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Shaw and Shal voltage-gated potassium channels mediate circadian changes in *Drosophila* clock neuron excitability

Abbreviated title: Shaw and Shal mediate circadian excitability

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1 **Abstract**

2 Like in mammals, *Drosophila* circadian clock neurons display rhythms of activity with higher
3 action potential firing rates and more positive resting membrane potentials during the day. This
4 rhythmic excitability has been widely observed but, critically, its regulation remains unresolved.
5 We have characterized and modeled the changes underlying these electrical activity rhythms
6 in the lateral ventral clock neurons (LNvs). We show that currents mediated by the voltage-
7 gated potassium channels Shaw (Kv3) and Shal (Kv4) oscillate in a circadian manner.
8 Disruption of these channels, by expression of dominant negative (DN) subunits, leads to
9 changes in circadian locomotor activity and shortens lifespan. LNv whole-cell recordings then
10 show that changes in Shaw and Shal currents drive changes in action potential firing rate and
11 that these rhythms are abolished when the circadian molecular clock is stopped. A whole-cell
12 biophysical model using Hodgkin-Huxley equations can recapitulate these changes in electrical
13 activity. Based on this model and by using dynamic clamp to manipulate clock neurons directly,
14 we can rescue the pharmacological block of Shaw and Shal, restore the firing rhythm, and thus
15 demonstrate the critical importance of Shaw and Shal. Together, these findings point to a key
16 role for Shaw and Shal in controlling circadian firing of clock neurons and show that changes in
17 clock neuron currents can account for this. Moreover, with dynamic clamp we can switch the
18 LNvs between morning-like and evening-like states of electrical activity. We conclude that
19 changes in Shaw and Shal underlie the daily oscillation in LNv firing rate.

20 **Introduction**

21 All organisms are subject to daily environmental changes caused by the earth's rotation;
22 therefore, they have evolved circadian clock mechanisms that regulate changes in behaviour,
23 physiology and metabolism to ensure their timely occurrence and thus allowing environmental
24 adaption. The central circadian clock maintains a ~24-hour rhythm and in turn synchronizes the
25 clock of peripheral tissues. Each clock neuron expresses core components of the molecular
26 oscillator that switches itself on and then off roughly every 24-hours by a mechanism that is
27 conserved from *Drosophila* to mammals including humans (Panda et al. 2002). Clock neurons
28 show activity rhythms with higher spike firing rates and more depolarized membrane potentials
29 during the day however the mechanisms driving this are not yet fully understood. Here, using a
30 combination of electrophysiology, pharmacology, behaviour and computational modelling, we
31 identify the voltage-gated potassium channels Shaw and Shal as underlying these rhythmic
32 properties.

33 We studied this in the lateral ventral neurons (LNvs), a subset of the 150 *Drosophila* clock
34 neurons that subdivides into large (l-) and small (s-)LNvs, broadly expressing the neuropeptide
35 pigment-dispersing factor (PDF) barring the 5th s-LNv that is PDF-negative (Helfrich-Förster et
36 al. 2007). PDF has an important role in morning anticipation and coordinating oscillations of the
37 clock neurons with *pdf⁰¹* null mutants being arrhythmic in constant darkness (DD) (Taghert and
38 Shafer 2006). l-LNvs are known as wake-promoting arousal neurons (Parisky et al., 2008),
39 while the s-LNvs are required to drive DD circadian rhythmicity (Helfrich-Förster et al. 1998);
40 like other clock neurons they are also more depolarized and have a higher firing rate in the
41 morning and these changes in membrane excitability are driven by the molecular clock (Sheeba
42 et al. 2008; Cao and Nitabach 2008; Buhl et al. 2016).

43 Excitability of the LNvs is also important in regulating circadian locomotor behavioral rhythms.
44 LNv hyperexcitation has been shown to disrupt nocturnal sleep (Sheeba et al. 2008).
45 Conversely, electrical silencing of LNvs disrupts circadian rhythms (Wu et al. 2008) and

46 imposing specific electrical activity patterns drives the transcriptional state of the neurons
47 towards morning- or evening-like states (Mizrak et al., 2012). These rhythmic changes in
48 membrane excitability are therefore important for sustaining the molecular clock, synchronizing
49 molecular oscillations in different clock neuron groups and communicating circadian information
50 from the clock to other regions of the brain and body that mediate circadian changes in
51 physiology and behavior (Allada and Chung, 2010; Allen et al., 2017; Belle and Allen, 2018;
52 Colwell, 2011; Flourakis et al., 2015; Kudo et al., 2011; Meijer and Michel, 2015; Mizrak et al.,
53 2012; Nitabach et al., 2005).

