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Balance of activity between LNvs and glutamatergic dorsal clock neurons promotes robust circadian rhythms in *Drosophila*

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Summary

Circadian rhythms offer an excellent opportunity to dissect the neural circuits underlying innate behavior because the genes and neurons involved are relatively well-understood. We first sought to understand how *Drosophila* clock neurons interact in the simple circuit that generates circadian rhythms in larval light avoidance. We used genetics to manipulate two groups of clock neurons, increasing or reducing excitability, stopping their molecular clocks and blocking neurotransmitter release and reception. Our results revealed that Lateral Neuron (LN_v) clock neurons promote and Dorsal Neurons (DN_1s) inhibit light avoidance, that these neurons probably signal at different times of day, and that both signals are required for rhythmic behavior. We found similar principles apply in the more complex adult circadian circuit that generates locomotor rhythms. Thus the changing balance in activity between clock neurons with opposing behavioral effects generates robust circadian behavior and likely helps organisms transition between discrete behavioral states such as sleep and wakefulness.

Introduction

A major goal of Neuroscience is to understand how the nervous system functions at multiple different levels (from genes to neural circuits) to generate behavior. Innate behaviors are particularly attractive to study since they are hardwired into the nervous system and are very similar between individual animals. The control of circadian (~24hr) rhythms offers an excellent opportunity to genetically dissect neural circuits since dedicated clock genes have been identified. This enabled the identification of pacemaker neurons where clock genes function to modulate multiple innate behaviors including sleep, courtship and drug sensitivity (reviewed by Allada and Chung, 2010).

Although recent studies have shown the importance of neuronal communication in synchronizing and strengthening molecular behavioral rhythms (Hogenesch and Herzog, 2011; Nitabach and Taghert, 2008), the nature of the signals between clock and neurons and their effects on neuronal activity are unclear. To address this, we utilized the “minimal”

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circadian network in *Drosophila* larvae, which has only 9 clock per brain that neurons lobe, with the idea general principles of circadian neural circuits in larvae would also apply to adult flies and perhaps even in mammals. *Drosophila* larvae show circadian rhythms light sensitivity, which is measured by assaying how well larvae avoid light on a half light-half dark agar plate (Mazzoni et al., 2005). This requires both the larval visual system (Bolwig's Organ) and clock neurons (Keene et al., 2011). Bolwig's Organ likely innervates the 5 larval Lateral neurons (LN_vs) (Keene et al., 2011; Klarsfeld et al., 2011), including the 4 LN_vs which express the neuropeptide Pigment Dispersing Factor (PDF). Consistent with direct innervation, light transmitted via Bolwig's Organ rapidly increases neuronal activity of the PDF-expressing LN_vs (Yuan et al., 2011).

We used the spatial precision of the Gal4/UAS system (Brand and Perrimon, 1993) to target specific groups of clock neurons. This approach is extremely powerful when combined with transgenes that increase or decrease neuronal excitability. The specific neurotransmitters and neuropeptides produced by different neurons can also be manipulated relatively easily, as can the receptors that mediate the responses of downstream neurons. Armed with these genetic tools, we set out to decode the logic and function of the network interactions between clock neurons.

We found that LN_vs and a group of dorsal larval clock neurons (DN_1s) have opposite behavioral effects: LN_vs promote whilst DN_1s inhibit larval light avoidance. We also found that the similarly phased molecular clocks in LN_vs and DN_1s have opposite relationships to neuronal activity: low CLK/CYC activity, which normally occurs at dawn, makes LN_vs highly excitable but decreases DN_1 signaling. Thus the cells which become adult Morning cells (Grima et al., 2004; Stoleru et al., 2004) are most excitable in the morning, while the DN_1s, which become the adult DN_{1a}s, a subset of adult Evening cells (Grima et al., 2004; Stoleru et al., 2004), seem most excitable in the evening. Our data also reveal that the morning peak of light avoidance requires that DN_1s signal minimally at dawn. DN_1s therefore seem to gate LN_v activity, which could be a general mechanism for the dual oscillator model underlying circadian rhythms (Pittendrigh and Daan, 1976). Finally we show that rhythmic light avoidance requires glutamatergic inhibitory inputs from the two larval DN_1s, received on LN_vs via GluCl, a glutamate-gated chloride channel that inhibits LN_v Our studies of the circuit activity.

Our studies of the circuit interactions between larval LN_vs and DN_1s lead to simple principles that hold true in adult flies: Signaling from non-LN_v clock neurons promotes circadian rhythms by inhibiting the outputs of the master LN_v pacemaker neurons. This presumably narrows the morning peak of locomotor activity and helps sharpen the behavioral transition from inactivity (sleep) to activity (wakefulness). These data add to the emerging concept that the precision and robustness of whole animal behavioral rhythms arise from network interactions between individual clock neurons and offer a cellular mechanism for how clock neurons are coupled.

Results

Pre- and post-synaptic DN_1 terminals are located close to LN_v axonal termini

Adult E cells are labeled by the *cry13-Gal4* driver in combination with a *Pdf-Gal80* transgene, and, along with LN_vs, are required to generate normal behavioral rhythms in 12h Light: 12h Dark (LD) cycles (Stoleru et al., 2004). We found that this driver combination only labeled the two larval DN_1s (Fig 1A and data not shown). Although expression of GFP was often difficult to detect simultaneously in both larval DN_1s (as in Fig 1A), expression of *UAS-Diphtheria toxin* (*UAS-Dti*) always ablated both larval DN_1s, while the PDF+ LN_vs, the 5^{th} PDF- LN_v and the 2 DN_2s were unaffected, as judged by clock protein staining (data

not shown). This is consistent with larval DN_1s becoming the adult DN_1a neurons, a subset of adult E cells (Grima et al., 2004; Stoleru et al., 2004).

GFP-labeled DN_1 projections terminate in the vicinity of the PDF+ LN_v axonal termini (Fig 1A). Since the GFP derivative used is a post-synaptic marker (*Dscam17.1-GFP*, (Wang et al., 2004), larval DN_1 projections could receive inputs in this region, including from LN_vs. To localize DN_1 pre-synaptic termini, we used *UAS-Synaptotagmin-HA* (*UAS-Syt-HA,* (Robinson et al., 2002) expressed via the stronger *cry16-Gal4* driver in combination with *Pdf-Gal80* since *cry13-Gal4* expression zof Syt-HA was undetectable. The two larval DN_1s marked by CD8-GFP expression project to the LN_v termini where Syt-HA is detectable in several foci, some of which are very close to LN_v axons (Fig 1B–C). Thus DN_1s could signal to LN_vs and receive their inputs. This is consistent with electron microscopy studies of adult s-LN_vs that revealed input synapses to s-LN_v projections in the dorsal protocerebrum, the location of adult DNs (Yasuyama and Meinertzhagen, 2010).

We also detected low levels of CD8-GFP and Syt-HA expression in LN_vs when expressed with the *cry16-Gal4; Pdf-Gal80* combination, presumably because *cry16-Gal4* is not completely repressed by *Pdf-Gal80*. Since *cry16-Gal4* also labels a few non-clock neurons in the brain (data not shown), we did not use *cry16-Gal4* in subsequent behavioral experiments.

LNs and DN_1s have opposite roles in light avoidance

Given the possibility that DN_1s signal to LN_vs, we first characterized the contributions of these different groups of clock neurons to light larvae raised in 12:12 LD cycles at 25°C. In this assay, 15 larvae are placed on a half-covered Petri dish and the number of larvae on the dark side is counted after 15min. At 750lux, ~70% of wild-type larvae are in the dark at the end of the assay and this requires the clock genes *period* (*per*) and *timeless* (*tim*) (Gong, 2009; Keene et al., 2011; Mazzoni et al., 2005). In the *Drosophila* clock, *per* and *tim* expression is activated by the Clock (CLK) and Cycle (CYC) transcription factors. PER and TIM proteins then feedback to inhibit CLK/CYC activity (reviewed by (Hardin, 2011)). Strikingly, *Clk* and *cyc* mutant larvae have the opposite light avoidance phenotype to *per* and *tim* mutants: At 150lux, wild-type larvae cannot distinguish between light and dark but *cyc* and *Clk* mutant larvae display robust levels of light avoidance at this lower light intensity. Thus clock genes strongly modulate light avoidance (Mazzoni et al., 2005). At these light intensities, light avoidance is mediated by the Rh5-expressing subset of Bolwig's Organ photoreceptors (Keene et al., 2011) and is independent of the larval body wall photoreceptors (Xiang et al., 2010).

