Reciprocal cholinergic and GABAergic modulation of the small ventrolateral pacemaker neurons of *Drosophila*’s circadian clock neuron network

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***Lelito KR, Shafer OT.*** Reciprocal cholinergic and GABAergic modulation of the small ventrolateral pacemaker neurons of *Drosophila*’s circadian clock neuron network. *J Neurophysiol* 107: 2096–2108, 2012. First published January 25, 2012; doi:10.1152/jn.00931.2011.—The relatively simple clock neuron network of *Drosophila* is a valuable model system for the neuronal basis of circadian timekeeping. Unfortunately, many key neuronal classes of this network are inaccessible to electrophysiological analysis. We have therefore adopted the use of genetically encoded sensors to address the physiology of the fly’s circadian clock network. Using genetically encoded Ca$^{2+}$ and cAMP sensors, we have investigated the physiological responses of two specific classes of clock neuron, the large and small ventrolateral neurons (l- and s-LNvs), to two neurotransmitters implicated in their modulation: acetylcholine (ACh) and γ-aminobutyric acid (GABA). Live imaging of l-LNvs, cAMP and Ca$^{2+}$ dynamics in response to cholinergic agonist and GABA application were well aligned with published electrophysiological data, indicating that our sensors were capable of faithfully reporting acute physiological responses to these transmitters within single adult clock neuron somata. We extended these live imaging methods to s-LNvs, critical neuronal pacemakers whose physiological properties in the adult brain are largely unknown. Our s-LNvs experiments revealed the predicted excitatory responses to bath-applied cholinergic agonists and the predicted inhibitory effects of GABA and established that the antagonism of ACh and GABA extends to their effects on cAMP signaling. These data support recently published but physiologically untested models of s-LNvs modulation and lead to the prediction that cholinergic and GABAergic inputs to s-LNvs will have opposing effects on the phase and/or period of the molecular clock within these critical pacemaker neurons.

***THE CIRCADIAN CLOCK*** is an innate temporal program that rhythmically orchestrates most aspects of animal behavior and physiology (Aschoff 1981). Circadian clocks are a ubiquitous adaptation to the predictable and often severe daily and seasonal fluctuations that characterize much of the Earth’s surface. The proper entrainment of these endogenous clocks to local time is a prerequisite for the optimal orchestration of clock-controlled behavioral and physiological outputs with an ever-changing environment. In animals, the master circadian clock comprises a network of central brain neurons, each of which expresses a highly conserved molecular clockwork (Herzog et al. 2006). Subsets of these neurons, the large and small ventrolateral neurons (l-LNvs and s-LNvs), are critical for the control of sleep and arousal and for several aspects of circadian timekeeping (Chung et al. 2009; Parisky et al. 2008; Renn et al. 1999; Shang et al. 2008; Sheeba et al. 2008a; Yoshii et al. 2009). The s-LNvs are thought to be the dominant neuronal pacemaker of the circadian clock neuron network under light-dark cycles and under constant darkness and temperature (Grima et al. 2004; Rieber et al. 2006; Stoleru et al. 2004, 2005). Given the important roles that these neurons serve in timekeeping, an understanding of the physiological basis of their circadian function is critical to our understanding of the clock network in *Drosophila*. The l-LNvs—probably because of their large cell body diameters and superficial locations—have been the focus of detailed electrophysiological analysis within the intact adult clock neuron network (Fogle et al. 2011; McCarthy et al. 2011; Park and Griffith 2006; Sheeba et al. 2008b). In contrast, the electrophysiological analysis of s-LNvs is challenging, and, to our knowledge, only one electrophysiological experiment, which described the resting membrane potential of s-LNvs across the diurnal cycle, has been reported for this neuron type (Cao and Nitabach 2008). Thus the physiological properties of the critical adult s-LNvs remain largely unknown. Likewise, the physiology of the majority of clock neuron classes has not been addressed through electrophysiological observations, likely owing to inaccessibility. For this reason we have sought to employ live imaging methods for the measurement of Ca$^{2+}$ and cAMP signaling to extend the physiological analysis of the adult clock neuron network beyond the l-LNvs.

Acetylcholine (ACh) and γ-aminobutyric acid (GABA) have both been proposed as important neurochemical modulators of the clock neuron networks of both insects and mammals (Albus et al. 2005; Dahdal et al. 2010; Hamasaki et al. 2005; Liu and Reppert 2000). Electrophysiological studies of l-LNvs have established that these neurons are excited by ACh through...
nicotinic acetylcholine receptors (McCarthy et al. 2011) and are inhibited by GABA, likely via ionotropic GABA_A receptors (Chung et al. 2009; McCarthy et al. 2011; Parisky et al. 2008). A growing body of work indicates that the s-LN_s are important GABA-modulated regulators of sleep and arousal in the adult fly (Chung et al. 2009; Parisky et al. 2008; Shang et al. 2008; Sheeba et al. 2008a). Several lines of evidence suggest that, like l-LN_s, s-LN_s are reciprocally modulated by ACh and GABA (Chung et al. 2009; Dahdal et al. 2010; Hamasaka et al. 2005; Parisky et al. 2008; Wegener et al. 2004). Nevertheless, the predicted physiological effects of these transmitters on adult s-LN_s have not yet been directly tested in the adult brain.

Although live cAMP imaging has been previously conducted on adult s-LN_s (Shafer et al. 2008; Shang et al. 2011), previous attempts to address the acute Ca^{2+} responses of the s-LN_s to neurotransmitters have focused on dissociated and cultured LN_s of the larval brain (Dahdal et al. 2010; Hamasaka et al. 2005; Wegener et al. 2004). These neurons persist through metamorphosis to become the adult s-LN_s (Helfrich-Förster 1997) and may maintain many aspects of their larval physiology. Nevertheless, the circadian clock network of the adult brain is radically different from the relatively simple larval clock network, and the s-LN_s neurons of the adult likely acquire a large number of new synaptic inputs and neuronal targets during metamorphosis (Helfrich-Förster et al. 2007; Kaneko and Hall 2000; Kaneko et al. 1997). Thus it is likely that some aspects of s-LN_s physiology will differ between the larval and adult stages of Drosophila. For this reason it is critical that models of connectivity and modulation be tested in the intact adult clock neuron network. Here we test the efficacy and limitations of genetically encoded Ca^{2+} and cAMP sensors for measuring the physiological responses of deeply situated neurons of the adult circadian clock neuron network and use these tools to investigate the physiological effects of ACh and GABA on the critical s-LN_pacemakers of the adult Drosophila brain.

METHODS

Fly strains. Expression of the GCaMP3.0 and Epac1-camps sensors was achieved with the previously described; UAS-GCaMP3.0; (Tian et al. 2009) and ; UAS-Epac1-camps50A; (elements (Shafer et al. 2008). We created stable lines expressing these sensors in l- and s-LN_s, by combining each of these second chromosome UAS elements with the X-chromosome PDF driver Pdf(M)-GAL4 (Renn et al. 1999). These Pdf-GAL4; UAS-GCaMP3.0; and Pdf-GAL4; UAS-Epac1-camps flies were reared under a 12:12-h light-dark cycle at 25°C on cornmeal-yeast-sucrose media. Male flies were used for all live imaging experiments and were dissected and imaged 2–4 days after adult emergence. Only flies dissected during the day were used for our experiments.

