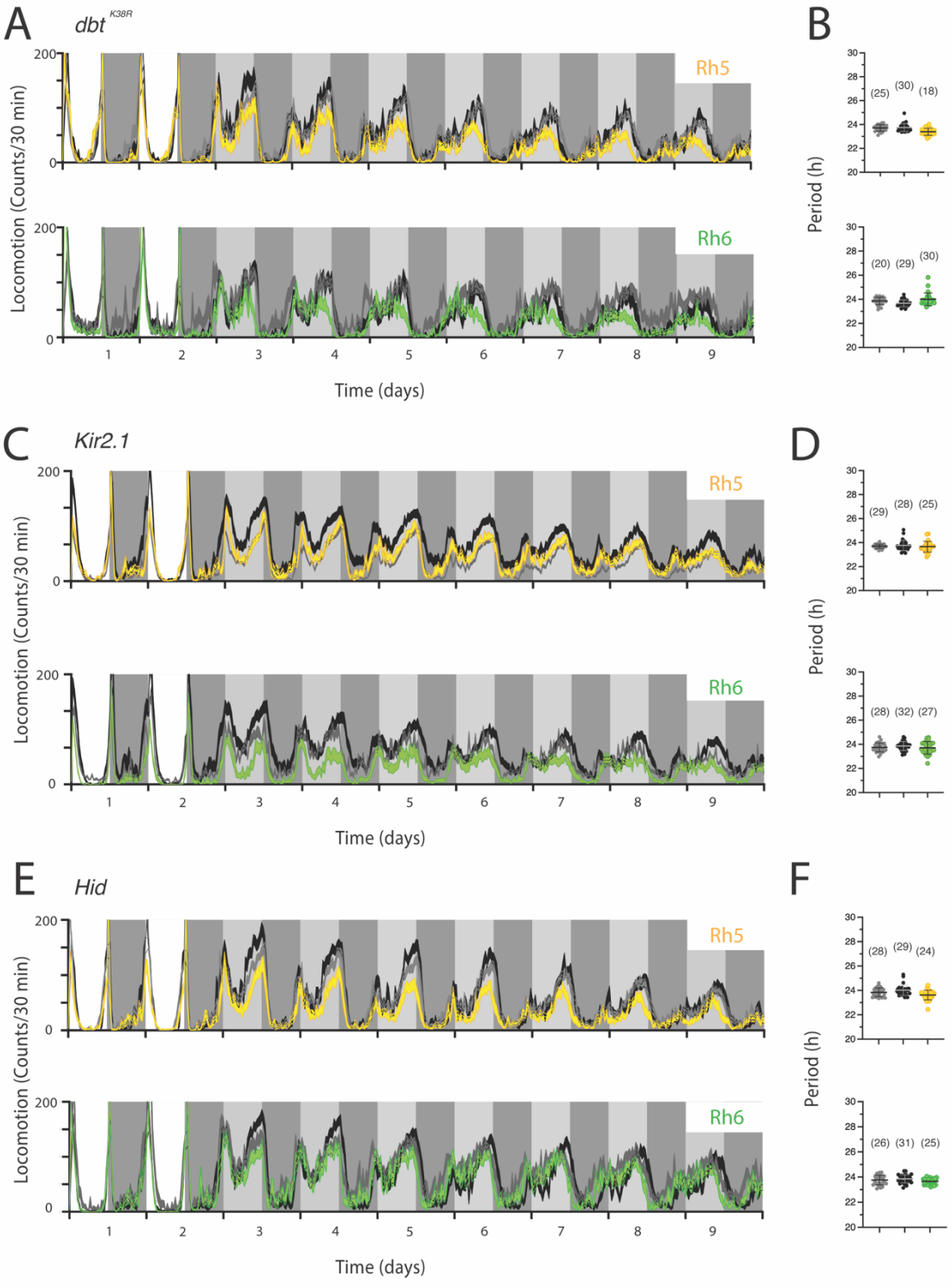

A
dbt K38R
Rh5
Rh6
Locomotion (Counts/30 min)
Time (days)
B
Period (h)
(25) (30) (18)
(20) (29) (30)
C
Kir2.1
Rh5
Rh6
Locomotion (Counts/30 min)
Time (days)
D
Period (h)
(29) (28) (25)
(28) (32) (27)
E
Hid
Rh5
Rh6
Locomotion (Counts/30 min)
Time (days)
F
Period (h)
(28) (29) (24)
(26) (31) (25)

**Figure 11: Locomotor activity in flies expressing mutations in retinal cells R7-8 and the HB eyelet**

Activity rhythms of flies were assessed when expressing mutants in photoreceptor cells R7 and R8 of the eye, and the HB eyelet. (**A)** Clock transgene UAS-*dbt*$^{K38R}$ expressed in the R7 cells (Rh5-Gal4, yellow), and R8 cells and HB eyelet (Rh6-Gal4, green) (**B)** Period analysis of activity rhythms +/- SD. (**C)** UAS-*Kir2.1* expressed in the R7 cells (Rh5-Gal4, yellow), and R8 cells and HB eyelet (Rh6-Gal4, green) (**D)** Period analysis of the activity rhythms +/- SD. (**E)** UAS-*hid* expressed in the R7 cells (Rh5-Gal4, yellow), and R8 cells and HB eyelet (Rh6-Gal4, green). (**F)** Period analysis of the activity rhythms +/- SD. White and dark grey backgrounds (days 1-2) represent day and night. Light grey and dark grey backgrounds (days 3-9) represent subjective day and subjective night, respectively. All grey curves represent controls using only the Gal4 drivers (Rh5-Gal4 or Rh6-Gal4 > +). All black curves represent controls using only the transgene (+ > UAS-*dbt*$^{K38R}$ or UAS-*Kir2.1* or UAS-*hid*). Locomotion counts are averaged into 30 min bins, and the mean of 18-32 flies presented, +/- SEM (thickness of curve). n= 18-32.