To test the role of LN_vs and DN_1s in light avoidance, we tested larvae at 150lux since starting from a basal level of light avoidance allowed us to identify manipulations that induce light avoidance and bypass redundancies in the system (Keene et al., 2011). Larvae were taken during the light phase of an LD cycle between Zeitgeber Times 3–6 (ZT, where ZT0=lights on and ZT12=lights off). We used *Pdf-Gal4* (abbreviated as *Pdf>* hereafter) and *cry-Gal4; Pdf-Gal80* (*DN_1>*) to target expression to larval LN_vs and DN_1s respectively. We first tested the effect of ablating LN_vs or DN_1s or altering their electrical excitability.

We found that hyperpolarizing LN_vs through *dORKΔC* or ablation via *Dti* had no effect on light avoidance (Fig 2A) compared to *Pdf>dORKΔNC* control larvae, which express a non-conducting version of *dORKΔC* (Nitabach et al., 2002). However, LN_v expression of *NaChBac*, a bacterial voltage-gated Na^+ channel that increases adult LN_v excitability (Nitabach et al., 2006; Sheeba et al., 2008a) and larval LN_v responses to light (Yuan et al., 2011) increased light avoidance scores (Fig 2A). Since hyper-exciting LN_vs increases light avoidance, we conclude that LN_vs promote light avoidance.

Expression of these same transgenes in DN_1s yielded opposite results (Fig 2B). Compared with *DN1 > dORKΔNC* control larvae, light avoidance levels increased significantly when DN_1s were hyperpolarized with either *dORKΔC* or *mKir2.1* or ablated with *Dti*. Thus, LN_vs promote and DN_1s inhibit light avoidance, with the difference between their excitability presumably determining overall levels of light avoidance.

Low CLK/CYC activity in LN_vs or DN_1s increases light avoidance

Larvae would be unlikely to avoid light if LN_vs and DN_1s released their conflicting signals simultaneously. Therefore we hypothesized that LN_vs and DN_1s signal at different times of day. Since the molecular clocks in LN_vs and DN_1s are similarly phased, we speculated that relationship between their molecular clocks and excitability must differ in LN_vs and DN_1s.

To test this, we used transgenes that encode dominant negative forms of CLK (*UAS-Clk^{DN}*) or CYC (*UAS-cyc^{DN}*) that block CLK/CYC-activated transcription (Tanoue et al., 2004). We found that expressing *Clk^{DN}* or *cyc^{DN}* in LN_vs (Fig 3A) or DN_1s (Fig 3B) significantly increased light avoidance compared to control larvae.

Since *Pdf> Clk^{DN}* and *Pdf> cyc^{DN}* larvae had similar light avoidance phenotypes as hyperexciting LN_vs via *NaChBac*, we infer that low CLK/CYC activity *increases* LN_v excitability which in turn promotes light avoidance. Conversely, since expressing *Clk^{DN}* or *cyc^{DN}* in DN_1s has a similar light avoidance phenotype to hyperpolarizing DN_1s via *dORKΔC* or *Kir2.1*, we infer that low CLK/CYC activity *decreases* DN_1 excitability and consequently increases light avoidance by reducing DN_1-mediated inhibition.

To test this further, we asked whether the increased light avoidance caused by expression of *cyc^{DN}* in LN_vs or DN_1s could be reduced by altering neuronal electrical excitability. We found that co-expressing *dORKΔC* with *cyc^{DN}* in LN_vs (Fig 3A) or *NaChBac* with *cyc^{DN}* in DN_1s (Fig 3B) rendered larvae as insensitive to light at 150lux as wild-type larvae. However, co-expressing *NaChBac* with *cyc^{DN}* in LN_vs (Fig 3A) did not reverse the increased sensitivity caused by expressing *cyc^{DN}*. These results are consistent with low levels of CLK/CYC activity increasing LN_v excitability and thus light avoidance levels – and this is rescued by hyperpolarizing LN_vs. Conversely, low CLK/CYC activity seems to decrease DN_1 excitability, which also increases light avoidance – and this is rescued by hyperexciting DN_1s.

Since the phenotypes caused by *cyc^{DN}* can be rescued by altering the excitability of LN_vs and DN_1s, it seems unlikely that the behavioral phenotypes caused by *cyc^{DN}* arise from putative developmental defects caused by reduced CLK/CYC activity during development (Goda et al., 2011). Furthermore, we found that expressing *cyc^{DN}* in differentiated larval LN_vs for only the 24hr immediately prior to assaying behavior still increased light avoidance (Fig S1).

High CLK/CYC activity likely increases DN_1 and decreases LN_v excitability

The *per^{01}* mutation stops the clock with constitutively high levels of CLK/CYC activity, allowing us to test how high levels of CLK/CYC activity affect LN_v and DN*1* excitability. Since *per^{01}* larvae display low levels of light avoidance at 750lux (Mazzoni et al., 2005), we tested whether light avoidance in *per^{01}* mutants could be restored to wild-type levels by manipulating LN_v and DN_1 excitability. We found that hyperexciting LN_vs in a *per^{01}* background via *NaChBac* significantly increased levels of light avoidance, while hyperpolarizing LN_vs through *dORKΔC* expression had no effect (Fig 3C), suggesting that *per^{01}* LN_vs have reduced excitability. Conversely, *dORKΔC* expression in DN_1s of *per^{01}* mutants significantly increased light avoidance, whereas *NaChBac* expression had no effect (Fig 3C), suggesting that *per^{01}* DN_1s have increased excitability. From this, we conclude

that *per*^{01} mutants display low levels of light avoidance because high CLK/CYC activity in *per*^{01} mutants simultaneously reduces LN_v excitability and increases DN_1 excitability.

These experiments indicate that CLK/CYC activity levels have opposite effects on LN_v and DN_1 excitability, with LN_vs most excitable when CLK/CYC activity is low, and DN_1s most excitable when CLK/CYC activity is high. The normal daily rhythm in CLK/CYC activity would then make LN_vs and DN_1s most likely to signal around dawn and dusk respectively. These conclusions for larval LN_vs arrived at via genetic manipulations parallel electrophysiological recordings that reveal adult LN_vs to be most excitable around dawn (Cao and Nitabach, 2008; Sheeba et al., 2008b), and are consistent with the role of adult s-LN_vs in promoting morning locomotor activity (Grima et al., 2004; Stoleru et al., 2004). Although no recordings have been made from non-LN_v clock neurons, increased excitability at dusk in larval DN_1s is consistent with adult E cells promoting evening locomotor activity (Grima et al., 2004; Stoleru et al., 2004).

DN_1s are essential for circadian rhythms in light avoidance

Larvae become more sensitive to light after several hours in darkness and wild-type larvae display circadian oscillations in avoiding 150lux light. This rhythm peaks at dawn (CT24, CT: circadian time, time in constant darkness) and is lowest at dusk (CT12) (Mazzoni et al., 2005). Our data from larvae taken from LD cycles suggest a mechanism for generating circadian rhythms in light avoidance: When CLK/CYC activity is low, around dawn, LN_vs are most excitable and promote light avoidance with minimal inhibition by DN_1s. Conversely, when CLK/CYC activity is high, around dusk, reduced LN_v activity coupled with increased DN_1 inhibition results in low levels of light avoidance.

To test this model, we first asked whether DN_1s are required for rhythmic light avoidance. Larvae were entrained to at least 3 LD cycles before transfer to DD, with light avoidance assayed on days 2–3 in DD. Control (*UAS-Dti* / +) larvae displayed a rhythm in light avoidance at 150lux, with levels higher at subjective dawn than at subjective dusk (Fig 4A). However, no rhythm was detected in DN_1-ablated (*DN_1 > Dti*) larvae, with light avoidance levels constitutively high (Fig 4A). Since light avoidance levels were elevated when DN_1s were ablated, we tested these larvae at a lower light intensity (50lux), but were still unable to detect any rhythm in light avoidance (Fig 4A). Therefore, we conclude that DN_1s are necessary for circadian rhythms of light avoidance.

