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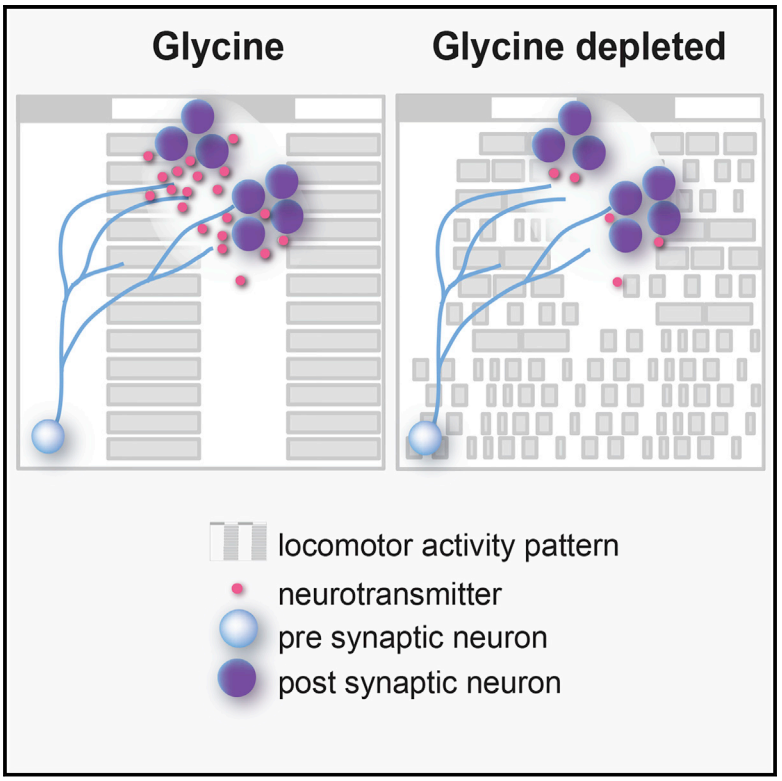
Organization of Circadian Behavior Relies on Glycinergic Transmission

Graphical Abstract



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In Brief

Frenkel et al. discovered that glycine, a fast inhibitory neurotransmitter, plays a key role in the organization of daily patterns of locomotor activity in *Drosophila.* Thus, circadian changes in tonic inhibition provide a time-of-day switch that rapidly turns off specific targets to keep the circadian network in tune.

Highlights

- LNvs are glycinergic neurons
- Glycine is an inhibitory neurotransmitter in the adult fly brain
- Glycine depletion renders the circadian network susceptible to environmental changes
- Glycine provides coherence to the circadian network

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Organization of Circadian Behavior Relies on Glycinergic Transmission

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SUMMARY

The small ventral lateral neurons (sLNvs) constitute a central circadian pacemaker in the *Drosophila* brain. They organize daily locomotor activity, partly through the release of the neuropeptide pigment-dispersing factor (PDF), coordinating the action of the remaining clusters required for network synchronization. Despite extensive efforts, the basic principles underlying communication among circadian clusters remain obscure. We identified classical neurotransmitters released by sLNvs through disruption of specific transporters. Adult-specific RNAi-mediated downregulation of the glycine transporter or impairment of glycine synthesis in LNv neurons increased period length by nearly an hour without affecting rhythmicity of locomotor activity. Electrophysiological recordings showed that glycine reduces spiking frequency in circadian neurons. Interestingly, downregulation of glycine receptor subunits in specific sLNv targets impaired rhythmicity, revealing involvement of glycine in information processing within the network. These data identify glycinergic inhibition of specific targets as a cue that contributes to the synchronization of the circadian network.

INTRODUCTION

The ~24-hr rhythm imposed by the rotation of the Earth around its own axis gives rise to the perpetual repetition of day/night cycles. On Earth, the circadian clock evolved under the pressure to anticipate this timing sequence. In *Drosophila*, the central clock comprises ~150 neurons, organized in ventral (i.e., the small and large lateral neurons ventral [sLNvs and lLNvs, respectively] and dorsal [lateral neurons dorsal, lateral posterior neurons, and dorsal neurons 1–3 (LNds, LPNs, and DN1–3 s, respectively)]) clusters (Shafer et al., 2006). Much is known about the molecular ~24-hr self-sustained oscillations taking place within clock neurons (Ozkaya and Rosato, 2012), but how these heterogeneous clocks communicate to each other is almost an enigma. Some neuropeptides are known to play a crucial role; among them, pigment-dispersing factor (PDF) released from sLNvs is essential, as it sets the phase of several other circadian clocks in the brain (Shafer and Yao, 2014).

But regarding fast classical neurotransmission from central pacemakers, even less is known: a screen for biogenic amines in the sLNvs did not provide indication for the presence of dopamine, serotonin, or histamine (Hamasaka and Nässel, 2006), although serotonin and dopamine modulate circadian entrainment by affecting light sensitivity (Hirsh et al., 2010; Yuan et al., 2005), and aminergic systems affect locomotor activity downstream of pacemaker neurons (Chen et al., 2013). Aside from input- and output-related components, less than 10% of the core pacemakers have been ascribed a classical neurotransmitter; in each hemisphere, two DN1as express the vesicular glutamate transporter vGlut (Collins et al., 2012; Daniels et al., 2008; Hamasaka et al., 2007) and four LNds the vesicular acetylcholine transporter vAChT (Beckwith and Ceriani, 2015a; Johard et al., 2009).

In an attempt to solve the puzzle of how cellular clocks interact with each other, we developed a simple method to uncover the nature of fast neuronal communication. We initially tested the efficiency of this approach in the LNvs. The well-documented hierarchy of the sLNvs in the temporal organization of locomotor activity makes them particularly appealing (Renn et al., 1999; Stoleru et al., 2005). Moreover, the presence of typical output synapses with active zones accompanied by small clear vesicles (Yasuyama and Meinertzhagen, 2010) and the perturbing effect of blocking fast synaptic transmission (Kilman et al., 2009; Umezaki et al., 2011; Wülbeck et al., 2009) are two pieces of evidence supporting the anatomical and functional presence of classical neurotransmitters within LNvs neurons. Along this work, we identified glycine as the neurotransmitter employed by the

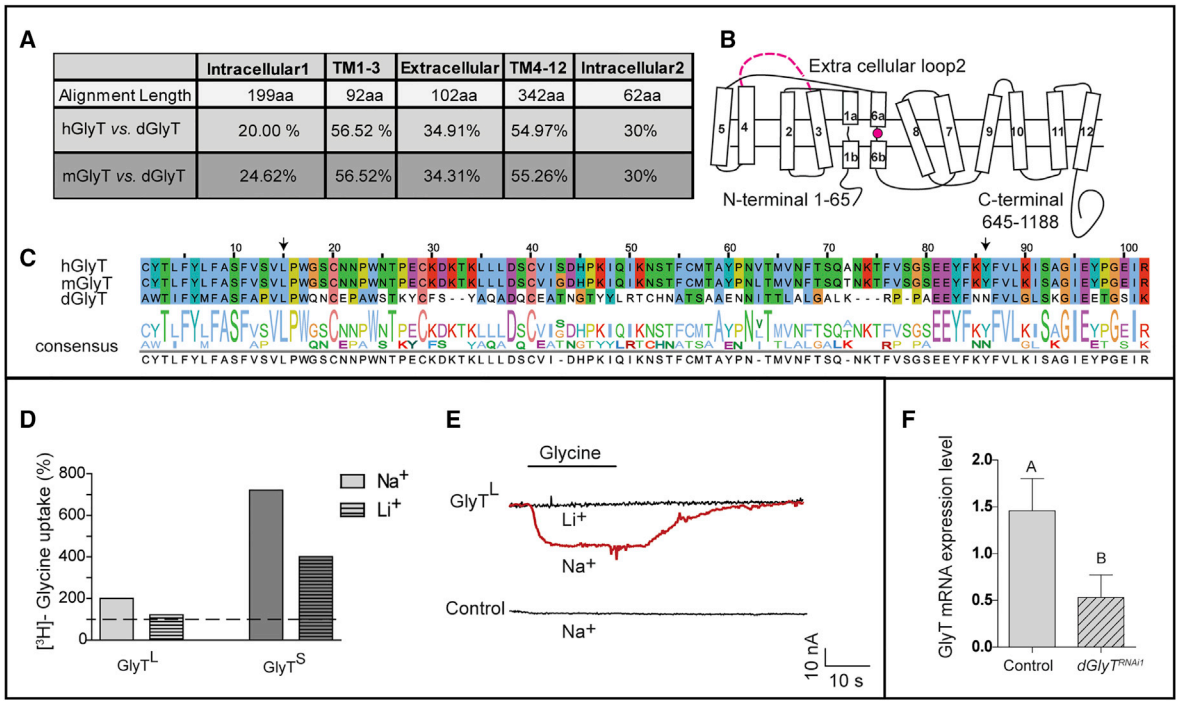


Figure 1. Identification of the *Drosophila* Glycine Transporter

(A) Percentage of identity between different domains of the human and mouse neuronal glycine transporter (SC6A5) and the protein encoded by *CG5540*, dGlyT. dGlyT contains a large C-terminal domain (600 amino acids long) that is not present in the mammalian orthologs.