54 Rhythmic changes in the membrane excitability of clock neurons are mediated by ion channels
55 endogenously expressed in clock neurons (Itri et al., 2010; Meijer and Michel, 2015). Various
56 channels have been implicated in the rhythmic changes in membrane excitability of clock
57 neurons such as the sodium leak channel *narrow abdomen* which acts with a voltage-gated
58 potassium channel (Flourakis et al. 2015) in the dorsal clock neurons (DN1) of *Drosophila*. In
59 the LNV, the inward rectifier potassium channel, Kir, expression varies circadianly, peaking at
60 dusk, in line with the hyperpolarization of the clock neurons at this time, Kir overexpression
61 dampened the molecular clock and resulted in arrhythmic flies in constant conditions (Mizrak et
62 al., 2012). While LNV expression of a tethered toxin to paralytic (voltage-gated sodium channel
63 (Nav1)) has shown its importance in the phase of PDF release controlling rhythmic behavior
64 (Wu et al., 2008). The photoreceptor, Cryptochrome when activated by blue light depolarizes L-
65 LNV via a voltage-gated potassium channel β -subunit, hyperkinetic assembling with pore
66 forming subunits of voltage-gated potassium channels (Fogle et al., 2015). In the mammalian
67 clock called the suprachiasmatic nucleus (SCN), the large-conductance calcium-sensitive
68 potassium channel BK is important for the rhythmic changes in membrane excitability of clock
69 neurons, protein levels vary circadianly and BK mutants change circadian behavior (Kent and
70 Meredith, 2008; Meredith et al., 2006; Whitt et al., 2016), while the small-conductance calcium-
71 sensitive potassium channel SK also controls rhythmic firing of clock neurons (Belle et al. 2009).

72 Like in flies, Kir channels are also important for rhythmic changes in membrane excitability of
73 clock neurons (Hablitz et al., 2014) and Nav1.1 is also expressed in SCN, with loss of function
74 resulting in impaired SCN communication and circadian rhythms (Granados-Fuentes et al.,
75 2012). Voltage-gated L-type calcium channels are also expressed in the SCN and control the
76 rhythmic changes in membrane excitability of clock neurons (Diekman et al. 2013; McNally et
77 al., 2019; Pennartz et al., 2002).

78 Transcriptomic studies of the LNVs have shown that mRNA levels of Shaker, Shab, Shaw, and
79 Shal which are the *Drosophila* orthologues of the mammalian Kv1, Kv2, Kv3, and Kv4
80 respectively (Coetzee et al. 1999) vary circadianly with Shaw and Shal reported to vary
81 transcriptionally (mRNA level of LNV clock neurons) with Shaw highest in the morning (ZT0)
82 and Shal highest in the evening (ZT12) (Kula-Eversole et al. 2010). Kv3 and Kv4 mRNA, protein
83 levels and currents were also both found to vary in the SCN, with mouse knock outs of the
84 genes resulting in loss of rhythmic changes in membrane excitability of clock neurons and loss
85 of behavioral rhythms (Itri et al. 2010; Itri et al. 2005; Kudo et al., 2011).

86 Shal/Kv4 is an A-type channel regulating neuronal firing (Gasque et al. 2005) including SCN
87 firing rates and affects circadian rhythms with knock-down having greater effect at night
88 (Hermansteyne et al. 2017; Itri et al., 2010). Kv4.2 (Shal) and Kv1.4 (Shaker) are expressed in
89 the SCN and loss of function mutants disrupt the clock neuron neuronal firing, circadian
90 behavior and circadian period of PER2 expression, reiterating the importance and
91 interdependence of the rhythmic changes in membrane excitability of clock neurons and the
92 molecular clock (Granados-Fuentes et al., 2015; Granados-Fuentes et al., 2012; Hermansteyne
93 et al., 2017). Additionally, block of Shal function by a dominant negative (DN) transgene in
94 *Drosophila* has been associated with clock neuronal hyperexcitation, preferentially increasing
95 clock neuron firing rates around ZT13 when rhythmic change in resting membrane potential
96 and firing rate are low, disrupting PDF signaling to DN1 clock neurons (Feng et al. 2018).
97 Shaw/Kv3 is critical for circadian modulation of SCN neuron activity, with the fast-delayed