A functional LN_v or DN_1 clock is sufficient for light avoidance rhythms

To test whether a functional molecular clock in LN_vs or DN_1s is sufficient to generate circadian rhythms in light avoidance we used a *UAS-per* transgene (Yang and Sehgal, 2001) to restore *per* expression to either LN_vs or DN_1s in *per*^{01} mutant larvae (Fig 4B). We confirmed that these manipulations at least partly rescued molecular clock oscillations in the relevant cells (Fig S2). Control (*per*$^{+}$ *UAS-per*) larvae showed higher light avoidance scores at CT24 than CT12, while *per*^{01} mutant larvae carrying the *UAS-per* transgene but no Gal4 driver displayed low levels of light avoidance at both CT12 and CT24 with no significant rhythm. We found that restoring *per* expression to either LN_vs or DN_1s rescued rhythmic light avoidance (Fig 4B).

We propose that a rhythmic molecular clock in the DN_1s of *per*^{01}*; DN_1 > per* larvae drives rhythmic signals from DN_1s, that regulate LN_v neuronal activity. Since DN_1s seem to be most active at dusk, this would allow LN_vs to promote light avoidance at dawn even in the absence of their own functional clock. This result directly parallels observations from adult flies, where restoring *per* to only non-LN_v clock neurons in *per*^{01} mutant flies restored the morning peak of locomotor activity (Stoleru et al., 2004). Conversely, we propose that

larvae lacking *per* expression in DN_1s (*per*^{01}*; Pdf > per*, Fig 4B), remain rhythmic because high CLK/CYC activity in *per*^{01} DN_1s (Fig 3C) renders them excitable and able to release their essential signal, whilst the functional LN_v clock controls the timing of behavior. This contrasts with DN_1 ablation, which prevents rhythms (Fig 4A). Therefore the DN_1 signal is both necessary (ablated DN_1s, Fig 4A) and sufficient (*per*$^{+}$ DN_1s with *per* mutant LN_vs, Fig 4B) for light avoidance rhythms.

If CLK/CYC activity regulates DN_1 excitability (Fig 3), low CLK/CYC activity should block release of the essential DN_1 signal and be phenotypically similar to ablating DN_1s. To test this, we assayed the effect of stopping the DN_1 molecular clock with low CLK/CYC activity on light avoidance rhythms at 150lux (Fig 4C). We found that *DN*$_1$ > *Clk*^{DN} larvae lost light avoidance rhythms, with larvae constitutively sensitive to light at both 150lux and 50lux, similar to DN_1 ablation. It should be noted that the experiments in Fig 4B and 4C are complementary rather than identical since expression of *Clk*^{DN} or *cyc*^{DN} in a single neuronal group blocks the clock in those cells but leaves the other clock neurons wild-type, whereas restoration of *per* to a single neuronal group leaves the rest of the larva in a mutant *per*^{01} state. Overall, our LD and DD data suggest that the DN_1 molecular clock regulates DN_1 neuronal activity, with DN_1s least active when CLK/CYC activity is lowest at dawn.

Transient activation of DN_1s at dawn blocks light avoidance rhythms

Next we sought to directly test when DN_1s normally signal using a transgene that expresses the heat-activated cation channel, TrpA1 (Hamada et al., 2008) Since TrpA1 is activated at temperatures >25°C, it can be used to transiently activate neurons in which it is expressed (Pulver et al., 2009). We used TrpA1 to transiently stimulate DN_1s at CT12 and CT24 and measure the effect on light avoidance (Fig 4D). At 20°C, *DN*$_1$ > *TrpA1* larvae displayed normal light avoidance rhythms. However, activating DN_1s via TrpA1 at 26°C blocked the rhythm, with levels of light avoidance constitutively low at both CT12 and CT24. No reduction in light avoidance at CT24 was observed between 20°C and 26°C for either *UAS-TrpA1* / + or *DN*$_1$ / + control larvae (Fig S3).

Since TrpA1 activation of DN_1s did not affect light avoidance at CT12, we conclude that DN_1s are already active at CT12. However, as DN_1 activation reduces light avoidance at CT24, we conclude that DN_1s are usually inactive at CT24. These data are consistent with the model that DN_1s are much more active when CLK/CYC activity is high (CT12) than when CLK/CYC activity is low (CT24). Taking all these experiments together, we conclude that CLK/CYC activity regulates DN_1 neuronal activity, peaking at dusk.

Glutamate is the inhibitory neurotransmitter produced by larval DN_1s

One mechanism that could explain these data is that DN_1s regulate light avoidance by inhibiting LN_v neuronal activity. This is consistent with the inhibition of light avoidance at CT24 through TrpA1 activation of DN_1s (Fig 4D) and with possible axo-axonal synapses between the DN_1 projections and LN_v axonal termini (Fig 1). Without the ability to conduct paired recordings between LN_vs and DN_1s, we sought to identify the relevant signal released by DN_1s and its receptor on LN_vs.

Larval DN_1s produce the neuropeptide IPNamide (Shafer et al., 2006) and the vesicular glutamate transporter, suggesting that they are also glutamatergic (Hamasaka et al., 2007). Glutamate is a good candidate for the DN_1 signal since larval LN_v activity can be inhibited by directly applying glutamate to dissociated LN_vs (Dahdal et al., 2010; Hamasaka et al., 2007).

We used two independent methods to genetically alter glutamate signaling. First, we used RNAi to reduce expression of the vesicular glutamate transporter (*VGlut*), using the strong

tim-Gal4 driver. (All RNAi experiments co-expressed UAS-dicer-2 (dcr-2), to increase RNAi efficacy, but this is omitted from written genotypes for simplicity.) Although *tim-Gal4* is expressed in all clock neurons, DN_1s are the only larval clock neurons expressing *VGlut* (Hamasaka et al., 2007). We found that *tim > VGlut*^{RNAi} larvae displayed increased light avoidance in LD at 150lux (Fig 5A), as seen for hyperpolarizing or ablating DN_1s (Fig 2) and also lost circadian rhythms in light avoidance (Fig S4A).

Next, we followed the method of Featherstone et al (2002) who ectopically expressed *Glutamate decarboxylase 1* (*Gad1*) in glutamatergic neurons. Although *Gad1* is normally used by GABAergic neurons to synthesize GABA from glutamate, *Gad1* expression in a glutamatergic neuron phenocopies the effect of mutants defective in glutamate synthesis and reduces pre-synaptic glutamate levels (Featherstone et al., 2002). Since larval DN_1s are not GABAergic (Hamasaka et al., 2005) and do not normally produce Gad1 (data not shown), they are unlikely to express the vesicular GABA transporter and so should be unable to load the GABA produced by *Gad1* mis-expression into synaptic vesicles.

We found that *DN_1 > Gad1* larvae also showed increased levels of light avoidance in LD at 150lux (Fig 5B), again similar to DN_1 hyper-polarization or ablation. DN_1s in *DN_1 > Gad1* larvae still display normal TIM oscillations, indicating that *Gad1* mis-expression does not affect DN_1 viability or molecular clock function (Fig S4B). The identical phenotypes from these two independent manipulations of glutamatergic signaling lead us to conclude that glutamate is the inhibitory signal released by DN_1s to modulate light avoidance.

DN_1 glutamate regulates LN_v activity via GluCl

Hamasaka et al. (2007) proposed that glutamate inhibits LN_v activity via the metabotropic mGluRA glutamate receptor. They also showed that light avoidance levels are increased in *mGluRA* mutant larvae, although they did not determine the relevant cells (Hamasaka et al., 2007). However, our gene expression profiles from purified larval LN_vs revealed that they also express the glutamate-gated chloride channel *GluCl* ~2.5-fold more highly than in Elav + neurons (M. Ruben & JB, data not shown). Adult l-LN_vs also have functional GluCl channels, although its behavioral role is unknown (McCarthy et al., 2011).