(B) Topology of the secondary structure of dGlyT modified from Yamashita et al. (2005). Extracellular loop 2 (pink) is involved in glycine recognition. The pink circle in TM loop 6 indicates W343, distinctive of glycine transporters.

(C) Amino acid alignment of extracellular loop 3; the consensus sequence is shown at the bottom. Arrows indicate the key residues L172 and N237 in dGlyT.

(D and E) *dGlyT* encodes a bona fide glycine transporter. Controls or oocytes expressing the long and short version of CG5549, named dGlyT^{L} and dGlyT^{S}, respectively, were assayed for [^{3}H]-glycine transport (100 μM final concentration, specific activity 1.1 MBq·mmol^{-1}) over 5 min in external solution containing NaCl or LiCl (n = 3 per condition) (D). dGlyT^{L} encodes the full-length fly *CG5549* (3,567 bp). dGlyT^{S} (2,016 bp) lacks the extended C-terminal domain. Bars represent the percentage of [^{3}H]-glycine uptake relative to a water-injected control (dashed line). Quantitative analysis revealed that dGlyT^{L} increased glycine uptake ~2-fold, while dGlyT^{S} increased it nearly eight times (plain bars). Na^{+} to Li^{+} replacement led to a ~40% decrease in glycine uptake (stripped bars). Two electrode voltage-clamp recordings showed that glycine application elicited reversible Na^{+} inward currents, which were inhibited upon replacement of Na^{+} to Li^{+} (E). A representative experiment is shown (n = 5). Control stands for a water-injected oocyte in a Na^{+} external solution upon glycine application.

(F) Real-time qPCR analysis of d*GlyT* mRNA levels in fly heads. *tubulin* was used as the normalization control. Bars represent the mean ± SEM of three independent experiments. Control stands for *GMRG4* > + flies. *dGlyT* mRNA levels decrease upon *dGlyT*^{RNAi} expression in the eyes driven by *GMRG4*. A *UAS-dicer2* transgene was included to potentiate the effect of the RNAi (t test, T = 9.18, $p < 0.01$).

LNvs and report its role in the *Drosophila* brain. We also showed that glycine inhibits action potential firing in LNv postsynaptic neurons and looked for putative receptor subunits. Our results uncovered the contribution of both stable and dynamic synaptic partners of the LNvs that are revealed upon disruption of glycinergic transmission.

RESULTS

A Genetic Strategy to Define the Neurotransmitter Identity of a Circadian Neuronal Cluster

Neuron identity is defined by its morphology, the expression of receptors, and the release of neurotransmitters. Given the relevance of the LNvs within the circadian network, we carried out a screen aimed at identifying their classical neurotransmitters. We reasoned that because each neurotransmitter system is characterized by distinctive features, we could identify it through disruption of membrane and vesicular transporters in a cell-autonomous fashion via RNAi expression. Bioinformatic analysis within the fly genome (http://flybase.org) retrieved 17 potential candidates (Table S1). Constitutive downregulation of some of them triggered subtle effects under free-running conditions (constant darkness [DD]), mostly on rhythmicity. A consistent increase in period length was observed when downregulating *CG5549*, a putative glycine transporter (Thimgan et al., 2006). *CG5549* had not been characterized functionally; however, in situ hybridization showed that it is broadly expressed in the CNS, especially in photoreceptors and optic neuropils (Thimgan et al., 2006). The analysis of topological domains revealed a 50% identity among *Drosophila* and mammalian glycine transporters in the 12 transmembrane (TM) regions (Figure 1A). Moreover, the characteristic residue W482 found in glycine transporters at TM6 (Rees et al., 2006) is also present in the *Drosophila* ortholog (W343; Figure 1B). In humans, the extracellular loop 2 is involved

in glycine recognition, particularly residues L306 and Y377 (Rees et al., 2006); *Drosophila CG5549* also contains a leucine (L172) and a conservative charged residue, N237, in equivalent positions (Figure 1C), suggesting an additional layer of conservation.

CG5549 Mediates Glycine Transport

Diverse membrane amino acid transporters, part of the solute carrier (SLC) family, were characterized using *Xenopus laevis* oocytes (Guastella et al., 1992; Lin et al., 2015). In order to deorphanize *CG5549*, we measured direct glycine uptake and the Na^+ currents underlying this symporter activity. Oocytes were injected with RNA encoding both a long (full-length) and a short (truncated) variant of the putative glycine transporter ($dGlyT^L$ and $dGlyT^S$; see the Figure 1D legend for details), which were tested for their ability to take up [^{3}H]-glycine. As oocytes possess an endogenous transport system for glycine (Guastella et al., 1992), basal levels of [^{3}H]-glycine uptake were detected in water-injected oocytes. Accumulation of [^{3}H]-glycine in oocytes expressing $dGlyT^L$ was increased 2-fold compared to basal levels, suggesting that *CG5549* encodes a functional glycine transporter. Notably, $dGlyT^S$ resembling mammalian GlyTs appeared to be even more active (>7-fold). Replacement of Na^+ by Li^+ in the bath solution decreases activity of both versions to approximately half (Figure 1D); such Na^+ dependence is a conserved feature of the SLC6 family (Lin et al., 2015).

We next took advantage of the Na^+ current generated by the glycine symport activity to further characterize $dGlyT^L$ through two electrode voltage-clamp recordings. Glycine applications evoked inward currents that were reversible by washout and severely impaired when replacing Na^+ by Li^+ in the recording buffer (Figure 1E). These responses are compatible with mammalian glycine transporters (Edington et al., 2009; Vandenberg et al., 2007). In sum, these results confirm that *CG5549* indeed encodes a glycine transporter.

Glycinergic Transmission Contributes to Set Period Length

Traditional approaches to assess the relevance of a gene of interest on any given behavior include mutant analysis, despite potential developmental defects or even compensatory mechanisms triggered by chronic depletion; in the case of *dGlyT*, only a deficiency (spanning a large deletion lethal in homozygosis) was available. We monitored daily locomotor activity in a heterozygote *dGlyT* deficiency and found no changes on circadian parameters; consistently, qPCR analysis showed no significant differences on *dGlyT* mRNA level (Table S2). Thus, RNAi became the only genetic tool available to analyze glycinergic transmission.

We examined the silencing efficiency of a specific $CG5549^{RNAi}$ in the tissue in which it is most abundant (Thimgan et al., 2006). The *dGlyT* mRNA level was assessed in flies expressing *UAS-dicer2*; $UAS\text{-}CG5549^{RNAi}$ (from now on, $dGlyT^{RNAi1}$) in visual neuropils by real-time qPCR. *dGlyT* was detected in head extracts, and, importantly, overall levels were decreased to ~30% in flies expressing $dGlyT^{RNAi1}$ (Figure 1F).

We next addressed the role of glycinergic transmission reducing glycine availability in the small and large LNvs (Figures 2A and 2B). d*GlyT* mRNA downregulation was restricted to the adult stage employing the inducible *pdfGeneSwitch* line (Depetris-Chauvin et al., 2011). Daily activity patterns of *pdfGS* > $dGlyT^{RNAi1}$ flies (LNvs > $dGlyT^{RNAi1}$*RU*, or $dGlyT^{RNAi1}$) were similar to those of control flies (LNvs > +RU), displaying wild-type light-dark (LD) behavior (Figure 2C). However, adult-specific *dGlyT* mRNA downregulation resulted in a close to 1-hr lengthening of the free-running period compared to both controls (LNvs > +RU and LNvs > $dGlyT^{RNAi1}$ in the presence of vehicle, referred to as controls 1 and 2; Figure 2D). Additional analysis showed that rhythmicity is intact under these conditions, since both fast Fourier transform (FFT) and the power of the peak analyses retrieved similar values for all experimental groups (Table S3). Downregulation with independent RNAi lines rendered similar results: an increase in period length with no effect on rhythmicity (Figure S1; Table S3).