*3.8 Proposed Models*

Our data has given us a better understanding of the connection between the eye clocks and the brain clocks of the fly. First, we revealed that the eye clock regulates morning anticipation (**Figure 3**). Additionally, we demonstrated that circadian clock of the LNds and the l-LNvs are responsive to the eye clock (**Figure 4-6, blue and purple**). When modifying the circadian clock of the LNvs, the LNd clock was not affected suggesting that the LNds are not responsive to the circadian clock of the LNvs (**Figure 7**). We therefore proposed two communication pathways from the eye clock to the brain clocks, and how these pathways could regulate specific aspects of behaviour (**Figure 12**). In both models, the eye clock communicates to the LNd and LNv clocks. Locomotion results suggest that neuronal firing from the eye is also important in regulating morning anticipation (**Figure 10, teal**). Therefore, we hypothesize that the circadian clock within the photoreceptor cells of the retina is crucial in triggering the firing of their own cells (see discussion). There is no evidence of a synaptic connection between the photoreceptor cells of the eye and the neuronal clusters, which leads us to believe there could be intermediate components that channel these signals from the eye to communicate to downstream neurons. Studies suggest that a region called the accessary medulla (aMe) connects with the circadian neuronal clusters (Li et al. 2018, Helfrich-Förster 2007, Shafer 2006, Yoshii et al. 2008), and this could possibly be the region that channels the signals from the eye to the CNN (see discussion).

Given that the LNvs are claimed to be the master pacemaker neurons (Stoleru et al. 2004, Grima et al. 2004), we believe there is a possibly that the LNvs and LNds are part of one simple network, with the LNvs at the top of the circadian neuronal hierarchy (**Figure**

**12A**). The eye may communicate to the LNd clock through the LNv clock (**Figure 12A, dashed arrows**), or through LNv neuronal firing (**Figure 12A, solid arrow**). We favour the possibility of LNv neuronal firing, given that we demonstrated no LNd response when modifying the LNv clock (**Figure 7**). Our locomotion results suggest that the s-LNv clock regulates morning anticipation behaviour (**Figure 8C, orange**) and that l-LNv neuronal firing plays a role in morning anticipation (**Figure 9A, maroon**), suggesting that both LNv clusters can receive information from the eye clock. However, given these differences between the two LNv clusters, further work will be required to distinguish between the two clusters in how they communicate information from the eye to the LNds. Interestingly, our results reveal that the s-LNv clock may not be responsive to the eye clock (**Figure 4-6, orange**), but the l-LNv clock is responsive (**Figure 6, purple**). We believe there is a possibility that the s-LNv clock could function even in the absence of the eye clock (see discussion). If the LNvs and LNds are a part of a linear network, it is possible that the LNvs instruct the LNds through the release of PDF, given that there have been studies demonstrating a connection between these two clusters through PDF signaling (Hyun et al. 2005, Lear et al. 2005) (see discussion). Further work will be required in our lab to confirm the role of PDF in this communication pathway. This proposed model regulates morning anticipation behaviour, however the LNds do not regulate morning anticipation, but rather evening anticipation (Stoleru et al. 2004, Grima et al. 2004) (**Appendix B**). It is possible that it is not the LNds that contribute to morning anticipation, but rather the LNd response to PDF (see discussion).

Our second proposed model involves the LNv and LNd clocks responding to the eye independently from each other (**Figure 12B**). The LNv (more specifically the l-LNvs)

and LNd clocks are affected when modifying the eye clock (**Figure 5-6**), and the LNd clock may not be responsive to the LNv clock (**Figure 7**). These two clocks also regulate different aspects of circadian behaviour (LNvs regulate morning anticipation; LNds regulate evening anticipation), supporting the idea that these clocks are part of two separate networks. Similar to our first proposed model (**Figure 12A**), the s-LNvs could be functioning independently of the eye. It will be crucial to investigate the connection between the LNvs and LNds through neuronal firing. It will be necessary to conduct experiments where we inhibit neuronal firing of the LNvs and measuring the effect on the LNd clock, to confirm whether or not the LNds are responsive to the LNvs, and to confirm which proposed model is true.