Dissection and solutions. Flies were anesthetized on ice, and the brains were dissected directly into ice-cold Tübingen and Düsseldorf Drosophila Ringer solution consisting of (in mM) 46 NaCl, 182 KCl, 3 CaCl_2, and 10 Tris, pH 7.2 (Sullivan et al. 2000). All cuticle, compound eye tissue, and large trachea were removed from the dissected brains. Brains were mounted anterior surface up in drop of hemolymph-like saline (HL3) consisting of (in mM) 70 NaCl, 5 KCl, 1.5 CaCl_2, 20 MgCl_2, 10 NaHCO_3, 5 trehalose, 115 sucrose, and 5 HEPES, pH 7.1 (Stewart et al. 1994) placed on the center of a 35-mm Falcon dish (Becton Dickerson Labware, Franklin Lakes, NJ). A petri dish insert for a PS-8H perfusion system (Bioscience Tools, San Diego, CA) was lowered around the brain. Brains were allowed to recover for 5–10 min before the start of imaging experiments. HL3 flow was established across the brain at the beginning of each experiment with the gravity-fed PS-8H perfusion system. Test compounds were applied by switching perfusion flow from the main HL3 line to a second line containing test compound for 30 s, followed by a return to HL3 flow. For vehicle controls, we switched to a second HL3 perfusion line for 30 s, followed by a return to the main HL3 line. All test compounds were purchased from Sigma-Aldrich (St. Louis, MO) and were dissolved in HL3.

For both GCaMP3.0 and Epac1-camps imaging experiments, single brains were treated with multiple doses of agonist and with vehicle controls unless otherwise noted. A typical brain received two to five agonist stimulations of differing concentrations and was allowed to recover for 5–10 min between stimulations with continuous washout with HL3 saline. Thus, for dose-response experiments, multiple concentrations of agonist and a control perfusion were delivered to each brain. Although this approach revealed dose-dependent effects of agonist treatments, the magnitudes of the individual responses were likely affected somewhat by previous treatments. We therefore used only single-agonist perfusions when comparing neuronal responses in the presence or absence of tetrodotoxin (TTX). We performed TTX treatments by diluting TTX in both the HL3 perfusion flow and in each solution of diluted agonist. To ensure that TTX had time to penetrate our intact brain preparation, brains were incubated in 2 μM TTX for 20 min before imaging and experienced 60–90 s of 2 μM TTX flow before being treated with agonist solutions containing 2 μM TTX.

Live imaging and analysis. Live imaging of the GCaMP3.0 and Epac1-camps sensors was conducted on an Olympus FV1000 laser scanning confocal microscope (Olympus, Center Valley, PA). LN_s were first located based on their basal sensor expression under epifluorescent illumination using green fluorescent protein (GFP) optics. The l- and s-LN_s were differentiated based on their cell body sizes and locations. We used a 60 × 1.0 NA W, FUMFLN objective with a dipping cone and correction collar for all live imaging experiments (Olympus). For GCaMP3.0 imaging experiments, frames were scanned with a 488-nm laser at 10 Hz for a recording duration of 5 min and GFP emission was directed to a photomultiplier tube using standard GFP optics. Epac1-camps FRET imaging was performed by scanning frames with a 440-nm laser at a frequency of 1 Hz for 5 min. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) emission were separated by a 510/10 nm dichroic mirror. In the l-LN_s, some CAMP responses to GABA were observed to have relatively long latencies; Epac1-camps time courses for GABA treatments were therefore extended to a recording duration of 10 min. For both GCaMP3.0 and Epac1-camps experiments, regions of interest (ROIs) were selected over single cell bodies of l- and s-LN_s within a single focal plane. To reduce movement artifacts for GCaMP3.0 experiments, the confocal aperture was increased to a diameter of 564 μm to increase the thickness of the optical section, and focal planes were focused on center of somata of interest. The ratiometric nature of Epac1-camps made these precautions unnecessary for our cAMP imaging experiments. Mean ROI pixel intensities for GCaMP3.0 fluorescence or Epac1-camps CFP and YFP fluorescence (value range: 0–4,095) were collected for each time point with Olympus Fluoview software (v. 10).

Raw GCaMP3.0 fluorescence traces were processed with custom analysis software written in MATLAB (MathWorks, Natick MA). GCaMP3.0 traces were first filtered with a 10-point moving average to reduce random noise, then normalized with respect to baseline fluorescence using the following equation: \( \frac{[F_{\text{p}} - F_{\text{b}}]/F_{\text{p}}]}{100} \) x 100, where \( F_{\text{p}} \) is the intensity value at each point in time and \( F_{\text{b}} \) is the baseline fluorescence calculated as the average fluorescence intensity recorded during the first 10 s of imaging. Mean ± SE traces were created based on all filtered and normalized GCaMP3.0 fluorescence traces for each neuronal class and treatment. The maximum increase in GCaMP3.0 fluorescence was determined for each filtered and normalized trace...
based on the entire 5-min duration of the recording. These values were then used to determine the mean maximum GCaMP3.0 fluorescence change for each treatment and neuron type. Rarely, individual neurons displayed rather large monotonic increases in GCaMP3.0 fluorescence over the course of an experiment. We defined outlier plots as monotonic plots with maximal changes that were two standard deviations or more from the mean maximal change for the vehicle treatment group. These plots were removed from the data set prior to the application of statistical tests.

Time course data collected for Epacl-camps experiments consisted of raw CFP and YFP values for each time point. The YFP channel was corrected to subtract CFP spillover into the YFP channel with the following equation: YFPnorm = YFP - (CFP × 0.444), where YFPnorm is the spillover-corrected YFP intensity, YFP and CFP are the raw intensity values, and 0.444 is the proportion of CFP emission that spills over into the YFP channel on our imaging system. The inverse FRET ratio, which is proportional to changes in cAMP, was calculated by taking the ratio CFP/YFPnorm. Raw inverse FRET traces were filtered with a 10-point moving average to remove high-frequency noise, and the initial time point for each trace was normalized to “1.0.” Filtered and normalized inverse FRET ratio traces were averaged to create average traces for each treatment and neuron type and were expressed as percent changes in inverse Epacl-camps FRET. The maximum increase or decrease in inverse Epacl-camps FRET was determined for each individual trace from the entire 5- or 10-min duration of the recording. These values were used to generate the mean maximum changes in inverse Epacl-camps FRET for each treatment and neuron type. For our nicotine experiments, the maximum change in inverse Epacl-camps FRET was determined for the first 3 min of the time course to compensate for the steadily increasing baseline observed for all vehicle control plots in this experiment.

For all comparisons of mean maximum change in GCaMP3.0 fluorescence or Epacl-camps inverse FRET ratio, a Kruskal-Wallis one-way ANOVA was performed on the maximum response magnitudes for each set of experimental compounds and neuron type and a Dunn’s multiple comparison test was performed to determine which treatments within the group of compounds tested produced responses significantly different from vehicle controls. Statistical significance was set at P < 0.05 for the ANOVA and multiple comparisons test. For pairwise comparisons of maximum changes in GCaMP3.0 fluorescence or inverse Epacl-camps FRET, we employed the Mann Whitney U-test. All data were statistically analyzed and plotted with Prism 5 (GraphPad, San Diego, CA). For all data, sample sizes are reported as two numbers, separated by a comma, representing the number of neurons and number of brains sampled, respectively.

RESULTS

s-LNvs retain their receptivity to cholinergic agonists in the adult brain. We first investigated the Ca2+2 responses of l-LNvs, which have been previously shown by electrophysiology to be responsive to ACh (McCarthy et al. 2011), to cholinergic agonists. For vehicle-treated l-LNvs, a gradual, steady, and relatively small decline in GCaMP3.0 fluorescence was typically observed (Fig. 1A). We found no evidence for spontaneous increases in GCaMP3.0 fluorescence in vehicle-treated or untreated l-LNvs, despite the fact that these neurons have been shown to fire spontaneous streams of action potentials, sometimes organized as low-frequency bursts (McCarthy et al. 2011; Sheeba et al. 2008b). However, given the relatively slow nature of the return to basal Ca2+ levels following bursts of action potentials in neurons (e.g., Irwin and Allen 2009) and the well-described kinetics of GCaMP3.0 responses to neuronal firing (Tian et al. 2009), we would not expect this sensor to detect such spontaneous activity.