To test whether glutamate regulates light avoidance in LN_vs via GluCl or mGluRA, we used RNAi to reduce expression of each receptor. Both transgenes reduce expression of their target (Hamasaka et al., 2007 and Fig S4C). We found that *Pdf > GluCl*^{RNAi} larvae had significantly increased light avoidance at 150lux, whereas *Pdf > mGluRA*^{RNAi} and control larvae did not avoid light (Fig 5C). Thus reducing GluCl in LN_vs phenocopies reducing glutamate release from DN_1s.

Next we tested the roles of *GluCl* and *mGluRA* in regulating circadian behavior. Our data show that *Pdf > GluCl*^{RNAi} larvae had no light avoidance rhythm, with levels of light avoidance constitutively high (Fig 5D), whilst *Pdf > mGluRA*^{RNAi} larvae display rhythmic light avoidance (Fig 5D). Thus *GluCl* is required in LN_vs for rhythmic light avoidance. We propose that DN_1s rhythmically release glutamate, which is perceived via GluCl in LN_vs to mediate rhythmic inhibition of LN_v neuronal activity. We have subsequently found that *mGluRA* helps synchronize LN_v molecular oscillations (BC and JB in prep).

To directly test if GluCl can inhibit LN_v activity, we measured the responses of dissociated larval LN_vs expressing the intracellular Ca^{2+} sensor GCaMP1.6 (Reiff et al., 2005) to directly applied neurotransmitters. ACh produced by Bolwig's Organ is required for larval light avoidance (Keene et al., 2011). Applying ACh to dissociated LN_vs increased intracellular Ca^{2+} levels, as previously reported (Dahdal et al., 2010; Wegener et al., 2004), measured by increased GCaMP fluorescence (Fig 5E–F). ACh increases intracellular Ca^{2+}

in LN_vs by activating nicotinic ACh receptors to produce excitatory post-synaptic potentials, eventually causing depolarization. In turn, this increases cytoplasmic Ca^{2+} via voltage-gated Ca^{2+} channels (Dahdal et al., 2010; Wegener et al., 2004), which is observed as increased GCaMP fluorescence. Given the relative insensitivity of GCaMP1.6 to single action potentials (Pologruto et al., 2004), these Ca^{2+} transients in LN_vs likely reflect bursts of action potentials.

Co-applying 100μM glutamate completely blocked ACh-induced Ca^{2+} transients (see Fig 5E for a representative recording). We were unable obtain a narrowly defined IC_{50} value for glutamate, perhaps due to cell-to-cell variation in glutamate receptor content induced by dissociation. However, full inhibition of the response to 10μM ACh was produced with 10μM glutamate (n=6). To test if GluCl contributes to the inhibitory effects of glutamate on LN_vs, we repeated these experiments in a low chloride buffer (Fig 5E). This reduced glutamate inhibition of LN_v responses to ACh by 75% ± 13 (n=12 neurons). Therefore LN_vs require extracellular Cl- for the majority of glutamate-induced inhibition. We also found that applying 500nM ivermectin, an irreversible GluCl activator (Cully et al., 1994), blocked the response of LN_vs to ACh in the absence of glutamate (Fig 5F, n=4 neurons). These in vitro data parallel our in vivo data and support the idea that ACh released from the visual system can only fully activate LN_vs in the absence of DN_1 glutamatergic signals mediated via GluCl in LN_vs.

Taking all the larval data in Figures 1 to 5 together, we propose the following model for rhythmic light avoidance (Fig S5). Around dawn, low CLK/CYC activity increases LN_v excitability and reduces DN_1 activity. With DN_1s releasing minimal glutamate, the LN_vs respond strongly to ACh from the visual system and promote the dawn peak in light avoidance. Around dusk, high CLK/CYC activity reduces LN_v excitability but increases DN_1 activity, causing glutamate release and inhibition of the response of the LN_vs to ACh via GluCl, reducing light avoidance. Thus we propose a mechanism for the Morning and Evening dual oscillator model (Grima et al., 2004; Pittendrigh and Daan, 1976; Stoleru et al., 2004): Neuronal excitability peaks in antiphase between excitatory LN_vs and inhibitory DN_1s to generate robust behavioral rhythms.

Signals from non-LN_vs are required for robust adult behavioral rhythms

Although adult clock neurons are more numerous and control more behaviors than their larval counterparts, we sought to test if the principles we identified in larvae also operate in adult flies, focusing on locomotor activity rhythms in DD. Previous studies suggested that the neurons targeted by *cry13-Gal4; Pdf-Gal80* are dispensable for adult DD rhythms since their ablation leaves flies rhythmic, possibly because sufficient CRY- non-LN_vs remain to support rhythms (Stoleru et al., 2004). Therefore we used the *tim-Gal4*; *Pdf-Gal80* combination to target strong transgene expression to all clock neurons except LN_vs i.e. the dorsal Lateral Neurons (LN_ds) and the three groups of Dorsal Neurons. We also used the *tim-Gal4;cry-Gal80* combination to target the non-CRY expressing subset of adult clock neurons (DN_2s and subsets of LN_ds, DN_1s and DN_3s). *tim-Gal4; Pdf-Gal80* and *tim-Gal4; cry-Gal80* drivers both display robust rhythms when crossed to the *dORKΔNC* control transgene (Table 1; power > 500; see Experimental Procedures for a description of power).

To test the requirement for adult non-LN_v clock neuron signals in circadian behavior, we first reduced neuronal excitability using the *dORKΔC* transgene. We found that *tim-Gal4; Pdf-Gal80 > dORKΔC* flies have as low power rhythms in DD as *Pdf > dORKΔC* flies, whereas *tim-Gal4; cry-Gal80 > dORKΔC* flies display robust rhythms (Fig 6A–B and Table 1). Thus strong adult locomotor rhythms require signals from the CRY-expressing non-LN_v clock neurons. These include the DN_{1a}s which are descended from the larval DN_1s (Klarsfeld et al., 2004; Shafer et al., 2006).

Adult non-LN_vs can inhibit morning activity

TrpA1 activation of larval DN_1s at CT24 inhibited the morning peak of light avoidance (Fig 4D), suggesting that LN_vs can only promote light avoidance in the absence of DN_1 activity. Since the adult morning activity peak lasts for several hours, an equivalent experiment would require a prolonged temperature increase, which could complicate data interpretation because temperature is a potent zeitgeber (Glaser and Stanewsky, 2007). Instead, we analyzed the behavior of flies with hyper-excited non-LN_vs. We noticed that although *tim-Gal4;Pdf-Gal80 > NaChBac* flies had robust rhythms, their activity becomes unimodal after several days in DD and morning activity is lost (Fig 6C–E, Table 1). We infer that NaChBac increases non-LN_v excitability so that they now signal at the wrong time of day and block the morning peak of locomotor activity, normally promoted by LN_vs. Thus cessation of inhibitory signaling by non-LN_vs around dawn may be as important as excitatory signaling by LN_vs in generating the morning activity peak and non-LN_vs seem to gate LN_v activity in both larvae and adult flies.

As with dORKΔ expression, this phenomenon requires the CRY-expressing non-LN_v clock neurons since *tim-Gal4; cry-Gal80 > NaChBac* flies had reduced strength rhythms (Fig 6C, D, Table 1). Since this transgene combination targets a smaller subset of the non-LN_V clock neurons than *tim-Gal4;Pdf-Gal80*, these data suggest that the CRY- clock neurons do not contribute to the specific inhibition of morning activity in *tim-Gal4; Pdf-Gal80 > NaChBac* flies.

Overall, our broad manipulations to non-LN_v clock neurons indicate that, as in larvae, non-LN_v signals are required for robust circadian behavior (Fig 6A–B) and probably gate LN_v activity to refine the dawn peak of activity (Fig 6C–E).