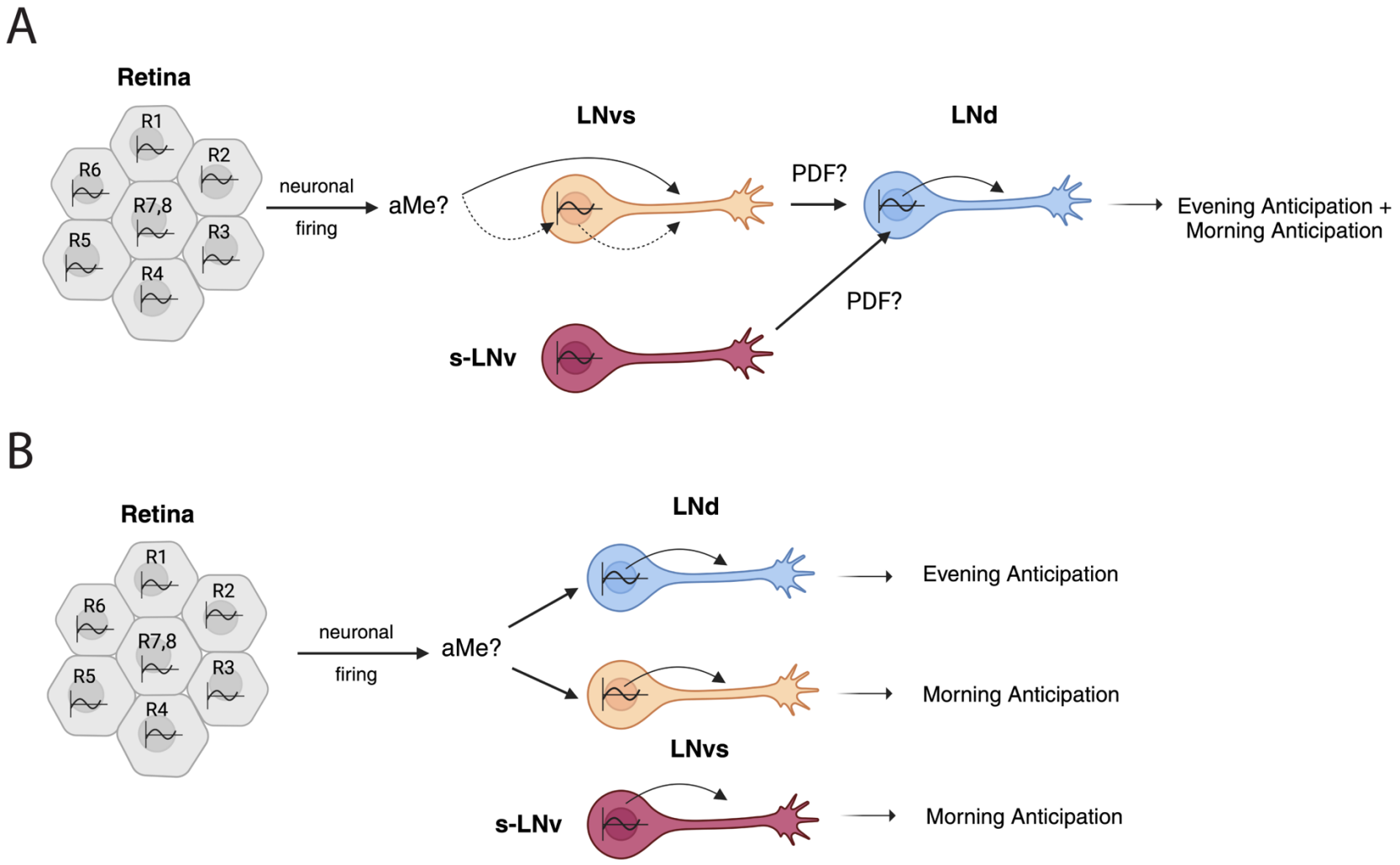

A
Retina
R1
R2
R6
R7,8
R3
R5
R4
neuronal
firing
aMe?
LNvs
PDF?
LNd
Evening Anticipation +
Morning Anticipation
PDF?
s-LNv
B
Retina
R1
R2
R6
R7,8
R3
R5
R4
neuronal
firing
aMe?
LNd
Evening Anticipation
Morning Anticipation
LNvs
s-LNv
Morning Anticipation

**Figure 12: Linear network vs. multi-network model from the eye to behaviour**

Created with BioRender. **(A)** The circadian clocks of the retinal photoreceptor cells (grey) trigger firing of their own cells, sending information to the LNvs (orange neuron). Communication from the eye to the LNvs may channel through the accessary medulla (aMe). Communication from the eye may signal through the LNv clock (dashed line), or by bypassing the LNv clock (solid line) to trigger neuronal firing and communicate to the LNd clock (blue neuron), regulating morning and evening anticipation behaviour. LNvs may communicate to LNds through PDF signaling. Between two LNv clusters, the s-LNvs could function independently from the eye (maroon neuron). **(B)** A multi-network pathway where the circadian clocks of the photoreceptor cells trigger neuronal firing of their own cells. LNv (orange neuron) and LNd (blue neuron) clocks receive input from the eye clocks independently from one another, and these signals from the eye may channel through the accessary medulla (aMe). The circadian clock of the LNds regulates evening anticipation and the circadian clock of the LNvs regulates morning anticipation. The s-LNvs (maroon neuron) could function independently from the eye (maroon neuron).