We first tested the responses of single l-LNvs to 30-s perfusions of 10−5, 10−4, 10−3, and 10−2 M ACh and vehicle. Only ACh delivered at 10−2 M resulted in a significant increase in GCaMP3.0 fluorescence compared with vehicle controls (Fig. 1B). Given the fact that electrophysiological analysis of l-LNvs has established that l-LNvs are directly receptive to ACh (McCarthy et al. 2011), we wondered whether the apparent low sensitivity of these neurons to bath-applied ACh might be due to degradation or uptake of the transmitter within our largely intact brain preparation. We therefore tested the responses of single l-LNvs to 30-s perfusions of the cholinergic agonist carbamoylcholine (CCh) (Fig. 1, A and B). Perfusion of 10−5 M CCh did not result in significant Ca2+ responses compared with vehicle controls in l-LNvs, but CCh concentrations of 10−4 M and greater produced significant increases in GCaMP3.0 fluorescence [Fig. 1, A and B, and Supplemental Movie S1; P < 0.05 by Kruskal-Wallis 1-way ANOVA with Dunn’s multiple comparisons test; for CCh: nveh = 21 neurons from 10 brains (21,10); n10−5 = 17,7; n10−4 = 27,11; n10−3 = 16,7; n10−2 = 10,5; for ACh: nveh = 29,9; n10−5 = 21,7; n10−4 = 21,7; n10−3 = 24,8; n10−2 = 32,11).1 Thus the l-LNvs displayed Ca2+ increases consistent with neuronal excitation in response to bath-applied cholinergic agonists, with CCh eliciting much larger responses than ACh (Fig. 1B). These results indicated that GCaMP3.0 is suitable for the measurement of excitatory Ca2+ responses within single clock neuron somata of the explanted adult brain.

We next asked whether the s-LNvs of the adult brain also respond to cholinergic agonists. Figure 1C shows the mean GCaMP3.0 traces of s-LNvs to 30-s perfusions of 10−5, 10−4, 10−3, and 10−2 M CCh and vehicle. The s-LNvs displayed significant increases in GCaMP3.0 fluorescence in response to CCh concentrations of 10−4 M and higher (Fig. 1D and Supplemental Movie S1; P < 0.05 by Kruskal-Wallis 1-way ANOVA with Dunn’s multiple comparisons test; for CCh: nveh = 24,9; n10−5 = 15,5; n10−4 = 15,5; n10−3 = 13,6). The response of s-LNvs to 10−4 M CCh was markedly bimodal, with a large peak preceded by a smaller peak (Fig. 1C). This bimodality was evident in individual plots and was not due to differing latencies among individual neurons (data not shown). Higher doses of CCh caused responses that were less bimodal and dominated by a single large peak of GCaMP3.0 fluorescence. Like l-LNvs, the s-LNvs displayed stronger responses to CCh than to ACh (Fig. 1D; for ACh: nveh = 20,7; n10−5 = 18,7; n10−4 = 18,7; n10−3 = 21,8; n10−2 = 29,11).

Given the largely intact nature of our explanted brain preparation and the widespread use of ACh as an excitatory neurotransmitter in the fly brain, it is possible that some or even all of the responses of the s-LNvs to cholinergic agonists were indirect responses mediated by neuronal intermediaries. A widely used approach to determining whether a neurophysiological response is due to direct excitation employs the voltage-gated Na+ channel blocker TTX (Soderlund 2005). We therefore asked whether the Ca2+ response of s-LNvs to 10−4 M CCh persisted in the presence of 2 μM TTX, which has been previously used for electrophysiological and live imaging experiments on the explanted fly brain (McCarthy et al. 2011; Shang et al. 2011; Sheeba et al. 2008b). In the presence of TTX, s-LNvs displayed bimodal GCaMP3.0 fluo-

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1 Supplemental Material for this article is available online at the Journal website.
response increases in response to $10^{-4}$ M CCh that were highly similar to those of −TTX controls (Fig. 1E). In fact, there was no significant difference in the magnitudes of the CCh-induced GCaMP3.0 fluorescence increases displayed by s-LNvs in the presence and absence of TTX, although the +TTX responses trended toward lower magnitudes (Fig. 1F; $P = 0.2775$ by Mann-Whitney U-test; $n = 10.5$ for −TTX and $n = 9.5$ for +TTX). Our results indicate that, like l-LNvs, s-LNvs are directly excited by cholinergic agonists in the adult brain.

Nicotinic ACh receptors mediate Ca$^{2+}$ responses of s-LNvs to cholinergic agonists. Previous studies have shown that nicotinic receptors mediate the cholinergic response of adult l-LNvs (McCarthy et al. 2011). Furthermore, work in the dissociated larval central nervous system (CNS) indicates that larval s-LNvs express nicotinic ACh receptors (Dahdal et al. 2010; Wegener et al. 2004), suggesting that adult s-LNvs will likewise express nicotinic receptors. We therefore measured the effects of bath-applied nicotine on Ca$^{2+}$ dynamics within single LNvs. Thirty-second perfusions of nicotine evoked clear increases in GCaMP3.0 fluorescence in both l- and s-LNvs (Fig. 2, A, B, E, and F; $P < 0.05$ for $10^{-4}$ M CCh in l-LNvs and for $10^{-5}$ and $10^{-4}$ M CCh in s-LNvs by Kruskal-Wallis 1-way ANOVA and Dunn’s multiple comparisons test; for l-LNvs, $n_{veh} = 12.5$; $n_{10^{-5}} = 12.5$; $n_{10^{-4}} = 12.5$; $n_{10^{-5}} = 12.5$; for s-LNvs, $n_{veh} = 12.5$; $n_{10^{-5}} = 12.5$; $n_{10^{-4}} = 12.5$; $n_{10^{-5}} = 12.5$). Thus both l- and s-LNvs displayed excitatory Ca$^{2+}$ increases in response to nicotine.

We wondered whether the activation of muscarinic ACh receptors might also have contributed to the LNv Ca$^{2+}$ responses to CCh. We therefore asked whether bath application of the musca-
Fig. 2. A nicotinic but not a muscarinic ACh receptor agonist causes Ca\(^{2+}\) increases in adult LN\(_v\)-s. A and B: mean ± SE GCaMP3.0 fluorescence traces recorded from single I-LN\(_v\) (A) and s-LN\(_v\) (B) somata from brains treated with 30-s (black bar) perfusions of 10\(^{-5}\), 10\(^{-4}\), and 10\(^{-3}\) M nicotine (Nic) and vehicle (Veh). For A: n\(_{\text{veh}}\) = 12 neurons from 5 brains (12.5); n\(_{10^{-5}}\) = 12.5; n\(_{10^{-4}}\) = 12.5; n\(_{10^{-3}}\) = 12.5. For B: n\(_{\text{veh}}\) = 12.5; n\(_{10^{-5}}\) = 12.5; n\(_{10^{-4}}\) = 13.5; n\(_{10^{-3}}\) = 12.5. Key in A also applies to B–D. C and D: mean ± SE GCaMP3.0 fluorescence traces recorded from single I-LN\(_v\) (C) and s-LN\(_v\) (D) somata in response to 30-s (black bar) perfusions of 10\(^{-5}\), 10\(^{-4}\), and 10\(^{-3}\) M pilocarpine (Pilo) and Veh. For C, n\(_{\text{veh}}\) = 12; n\(_{10^{-5}}\) = 12.5; n\(_{10^{-4}}\) = 12.5; n\(_{10^{-3}}\) = 12.5. For D, n\(_{\text{veh}}\) = 12.5; n\(_{10^{-5}}\) = 12.5; n\(_{10^{-4}}\) = 12.5; n\(_{10^{-3}}\) = 12.5. E: summary of mean ± SE maximum GCaMP3.0 fluorescence increases displayed by I-LN\(_v\) in response to 10\(^{-4}\) M Nic in the presence (magenta) or absence (green) of 2 \(\mu\)M TTX. H: summary of mean maximum GCaMP3.0 fluorescence increases displayed by s-LN\(_v\) in response to 10\(^{-4}\) M Nic in the presence (magenta) or absence (green) of TTX based on data shown in G. There was no significant difference in mean maximum fluorescence increases between the +TTX and −TTX conditions (P = 0.3793 by Mann-Whitney U-test).