Because neuronal neurotransmitter transporters at the membrane are involved in neurotransmitter recycling, *dGlyT* downregulation would be expected to decrease glycine availability within the LNv terminal. Thus, disrupting the enzyme responsible for the conversion from serine into glycine should have a similar effect on period length. We identified *CG3011* as the putative serine hydroxymethyltransferase (*Shmt*) encoded in the fly genome (Figure 2E). A putative *Shmt* insertional mutant enabled testing its impact on circadian behavior. While the *Shmt* insertional mutant showed period lengthening when compared to its genetic control, analysis of the activity profiles highlighted a major deconsolidation of the activity patterns, perhaps as a result of pleiotropic effects (Table S2), reinforcing the need for the specific expression of $Shmt^{RNAi}$ in the LNvs. During the initial phase of the experiment, daily activity patterns of *pdf*GS > $Shmt^{RNAi}$ (LNvs > $SHMT^{RNAi}$ RU, or $SHMT^{RNAi}$) flies were similar to those of control flies, ruling out potential defects associated to an overall depletion of glycine (Figure 2F). Interestingly, it also resulted in period lengthening (Figure 2G). As described for *dGlyT*, no effect on other circadian parameters was found upon downregulation of *Shmt* levels solely in LNvs (Table S3).

Taken together, these results suggest that decreasing glycine availability within the LNvs, either through decreased synthesis or reuptake, gives rise to increased period length; moreover, given that glycine depletion affects circadian period but does not trigger loss of rhythmicity, it is unlikely that neuronal viability is compromised; hence, we predict that LNvs are glycinergic neurons.

Glycinergic Transmission Enhances the Stability of the Circadian Network

Continuous light exposure destabilizes the molecular clock, thereby resulting in arrhythmic behavior. Moreover, internal desynchronization within the circadian network increases with light intensity, leading to an activity pattern that displays components that free-run with different periods (Rieger et al., 2006). Blockade of fast neurotransmitter release in LNvs in flies kept under constant light also elicits a complex circadian rhythm with two period components (Umezaki et al., 2011).

In order to test the effect of blocking glycinergic transmission in LNvs, we performed an extended recording of the locomotor activity profiles; decreased stability is often associated to the presence of more than one stable period component (termed complex

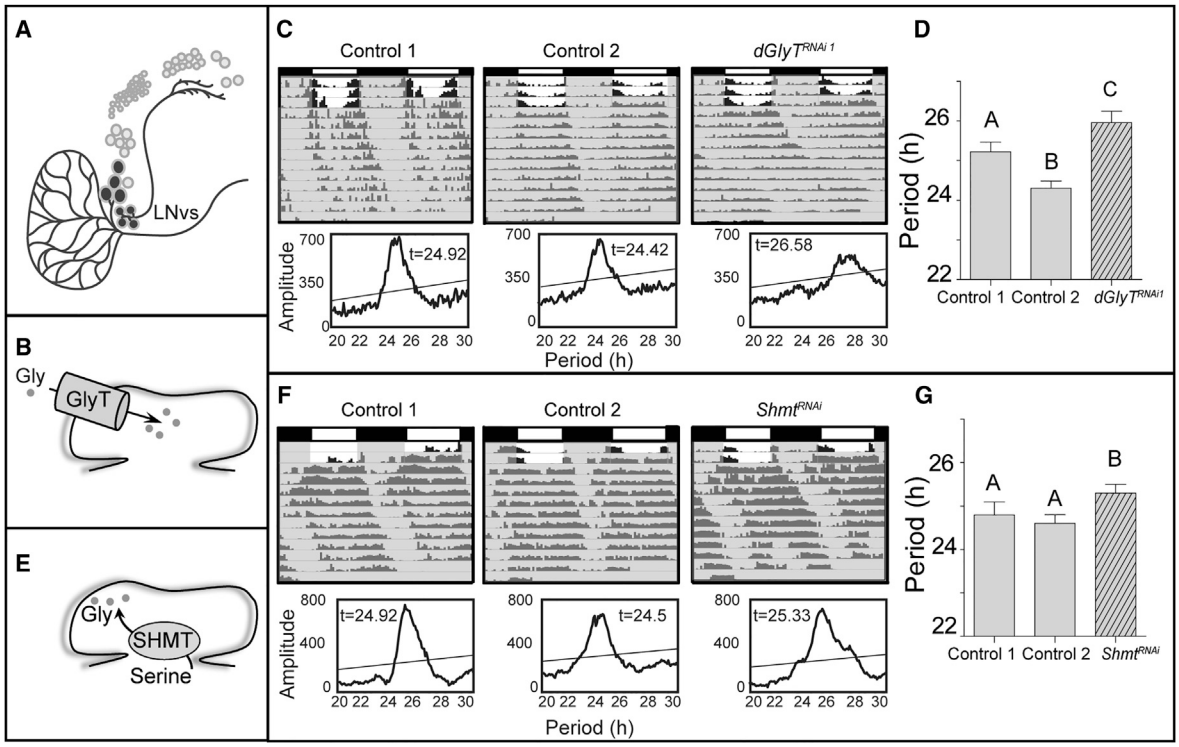


Figure 2. Glycine Depletion Increases Period Length

(A) Schematic diagram of a brain hemisphere describing all circadian neurons; somas colored in black represent the circadian group under treatment (as in Figures 3 and 6).

(B and E) Schematic diagram of a neuronal terminal indicating the protein under analysis.

(C and D) Downregulation of *dGlyT* alters endogenous period. In (C), top panels show representative double-plotted actograms of individual flies of the indicated genotypes. LNvs refers to the *pdf*GS driver. Flies were kept in LD for 3d then switched to DD for 9d (shaded gray area). Bottom panels depict endogenous period determined in DD. In (D), bar graph shows the quantitation of the free-running period for the indicated genotypes for each group of experiments. Analysis included a one-way ANOVA (for period: $F(3,12) = 10.01$, $p = 0.0041$, LSD Fisher, $\alpha = 0.05$). See also Figure S1 for additional experiments employing different RNAis.

(F and G) Downregulation of the enzyme converting serine into glycine triggers period lengthening. In (F), experiments were carried out as in (C). Representative actograms are shown. In (G), bar graph shows the quantitation of the endogenous period ($F(3,10) = 8.93$, $p = 0.009$, LSD Fisher, $\alpha = 0.05$). Experiments were independently repeated three to five times, with 20–32 flies analyzed per genotype/experiment. Rhythmicity, power, and FFT were analyzed, but no statistically significant differences were found (see Table S3). Data are presented as the mean ± SEM. Different letters indicate statistical significance.

Bars represent mean ± SEM. In Figures 2 and 3, control 1 refers to a single copy of the Gal4 driver in the presence of RU486, and control 2 is a non-induced genetic control.

rhythms). Locomotor activity patterns of control (LNvs > + RU and LNvs > $GlyT^{RNAi1}$ VEH, controls 1 and 2, respectively) and d*GlyT*-depleted (LNvs > $GlyT^{RNAi1}$ RU, $GlyT^{RNAi1}$) flies were examined under free-running conditions with increasing light. Initially, flies were released into constant dim light (moonlight, 0.1 lux) (Bachleitner et al., 2007; Rieger et al., 2009). While only ~30% of control animals showed complex rhythms, a large proportion (~66%) of the depleted d*GlyT* flies did so. In addition, they showed the most dramatic long-period component (Figure 3A).

Subsequently, flies were released into constant dim light (1 lux). Approximately 60%–70% of control animals showed complex rhythms, while most *dGlyT* depleted flies became arrhythmic (~80%; Figure 3B). These results reinforce the notion that glycinergic transmission contributes to the stability of the activity patterns, since a subtle increase in light intensity at night could trigger arrhythmicity. Thus, disrupting glycinergic transmission mimics the instability triggered upon exposure to constant low light.