# Chapter 4: Discussion

*4.1 Multi-network model vs. linear model*

One of our proposed models demonstrates the possibility of the LNd and LNv clocks receiving input from the eye clock independently from one another (**Figure 12B**). The LNvs are claimed to be the master pacemaker neurons because they are thought to receive external inputs and communicate information to the other neuronal clusters through the release of PDF (Park et al. 2000, Stoleru et al. 2005). However, different clusters can dominate in different environmental conditions (LNds: dominate in LL, DN1s: responsive to temperature) (Murad et al. 2007, Picot et al. 2007, Yadlapalli et al. 2018). Additionally, neuronal firing from these neurons in response to light can occur independently from each other (Li et al. 2018). In the absence of CRY, the LNvs or LNds can entrain behaviour through the visual input. This earlier study introduced a double mutant *pdf > Kir2.1; cry*$^{02}$. This double mutant silences neuronal firing of the PDF-expressing neurons (LNvs) and eliminates the function of CRY. The mutant flies were subjected to a normal LD regime for the first five days, then phase shifted the LD cycle to observe the fly's ability to re-entrain their locomotor behaviour to the light condition. They also used a double mutant *pdf>hid; cry*$^{02}$, which kills the PDF-expressing neurons, rather than solely eliminating neuronal firing. Both double mutants did re-entrain locomotor behaviour immediately when phase shifting the LD cycle, demonstrating that behaviour can be entrained independently of the LNvs. This supports our proposed model that the neurons can act independently from one another (**Figure 12B**). However, even though behaviour was re-entrained, there were clear changes in the morning and evening activity peaks. The double mutants expressed in the LNvs lost morning anticipation behaviour. This aligns with earlier studies that the LNvs are the morning neurons and regulate morning anticipation (Stoleru

et al. 2004, Grima et al. 2004). Interestingly, the evening peak of these double mutants disappear. When silencing neuronal firing of the LNvs (*pdf > Kir2.1*), the evening activity peak disappears after three days, and when killing the entire neuron (*pdf > Hid*), the evening peak disappears immediately. Given that the circadian clock of the LNds regulates evening activity (**Appendix B**) (Stoleru et al. 2004, Grima et al. 2004), these findings align with our proposed model in **Figure 12A** that neuronal firing of the LNvs is necessary to influence to circadian clock of the LNds. Importantly, when using $pdf^{01}$ or a PDF receptor mutant $Han^{5304}$, the same study showed that the LNds can still fire in response to light, meaning the LNds can fire independently of PDF signaling. This suggests that neuronal firing of the LNds is not dependent on LNvs firing, but the circadian clock of the LNds is influenced by LNv firing. We believe our linear model (**Figure 12A**) is the model that fits most with this available data.

*4.2 Differences between drivers*

Our locomotion data expressing $dbt^{K38R}$ in the s-LNvs (**Figure 8A, B, maroon**) and in both LNv clusters (**Figure 8A, B, orange**) revealed some differences in behaviour. When expressing this kinase transgene only in the s-LNvs, the behavioural period lengthened by ~1-1.5 h, and all the flies remained rhythmic. On the other hand, when expressing in both s-LNvs and l-LNvs with pdf-Gal4, 50% of flies became arrhythmic, and the rhythmic flies displayed a normal behavioural period of ~24 h. These two results are both contradictory to a study claiming that $dbt^{K38R}$ causes 62% arrhythmicity and those that remained rhythmic displayed a period of ~33-h (Muskus et al. 2007). However, this earlier study expressed $dbt^{K38R}$ in all *tim*-expressing cells using tim-Gal4. Given that we are targeting the clock of particular neuronal clusters, rather than all circadian cells, we believe

that expression of *dbt*$^{K38R}$ in all circadian tissues is necessary to create arrhythmic behaviour, which aligns with our results. Another more recent study supports this idea, demonstrating that knocking out the circadian clock in the LNvs maintains rhythmic behaviour (Deventhal et al. 2019).

Importantly, we suggest that our pdf-Gal4 driver may not be strong enough to observe accurate results and to properly compare with our other drivers. If pdf-Gal4 is not efficiently expressing our transgenes, this may leave endogenous genes, allowing the fly to behave more like WT. In order to eliminate this possibly, we can use a different driver specific to the LNvs or use an absolute method to eliminate the gene completely. For example, a Gal4-based CRISPR method to knock out *per* and *tim* genes in target neurons may be necessary, similarly to an earlier study (Deventhal et al. 2019). This assures that the gene is knocked out completely, leaving no residual endogenous genes.

*4.3 Differences between transgenes*

Our locomotion data in the R1-6 photoreceptor cells reveal that the eye clock regulates morning anticipation (**Figure 3**). However, evening activity is noticeably affected as well, but only in flies expressing the kinase transgenes (*dbt*$^{K38R}$ and *Tik*). When expressing *tim*RNAi, eliminating the clock, evening activity does not appear to be affected. *dbt*$^{K38R}$ and *Tik* are variants of the DBT and CK2 kinases. These kinases are responsible for maintaining stability of the repressor proteins PER and TIM, as well as regulating their nuclear entry (CK2) and degradation (DBT), which are steps that occur at specific times of the ~24-h cycle of the circadian clock (Top et al. 2016, Lin et al. 2005, Allada & Meissner 2005, Meissner et al. 2008, Harms et al. 2004, Martinek et al. 2001, Harms et al. 2004, Cyran et al. 2005, Edery et al. 1994, Kloss et al. 1998, Kloss et al. 2001). Therefore, the