The muscarinic agonist pilocarpine had no measurable effects on the Ca\(^{2+}\) dynamics in I- and s-LN\(_v\)-s. Thirty-second perfusions of 10\(^{-5}\) to 10\(^{-3}\) M pilocarpine had no significant effects on GCaMP3.0 fluorescence in either I- or s-LN\(_v\)-s (Fig. 2, C–F; P > 0.05 for all pairwise comparisons of pilocarpine treatments with vehicle control by Kruskal-Wallis 1-way ANOVA with Dunn’s multiple comparisons test; for I-LN\(_v\), n\(_{\text{veh}}\) = 12.5; n\(_{10^{-5}}\) = 12.5; n\(_{10^{-4}}\) = 12.5; n\(_{10^{-3}}\) = 12.5; for s-LN\(_v\), n\(_{\text{veh}}\) = 12.5; n\(_{10^{-5}}\) = 12.5; n\(_{10^{-4}}\) = 12.5; n\(_{10^{-3}}\) = 12.5). Thus the muscarinic agonist had no measurable effects on the Ca\(^{2+}\) dynamics of I- and s-LN\(_v\)-s, consistent with previous work on dissociated larval LN\(_v\)-s indicating that these neurons are not sensitive to pilocarpine (Wegener et al. 2004).

We next confirmed that the effects of nicotine on Ca\(^{2+}\) in the s-LN\(_v\)-s were direct by comparing the GCaMP3.0 responses of s-LN\(_v\)-s treated with 10\(^{-4}\) M nicotine in the presence or absence of 2 \(\mu\)M TTX. Under both conditions, 30-s perfusions of nicotine caused clear increases in GCaMP3.0 fluorescence whose maximum changes were not significantly different (Fig. 2, G and H; for −TTX, n = 25.8; for +TTX n = 27.10; P = 0.3793 by Mann-Whitney U-test). Thus s-LN\(_v\)-s are directly receptive to nicotine, further supporting the conclusion that these critical pacemaker neurons express nicotinic ACh receptors in the adult brain.

Carbamoylcholine causes acute cAMP increases in ventrolateral neurons. G protein and cAMP signaling is critical for the maintenance of normally paced circadian rhythms in animals (Dahdal et al. 2010; O’Neill et al. 2008). We therefore wondered whether cholinergic excitation of adult LN\(_v\)-s had acute effects on cAMP signaling. To further characterize the nature of the cholinergic responses in these neurons, we used the genetically encoded cAMP sensor Epac1-camps to measure the effects of bath-applied cholinergic agonists on cAMP signaling. Figure 3A shows the mean inverse Epac1-camps FRET traces for single I-LN\(_v\)-s treated with 30-s perfusions of 10\(^{-5}\), 10\(^{-4}\), 10\(^{-3}\), and 10\(^{-2}\) M CCh and vehicle. Concentrations of 10\(^{-4}\) and 10\(^{-3}\) M CCh evoked significant increases in Epac1-camps FRET ratio in I-LN\(_v\)-s compared with vehicle controls (Fig. 3C; P < 0.05 by Kruskal-Wallis 1-way ANOVA with Dunn’s multiple comparisons test; n\(_{\text{veh}}\) = 15.6; n\(_{10^{-5}}\) = 17.7; n\(_{10^{-4}}\) = 17.7; n\(_{10^{-3}}\) = 17.7; n\(_{10^{-2}}\) = 17.7). The Epac1-camps responses to CCh appeared to be biphasic, with a small and brief decrease in inverse FRET followed by a large increase in the inverse FRET ratio (Fig. 3A). However, this initial loss of inverse FRET may simply have been a reflection of a steady loss of inverse FRET, which was apparent in the vehicle controls, preceding the large CCh-induced cAMP increase (Fig. 3A). The 10\(^{-2}\) M CCh treatment did not result in significant cAMP increases compared with vehicle controls, consistent with previous work in dissociated larval neurons, which revealed decreased Ca\(^{2+}\) responses at very high ACh doses (Wegener et al. 2004) (Fig. 3, A and C).

The s-LN\(_v\)-s displayed a highly similar pattern of Epac1-camps responses to perfusions of 10\(^{-5}\), 10\(^{-4}\), 10\(^{-3}\), and 10\(^{-2}\)
M CCh (Fig. 3B). Concentrations of $10^{-4}$ and $10^{-3}$ M CCh evoked significant increases in Epac1-camps FRET ratio in s-LN$_v$s compared with vehicle controls (Fig. 3C; $P < 0.05$ by Kruskal-Wallis 1-way ANOVA with Dunn’s multiple comparisons test; $n_{veh} = 12.5; n_{10^{-4}} = 15.6; n_{10^{-5}} = 15.6; n_{10^{-3}} = 15.6; n_{10^{-2}} = 15.6$). Like l-LN$_v$s, the s-LN$_v$s displayed biphasic responses to $10^{-4}$ and $10^{-3}$ M CCh and failed to display significant responses to the $10^{-2}$ M treatment relative to vehicle controls (Fig. 3B; $P > 0.05$ by Kruskal-Wallis with Dunn’s multiple comparisons test). For s-LN$_v$s the bimodality of the cAMP response was less equivocal than that of l-LN$_v$s, as the vehicle controls displayed no obvious loss of inverse FRET immediately following perfusion. We do not know the cellular basis for this bimodality or how it might relate to the bimodality observed in the s-LN$_v$s Ca$^{2+}$ response to $10^{-4}$ M CCh (Fig. 1C), but it might reflect the nature of the coupling of acute ionotropic excitation to cAMP increases in these neurons. Nevertheless, these results suggest that cholinergic excitation is coupled to cAMP increases in the l- and s-LN$_v$s of the adult brain.

To confirm that the cAMP responses of the critical s-LN$_v$ pacemakers were in fact a direct response to CCh and not an indirect modulatory effect of other CCh-stimulated pathways, we asked whether CCh-induced cAMP increases in s-LN$_v$s persisted in the presence of TTX. Indeed, the cAMP responses of s-LN$_v$s to CCh was direct, as the increase in inverse Epac1-camps FRET caused by $10^{-4}$ M CCh persisted in the presence of 2 $\mu$M TTX (Fig. 3, D and E). There was no significant difference in the maximum increases in inverse Epac1-camps FRET between the +TTX and −TTX conditions (Fig. 3E; $P = 0.4074$ by Mann-Whitney U-test; $n = 25,10$ for +TTX and $n = 34,10$ for −TTX). These results indicate that direct cholinergic excitation is coupled to cAMP increases in the critical s-LN$_v$s pacemakers of the adult brain.