Glycine Inhibits a Postsynaptic Target of the sLNvs

Once we established that the LNvs are glycinergic neurons, we inquired about its effect on one of the well-characterized sLNv postsynaptic targets, the DN1ps (Cavanaugh et al., 2014; Gorostiza et al., 2014; Seluzicki et al., 2014). Electrophysiological recordings were performed applying an external solution supplemented with glycine. As glycine receptors are ligand-gated chloride channels (Finger, 1982; Hamill et al., 1983; Sakmann et al., 1983), the predicted result was that glycine application would inhibit DN1ps action potential firing. Indeed, bath application of glycine greatly diminished the firing frequency of DN1p neurons (Figures 4A and 4B). Spiking frequency was recovered almost completely upon glycine washout. To test whether there was a direct effect onto the DN1ps, we applied glycine specifically onto this neuronal cluster. A representative loose patch recording of a DN1p cell is included (Figure 4C). Reduction of DN1p firing upon glycine administration was consistent in all recorded neurons. Thus, glycine acts as an inhibitory

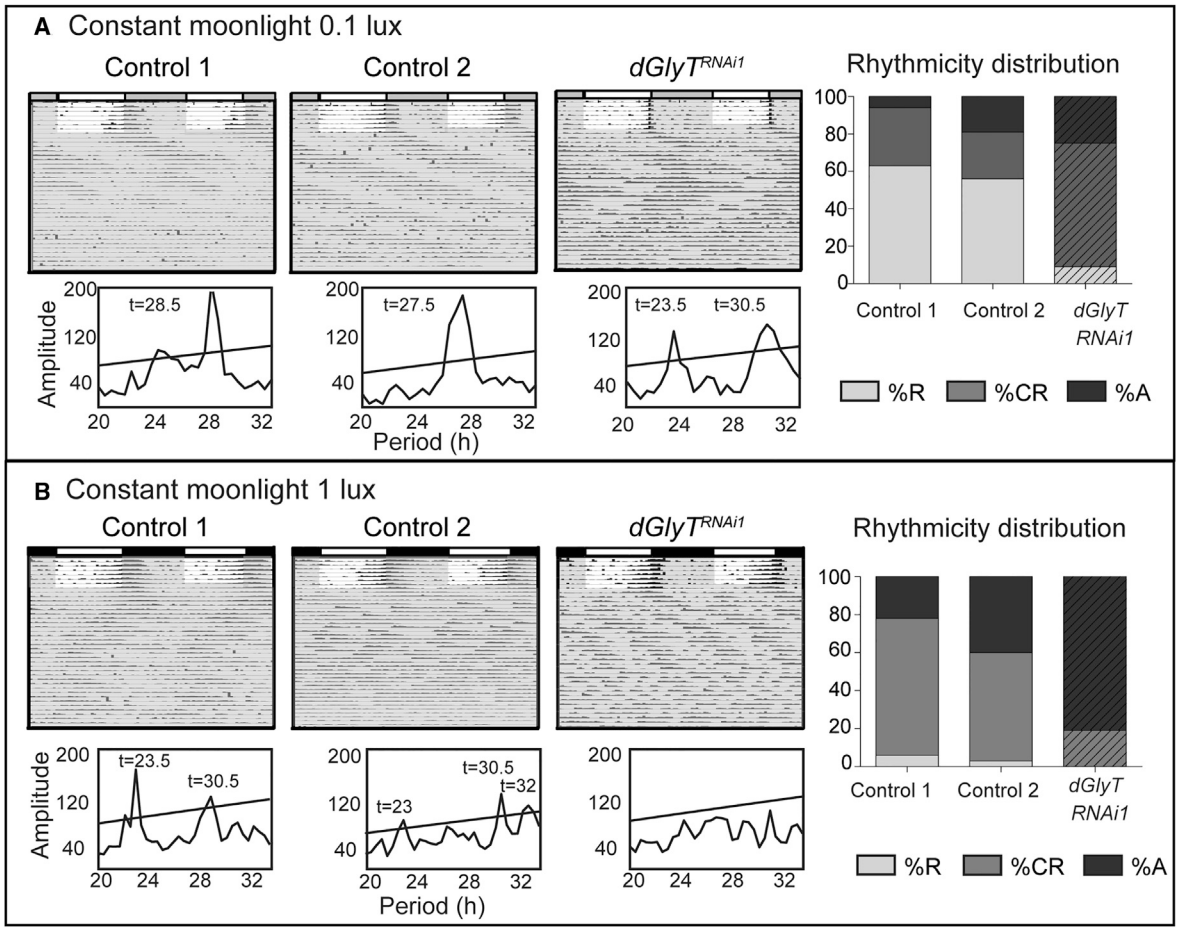


Figure 3. Glycine Depletion Destabilizes Circadian Rhythms

(A) Representative double-plotted actograms and periodograms of individual flies of the indicated genotypes (left). Flies were kept in LD for 7 days and then switched to continuous moonlight (MM; 0.1 lux, shaded gray area) and monitored for 30 days at 20°C. Rhythmicity distribution in MM 0.1 lux (right). Bar graphs indicate percentages of rhythmic (R), complex rhythmic (CR), and arrhythmic (A) flies.

(B) Experiments were performed as indicated in (A) but under MM 1 lux (shaded gray area). In MM 10 lux, flies became mostly arrhythmic; no free-running period could be determined.

neurotransmitter on DN1ps, decreasing firing frequency, suggesting an additional layer of control to the cell-autonomous mechanisms dictating the circadian changes of firing properties of the DN1ps (Flourakis et al., 2015).

Identifying Putative Glycine Receptor Subunits

Glycine's effect is mediated by receptors of the Cys-loop ligand-gated ion channel family, which leads to a fast inhibitory response in the spinal cord and brainstem in mammals (Lynch, 2004; Talwar and Lynch, 2015; Zhang et al., 2015). In general, heteromeric pentamers comprise alpha and beta subunits; several receptors can be assembled, depending on those recruited. No information regarding fly orthologs was available. Mining flybase.org with the words "glycine receptor alpha subunit" retrieved *CG7446* (*Grd*) as the putative GABA/glycine-like receptor of *Drosophila*. In addition, other loci potentially fulfilling this role could be identified: *CG12344* and *CG7589*, as well as *CG10537* (*Rdl*) and *CG17336* (*Lcch3*). Both *Rdl* and *Lcch3* were characterized as GABA-A receptor subunits (Dupuis et al., 2010; Tsang et al., 2007), but no functional characterization of the remaining ones was available.

To shed light on the relationship of the potential receptor subunits, a bioinformatics analysis was performed. Human GABA-A and glycine receptors have the same topological domains, three-dimensional structure, and general function. We reasoned that the same could be inferred for the *Drosophila* ligand-gated Cl^- channel-like proteins, as they share sequence identity ranging from 20% to 40% with their human counterparts. In fact, the same domain arrangement is annotated for *Rdl*, *Lcch3*, and *Grd*; that is, an extracellular domain followed by three TM next to an intracellular one along with a fourth TM domain, according to Uniprot. In addition, several residues have been proposed to be specific for either GABA or glycine receptors (Breitinger and Becker, 1998; Lynagh and Pless, 2014; Lynch, 2004; Vandenberg et al., 1992). Multiple sequence alignment showed that 30%–50% of glycine-specific residues are present

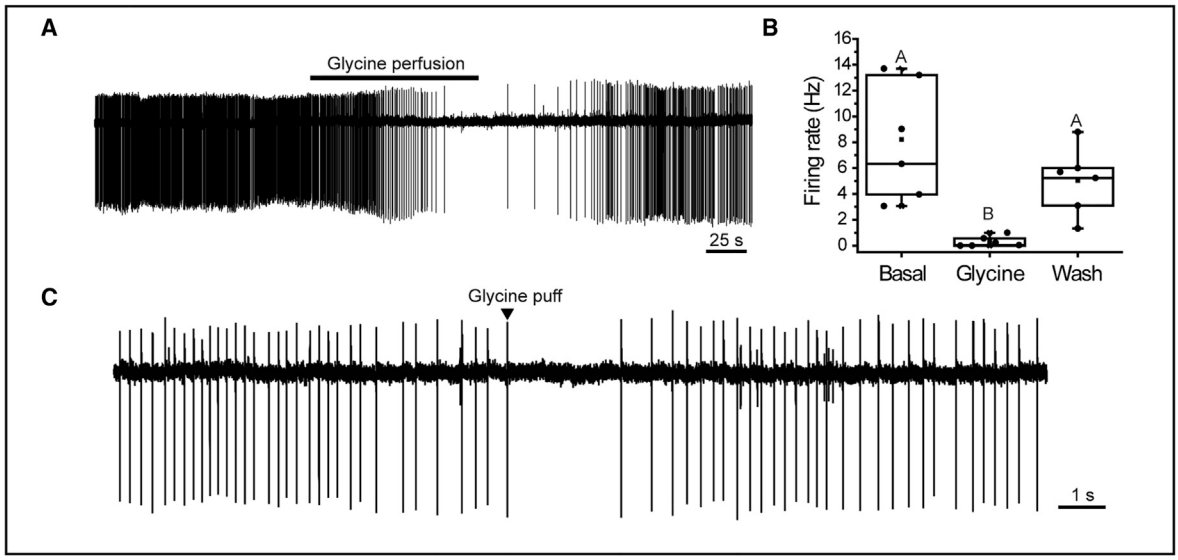


Figure 4. Glycine Affects DN1p Physiology

(A) Representative loose cell-attached recording of a DN1p; the bar indicates the time for 20 mM glycine application (a 1-min delay is accounted for the perfusion system).

(B) Firing rate quantification of the effect shown in (A). Box represents percentiles (25th and 75th), and the mean is represented by the square (n = 6). Different letters indicate statistically significant differences ($p < 0.002$) after glycine treatment (one-way ANOVA with Tukey post hoc test).