*dbt*$^{K38R}$ and *Tik* transgenes are modifying particular steps/ functions of the clock. This could explain the behavioural differences seen between the kinase variants and *tim*RNAi. The kinase variants are altering the function of the clock, and thus circadian transcription, which could be signaling through a neuronal pathway to affect evening activity. On the other hand, when the circadian clock is eliminated with *tim*RNAi, there is a possibility that this neuronal pathway could function to regulate evening activity even when signals from the eye clock are eliminated. In summary, different neuronal pathways may be activated depending on how the eye clock is affected. These observations may be correlated to the luminescence oscillations measured in distinct neuronal clusters when modifying the clock of the eye with the kinase variants (*dbt*$^{K38R}$ and *Tik*) (**Figure 4, 5**) and *tim*RNAi (**Figure 6**). Oscillations in the l-LNvs were only affected when eliminating the eye clock with *tim*RNAi (**Figure 6, purple**), however expression of the kinase variants in the eye did not affect the l-LNv oscillations (**Figure 5, 6, purple**). Therefore, expressing kinase variants to modify the eye clock does not affect the clock oscillations of the l-LNvs, however eliminating the eye clock is a strong enough modification to affect the l-LNv clock.

*4.4 Retinal clocks are necessary to trigger firing of retinal cells*

Expressing circadian clock mutants in the eye affected the oscillations in downstream neuronal clusters, the LNds and l-LNvs (**Figure 5, 6**). However, the clock oscillations in these affected neurons returned to a WT rhythm at days ~6-7. The reason behind this is not clear. However, we suspect that this observation can be explained by a loss of retinal function efficacy in constant darkness over time. Our locomotion results with Rh1-Gal4 demonstrate that when expressing *dbt*$^{K38R}$, *Tik* and *tim*RNAi in the eye (**Figure**

**3**), morning anticipation behaviour is lost. Similarly, when silencing neuronal firing of the photoreceptor cells with *Kir2.1*, morning anticipation is lost (**Figure 10, teal**). However, when killing the photoreceptor cells of the eye with mutant *hid* (**Figure 10, pink**), behaviour was surprisingly not affected. We believe this may be due to a "dominant negative" effect of the eye, in that the circadian clocks of the brain can function independently of the eye. In other words, when the eye clock is altered, it can send downstream signals to the l-LNvs and LNds to alter their clocks, but when the photoreceptor cells are ablated and send no signal, the l-LNvs and LNds can still function normally. Considering the eye clock and firing from the eye cells both contribute to the regulation morning anticipation behaviour (**Figure 3, 10**), we suggest that the circadian clocks of the photoreceptor cells of retina are necessary to trigger the firing of their own cells to send signals to the CNN. The clock oscillations in the l-LNvs and LNds returned to a WT rhythm at days ~6-7, therefore we hypothesize that the expression of circadian transgenes in the eye slowly weakens/decreases the firing of the eye cells and after a few days in DD, the firing completely diminishes. Further work will be necessary to investigate the effect of the eye clock on self-firing of the cell.

*4.5 Indirect connection between the eye and circadian neuronal clusters*

There are potential signaling pathways from the eye that communicate to the CNN (Bloomquist et al. 1988, Montell 2012, Hardie & Juusola 2015). However, there is no evidence of a direct synaptic connection between the retinal cells and the circadian neuronal clusters. Given that our results demonstrate a connection between the eye clock and the clock of the l-LNvs and LNds (**Figure 5, 6**), we believe this may be due to an

indirect connection, and there may be mediators that receive initial input from the eye to then relay the information to the CNN. A possible mediator is a region in the *Drosophila* brain called the accessary medulla (aMe). The aMe is a small neuropil adjacent to the frontomedial edge of the medulla (Helfrich-Förster 1997, Helfrich-Förster & Homberg 1993). Circadian neuronal clusters that respond to light can send their dendrites to the aMe, revealing a function of the aMe as a hub to channel light information to the neuronal clusters (Li et al. 2018). This is in agreement with other studies demonstrating a connection between the aMe and the circadian neuronal clusters (Helfrich-Förster. 2007, Shafer 2006, Yoshii et al. 2008), however there is a possibility of other unknown mediators involved in this communication pathway. We believe from our data and from earlier studies that the circadian clock of the eye is necessary to trigger the firing of the cell, which channels through a mediator (possibly the aMe) and communicates to the circadian clock of the l-LNvs and LNds to regulate morning anticipation.

*4.6 Connections between the eye and the l-LNvs*

Our results demonstrate a connection between the eye clock and the l-LNvs (**Figure 6, purple**), but not with the s-LNvs (**Figure 6, orange**). Our conclusions are in agreement with earlier studies demonstrating that input from the visual system is communicated through the l-LNvs to influence the circadian neuronal network (CNN) (Helfrich-Förster 2002, Malpel et al. 2002, Schlichting et al. 2014, Schlichthing et al. 2016, Muraro and Ceriani, 2015). There is evidence that the fly retina communicates to the CNN through the stimulation of $Ca^{2+}$ and cAMP in the LNvs. Addition of a cholinergic agonist triggers the spike in $Ca^{2+}$ and cAMP levels in the LNvs (Wegener et al. 2014, Lelito & Shafer 2012).