Nicotinic ACh receptors mediate cAMP responses of s-LN$_v$s to cholinergic agonists. The finding that cholinergic excitation is coupled to cAMP increases in the LN$_v$s has important implications for how the molecular clock within these neurons might be affected by synaptic inputs, as cAMP signaling is thought to be a central component of circadian timekeeping in animals (Levine et al. 1994; O’Neill et al. 2008). We therefore sought to characterize the cholinergic stimulation of cAMP in the LN$_v$s by pharmacologically addressing the nature of the ACh receptors that underlie it. Bath-applied $10^{-4}$ and $10^{-5}$ M nicotine, doses that caused consistent Ca$^{2+}$ responses in LN$_v$s (Fig. 2, A and B), also caused inverse Epac1-camps FRET increases in both l- and s-LN$_v$s (Fig. 4, A and B). The l-LN$_v$s also responded to $10^{-5}$ M nicotine (Fig. 4A). Because of constantly increasing inverse FRET levels in vehicle-treated and nonresponding neurons, our conservative statistical analysis indicated significant cAMP responses only at the $10^{-4}$ M dose for l-LN$_v$s and at the $10^{-5}$ M dose for s-LN$_v$s (Fig. 4, E and F; $P < 0.05$ for these 2 treatments vs. vehicle controls by Mann-Whitney U-test).
A nicotine but not a muscarinic receptor agonist causes cAMP increases in adult LNvs. A and B: mean ± SE inverse Epac1-camps FRET traces recorded from single I-LNv, (A) and s-LNv, (B) somata from brains treated with 30-s perfusions (black bar) of 10⁻⁵, 10⁻⁴, and 10⁻³ M nicotine (Nic) and vehicle (Veh). For A, nₑᵥ = 18 neurons from 5 brains (18,5); n₁₀⁻⁵ = 18,5; n₁₀⁻⁴ = 18,5; n₁₀⁻³ = 17,5. For B, nₑᵥ = 14,5; n₁₀⁻⁵ = 14,5; n₁₀⁻⁴ = 14,5; n₁₀⁻³ = 14,5. Key in A also applies to B–D. C and D: mean ± SE inverse Epac1-camps FRET traces recorded from single I-LNv, (C) and s-LNv, (D) somata from brains treated with 30-s perfusions of 10⁻⁵, 10⁻⁴, and 10⁻³ M pilocarpine (Pilo) and Veh. For C, nₑᵥ = 18,5; n₁₀⁻⁵ = 21,6; n₁₀⁻⁴ = 18,5; n₁₀⁻³ = 18,5. For D, nₑᵥ = 14,5; n₁₀⁻⁵ = 14,5; n₁₀⁻⁴ = 14,5; n₁₀⁻³ = 14,5. Scale bars in D apply to A–D.

E: summary of mean ± SE maximum increases in inverse Epac1-camps FRET for I-LNv responses to Nic, Pilo, and Veh shown in A and C. F: summary of mean maximum increases in inverse Epac1-camps FRET for s-LNv responses to Nic, Pilo, and Veh shown in B and D. For E and F, asterisks indicate a mean maximum change in inverse FRET that was significantly different from the change evoked by Veh (P < 0.05, by Kruskal-Wallis ANOVA and Dunn’s multiple comparisons test). G: mean ± SE inverse Epac1-camps FRET traces recorded from single s-LNv somata from brains treated with 30-s perfusions of 10⁻³ M Nic in the presence (magenta; n = 14,6) or absence (green; n = 13,6) of 2 μM TTX. H: summary ± SE of mean maximum increases in inverse FRET displayed by s-LNv soes based on data in G. Mean maximum increase in inverse FRET did not differ significantly between the +TTX and −TTX conditions (P = 0.2541 by Mann-Whitney U-test).

Kruskal-Wallis 1-way ANOVA and Dunn’s multiple comparisons test, P > 0.05 for all remaining doses of nicotine vs. vehicle perfusion; for I-LNv, nₑᵥ = 18,5; n₁₀⁻⁵ = 18,5; n₁₀⁻⁴ = 18,5; n₁₀⁻³ = 17,5; for s-LNv, nₑᵥ = 14,5; n₁₀⁻⁵ = 14,5; n₁₀⁻⁴ = 14,5; n₁₀⁻³ = 14,5. The steady increase in the inverse Epac1-camps FRET observed for vehicle controls was due to an uneven photobleaching of YFP and CFP, with greater bleaching for the former fluorophore, as has been reported in previous studies using Epac1-camps (Börner et al. 2011). It was not clear why this occurred in some experiments but not others. Nevertheless, these results indicate that bath-applied nicotine causes increases in cAMP in I- and s-LNv.

We wondered whether the activation of muscarinic ACh receptors might also have contributed to the LNv cAMP responses to Ch (Fig. 3, A–C). We therefore tested the effects of pilocarpine perfusion on inverse Epac1-camps FRET levels in I- and s-LNv. Thirty-second perfusions of 10⁻⁵ to 10⁻³ M pilocarpine had no measurable effects on inverse Epac1-camps FRET in I- or s-LNv (Fig. 4, C–F; P > 0.05 for all comparisons of pilocarpine dose and vehicle control by Kruskal-Wallis 1-way ANOVA and Dunn’s multiple comparisons test; for I-LNv, nₑᵥ = 18,5; n₁₀⁻⁵ = 21,6; n₁₀⁻⁴ = 18,5; n₁₀⁻³ = 18,5; for s-LNv, nₑᵥ = 14,5; n₁₀⁻⁵ = 14,5; n₁₀⁻⁴ = 14,5; n₁₀⁻³ = 14,5). These results suggest that muscarinic pathways do not significantly affect cAMP levels in adult LNv.

To confirm that the cAMP responses of s-LNv to nicotine were direct responses rather than indirect modulatory effects of other nicotine-sensitive pathways, we asked whether nicotine-induced cAMP increases in s-LNv persisted in the presence of TTX. Indeed, the cAMP responses of s-LNv to nicotine were direct, as significant increases in inverse Epac1-camps FRET in response to 30-s perfusions of 10⁻⁴ M nicotine persisted in the presence of 2 μM TTX (Fig. 4, G and H). The s-LNv appeared to display slightly smaller and briefer cAMP responses in the presence of toxin (Fig. 4G), but a statistical comparison of the maximum changes in inverse Epac1-camps FRET displayed in the +TTX and −TTX conditions revealed no significant differences in the mean maximum increases (Fig. 4H; P = 0.2541 by Mann-Whitney U-test; n = 14,6 for +TTX and n = 13,6 for −TTX). Taken together, our results indicate that the direct activation of nicotinic ACh receptors is coupled to cAMP increases in s-LNv of the adult brain.

GABA antagonizes cholinergic excitation in adult LNv. We next tested the ability of the GCaMP3.0 and Epac1-camps sensors to detect inhibitory responses in single adult LNv soma. The I-LNv expresses the GABA_A receptor RDL, an inhibitory ligand-gated Cl⁻ channel (Chung et al. 2009; Parisky et al. 2008), and electrophysiological analysis has established that bath application of GABA inhibits I-LNv action potentials, likely via GABA_A receptors (McCarthy et al. 2011). We therefore asked whether GCaMP3.0 imaging could detect acute GABAergic inhibition in I-LNv. Thirty-second perfusions of GABA alone at concentrations of 10⁻⁴, 10⁻³, and 10⁻² M had
no significant effects on GCaMP3.0 fluorescence compared with vehicle controls (Fig. 5, A and C; P > 0.05 for all 3 concentrations by Kruskal-Wallis 1-way ANOVA and Dunn’s multiple comparisons test; $n_{\text{veh}} = 14.5; n_{10^-3} = 14.5; n_{10^-2} = 14.5; n_{10^-1} = 14.5$). It is possible that GABA alone might not alter Ca$^{2+}$ levels significantly, particularly if Ca$^{2+}$ was already at or near baseline levels, or that GCaMP3.0 is insufficiently sensitive to detect inhibitory effects on intracellular Ca$^{2+}$. We wondered whether GCaMP3.0 could detect GABAergic inhibition when GABA is coapplied with an excitatory stimulus, as has been shown previously for dissociated larval LN$s$ (Dahdal et al. 2010). We therefore tested whether coaplication of GABA with CCh would reduce or abolish the direct CCh response of I-LN$s$. In the presence of 2 μM TTX, coapplication of 10$^{-3}$ M GABA and 10$^{-4}$ M CCh significantly reduced the Ca$^{2+}$ response to CCh by the I-LN$s$ (Fig. 5, D and F; $P = 0.0096$ by Mann-Whitney U-test; $n_{\text{CCh}} = 12.5$ and $n_{\text{CCh\&GABA}} = 12.5$).