(C) Representative loose cell-attached recording of a DN1p where a 10-ms (pressure 20 psi) 500-mM glycine solution was puffed (from a distance of three to five somas) using a pressure system (arrowhead). All DN1ps were silenced after glycine puffing (n = 8). A mechanical artifact was ruled out, since puffing solution without glycine had no effect on firing activity. Genotype: DN1ps > CD8-Cherry. Flies were raised at 25°C, 12:12-hr LD. Recordings were performed within ZT2–ZT9.

at equivalent positions in the mammalian GABA-A receptor subunits, underscoring that these are not truly specific for glycine receptors. We next analyzed the *Drosophila* proteins encoded by these CGs and found that although some residues were still conserved, the overall percentage did not differ from those shared with the GABA-A subunits (Figure S2A). We conclude that these *Drosophila* proteins can recognize both glycine and GABA.

We next built a sequence similarity network, which retains basic clustering and topology information present in phylogenetic trees but is a better representation of protein family sequence and structural interrelationships (Atkinson et al., 2009). *Drosophila* putative Gly/GABA receptor subunits were analyzed along with human cys-loop receptor subunits; networks were built at different thresholds (Figure S2B). Interestingly, acetylcholine receptors separated at lower ones (bit score of 80). Subunits encoded by *CG12344* and *CG7589* are the most dissimilar of the group, and after stringency increases they split out (bit scores of 85 and 107, respectively). GABA-A receptors showed enough differences to cluster into two groups (alpha and beta subunits, bit score of 175). Interestingly, even at this stringent threshold, GRD showed similarity to both GABA-A and glycine receptor subunits. Instead, RDL and LCCH3 resembled GABA-A receptors only. In a nutshell, the similarity network analysis also suggested that *CG12344*, *CG7589*, and *Grd* encode ligand-gated chloride channel subunits, consistent with a putative role as glycine receptors (Figure 5A).

The Circadian Network as a Glycinergic Target

To investigate their contribution in the control of rhythmic behavior, we resorted to downregulation of putative glycine receptor subunits (*Grd*, *CG12344*, and *CG7589*). As a first step, real-time qPCR analysis confirmed that the mRNAs encoding the putative dGlyR subunits were downregulated to 30%–50% of endogenous levels (Figures 5B and S5).

We next examined the impact on the temporal organization of locomotor behavior of knocking down putative receptor subunits. Surprisingly, downregulation of single subunits in all circadian clusters (driven by $Clk^{856}G4$ expression) resulted in subtle circadian phenotypes (i.e., a shorter period upon downregulation of *CG12344*; Figures 6A and 6B; Table S4) and more consolidated activity patterns than controls. The possibility that those modest phenotypes could be the outcome of disparate effects on each of the circadian clusters prompted us to silence putative receptor subunits in specific subsets of circadian neurons.

DN1ps contact the sLNvs throughout the day (Gorostiza et al., 2014) and acutely respond to glycine (Figure 4). Control animals (DN1ps > +, described as d*Clk4.1G4*) displayed a clear rest/activity pattern and very robust rhythmicity. Surprisingly, a subtle but statistically significant period lengthening was observed in the DN1ps > Grd^{RNAi1} group (Figures 6C and 6D; Table S4), which was independently confirmed employing alternative Grd^{RNAi2} and $CG12344^{RNAi2}$ lines (Figure S3); it is worth noting that this period-lengthening phenotype is reminiscent of the one observed upon reducing glycine release from the LNvs (Figure 2). Downregulation of any putative d*GlyR* subunit in

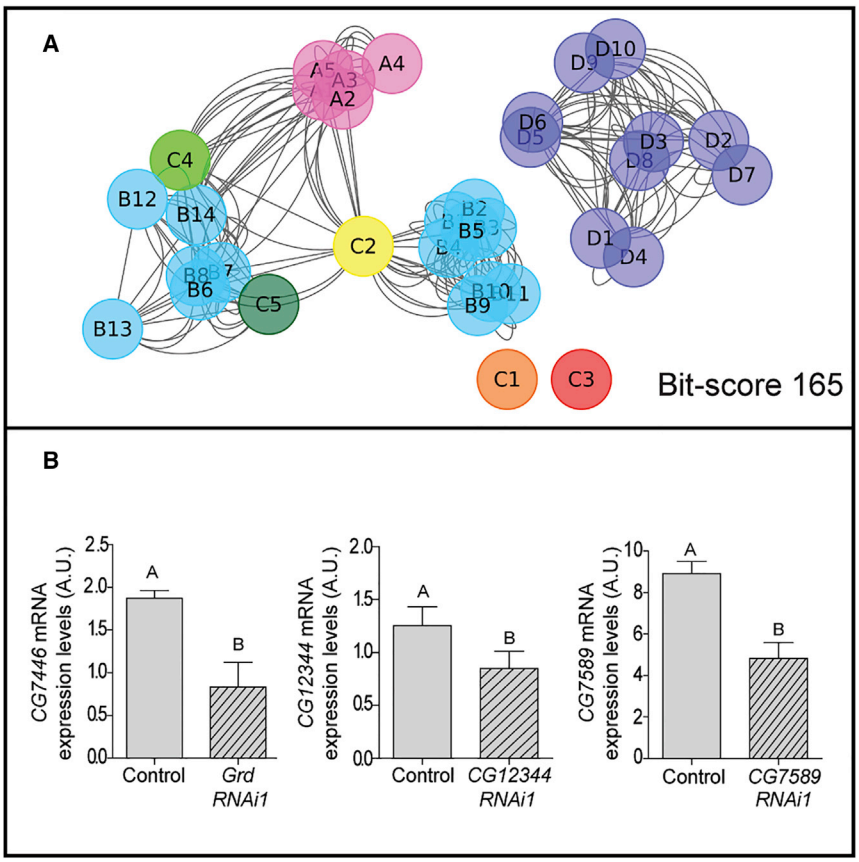


Figure 5. Analysis of Putative Glycine Receptor Subunits

(A) Sequence similarity networks defined the relationship between the *Drosophila* putative glycine/GABA subunits and human orthologs. Even when the network was thresholded at a BLAST bit score of 165 (E-value = 1.e-53, 35%–39% identity), GRD shows similarity to both human glycine and GABA-A receptor subunits. Conversely, CG12344 and CG7589 separate from both at 85 or 107 bit score. Glycine receptors, pink; acetylcholine receptors, violet; GABAA receptors, light blue; Grd, yellow; Rdl,: light green; Lcch3, dark green; CG12344, orange; and CG7589, red. Human GlyR nicotinic and GABAR and *Drosophila* subunits are named as described in Figure S2.