The compound eye has demonstrated a role in mediating l-LNv excitation through acetylcholine release (Murano & Ceriani 2015, Schlichting et al. 2015). Importantly, there is no evidence of an acetylcholine receptor expression in the s-LNvs. Taking together these studies, acetylcholine is a possible neurotransmitter involved in the transmission of information from the eye clock to the l-LNv clock. However, in our study, we aimed to understand the importance of the photoreceptor cells (R1-6, mainly) of the eye in communicating to the brain clocks. These photoreceptor cells are claimed to influence the CNN through the activation of rhodopsins (Hofbauer & Buchner 1989, Helfrich-Förster et al. 2001), which involves the release of histamine (Bloomquist et al. 1988, Montell 2012, Hardie & Juusola 2015). This makes histamine another potential candidate as a neurotransmitter that is communicated from the retinal cells to the l-LNvs, and further work will be necessary to discover the neurotransmitters involved.

*4.7 s-LNv clock function is independent from the eye*

We proposed a model when the LNvs receive input from the retinal cells and transmit this information to the LNd clock (**Figure 12A**). Our results demonstrated that when modifying the circadian clock of the eye, the LNd clock is affected, suggesting that the two clocks are linked (**Figure 5, 6, blue**). The l-LNv clock was also affected (**Figure 6, purple**), further supporting our model that the LNvs and LNds are part of the same network. However, when measuring oscillations of both LNvs, this revealed little to no effect when modifying the eye clock (**Figure 4-6, orange**). We believe this could be explained by the s-LNv clock functioning independently of the eye clock. This idea is supported by an earlier study demonstrating that oscillations in the s-LNvs can still be

entrained independently of the photoreceptors CRY and the retinal cells (Ogueta et al. 2018). When measuring PER expression levels in the s-LNvs in double mutants lacking retinal signaling pathways (*norpA*$^{P41}$ mutation) and CRY (*cry*$^{b}$), PER cycling remained normal. On the other hand, PER cycling in the l-LNvs was completely disrupted in the double mutant flies. These results are in agreement with our data that suggest that the l-LNv clock is more responsive to the eye comparatively to the s-LNvs. However, it is important to note that the earlier study eliminated the norpA signaling pathway in the eye, whereas in our study, we are unsure if this norpA signaling pathway is linked to the circadian clock of the eye. Further studies will be required to understand how instructions from the eye clock are communicating to the LNvs. It will be necessary to measure circadian clock oscillations in the LNvs (and other neurons) when silencing neuronal activity of the eye, to potentially observe differences comparatively to modifying the circadian clock of the eye. Additional results from our study reveal an independent role of the s-LNvs in regulating morning anticipation. Our locomotion data demonstrated that the circadian clock of the s-LNvs is crucial in regulating morning anticipation in LD conditions (**Figure 8A, maroon**). However, neuronal firing is not (**Figure 9A, maroon**). This suggests that only the s-LNv clock is important for morning anticipation in LD. Given that the s-LNv clock does not seem to be responsive to the eye clock (**Figure 4-6, orange**), the regulation of morning anticipation of the s-LNv could be independent of the eye clock's role in morning anticipation. These findings are supported by earlier studies revealing a role of the LNv clock in regulating morning anticipation in LD (Stoleru et al. 2004, Grima et al. 2004). Additionally, our conclusion that LNv neuronal firing is not important for morning anticipation behaviour is supported by other work (Wu et al. 2008), revealing that

the expression *Kir2.1* in the pdf+ neurons (LNvs) maintains rhythmic behaviour and morning anticipation remains intact.

*4.8 PDF signaling from LNvs to LNds*

After modifying the circadian clock of the LNvs, we measured clock oscillations in the LNds and found that the LNd clock was not responsive to the LNv clock (**Figure 7**). This suggests that the two clocks are not linked. However, this does not eliminate the possibility of other forms of connections between the neurons. In fact, it is claimed that the LNvs do interact with the LNds through PDF signaling. Both LNvs express and release PDF, and most of the circadian neuronal clusters contain a receptor for PDF (PDFR) (Im & Targhert 2009, Shafer et al. 2008). Mutants of PDF and/or the PDFR cause a reduction in the morning peak of activity, and a phase shift of the evening peak (Hyun et al. 2005, Lear et al. 2005). However, when expressing the PDFR only in the evening cells (LNds) of *pdfr-* flies, all deficiencies were rescued. Given that PDF signaling is crucial for maintaining rhythmic behaviour, we believe there is a connection between the LNvs and LNds through neuropeptide signaling, even though our results suggest that the LNv clock may not be involved in this pathway. Importantly, circadian clock activity of the LNvs can trigger the release of PDF (Park et al. 2000), but neuronal activity of the LNvs can as well (Schlichting et al. 2019). This means PDF could be released through LNv neuronal firing (bypassing its clock) to communicate to the LNd clock, which supports our proposed model (**Figure 12A**). It will be necessary to conduct experiments where we measure clock oscillations of the LNds after eliminating PDF, to identify the role of PDF in this pathway.