We next examined GABA’s effects on s-LN$s$, whose physiological responses to GABA have not been previously addressed in the adult brain. As for I-LN$s$, perfusion of GABA alone had no significant effects on GCaMP3.0 fluorescence in s-LN$s$ (Fig. 5, B and C; P > 0.05 for all 3 concentrations by Kruskal-Wallis 1-way ANOVA and Dunn’s multiple comparisons test; $n_{\text{veh}} = 17.5; n_{10^-3} = 17.5; n_{10^-2} = 17.5; n_{10^-1} = 17.5$), but coaplication of GABA and CCh revealed that GABA significantly reduced the s-LN$s$ response to CCh in the presence of 2 μM TTX (Fig. 5, E and F; $P = 0.0122$ by Mann-Whitney U-test; $n_{\text{CCh}} = 11.5$ and $n_{\text{CCh\&GABA}} = 11.5$). Thus, as predicted by studies on dissociated larval LN$s$ (Dahdal et al. 2010), adult s-LN$s$ were directly inhibited by bath-applied GABA.

**GABA causes acute reduction in cAMP levels in adult LN$s$.** There is evidence for the expression of both ionotropic GABA$_A$ receptors and metabotropic GABA$_B$ receptors by the PDF-positive I- and s-LN$s$ (Chung et al. 2009; Dahdal et al. 2010; Hamasaka et al. 2007; Parisky et al. 2008). In mammals GABA$_B$ receptors are negatively coupled to adenylate cyclase through $G_o$ signaling, and *Drosophila* GABA$_B$ receptors expressed in mammalian cell lines can elicit decreases in cAMP (Bettler et al. 2004; Mezler et al. 2001). If GABA$_B$ receptors signal through $G_o$ in *Drosophila* and are indeed present in the LN$s$, we would predict that GABA application would result in a reduction in cAMP levels. Thirty-second perfusions of GABA alone at 10$^{-4}$ to 10$^{-2}$ M resulted in significant reductions in inverse Epac1-camps FRET in I-LN$s$ relative to vehicle controls, consistent with a GABA-induced loss of cAMP in these neurons (Fig. 6, A and C; $P < 0.05$ for these concentrations compared with vehicle by Kruskal-Wallis 1-way ANOVA and Dunn’s multiple comparisons test; $n_{\text{veh}} = 30.11; n_{10^-2} = 17.6; n_{10^-3} = 17.6; n_{10^-1} = 17.7$). Likewise, 30-s perfusions of 10$^{-3}$ M GABA resulted in significant decreases in inverse Epac1-camps FRET in s-LN$s$ (Fig. 6, B and C; $P < 0.05$ for this dose compared with vehicle by Kruskal-Wallis 1-way ANOVA and Dunn’s multiple compar-

![Fig. 5. GABA alone has no acute effects on GCaMP3.0 fluorescence in adult LN$s$, but inhibits their CCh-induced Ca$^{2+}$ responses. A and B: mean ± SE GCaMP3.0 fluorescence traces recorded from single I-LN$\nu$ (A) and s-LN$\nu$ (B) somata from brains treated with 30-s (black bar) perfusions of 10$^{-4}$, 10$^{-3}$, and 10$^{-2}$ M GABA and vehicle (Veh). Scale bars in B apply to both A and B. For A, $n_{\text{veh}} = 14$ neurons from 5 brains (14.5); $n_{10^-4} = 14.5; n_{10^-3} = 14.5; n_{10^-2} = 14.5$. For B, $n_{\text{veh}} = 17.5; n_{10^-3} = 17.5; n_{10^-2} = 17.5; n_{10^-1} = 17.5$. Key in A also applies to B. C: summary of mean maximum GCaMP3.0 fluorescence changes for I-LN$\nu$ and s-LN$\nu$ responses to GABA. Values are based on data shown in A and B. There was no significant difference between the effects of any concentration of GABA perfusion and Veh controls ($P > 0.05$ by Kruskal-Wallis ANOVA and Dunn’s multiple comparisons test). D and E: mean ± SE GCaMP3.0 fluorescence traces recorded from single I-LN$\nu$ (D) and s-LN$\nu$ (E) somata from brains treated with 30-s perfusions of 10$^{-4}$ M CCh with (magenta) and without (green) coaplication of 10$^{-3}$ M GABA. These experiments were conducted in the presence of 2 μM TTX. Sample sizes were $n_{\text{CCh}} = 12.5$ and $n_{\text{CCh\&GABA}} = 12.5$ for D and $n_{\text{CCh}} = 11.5$ and $n_{\text{CCh\&GABA}} = 11.5$ for E. F: summary of mean ± SE maximum GCaMP3.0 fluorescence intensity increases recorded from I-LN$\nu$ and s-LN$\nu$, in response to 10$^{-4}$ M CCh with or without coaplication of 10$^{-3}$ M GABA in the presence of 2 μM TTX. Values are based on data shown in D and E. Asterisks indicate a significant difference in mean maximum CCh-induced increase in GCaMP3.0 fluorescence recorded in the presence or absence of GABA ($P_{\text{I-LN$\nu$}} = 0.0096, P_{\text{s-LN$\nu$}} = 0.0122$ by Mann-Whitney U-test).
isons test, \( P > 0.05 \) for the remaining concentrations; \( n_{\text{veh}} = 26,11; n_{10^{-4}} = 26,11; n_{10^{-3}} = 14,5; n_{10^{-2}} = 11,5 \). The GABA-induced loss of inverse FRET displayed by l- and s-LN \(_s\) was due to a reciprocal increase in YFP and decreases in CFP emission (i.e., caused by bona fide FRET changes) and not due to uneven photobleaching (data not shown).

Although both classes of LN \(_n\) neurons responded to GABA with cAMP decreases, the character of their responses differed. The l-LN \(_n\) cAMP response was relatively large and long lasting, with cAMP levels remaining low for the duration of the 10-min time course experiment (Fig. 6A). The s-LN \(_n\) response to 10\(^{-3}\) M GABA, in contrast, was relatively small, and transient compared with the l-LN \(_n\) responses (compare Fig. 6, A and B). Furthermore, in contrast to l-LN \(_n\), s-LN \(_n\) displayed no apparent response to 10\(^{-2}\) M GABA, whereas l-LN \(_n\) displayed large cAMP decreases in response to this dose (compare Fig. 6, A and B). These results suggest that bath-applied GABA causes significant reductions of cAMP in both l- and s-LN \(_n\) and that these two neuronal classes may differ in their transduction of GABAergic input.

Anatomical and genetic evidence suggests that the responses of s-LN \(_n\) to GABAergic inputs are the result of the direct effects of agonist binding receptors expressed by s-LN \(_n\) (Chung et al. 2009; Dahdal et al. 2010; Hamasaka et al. 2007; Parisky et al. 2008). We therefore asked whether GABA-induced cAMP decreases persisted in s-LN \(_n\) in the presence of 2 \( \mu \)M TTX. Thirty-second perfusions of 10\(^{-3}\) M GABA produced clear decreases in inverse Epac1-camps FRET in the presence of TTX with magnitudes that were not significantly different from those displayed in the absence of toxin (Fig. 6, D and E; \( P = 0.0503 \) by Mann-Whitney \( U \)-test; \( n = 34,12 \) for +TTX and \( n = 30,11 \) for −TTX). We conclude that GABA’s inhibitory effect on cAMP in the s-LN \(_n\) was direct.