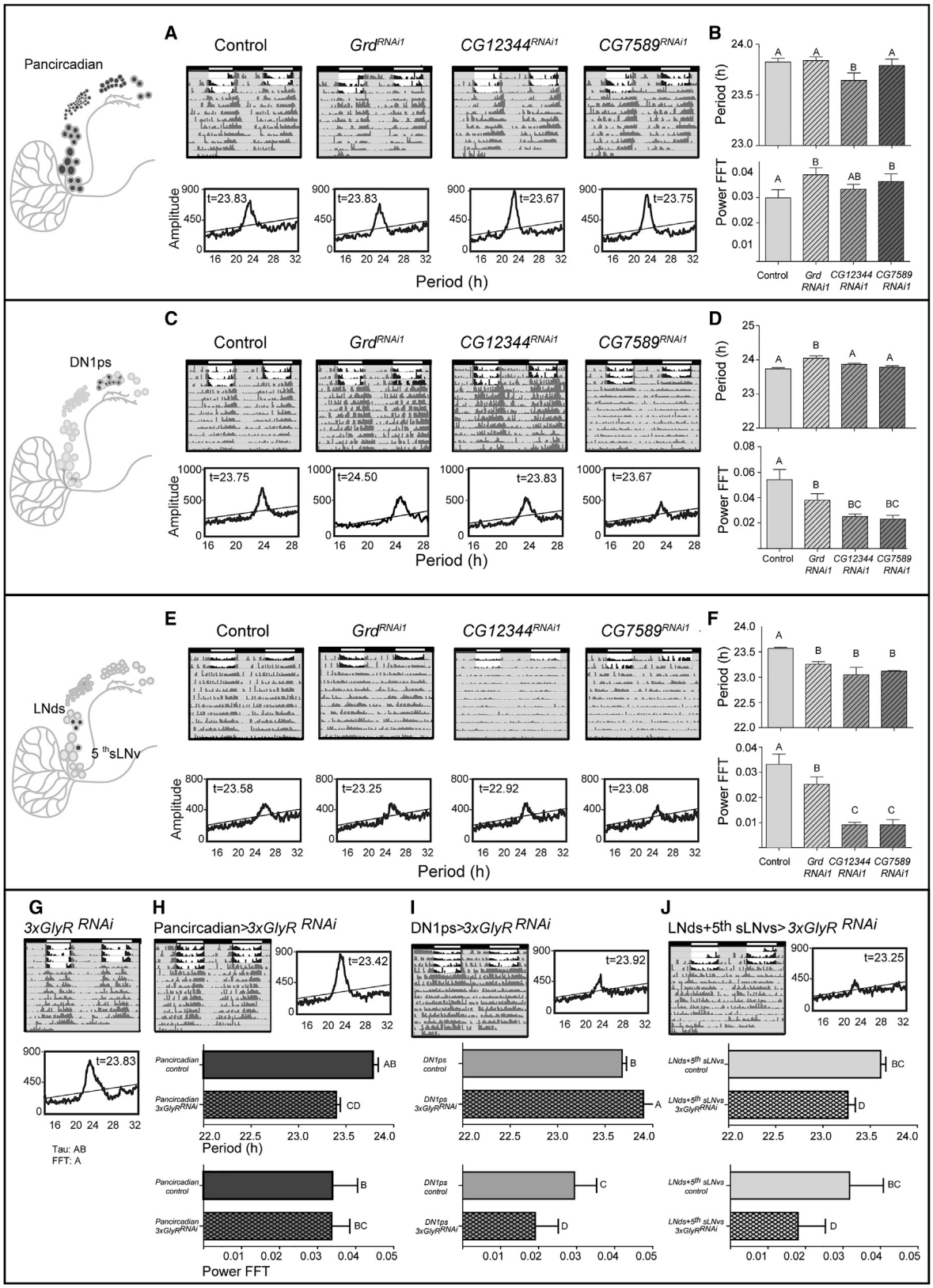
(B) Real-time qPCR analysis of putative d*GlyR* subunit mRNAs in fly heads. *tubulin* was used as the normalization control. Bars represent the mean ± SEM of three independent experiments. Putative *dGlyR* mRNA levels are reduced to different degrees upon RNAi expression in the eye (driven by *GMRG4*). Control stands for *GMRG4* > + flies. *CG12344*-specific RNAi reduced levels by ~30%; RNAi directed to *Grd* and *CG7589* reduced levels by ~50% (*Grd* t test, T = 5.24, $p < 0.05$; *CG12344* t test, T = 29.87, $p < 0.001$; *CG7589* t test, T = 3.87, $p < 0.05$).

DN1p neurons (DN1ps > Grd^{RNAi1}, DN1ps > $CG12344^{RNAi1}$, and DN1ps > $CG7589^{RNAi1}$) also led to deconsolidation of the activity pattern (Figures 6C and 6D; Table S4).

Next, we downregulated putative subunits on a different circadian domain, a subset of DN1ps and LNds expressing CRYPTOCHROME (CRY+, the *cry19G4;pdfG80* genetic combination). Flies expressing $CG12344^{RNAi}$ or $CG7589^{RNAi}$ in this heterogeneous group displayed less organized patterns of activity. Grd^{RNAi}, on the other hand, did not significantly affect rhythmicity, but it triggered a subtle period lengthening, reminiscent of what was observed when silencing *Grd* in DN1ps (Figures S4A–S4D; Table S4). We wondered whether these CRY+ neurons could also contact the sLNvs across the day. To address this possibility, we employed GFP reconstitution across synaptic partners (GRASP) to label contacts between adjacent membranes (Feinberg et al., 2008). Contacts between the sLNvs and the CRY+ group were detectable at all times (ZT2, ZT14, and ZT22), but there appeared to be some region-specific circadian component to the connectivity, opening the possibility that independent clusters could be contacting the sLNvs within defined windows (Figures S4E and S4G). Since the reconstituted GFP signal was sparse, it was possible to analyze separately when and where GFP reconstitution took place along the projections. In the lateral horn, it was widely detected through the night (ZT14 and 22), and in the dorsal projections, it was mostly detectable late at night, as reported for the LNd+5^{th} sLNv group (Gorostiza et al., 2014).

Since the E-oscillator makes time-of-day-dependent synaptic contacts with the sLNv (Gorostiza et al., 2014), we investigated the impact of blocking glycinergic transmission in this cluster (Mai179*G4;pdfG*80 driver, referred to as LNds+5^{th}). Downregulation of each putative *dGlyR* subunit in the E-oscillator triggered a modest but consistent decrease in period length, reinforcing the possibility of a feedback toward the sLNvs (Stoleru et al., 2004; Yao and Shafer, 2014). In addition, they also led to deconsolidated locomotor behavior (Figures 6E and 6F; Table S4). In a nutshell, reducing dGlyR single subunits in specific clusters affected the temporal organization of locomotor activity; remarkably, subtle but significant period changes were found, perhaps uncovering a distinct contribution of each cluster to the dynamic operation of the circadian network.

Partial knockdown of single subunits in restricted circadian neurons triggered either a short or long period, depending on the cluster affected. We reasoned that downregulation of all three *dGlyR* subunits would lead to more striking phenotypes. Interestingly, pancircadian co-expression of Grd^{RNAi}, $CG12344^{RNAi}$, and $CG7589^{RNAi}$ resulted in shortening of the period of locomotor activity, with no effect on other rhythmic parameters, closely resembling the phenotype derived from the downregulation of a single subunit (*CG12344*; Figures 6B and 6H). A similar scenario was observed when silencing all three subunits in the DN1ps, that is, a tendency to display a longer period associated with more deconsolidated activity patterns (Figures 6D and 6I). Likewise, silencing all three subunits in the



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E-oscillator triggered a statistically significant period shortening along with a clear effect on rhythmicity (Figures 6F and 6J); however, this phenotype does not appear any different from the one observed upon silencing single subunits, perhaps indicating the extent of period change to be uncovered upon reducing inhibition onto those cells in the context of an operational network. Taken together, these results show that glycine is a relevant classical neurotransmitter in the circadian network and also uncover the complex interactions among different clusters coordinated by the glycinergic inhibitory tone.

Grd, CG12344, and CG7589 Mediate Glycinergic Transmission in DN1ps

DN1ps stop firing action potentials in response to glycine. To investigate whether GRD, CG12344, and CG7589 mediate inhibition in a cell-autonomous fashion, we tested the response to glycine in control (DN1p > *CD8:GFP*) and $GlyR^{RNAi}$-expressing DN1ps. Combined RNAi expression (DN1p > *CD8:GFP,* Grd^{RNAi}, $CG12344^{RNAi}$, $CG7589^{RNAi}$) did not affect the basal firing rate. Control DN1ps were always silenced in response to glycine; however, multiple responses were observed in those expressing the RNAis (Figure 7), likely underscoring heterogeneity within the DN1p cluster (Yoshii et al., 2009). Approximately half of the recorded neurons did not silence in response to glycine, indicating that they normally assemble functional GlyRs through recruitment of these subunits. On the other hand, a subset of DN1ps still responded to glycine, displaying a marked decrease in action potential firing rate, implying that other GlyR subunits are employed. As the response to glycine is different in the two genotypes, it depends on GlyR expression. These results support the notion that different combinations of GRD, CG12344, and CG7589 could assemble native glycine receptors; furthermore, these data also underscore that additional GlyR subunits mediate this neurotransmitter activity. In sum, our work demonstrates that central pacemaker neurons make use of glycine to communicate time-of-day-dependent information to orchestrate the daily activity of circadian clusters.

DISCUSSION

As we were interested in solving the puzzle of how neuronal clocks communicate with each other, we challenged them by impairing fast neurotransmission. We provide functional evidence of the relevance of uncharacterized genes related to glycine transmission and thus open the opportunity to study glycinergic communication in a powerful genetic model.

Downregulation of Transporters to Uncover Neurotransmitter Identity

Anatomical and functional evidence indicates that classical neurotransmitters participate along with PDF in the output of the LNvs (Umezaki et al., 2011; Yasuyama and Meinertzhagen, 2010). In an attempt to define their classical neurotransmitter, we manipulated the level of membrane or vesicular transporters of known neurotransmitter systems exclusively in this neuronal group. Downregulation of *CG5549*, the glycine transporter (dGlyT) responsible for recycling this transmitter from the extracellular space, triggered a consistent change in the endogenous period. This effect is specific, as downregulation of *List* (*CG15088*, the lithium-inducible SLC6 transporter) did not change any circadian parameter (Table S1). On the other hand, downregulation of *CG3011* (*dShmt*) also gives rise to period lengthening, consistent with an effect on the circadian clock as opposed to a general effect on LNv viability, which results in progressive loss of rhythmicity (Renn et al., 1999). Interestingly, Nitabach and colleagues showed that expression of a tethered version of PDF in the LNvs in a condition where neurotransmitters cannot be released increases in almost an hour the endogenous circadian period, suggesting that neurotransmission contributes to rhythm acceleration (Choi et al., 2012), lending further support to our findings showing that glycine plays such a role in the adult brain.