*4.9 LNds response to PDF regulates morning anticipation*

Earlier studies from our lab (**Appendix B**) and others (Stoleru et al. 2004, Grima et al. 2004) demonstrates a role of the LNds in regulating evening anticipation behaviour. We proposed a model where the LNvs and LNds are a part of the same network receiving input from the eye to regulate morning anticipation (**Figure 12A**), however our locomotion results reveal that the LNds do not play a role in morning anticipation (**Figure 8, 9, blue)** (**Appendix B**). We believe that, if the LNds are a part of this linear network, it is not LNd activity that regulates morning anticipation, but rather the LNd response to PDF signaling from the LNvs. There is evidence that suggests that LNd response to PDF facilitates morning anticipation. When restoring PDFR in a *pdfr* -/- background, only in the LNvs, morning anticipation remains lost, similar to a *pdfr*- or $pdf^{01}$ fly. However, when restoring PDFR in both LNv and LNd cells, morning anticipation returns to normal (Renn et al. 1999, Lear et al. 2009, Im & Taghert 2010). Additionally, expressing membrane-tethered PDF in the LNds is sufficient to restore morning anticipation, but when expressing in the LNvs, morning anticipation is not restored (Choi et al. 2012). Others have observed arrhythmic behaviour in DD after ablation of the LNds, similar to results observed when ablating the s-LNvs (Stoleru et al. 2004). These studies suggest that the LNvs regulate morning anticipation by communicating to the LNds through PDF release. This aligns with our proposed model of a linear network from eye input to morning anticipation behavioural output (**Figure 12A**).

*4.10 Possible role of HB eyelet in communicating to neuronal clocks*

Expressing $dbt^{K38R}$ and *Kir2.1* with Rh6-Gal4 decreased evening activity (**Figure 11, green**). We were mainly focused on the role of the retinal cells R1-8 in this study, however Rh6 is also expressed in the HB eyelet. The eye of *Drosophila* is made up of different structures: three ocelli, two compound eyes and two HB eyelets. Our work and previous studies have demonstrated an important role for the compound eye in communicating with the CNN and influencing behaviour (Helfrich-Förster et al. 2001, Saint-Charles et al. 2016, Rieger et al. 2003, Veleri et al. 2007). The HB eyelet, however, does demonstrate a role in circadian rhythms. The HB eyelet is proposed to be a circadian photoreceptive organ because they project to the fly's circadian pacemaker center: the aMe (Hofbauer & Buchner, 1989, Yasuyama & Meinertzhagen 1999). A number of studies support this hypothesis by revealing a potential synaptic connection between the HB eyelet and the PDF-expressing neurons, the LNvs (Malpel et al. 2002). By staining whole-mounted larvae brains, that the Bolwig nerve (the precursor for the HB eyelet) makes connections with the lateral neurons (LNs) in the embryonic fly brain. Additionally, a loss of a functional Bolwig nerve affected the dendritic arborization of the LNs. Later in the fly's development, visual fibers from the HB eyelet indeed showed contact with the LNvs. The terminals of the Bolwig's nerve differentiate in close proximity to the LNvs (Helfrich-Förster et al. 2002). Rh6 in the HB eyelet is known to be involved in light entrainment, forming close connections with the pdf+ neurons (LNvs) (Schlichting et al. 2016). When staining the HB eyelet with Rh6-GFP, it was observed that the axons of the HB eyelet directly innervate the aMe and overlap with the PDF arborizations. When eliminating both CRY and signaling from the photoreceptor cells (double mutant *cryb*; $norpA^{P41}$), this

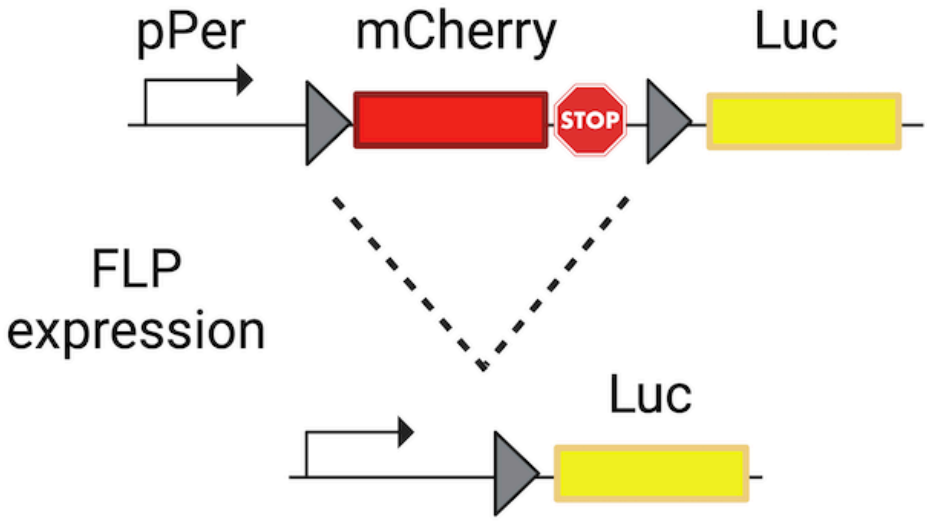
disrupts the fly's ability to resynchronize to different light regimes. However, the resynchronization was not completely abolished, suggesting that there are other inputs that help to communicate to the CNN. Expressing tetrodotoxin (*ttx*) (*ttx:* blocks synaptic transmission) in the HB eyelet of $norpA^{P41}$; *cryb* flies disrupts behaviour resynchronization even further (Veleri et al. 2007), demonstrating a role for the HB eyelet in behavioural synchronization. This supports our results that mutants being expressed with Rh6-Gal4 influence evening activity (**Figure 11, green**). An important feature that has been discovered in the HB eyelets is that they also use a *norpA* signaling pathway, communicating through acetylcholine and histamine release (Schlichting, et al. 2016). Both LNvs are receptive to acetylcholine (McCarthy et al. 2011, Lelito & Shafer 2012), and firing of the l-LNvs require cholinergic input from the visual system (Muraro & Ceriani 2015). Rh6-expressing photoreceptors also cause an increase of $Ca^{2+}$ and cAMP in the s-LNvs, and the l-LNvs respond to histamine (Schlichthing et al. 2016). These studies support the possibility of the HB eyelet playing a crucial role in communicating to the CNN.