Non-LN_v glutamate signals are required for robust locomotor activity rhythms

Finally we tested whether glutamate released from adult non-LN_v clock neurons is required for circadian behavior. Reducing *VGlut* expression in all clock neurons (*tim > $VGlut^{RNAi}$*) significantly reduced the strength of locomotor activity rhythms compared to controls (Fig 7A–C, Table 1). A second insertion of the same transgene and an independent $VGlut^{RNAi}$ transgene gave similar reductions in rhythm strength (Table 1). This phenotype is likely due to glutamate released from non-LN_v clock neurons because *VGlut* is only expressed in subsets of DN_1 and DN_3 neurons in the adult clock network (Hamasaka et al., 2007), and the strength of rhythms in *Pdf > $VGlut^{RNAi}$* flies was not reduced (Table 1).

To independently test a role for glutamate in the generation of adult rhythms in DD, we mis-expressed *Gad1*, as in larvae (Fig 5), to reduce pre-synaptic glutamate. This specifically affects glutamate levels since no adult clock neurons are GABAergic (Dahdal et al., 2010; Hamasaka et al., 2005). *tim-Gal4; Pdf-Gal80 > Gad1* flies had lower power rhythms than control flies whereas *tim-Gal4; cry-Gal80 > Gad1* flies had robust DD rhythms (Fig 7D–F and Table 1). Thus two independent manipulations of glutamate signaling indicate that glutamate released from CRY+ non-LN_v clock neurons is required for robust locomotor activity rhythms. However, the rhythms of *tim > + $VGlut^{RNAi}$* and *tim-Gal4; Pdf-Gal80 > Gad1* flies are both stronger than *tim-Gal4; Pdf-Gal80 > dORKΔC* flies, suggesting that additional signals from non-LN_vs contribute to rhythmic behavior. This interpretation makes sense given the diversity of *Drosophila* adult clock neurons and the incomplete arrhythmicity of even mutants in *Pdf*, the major circadian neuropeptide (Renn et al., 1999).

Taking all of the adult data together, we find evidence that the principles we identified in the larval circadian network may also operate in adult flies. Specifically our broad manipulations to adult non-LN_v clock neurons indicate that non-LN_v signals: (i) are

important for strong adult rhythms; (ii) may gate LN_v outputs to shape activity at dawn; and (iii) include glutamate.

Discussion

We identified some of the network logic that helps generate a simple rhythmic behavior through precise genetic manipulations of the larval circadian circuit and extended these findings to the more complex adult circadian network. Previous studies have shown that intercellular signaling in clock neuron networks promotes molecular clock synchrony (Lin et al., 2004; Maywood et al., 2006; Stoleru et al., 2005) and can strengthen genetically weak molecular clocks (Liu et al., 2007). Our study increases the importance of circadian neural networks by finding that non-LN_v clock neurons are as important as the “master' pacemaker LN_v clock neurons for rhythmic behavior in both larvae and adult flies. However, LN_vs can still be considered pacemakers in DD since most manipulations to non-LN_v clock neurons do not affect period length.

Non-LN_v signals appear to gate pacemaker neuron activity. Why is this necessary when LN_vs have their own intrinsic excitability rhythms? We propose that the interaction of two oscillators with opposite signs helps reduce the time when LN_vs signal. Without signaling from non-LN_vs, adult locomotor activity rhythms are weak and activity is distributed throughout the day and night as in *tim-Gal4; Pdf-Gal80 > dORKΔC* flies. In contrast, in *tim-Gal4; Pdf-Gal80 > NaChBac* flies, the timing of locomotor activity is narrowed. Thus the gating of LN_v activity by non-LN_vs may help turn gradual changes in the excitability of each neuronal group into thresholds that promote a switch in overall output and allow flies to abruptly transition from inactivity to activity.

This gating system can only function if LN_vs and non-LN_vs have differently phased neuronal activity. However, most *Drosophila* clock neurons have similarly phased molecular clocks. We propose that molecular clocks in different clock neurons regulate divergent sets of output genes to generate distinct phases of neuronal excitability. This would be analogous to the mammalian circadian system where molecular clocks in different tissues drive tissue-specific outputs (e.g. Storch et al., 2002). In summary, our genetic dissection of a circadian neural circuit reveals an unexpected and essential role for inhibitory signals from non-LN_vs (E cells) in shaping activity profiles at dawn and a novel mechanism for how clock neurons couple together to promote robust rhythms.

Experimental procedures

Fly stocks

For a complete list of fly stocks used in this paper see Supplemental experimental procedures.

Larval light avoidance

For LD experiments, larvae were entrained to 5 days of 12:12 LD cycles at 25°C and tested on the 6th day as 3rd instar larvae. For DD experiments, larvae were entrained to 12:12 LD at 25°C for 3–4 days and tested on the second or third days in DD. Larvae were removed from LD or DD immediately prior to testing. ~15 larvae were placed in the middle of an 8.5 cm diameter agar-filled Petri dish and the number of larvae in the light and dark was recorded after 15 min as in Mazzoni et al (2005) with the following minor modifications: (i) To speed up scoring, any larvae visible through the lid of the plate were recorded as being on the light side even if crossing the midline; (ii) As larvae could be found on the walls lid on both the light and dark sides of the plate, they were included in the scoring; (iii) Light intensity was reduced by moving the light source away from the plate rather than adding filters; and (iv)

The light source used was a circular fluorescent 22W GE Cool White bulb. Data are plotted as `% larvae in the dark'. Each data point is the average of 3 or more experiments, with each experiment consisting of ~45 larvae on 3 plates assayed simultaneously, except when insufficient larvae of the required genotype were obtained from individual crosses. In this case, data from separate experiments were added in chronological order to reach a total of ~45 larvae. All experiments on larvae in LD were carried out between ZT3-6, and in DD between CT11.5 and CT13 ("CT12") and CT23.5 and CT1 ("CT24"). For TrpA1 experiments, larvae were entrained to LD cycles at 20°C for 7 days then moved to DD and tested on the second day in DD. Larvae were at 26°C for only the duration of the assay. Statistical comparisons were made using Student's t-test (for pairwise comparisons), ANOVA with Tukey's Post-hoc tests (for multiple comparisons within a single data set), or Two-way ANOVA (for comparisons between genotypes across multiple time points), as stated in the figure legends.

Dissociated larval LN_v recordings

Recordings from dissociated LN_vs Expressing *GCaMP1.6* were carried out as in Dahdal et al (2010). Briefly, 30–60 larval brains were dissociated by treatment with 2 Units/mL dispase II and manual trituration. GCaMP fluorescence from individual neurons was imaged on an inverted epifluorescence microscope (TE2000U, Nikon) via a standard GFP filter set. Cells were continuously superfused at 2 ml/min with standard saline (in mM: NaCl 128, KCl 2, $MgCl_2$ 4, $CaCl_2$ 1.8, sucrose 36, HEPES 5, pH 7.1) to which compounds were added as indicated. For low chloride experiments, standard saline was modified to reduce Cl- to 13.6 mM by replacement of NaCl with sodium gluconate.

Adult locomotor activity

For locomotor activity experiments, adults were entrained to 12:12 LD cycles at 25°C for at least 3 days before transfer to DD. Locomotor activity was recorded using the DAM system (TriKinetics, Waltham, MA). We used χ^2 analysis in ClockLab (Actimetrics, Wilmette, IL) to derive a power and significance for each rhythm over 10 days in DD. We subtracted the significance score from the power to calculate the strength of each rhythm (presented as `power' in the text). Using this analysis, control lines have average powers ranging from ~270–580 ("Rhythmic", see Table 1) while classical clock mutants (per^{01}, Clk^{Jrk}, Clk^{ar}) have powers from 10–40 ("Arrhythmic"). *Pdf > dORKΔC* flies, previously described as ~70% arrhythmic / 30% weakly rhythmic (Nitabach et al., 2002; Wu et al., 2008), have an average power of 91, establishing a baseline for the effect of manipulations of electrical excitability. All statistical comparisons were made by ANOVA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Findings LN_V and DN_1 clock neurons have opposite effects on light avoidance

LN_V and DN_1s likely signal at opposite times of day despite similarly-phased clocks

Larval rhythms need inhibitory glutamatergic DN_1 signals that gate LN_V activity

Non-LN_V signals also required for circadian locomotor activity rhythms in adults

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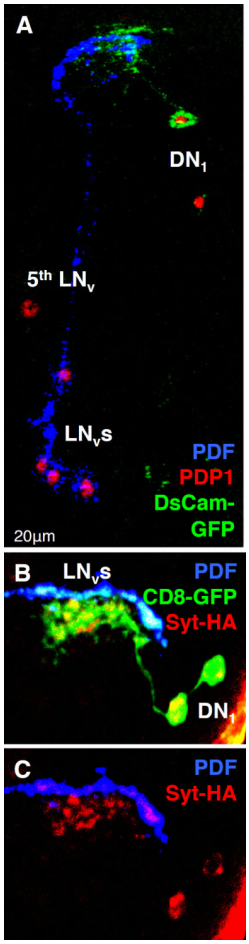

Figure 1. Pre- and post-synaptic DN_1 terminals are located close to LN_v axonal termini

(A) The nuclei of larval clock neurons were marked with the circadian transcription factor Par Domain Protein 1 (PDP1, red). LN_vs were co-labeled with PDF (blue). *cry13-Gal4; Pdf-Gal80* driven expression of the *Dscam17.1-GFP* post-synaptic marker (green) labels DN_1 projections very close to LN_v axons. The 5^{th} LN_v was identified by lack of PDF and GFP staining and its location.