Figure 6. Reducing Glycinergic Transmission Triggers Circadian Phenotypes

Locomotor behavior experiments were performed as in Figure 2. Control refers to a single copy of the Gal4 driver. Data are presented as mean ± SEM. Different letters indicate statistical significance. See Table S2 for additional circadian parameters.

(A and B) Downregulation in all circadian neurons (driven by *Clk856*G4). In (A), the top panel shows representative double-plotted actograms of pancircadian > +, pancircadian > Grd^{RNAi1}, pancircadian > $CG12344^{RNAi1}$, and pancircadian > $CG7589^{RNAi1}$, whereas the bottom panel shows periodogram analysis under free-running conditions. In (B), the top panel shows period quantitation. Analysis included a one-way ANOVA ($F(1,15) = 3.93$, $p < 0.05$, LSD Fisher test, $\alpha = 0.05$). The bottom panel shows quantitation of the FFT power. Analysis included a one-way ANOVA FFT ($F(1,15) = 4.57$, $p = 0.032$).

(C and D) Downregulation in the DN1ps. In (C), the top panel shows DN1ps > +, DN1ps > Grd^{RNAi1}, DN1ps > $CG12344^{RNAi1}$, and DN1ps > $CG7589^{RNAi1}$ (DN1ps refers to the d*Clock4.1G4* driver), as in (A). In (D), the top panel shows the average period quantitation. Statistical analysis included a one-way ANOVA ($F(1,15) = 8.56$, $p = 0.005$, LSD Fisher test, $\alpha = 0.05$). The bottom panel shows the FFT power for the genotypes shown in (C). Statistical analysis included a one-way ANOVA ($F(1,15) = 10.67$, $p = 0.0025$, LSD Fisher, $\alpha = 0.05$).

(E and F) Downregulation in LNds+5^{th} sLNv (driven by *Mai*179G4;*pdf*G80). In (E), the top panel shows LNds+5^{th} > +, LNds+5^{th} > Grd^{RNAi1}, LNds+5^{th} > $CG12344^{RNAi1}$, and LNds+5^{th} > $CG7589^{RNAi1}$, as in (A). In (F), the top panel shows period quantitation. Analysis included a one-way ANOVA ($F(1,13) = 7.44$, $p < 0.014$, LSD Fisher test, $\alpha = 0.05$). The bottom panel shows quantitation of the FFT power for the indicated genotypes. Analysis included a one-way ANOVA FFT ($F(1,13) = 46.18$, $p = 0.0001$).

(G–J) Simultaneous downregulation of *Grd*, *CG12344*, and *CG7589*. Control flies combining the three RNAis show no circadian phenotype (Grd^{RNAi1}, $CG12344^{RNAi3}$, and $CG7589^{RNAi1}$) (G). To avoid effects derived from two insertions within the same locus, a new $CG12344^{RNAi}$ was employed. (H)–(J) show combined expression of RNAis targeting all three subunits in all circadian neurons (H), the DN1ps (I), or the E-oscillator (J). All panels display a representative actogram and the corresponding periodogram (top), a bar graph showing period analysis (middle), and FFT power (bottom) for controls and experimental groups. Pancircadian expression of putative *dGlyR* RNAis triggers a significant period shortening with no effect on any rhythmic parameter (H). Downregulation in DN1ps tends to lengthen the period and deconsolidate the activity (I). Silencing putative *dGlyRs* in the E-oscillator shortens free-running period and affects rhythmic parameters (J). Statistical analysis included a two-way ANOVA for period ($F(10,13) = 15.40$, $p < 0.05$, LSD Fisher test, $\alpha = 0.05$) and FFT ($F(10,13) = 65.25$, $p < 0.005$, LSD Fisher test, $\alpha = 0.05$).

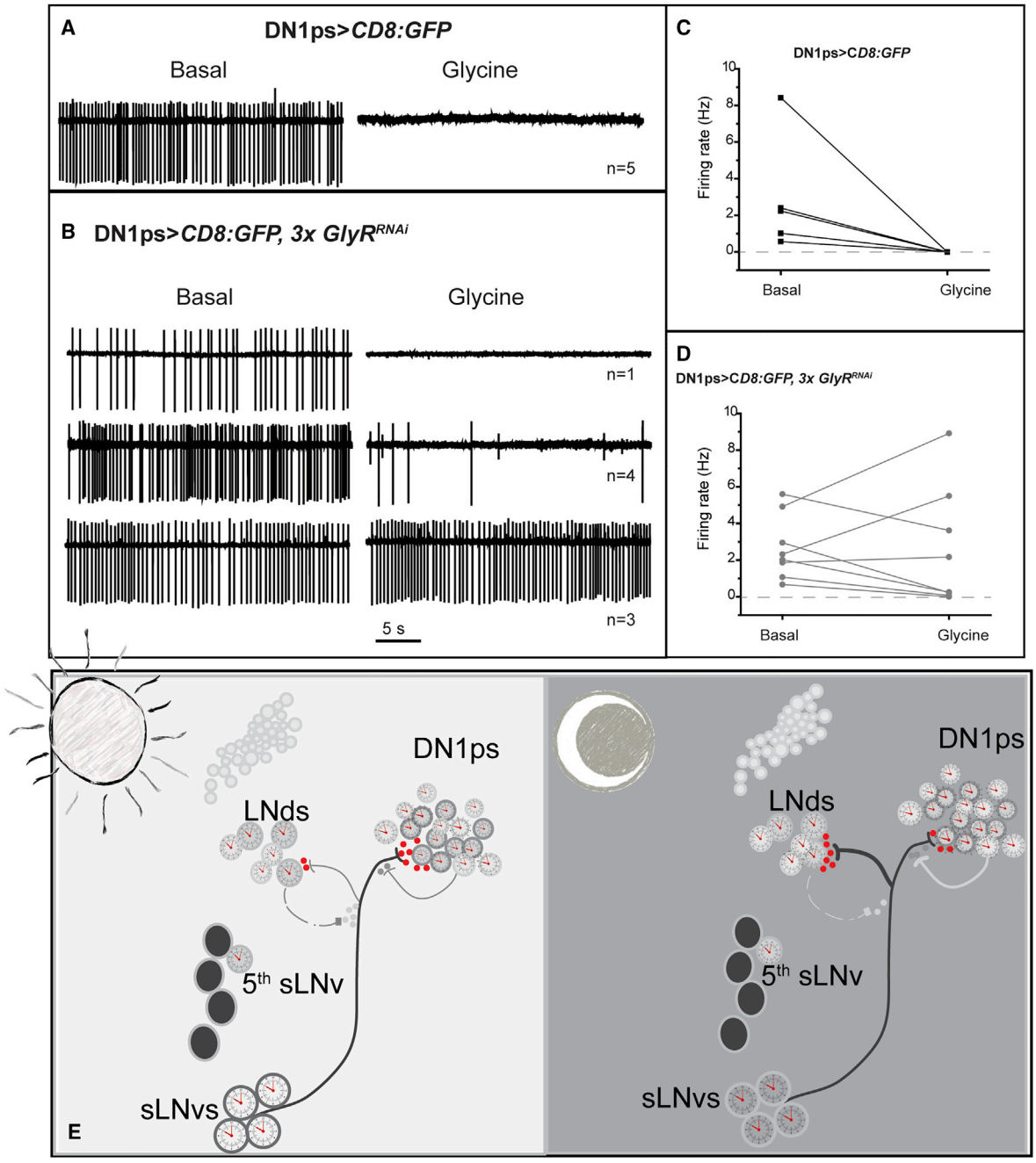

Figure 7. GRD, CG12344, and CG7589 Mediate Glycine Response in a Subset of DN1ps

(A and B) Representative loose cell-attached recordings of DN1ps in basal or after 5 mM glycine solution for a control (A) and DN1ps > CD8:GFP, Grd^{RNAi1}, $CG12344^{RNAi1}$, $CG7589^{RNAi1}$ (B). Glycine invariably stops action potential firing in controls but leads to a heterogeneous response on DN1ps expressing the combined RNAis. Only 12.5% got silenced (one out of eight), 50% showed a reduction of firing rate (four out of eight), and 37.5% showed no reduction (or even a slight increase) of firing rate (three out of eight).