GABA antagonizes cholinergic cAMP increases in s-LN \(_n\). Our GCamp3.0 imaging experiments indicated that GABA antagonizes CCh’s effects on Ca\(^{2+}\) in l- and s-LN \(_n\). We next asked whether GABA also inhibits the excitatory cAMP increases caused by CCh in these neurons. Coapplication of 10\(^{-3}\) M GABA with 10\(^{-4}\) M CCh did not significantly reduce the CCh-induced increase in cAMP in the l-LN \(_n\) (Fig. 7, A and C; \( P = 0.0915 \) by Mann-Whitney \( U \)-test; \( n_{\text{CCh}} = 17,5 \) and \( n_{\text{CCh}+\text{GABA}} = 17,5 \), although in some single-neuron traces a residual decrease in cAMP that resembled an inhibitory cAMP response to GABA was observed following the excitatory CCh response (data not shown). In contrast, coapplication of 10\(^{-3}\) M GABA significantly reduced the cAMP response to 10\(^{-4}\) M CCh in s-LN \(_n\) (Fig. 7, B and C; \( P = 0.0048 \) by Mann-Whitney \( U \)-test; \( n_{\text{CCh}} = 21,7 \) and \( n_{\text{CCh}+\text{GABA}} = 21,8 \)). The inhibitory effect of GABA on CCh-induced cAMP increases was direct, as coapplication of 10\(^{-3}\) M GABA significantly reduced the magnitude of the cAMP response to CCh in the presence of 2 \( \mu \)M TTX (Fig. 7, D and E; \( P = 0.0173 \) by Mann-Whitney \( U \)-test; \( n_{\text{CCh}+\text{TTX}} = 12,5 \) and \( n_{\text{CCh+GABA+TTX}} = 9,5 \)). These results suggest that GABAergic input directly antagonizes the effects of cholinergic input on cAMP levels within the s-LN \(_n\) in adult brain.

**Fig. 6.** GABA causes cAMP decreases in adult LN \(_n\); A and B: mean ± SE inverse Epac1-camps FRET traces recorded from single l-LN \(_n\), (A) and s-LN \(_n\), (B) somata from brains treated with 30-s perfusions (black bar) of 10\(^{-4}\), 10\(^{-3}\), and 10\(^{-2}\) M GABA. Scale bars in B also apply to A. For A, \( n_{\text{veh}} = 30 \) neurons from 11 brains (30,11); \( n_{10^{-4}} = 17,6; n_{10^{-3}} = 17,6; n_{10^{-2}} = 17,7 \). For B, \( n_{\text{veh}} = 26,11; n_{10^{-4}} = 26,11; n_{10^{-3}} = 14,5; n_{10^{-2}} = 11,5 \). Key in A also applies to B. C: summary of mean ± SE maximum decreases in inverse Epac1-camps FRET recorded from l- and s-LN \(_n\) in response to GABA perfusion. Values are based on data shown in A and B. Asterisks indicate a maximum decrease in inverse Epac1-camps FRET that was significantly different from the maximum decrease evoked by Veh \( (P < 0.05 \) by Kruskal-Wallis ANOVA and Dunn’s multiple comparisons test). D: mean ± SE inverse Epac1-camps FRET traces recorded from single s-LN \(_n\), somata from brains treated with 30-s perfusions (black bar) of 10\(^{-3}\) M GABA in the presence (magenta; \( n = 34,12 \)) or absence (green; \( n = 30,11 \)) of 2 \( \mu \)M TTX. E: summary of mean maximum decreases in inverse Epac1-camps FRET displayed by s-LN \(_n\) in response to 10\(^{-3}\) M GABA in the presence or absence of 2 \( \mu \)M TTX. Values are based on data displayed in D. There was no significant difference in mean maximum loss of inverse Epac1-camps FRET between the +TTX and −TTX conditions \( (P = 0.0503 \) by Mann-Whitney \( U \)-test).
DISCUSSION

In *Drosophila*, circadian timekeeping appears to be distributed across multiple classes of clock neuron and is thought to depend on physiological interactions between them (Grima et al. 2004; Lin et al. 2004; Peng et al. 2003; Picot et al. 2007; Rieger et al. 2006; Stoleru et al. 2004, 2005). Likewise, the synchronization of the circadian clock neuron network to the environment is maintained, at least in part, by inputs from peripheral sensory receptors, which must ultimately modulate the physiology and molecular timekeeping of the clock neuron network (Helfrich-Förster 2002; Helfrich-Förster et al. 2001; Klarsfeld et al. 2004; Sehadova et al. 2009). However, the physiological basis of clock network timekeeping, its modulation by sensory inputs, and the neurotransmitters employed by these pathways are largely unknown in the adult fly brain. The electrophysiological inaccessibility of much of the fly’s circadian clock neuron network is a fundamental barrier to understanding the neural basis of timekeeping in the fly. Recently developed genetically encoded sensors for Ca\(^{2+}\) and cAMP signaling make possible the measurement of physiological responses in electrophysiologically inaccessible neurons of the clock network. Here we have tested the performance of two of these sensors, the Ca\(^{2+}\) sensor GCaMP3.0 and the cAMP sensor Epac1-camps, within single deeply situated adult clock neurons and have used them to test the predicted effects of ACH and GABA on the critical s-LN\(\nu\) pacemakers of the adult circadian clock neuron network.

**Testing the sensitivity of genetically encoded sensors in single adult clock neuron somata.** The 1-LN\(\nu\)s are the only neurons of the adult clock neuron network that have been routinely analyzed electrophysiologically in the intact adult brain (Cao and Naitaba 2008; Fogle et al. 2011; McCarthy et al. 2011; Park and Griffith 2006; Sheeba et al. 2008b). We therefore tested the performance of GCaMP3.0 and Epac1-camps within the 1-LN\(\nu\) in response to modulators with established excitatory and inhibitory effects on these neurons. For single 1-LN\(\nu\)s, we find that GCaMP3.0 readily detects acute responses to excitatory agonists but fails to detect acute ionotropic inhibitory responses. Given the strong coupling of neuronal excitation with Ca\(^{2+}\) entry, it was not surprising that GCaMP3.0 could detect excitatory responses within single 1-LN\(\nu\)s. In contrast, the suitability of genetically encoded Ca\(^{2+}\) sensors for the measurement of acute inhibitory responses seems less straightforward. Nevertheless, GCaMP sensors can be used to detect inhibitory effects in the presence of excitation, as previously reported (Dahdal et al. 2010; Ignell et al. 2009).

Previous experiments on dissociated larval LN\(\nu\)s found that GABA application resulted in significant decreases in Ca\(^{2+}\) as reported by fura-2 imaging (Hamasaka et al. 2005). We suspect that this might reflect the high sensitivity of the synthetic Ca\(^{2+}\) dye relative to genetically encoded sensors. Indeed, experiments using an earlier version of GCaMP in a similar dissociated larval preparation were unable to detect these inhibitory responses to GABA (Dahdal et al. 2010). We therefore suspect that the Ca\(^{2+}\) changes induced by GABA application are too small to be detected by GCaMP3.0.