(B) *cry16-Gal4; Pdf-Gal80* driven expression of *UAS-CD8-GFP* (green) and the pre-synaptic marker *UAS-Syt-HA* (anti-HA, red) co-localize to DN_1 projections adjacent to LN_v axons (labeled with PDF, blue).

(C) Same image as (B) with GFP channel removed to show Syt expression in DN_1 projections adjacent to LN_v axons. This image shows a 20μm stack but single 4μm sections also show DN_1 projections adjacent to LN_v axons.

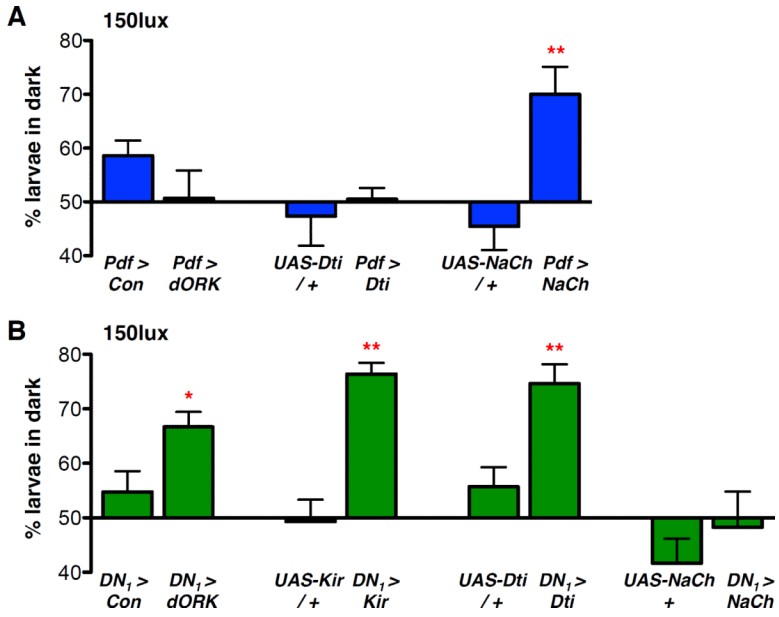

Figure 2. LN_vs promote and DN_1s inhibit larval light avoidance

Larval light avoidance was measured by counting the number of larvae on the dark sides of a Petri dish after 15min. Transgenes were targeted to either LN_vs using *Pdf-Gal4* (*Pdf* >), or DN_1s using *cry-Gal4; Pdf-Gal80* (DN_1 >). Control lines are either the *Gal4* line crossed to the non-conducting *UAS-dORKΔNC* transgene (Con) or the relevant *UAS-transgene* crossed to *y w* (*transgene* / +). Error bars show SEM. All statistical comparisons to the relevant control line were made using the students t-test. *p<0.05, **p<0.01.

(A) Light avoidance was assayed between ZT 3–6 at 150lux. Hyperpolarizing LN_vs with *UAS-dORKΔC* (*Pdf* > *dORK*) or ablating LN_vs with *UAS-Dti* (*Pdf* > *Dti*) had no significant effect on light avoidance vs control larvae. Hyperexciting LN_vs via *NaChBac* (*Pdf* > NaCh, p<0.005) increased larval light avoidance.

(B) Light avoidance was assayed as in (A). Hyperpolarizing DN_1s with *UAS-dORKΔC* or *UAS-mKir2.1* (*DN_1* > *dORK*, p<0.05 and *DN_1* > *Kir*, p<0.005), or DN_1-ablation (*DN_1* > *Dti*, p<0.01) significantly increased larval light avoidance. Hyperexciting DN_1s (*DN_1* > *NaCh*) had no significant effect on light avoidance.

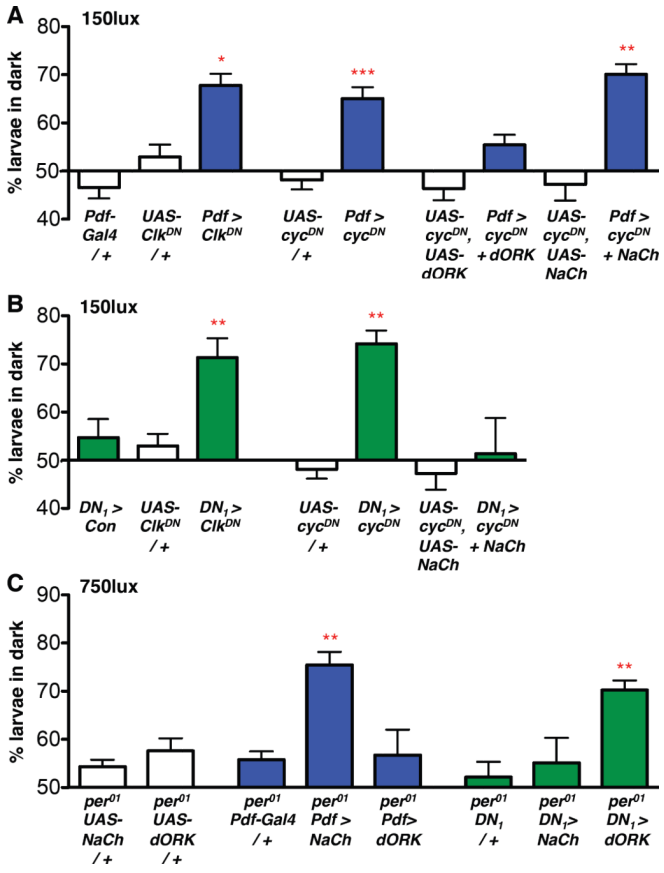

Figure 3. Altering CLK/CYC activity has opposite effects on LN_v and DN_1 excitability

All Statistical comparisons were made by ANOVA with Tukey's post-hoc test. *p<0.05, **p<0.01, ***p<0.001

(A) Light avoidance was assayed between ZT 3–6 in LD at 150lux. Expressing *Clk^{DN}* (p<0.05) or *cyc^{DN}* (p<0.001) in LN_vs increased larval light avoidance at 150lux compared to controls (*Pdf-Gal4* or *UAS-transgene* crossed to *y w*). Hyperpolarization of LN_vs expressing *cyc^{DN}* (*Pdf> cyc^{DN} + dORK*) restored light avoidance to wild-type levels while hyperexcitation of LN_vs expressing *cyc^{DN}* (*Pdf> cyc^{DN} + NaCh*) did not. See also Fig S1.

(B) Light avoidance was assayed as in (A). Expressing *Clk^{DN}* (p<0.01) and *cyc^{DN}* (p<0.01) in DN_1s increased larval light avoidance at 150lux compared to controls (*DN_1 > Con*, reproduced from Fig 2B, or *UAS-transgenes* crossed to *y w*, reproduced from Fig 3A). Hyperexcitation of DN_1s expressing *cyc^{DN}* (*DN_1 > cyc^{DN} + NaCh*) restored light avoidance to wild-type levels.