*4.11 Circadian rhythms and mental disorders*

Our results reveal a potential communication network regulating morning anticipation behaviour. We believe this may be important for the understanding of the molecular mechanisms underlying mental disorders, as they show links with circadian rhythms. For example, jetlag and/or shiftwork can cause sleep problems and changes in behaviour (e.g., depression, mania, bipolar episodes, etc…) in humans (Cingi et al. 2018, Inder et al. 2015, Lee et al. 2017, Jauhar & Weller 1986, Katz et al. 2002). Neuropsychiatric

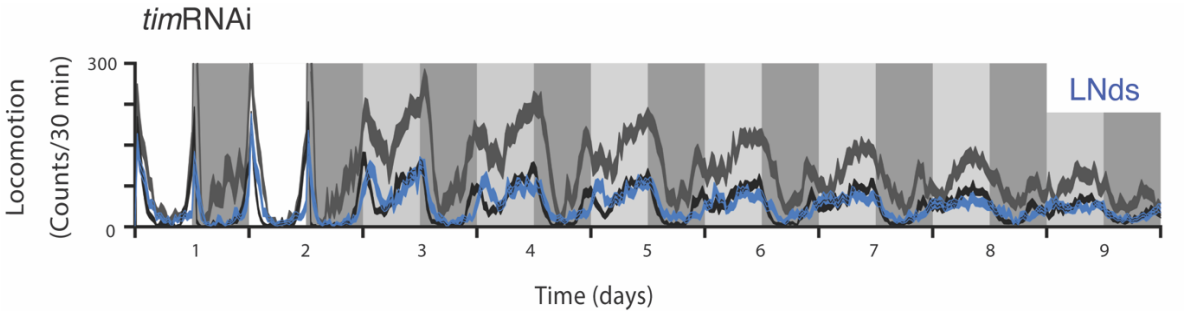
disorders such as schizophrenia (scz) and behavioural disorders like depression, bipolar disorder and seasonal affective disorder have also been linked to circadian rhythms (Logan & McClung 2019, Jones & Benca 2015, Walker et al. 2020). At the molecular level, associations between bipolar disorder and multiple genes of the circadian clock have been reported (Le-Niculescu et al. 2009, Bellivier et al. 2015). Studies related to schizophrenia have revealed that the rhythmic expression of mammalian circadian genes, such as *per2*, *clock* and *cry1*, was decreased or lost in scz patients (Johansson et al. 2016). However, treatments for such mental disorders remain limited. We believe that our inability to identify the specific genes that cause mental disorders is due to the differences in efficacy of mutations in the various regions of the brain, causing a loss of synchrony between them and ultimately, changes in behaviour. This is supported by studies demonstrating that circadian neuronal clusters respond differently to mutations (Johnstone et al. 2022, Top et al. 2016, Top & Young 2018). Our study suggests that the circadian clocks can use different neuronal pathways to communicate and regulate various aspects of behaviour, and investigation of these communication pathways could help to identify changes in genes when modifying the eye clock, which will help us to determine the regulatory pathways and neuronal functions that are responsive to the signals from the eye. We could then alter the expression of these genes in an attempt to reconstitute the behavioural changes we observe when manipulating the eye clock. We could additionally perform experiments where we rescue these genes after altering the eye clock in hopes of regaining wild-type behaviour. In conclusion, our work has opened to door to further understanding the link between circadian rhythms and behaviour, as well as the role of the circadian clocks in the brain and how their connections and collaborations can explain behavioural disorders.

# Appendix A




**Locally Activatable BioLuminescence (LABL).** Created with BioRender. LABL is a genetically encoded reporter that allows the measurement of transcriptional oscillations in target neurons in real time and *in vivo*. A *period* promoter is fused to *mCherry* and *luciferase* (*luc*) genes. *mCherry* is flanked by two FRT sites. When expressing of Flipase (FLP) in target neurons, *FRT*-flanked *mCherry* is excised, leaving luciferase under the regulation of the *period* promoter.

# Appendix B




**Locomotor activity of flies expressing *tim*RNAi in the LNds.** Activity rhythms of flies were assessed when expressing May179-Gal4; pdf-Gal80 > UAS-*tim*RNAi. White and dark grey backgrounds (days 1-2) represent day and night. Light grey and dark grey backgrounds (days 3-9) represent subjective day and subjective night, respectively. Grey curve represents control using only the Gal4 drivers (May179-Gal4; pdf-Gal80 > +). Black curve represents control using only the transgene (+ > UAS-*tim*RNAi). Locomotion counts are averaged into 30 min bins, and the mean of 25-32 flies presented, +/- SEM (thickness of curve). n=25-32.

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