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**Fig. 7.** GABA inhibits CCh-induced cAMP increases in s-LN\(\nu\)s but not in 1-LN\(\nu\)s. A and B: mean ± SE inverse Epac1-camps FRET ratio traces recorded from single 1-LN\(\nu\) (A) and s-LN\(\nu\) (B) somata from brains treated with 30-s perfusions of 10 \(^{-4}\) M CCh applied with (magenta) or without (green) 10 \(^{-4}\) M GABA. Scale bars in B also apply to A. For A, \(n_{CCh} = 17\) neurons from 5 brains (17.5); \(n_{CCh\&GABA} = 17.5\). For B, \(n_{CCh} = 21.7; n_{CCh\&GABA} = 21.8\). Key in A also applies to B. C: summary of mean ± SE maximum inverse Epac1-camps FRET increases recorded in 1- and s-LN\(\nu\)s in response to 10 \(^{-4}\) M CCh applied alone (green) or with 10 \(^{-4}\) M GABA (magenta). Values are based on data displayed in A and B. GABA did not significantly change the cAMP response to CCh in 1-LN\(\nu\)s (\(P = 0.0915\) by Mann-Whitney U-test). GABA significantly reduced the CCh-induced cAMP response in s-LN\(\nu\)s (\(P = 0.0048\) by Mann-Whitney U-test). D: mean ± SE maximum inverse Epac1-camps FRET traces recorded from single s-LN\(\nu\) somata from brains treated with 30-s perfusions of 10 \(^{-4}\) M CCh applied with (magenta; \(n = 9.5\)) or without (green; \(n = 12.5\)) 10 \(^{-4}\) M GABA in the presence of 2 \(\mu\)M TTX. E: summary of mean ± SE maximum increases in inverse Epac1-camps FRET displayed by s-LN\(\nu\)s in response to 10 \(^{-4}\) M CChs applied with (magenta) or without (green) 10 \(^{-4}\) M GABA in the presence of TTX. Values are based on data displayed in D. GABA significantly reduced the CCh-induced cAMP response of s-LN\(\nu\)s in the presence of toxin (\(P = 0.0173\) by Mann-Whitney U-test).
low for detection by GCaMP3.0 and that coapplication of putative inhibitors with excitatory agonists is necessary to address inhibition when using this sensor. We wonder if newly developed Ca²⁺ sensors (Markova et al. 2008; Waseem et al. 2010) might be more suitable than GCaMP3.0 for the detection of such acute inhibitory input within deep networks of the fly brain. In contrast to our results with GCaMP3.0 and Ca²⁺ signaling, we found that Epac1-camps was capable of detecting inhibitory effects on cAMP signaling. However, it is important to note that despite the fact that Epac1-camps is nearly 10 times more sensitive to cAMP than to cyclic guanosine monophosphate (cGMP) in vitro (Nikolaev et al. 2004), it appears to be sensitive to physiologically relevant cGMP levels in fly neurons (Shakiryanova and Levitan 2008).

The 1-LNc neurons displayed much stronger responses to CCh compared with ACh (Fig. 1). The relatively intact nature of our brain preparation may account for the relatively high efficacy of CCh in our study. ACh, a widespread excitatory neurotransmitter in the insect CNS, is tightly regulated in the intact brain via degradation by acetylcholinesterases (Treherne and Smith 1965a, 1965b). Consistent with this notion, previous studies of cholinergic responses in another dipteran CNS have reported a relatively high efficacy of CCh relative to ACh (Brotz et al. 1995).

**Testing predicted physiological responses of s-LNvs to cholinergic and GABAergic stimulation.** Having characterized the sensitivity of GCaMP3.0 and Epac1-camps for imaging excitatory and inhibitory responses within the physiologically well-studied 1-LNvs, we turned our attention to the s-LNvs. Unlike 1-LNvs, these neurons are critical for the maintenance of circadian rhythms and are thought to play a central role in the synchronization of the various clock neuron classes (Grima et al. 2004; Rieger et al. 2006; Shafer and Taghert 2009; Stoleru et al. 2004). Understanding the physiological basis of s-LNvs function is therefore critical to our understanding the clock network. The small diameters and deep brain locations of the s-LNvs have made electrophysiological analysis challenging (Cao and Nitabach 2008). We therefore sought to test the predicted effects of cholinergic and GABAergic agonists on s-LNvs using genetically encoded sensors.

Our results indicate that s-LNvs maintain their receptivity to cholinergic agonists via nicotinic ACh receptors, as predicted by previous work on the dissociated larval CNS (Dahdal et al. 2010; Wegener et al. 2004). Ca²⁺ and cAMP responses of s-LNvs to nicotine persisted in the presence of TTX, indicating that s-LNvs maintain direct receptivity to cholinergic input through nicotinic receptors after metamorphosis. Furthermore, we have found no evidence for sensitivity to muscarinic agonists by 1- and s-LNvs. CCh- and nicotine-induced Ca²⁺ and cAMP responses displayed similar time courses, suggesting a strong coupling of excitation and cAMP production in these neurons. Given the important role that cAMP signaling plays in the maintenance of circadian rhythms (Levine et al. 1994; O’Neill et al. 2008), these results suggest that cholinergic modulation of s-LNvs might contribute to the entrainment of the molecular circadian rhythms of these neurons through the modulation of cAMP levels.

ACh is a widespread excitatory neurotransmitter in the fly brain and serves as the transmitter of most sensory neurons (Buchner et al. 1986; Gorczyca and Hall 1987; Nässel 1991). Thus, there are many potential sources for the cholinergic modulation of the s-LNvs. One prime candidate in this regard is input from the Hofbauer-Buchner eyelet (H-B eyelet), an extraretinal light input pathway that has been implicated in the entrainment of circadian rhythms to light-dark cycles (Helfrich-Förster et al. 2002; Malpel et al. 2002; Rieger et al. 2003). The axons of the H-B eyelet express choline acetyltransferase and project to the accessory medulla, where they terminate near s-LNvs arborizations (Helfrich-Förster et al. 2002; Malpel et al. 2002; Yasuyama and Meinertzhagen 1999).

Our results also support the prediction, based on anatomical and genetic studies, that GABA inhibits adult s-LNvs (Chung et al. 2009; Dahdal et al. 2010; Hamasaka et al. 2005; Parisky et al. 2008). Furthermore, the acute GABAergic reduction of cAMP in s-LNvs is consistent with recent work suggesting that GABA receptors expressed by these neurons are an important component of circadian timekeeping (Dahdal et al. 2010; Hamasaka et al. 2005). A recent report has shown that Go signaling is required for the GABAergic inhibition of dissociated larval LNvs and that Go signaling via phospholipase C in adult LNvs is critical for the maintenance of normally paced locomotor rhythms (Dahdal et al. 2010). GABA receptors and Go signaling can also be negatively coupled to adenylyl cyclase, as has been reported for *Drosophila* GABA receptors in heterologous cell culture (Mezler et al. 2001), although some doubt has been cast upon the presence of such negative cAMP coupling in the fly brain (Dahdal et al. 2010; Ferris et al. 2006).

Like ACh, GABA is a widespread neurotransmitter in the adult fly brain (Küppers et al. 2003), and the potential sources of the GABAergic input to s-LNvs are manifold. GABA immunoreactivity is found in the accessory medulla where projections from the s-LNvs are found (Hamasaka et al. 2005), and GABA is released by many neurons of the visual system (Nässel 1991). Interestingly, GABA is thought to modulate light input to the accessory medulla of the cockroach (*Petri et al. 2002*), suggesting that GABA might play a similar modulatory role in the fly through the modulation of the s-LNvs response to input from external photoreceptors. One interesting possibility is that GABA might act to modulate the response of s-LNvs to cholinergic input coming from the H-B eyelet or other sensory inputs.

In conclusion, our results experimentally confirm the prediction—based on previously published experiments in the dissociated larval CNS and on genetic and anatomical experiments in adults—that s-LNvs are modulated antagonistically by cholinergic and GABAergic input. Importantly, our work establishes for the first time that this antagonism extends to cAMP signaling, which is known to be a central component of molecular timekeeping in animals (Levine et al. 1994; O’Neill et al. 2008). We therefore predict that acute cholinergic and GABAergic stimulation of s-LNvs have opposing effects on the phase and or period of the molecular clock within these critical neuronal pacemakers. Finally, the utility of the genetically encoded sensors used in this study within deeply situated adult clock neurons indicates that such physiological analysis can now be extended to the entire clock neuron network with these tools. These imaging methods, particularly when combined with the considerable genetic tools available in the fly, will be critical for advancing our understanding of circadian timekeeping in the brain.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: K.R.L. and O.T.S. conception and design of research; K.R.L. performed experiments; K.R.L. and O.T.S. prepared figures; K.R.L. and O.T.S. drafted manuscript; K.R.L. and O.T.S. edited and revised manuscript; O.T.S. approved final version of manuscript.

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