(C) Light avoidance was assayed between ZT 3–6 in LD at 750lux. Hyperexciting LN_vs (*per^{01}*; *Pdf> NaCh*, p<0.01) rescued the low levels of light avoidance of *per^{01}* larvae whilst hyperpolarizing LN_vs did not (*per^{01}*; *Pdf> dORK*). Hyperpolarizing DN_1s (*per^{01}*; *DN_1 > dORK*, p<0.01) also increased light avoidance whilst hyperexciting DN_1s (*per^{01}*; *DN_1 > NaCh*) did not.

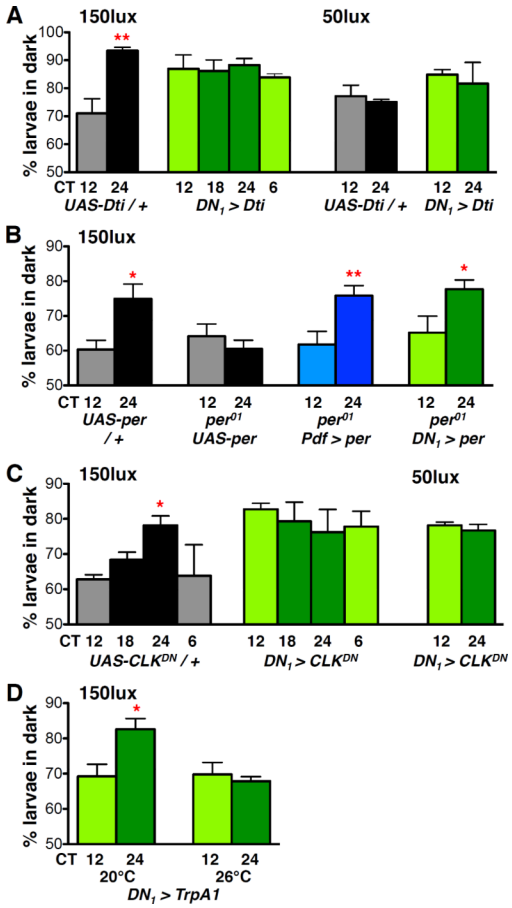

Figure 4. A signal from DN_1s is necessary and sufficient for light avoidance rhythms

All Statistical comparisons are as specified below. *p<0.05, **p<0.01, (A) Light avoidance was assayed on day 2 (CT12, 18, 24) or day 3 (CT6) of DD after prior LD entrainment. Control *UAS-Dti* / + larvae (grey) show time-dependent light avoidance at 150lux (CT12 vs CT24, t-test p<0.01). DN_1-ablated larvae (green) show no time-dependent light avoidance, (ANOVA p=0.79). 2-Way ANOVA between control and DN_1-ablated larvae for CT12 and 24 reveals a significant Genotype × Time interaction ($F_{1,11}$=8.53, p<0.05). No time-dependent differences in light avoidance were observed in control or DN_1-ablated larvae at 50lux (t-test).

(B) Light avoidance was assayed as in (A) at 150lux. All statistical comparisons by students t-test. Light avoidance scores were higher at CT24 than CT12 in control (*per^+ UAS-per*, p<0.05) but not in *per^{01}UAS-per* larvae. Rhythms were rescued by restoring per expression to LN_vs (blue, p<0.005) or DN_1s (green, p<0.05) in *per^{01}* mutants. See also Fig S2.

(C) Light avoidance was assayed as in (A). Light avoidance scores were lower at CT12 than at CT24 in control *UAS-CLK^{DN}* / + larvae at 150lux (t-test p<0.001). *DN_1 > CLK^{DN}* increased light avoidance compared to controls (2 Way ANOVA $F_{1,31}$=5.81, p<0.05), with no time-dependent differences in light avoidance observed at either 150lux (ANOVA) or 50lux (t-test).

(D) Light avoidance was assayed on day 2 in DD at 150lux using larvae reared at 20°C. Light avoidance scores were lower at CT12 than at CT24 in *DN_1 > TrpA1* larvae when assayed at 20°C (t-test p<0.01) but not 26°C. At 26°C, temperature-induced activation of DN_1s via TrpA1 reduces light avoidance at CT24 to CT12 levels (2 Way ANOVA, Temperature × Time interaction $F_{1,12}$=5.73, p<0.05). See also Fig S3

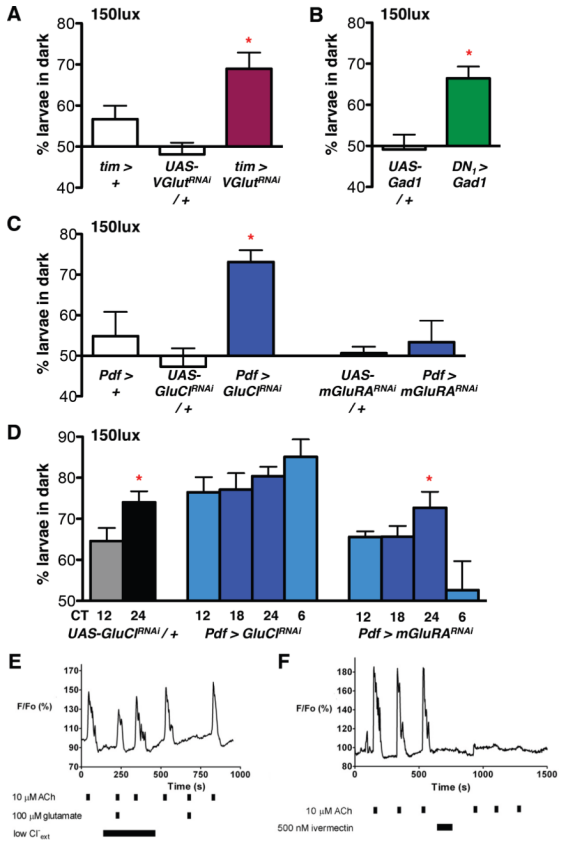

Figure 5. DN$_1$s release glutamate to inhibit light avoidance

For all RNAi experiments *UAS-dcr-2* was co-expressed to improve efficacy. The Gal4 control lines shown also express *UAS-dcr-2*. Statistical comparisons are as stated below. *p<0.05

(A–C) Larval light avoidance was measured as in Fig 2.

(A) Expression of a *VGlut-RNAi* transgene (GD2574) in all clock neurons (*tim* > *VGlut^{RNAi}* increased light avoidance at 150lux compared to control larvae. These data are significantly different (ANOVA p<0.005). Tukey's post-hoc comparison gives a significant difference only between *tim* > *VGlut^{RNAi}* and *UAS-VGlut^{RNAi}* / +. However, light avoidance in *tim* > *VGlut^{RNAi}* is higher than *tim* > + by t-test (p<0.05) and *tim* > *VGlut^{RNAi}* larvae also lose circadian rhythms in light avoidance (Fig S4A).

(B) Expression of *Glutamate decarboxylase* (*UAS-Gad1*) in DN$_1$s (*DN$_1$* > *Gad1*) significantly increased light avoidance at 150lux compared to *UAS-Gad1* / + control larvae (*Gad1* / +, t-test p<0.05). See also Fig S4B.

(C) A *GluCl-RNAi* transgene expressed in LN$_v$s (*Pdf* > *GluCl^{RNAi}*) significantly increased light avoidance at 150lux compared to control larvae (ANOVA p<0.05). An *mGluRA-RNAi* transgene expressed in LN$_v$s (*Pdf* > *mGluRA^{RNAi}*) had no effect on light avoidance compared to controls (ANOVA). See also Fig S4C.

(D) Light avoidance was assayed in DD at 150lux as in Fig 4. Light avoidance is higher at CT24 than CT12 in control larvae (*UAS-GluCl^{RNAi}* / +, which also contain a *UAS-dcr-2* transgene, t-test p<0.05). No rhythms in light avoidance were detectable when *GluCl-RNAi* was expressed in LN$_v$s (*Pdf* > *GluCl^{RNAi}*, ANOVA). Rhythmic light avoidance was still detectable in larvae expressing *mGluRA-RNAi* in LN$_v$s (*Pdf* > *mGluRA^{RNAi}*, ANOVA p<0.05). By 2 Way ANOVA comparison of CT12 and 24 time points, *Pdf* > *GluCl^{RNAi}* is

different to control ($F_{1,22}$=9.17, p<0.01) whilst *Pdf> mGluRA^{RNAi}* is not ($F_{1,24}$=0.00, p=0.9547)

(E) Glutamate-mediated inhibition of ACh-stimulated Ca^{2+} transients in dissociated larval LN_vs. Representative relative fluorescence (F/Fo) recordings are shown from dissociated larval LN_vs expressing *UAS-GCaMP1.6*. Solution changes, including neurotransmitter applications, are indicated by black bars. Lowering extracellular Cl^- to 13.6mM completely relieved glutamate-dependent inhibition. Glutamate completely blocked ACh-stimulated transients when physiological Cl^- was restored.