(C and D) DN1ps firing rate in basal or glycine condition for control (C) and DN1ps > CD8:GFP, $GlyR^{RNAix3}$; while all controls were inhibited, a proportion of the DN1ps did not respond to glycine, underscoring their heterogeneous nature (D). Basal firing rates were variable, and thus, the mean firing rate did not differ between control and experimental groups (control, 2.9 ± 1.6 Hz; experimental, 2.7 ± 0.7 Hz; not significant [NS] after t test, $p = 0.8$; $n_{CONTROL} = 5$, $n_{GlyR}^{RNAix3} = 8$). Chi-square distribution probability test between genotypes showed that the response to glycine is statistically different in DNp1 expressing or not $GlyR^{RNAix3}$ ($\chi^2_2 = 9.48$; $p = 0.008$).

(E) A proposed model of glycine action in the circadian network. Glycine (red) from sLNvs provides a tonic inhibition to DN1ps at all times during the day. Mostly at nighttime, sLNvs connect to LNds to coordinate their activity. As our results uncovered subtle period changes upon deregulation of putative GlyR subunits in specific dorsal clusters, we postulate that both DN1ps and LNds might feed back onto the LNvs through glutamate (gray) (Guo et al., 2016) and likely acetylcholine (dotted line) (Johard et al., 2009). This feedback could, in principle, take place at specific times during the day (Petsakou et al., 2015).

Glycine Is an Inhibitory Neurotransmitter in the Invertebrate Brain

Glycinergic inhibitory transmission plays a role in nociception (Zeilhofer et al., 2012) and motor control in the brainstem and spinal cord (Harvey et al., 2008). Glycine is also a modulator of neuronal excitation mediated by NMDA receptors at glutamatergic synapses in the central brain (Balu and Coyle, 2015). Here, we found glycine in the *Drosophila* CNS (specifically, in a subset of circadian pacemakers) and found that it stops action potential firing in a postsynaptic target. Interestingly, at least some neurons of the suprachiasmatic nucleus (SCN) are inhibited in the presence of glycine (Mordel et al., 2011), highlighting another layer of conservation among circadian clocks.

Identifying Glycinergic Targets among Circadian Neurons

Glycine receptors are part of the complex cys-loop receptor family of pentameric ligand-gated ion channels. Part of the complexity resides in their ability to assemble specific receptors depending on the subunits recruited, thus leading to both excitatory and inhibitory responses (Flint et al., 1998; Lynch, 2009). Little is known about the glycinergic ones, particularly in the fruit fly. Initially, a single gene was reported as the putative glycine receptor GRD (Harvey et al., 1994). To identify additional glycine subunits, we employed an in silico approach. Three putative glycine receptor subunits share conserved features present in ligand-gated chloride channel ones. Interestingly, combined downregulation of those genes in DN1ps revealed they mediate, in part, the response to glycine. Additional subunits are also required to form other types of glycine receptors. GRD was shown to assemble functional GABA receptors in *Xenopus* oocytes only in the context of *Lcch3* (Gisselmann et al., 2004), highlighting the underlying complexity. *Grd* is also involved in GABAergic transmission, as its downregulation in specific patterns attenuates the sleep-promoting effects of a GABA-A-R agonist (Dissel et al., 2015). Thus, depending on the collection of subunits expressed in a particular neuron, GRD takes part of receptors that respond to different neurotransmitters.

Differential Effects among Circadian Clusters

We envision several scenarios accounting for the mismatch between depleting glycine in PDF neurons and the inability to respond to it in circadian targets. If other circadian neurons communicate time-related information through this fast neurotransmitter, downregulating glycine availability in LNvs would surely give rise to a different behavioral output than chronic downregulation of a subset of GlyR subunits in the entire circadian network. Additional non-circadian neurons could also be relevant in defining the properties of locomotor behavior, and the resulting unbalance (derived from inhibition of certain circadian clusters while the non-circadian remain active) would be the cause for the desynchronization; candidate neurons would be the pars intercerebralis (Cavanaugh et al., 2014). Alternatively, since the three subunits analyzed herein do not assemble all functional GlyRs, a partial impairment of glycinergic transmission is ensured. In addition, neurons could express different spliced variants of each subunit hampering the efficiency of RNAi. In sum, modifying the stoichiometry among different receptor subunits in discrete neuronal subsets may alter the properties of native glycine receptors in an unpredictable manner (i.e., ligand affinity, ion conductance, and channel kinetics), influencing neuronal responsiveness to glycine and ultimately impinging on neuronal firing. Thus, behavioral complexity ultimately reflects the combination of functional changes in individual neuronal clusters and the orchestration resulting from network interactions.

Drastically altering neuronal excitability triggered shortening or lengthening of the circadian period, depending on the cluster (Dissel et al., 2014). Likewise, disrupting glycinergic transmission led to cluster-associated changes in the free-running period. Interestingly, downregulation of single receptor subunits in the DN1ps lengthened the period, further supporting their relevance (Zhang et al., 2010a, 2010b) and their ability to feed back onto the sLNvs (Guo et al., 2016). On the other hand, altering glycine transmission onto the LNds+5thsLNv gave rise to a shorter period, a phenotype also observed upon downregulation of all three subunits (Table S4), in either the E-oscillator or the whole circadian network. Interestingly, a short period phenotype accompanied by deconsolidation of rhythmic activity is a hallmark of *pdf01* mutants, in which the LNds are uncoupled from the sLNvs and run at a faster pace (Lin et al., 2004; Yoshii et al., 2009); these similarities open the provocative possibility that both PDF and glycine released from the sLNvs play a similar role onto the LNds. Altogether, these results support the notion that each cluster has a differential contribution to the dynamic operation of the circadian network.

Is Glycine Function Analogous to that of GABA in the SCN?

Meijier and colleagues suggested that electrical activity integrates phase information from endogenous oscillators within different regions of the SCN (Albus et al., 2005). In their model, the ventral SCN can shift the dorsal SCN and cause it to resynchronize to the new phase, and GABA is required for coupling those two regions. The fact that the sLNvs release an inhibitory neurotransmitter onto dorsal clusters is reminiscent of GABA's role. In the SCN, Cl^- reversal potential changes during the day in different circadian clusters, giving rise to either excitatory or inhibitory responses to GABA (De Jeu and Pennartz, 2002); if this were true in *Drosophila*, glycinergic responses could also change in a time-of-day fashion.

We do not necessarily envision glycine operating as a synchronizing cue to the molecular clocks, a role already shown to depend on PDF, but instead, it might coordinate the activity of independent clusters to provide coherence to the circadian network (Figure 7E). Under this scenario, we propose the that sLNvs are acting as an orchestra conductor that relies on at least two batons: one fast inhibiting signal (glycine) and a slower excitatory one (PDF). Thus, the sLNv could operate as a time-of-day switch that rapidly turns off specific targets to keep the circadian network synchronized.

EXPERIMENTAL PROCEDURES

Detailed protocols are included in Supplemental Experimental Procedures.

Strains and Fly Rearing

Flies were grown and maintained at 25°C in vials containing cornmeal medium under 12:12-hr LD cycles. A complete list of the fly strains employed is included in the Supplemental Information.

RNA Preparation, Oocyte Isolation, and Cell Injection

Drosophila cDNAs encoding a long and a short version of *CG4459,* codon optimized for *Xenopus* expression, were used as templates for in vitro transcription. Na^+ dependence of glycine transport was assessed through isotonic replacement of NaCl by LiCl.

Locomotor Behavior Analysis

Newly eclosed adult males entrained to 12-hr LD cycles were monitored for activity with infrared detectors and a computerized data collection system (TriKinetics) as previously described (Beckwith and Ceriani, 2015b). Daily locomotor rhythms under special light regimes were also recorded from 3- to 5-day-old male flies in the TriKinetics system as described previously (Hermann et al., 2012).

Electrophysiology

Loose cell-attached recordings were performed under external recording solution with perfusion and between ZT2 and ZT9 as reported elsewhere (Muraro and Ceriani, 2015).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.03.034.

AUTHOR CONTRIBUTIONS

L.F. and M.F.C. were responsible for conception and design of research. L.F., N.I.M., A.N.B.G., M.S.M., J.I.R., C.H.-L., C.M.-B., and D.J.C. performed experiments. L.F., N.I.M., A.N.B.G., M.S.M., C.H.-L., G.B., J.I.R., E.M.C., C.M.-B., C.H.-F., D.J.C., and M.F.C. analyzed data. L.F., N.I.M., M.S.M., C.M.-B., A.M.B.G., and D.J.C. prepared the figures. L.F. wrote the first draft of the manuscript. L.F. and M.F.C. wrote the final version of the manuscript.

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