(F) A 2min incubation of a larval LN_v with 500nM ivermectin irreversibly blocks subsequent ACh-induced Ca^{2+} transients.

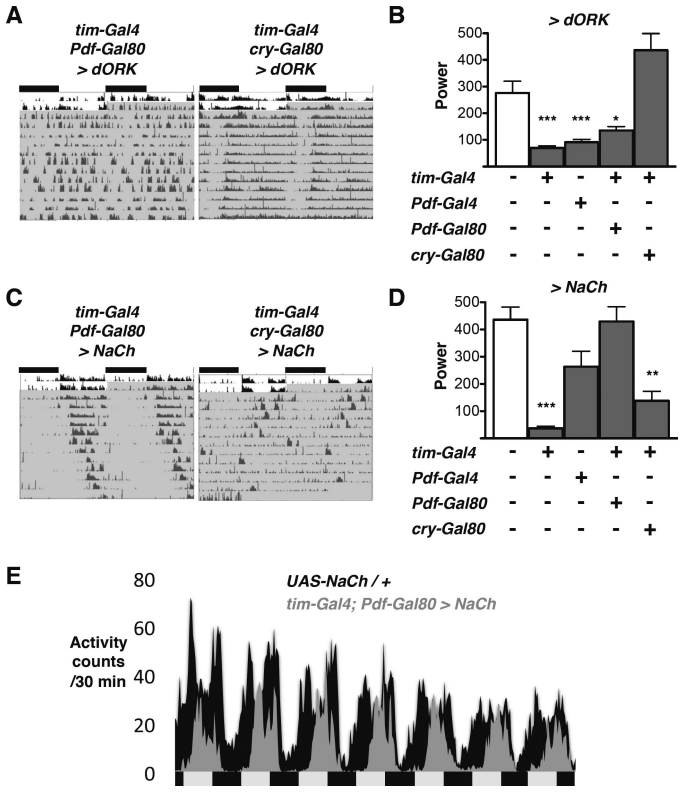

Figure 6. Adult CRY+ non-LN_vs are required for robust locomotor activity rhythms

Locomotor activity was recorded from flies entrained to 12:12 LD (white area of actogram), then transferred to DD (shaded). Comparisons to *UAS-Control* / + are by ANOVA with Tukey's post-hoc test. *p<0.05, **p<0.01, ***p<0.001

(A) Representative, double-plotted, normalized actograms are shown for *tim-Gal4*; *Pdf-Gal80 > dORK* and *tim-Gal4; cry-Gal80 > dORK* flies.

(B) The power of rhythms is plotted for flies expressing *UAS-dORK* under the control of *tim-Gal4, Pdf-Gal4, tim-Gal4; Pdf-Gal80* and *tim-Gal4; cry-Gal80* drivers. Power is significantly reduced in *Pdf > dORK, tim > dORK* and *tim-Gal4; Pdf-Gal80 > dORK* flies compared to *UAS-dORK* / + control flies.

(C) Representative, double-plotted, normalized actograms are shown for *tim-Gal4; Pdf-Gal80 > NaCh* and *tim-Gal4; cry-Gal80 > NaCh* flies.

(D) The power of rhythms is plotted for flies expressing *UAS-NaCh* under the control of *tim-Gal4, Pdf-Gal4, tim-Gal4; Pdf-Gal80* and *tim-Gal4; cry-Gal80* drivers. Power is significantly reduced in *tim-Gal4; cry-Gal80 > NaCh* flies compared to *UAS-NaCh* / + control flies.

(E) Average locomotor activity over the first 7 days in DD plotted for *UAS-NaCh* / + control (black) and *tim-Gal4; Pdf-Gal80 > NaCh* flies (grey). *tim-Gal4; Pdf-Gal80 > NaCh* fly activity is substantially reduced during the subjective morning. Each genotype shows the average of 16 flies from a single experiment.

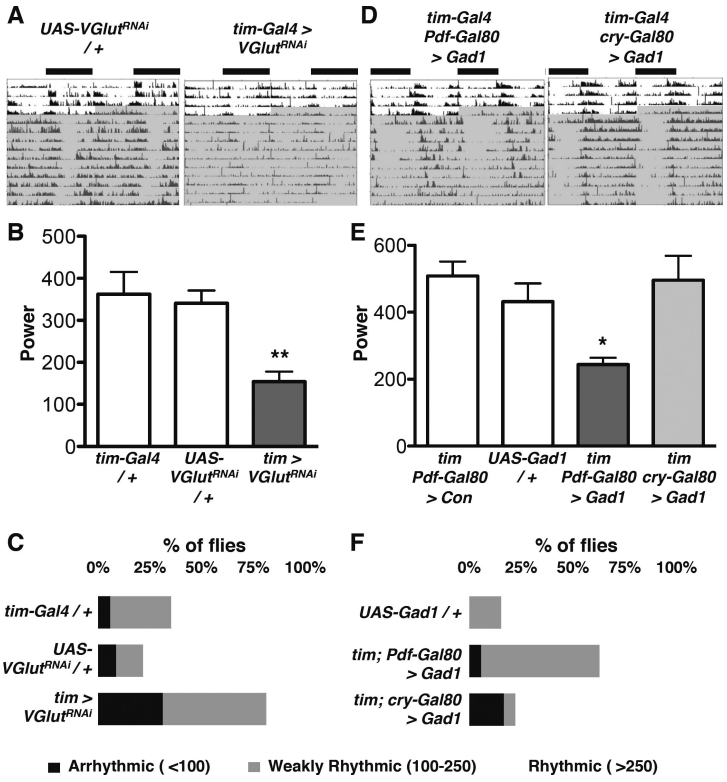

Figure 7. Glutamate signaling from Adult CRY+ non-LN_vs is required for robust locomotor activity rhythms

Locomotor activity was recorded from flies entrained to 12:12 LD (white area of actogram), then transferred to DD (shaded). Comparisons to *UAS-Control* / + by ANOVA with Tukey's post-hoc test. *p<0.05, **p<0.01

(A) Representative, double-plotted, normalized actograms are shown for *UAS-VGlut^{RNAi}* / + and *tim > VGlut^{RNAi}* flies. *UAS-dcr-2* was co-expressed to improve RNAi efficacy and the Gal4 control line also expresses *UAS-dcr-2*.

(B) The average power of rhythms is shown for *tim > dcr2 + VGlut^{RNAi}* flies and *tim > dcr2* and *UAS-VGlut^{RNAi}* / + control flies. Power is significantly reduced in *tim > VGlut^{RNAi}* flies compared to *tim > dcr2* and *UAS-VGlut^{RNAi}* control flies. Error bars represent SEM.

(C) Data from (B) plotted as % of flies that are “arrhythmic” (power >100) “weakly rhythmic” (power from 100–250) or “rhythmic” (power > 250).

(D) Representative, double-plotted, normalized actograms are shown for *tim-Gal4; Pdf-Gal80 > Gad1* and *tim-Gal4; cry-Gal80 > Gad1* flies.

(E) The average power of rhythms is shown for flies expressing *UAS-Gad1* with the *tim-Gal4; Pdf-Gal80* and *tim-Gal4; cry-Gal80* drivers. Power is significantly reduced in *tim-Gal4; Pdf-Gal80 > Gad1* flies compared to *UAS-Gad1* / + control or *tim-Gal4; Pdf-Gal80* crossed to the *UAS-dORKΔNC* control (con) flies. Error bars represent SEM.

(F) Data from (E) plotted as in (C)