98 rectifier protein and current it mediates being higher during the day than at night. It remains
99 higher during the day even during continuous darkness (DD) with blockade of Shaw/Kv3
100 preventing the rhythmic changes in membrane excitability of clock neurons (Itri et al., 2005).
101 Kv3 knock outs greatly reduced fast delayed rectifier current resulting in a reduction in
102 spontaneous activity in the day and reduced NMDA-evoked responses at night (Itri et al. 2005;
103 Kudo et al. 2011). In *Drosophila* Shaw mediates a voltage-gated potassium current, with clock
104 neuron expression of Shaw-DN and *RNAi*, increasing neuronal excitability and Shaw
105 overexpression decreasing excitability. Expression of the transgenes in clock neurons remove
106 the rhythmic changes in membrane excitability in terms of resting membrane potential and
107 spontaneous firing rate which result in animals with changes in rhythmic PDF signaling, sleep,
108 LD, DD and continuous light (LL) behavior (Hodge et al. 2005; Hodge and Stanewsky 2008;
109 Parisky et al. 2008; Buhl et al., 2016).

110 Here we use whole-cell patch-clamping to investigate the electrophysiological properties of the
111 LNvs and, using ion channel specific pharmacology, we show that Shaw and Shal display
112 circadian rhythms in their activity. Behavioral analysis shows that they affect locomotor activity
113 and lifespan. Finally, we build a computational model that, for the first time, allows the real-time
114 manipulation of the LNvs using dynamic clamp and thus switching the LNvs between morning
115 and evening states of neuronal activity.

116 **Materials and methods**

117 ***Fly husbandry***

118 Flies were raised in a 12 h:12 h light dark (LD) cycle with lights on at ZT0 (Zeitgeber time) on
119 standard *Drosophila* medium (0.7% agar, 1.0% soya flour, 8.0% polenta/maize, 1.8% yeast,
120 8.0% malt extract, 4.0% molasses, 0.8% propionic acid, 2.3% nipagen) at 25°C and collected
121 between 0-5 days post eclosion. The following flies used in this study were previously described
122 or obtained from the Bloomington or Vienna *Drosophila* stock centers: For wild type recordings,
123 *RFP* fused to upstream regulatory elements of the *PDF* gene, *PDF-RFP* (Ruben et al. 2012),
124 was used for identification of LNV neurons in all experiments. For channel manipulations, *PDF-*
125 *Gal4* (Park and Hall 1998) was crossed to *UAS-Shaw-Truncated* (Bloomington *Drosophila*
126 stock center (BDSC) stock 55748), a *Shaw dominant negative* (*Shaw DN*) transgene (Hodge
127 et al. 2005) or *UAS-Shal pore 14* mutant that generates a *Shal dominant negative* (*Shal DN*)
128 transgene (Ping et al. 2011). In order to block the molecular clock, *PDF-Gal4* was crossed to
129 *UAS-CLKΔ5* (BDSC 36318) or *UAS-CYCA103* (BDSC 36317) canonical clock gene mutant
130 transgenes (Tanoue et al. 2004). To validate the pharmacological blockers of Shaker and Shab,
131 *PDF-Gal4* was crossed to *UAS-Shaker RNAi* (Vienna *Drosophila RNAi* stock KK104474) or
132 *UAS-Shab RNAi* (BDSC 55682). For circadian rhythm experiments (Figure 4A-D) exclusively
133 males were used, all other experiments used both males and females.

134

135 ***Electrophysiological recordings***

136 Patch-clamp recordings were performed as described previously (Chen et al. 2015; Buhl et al.
137 2016). Briefly, brain explants of flies aged 0-5 days post-eclosion were dissected at several
138 time-points: ZT1-3 (ZT2 i.e. 2 h after lights came on/early morning), ZT7-9 (ZT8), ZT13-15
139 (ZT14 i.e. 2 h after lights went off/early night), and ZT19-21 (ZT20). For recordings in darkness,
140 dissections were conducted under red light illumination. Whole fly brains were dissected at
141 room temperature (20-22°C) in standard extracellular solution containing (in mM): 101 NaCl,

142 1 CaCl₂, 4 MgCl₂, 3 KCl, 5 glucose, 1.25 NaH₂PO₄, 20.7 NaHCO₃, pH 7.2 on a M205C
143 stereomicroscope (Leica, Wetzlar, Germany). The brain was positioned in the recording
144 chamber and secured ventral side up using a custom-made brain harp. Neurons were
145 visualized by RFP fluorescence using a 555 nm LED light source on an upright Zeiss
146 microscope (Examiner.Z1, Carl Zeiss Microscopy GmbH, Jena, Germany). LNV neurons were
147 identified on the basis of their fluorescence, position and size as the l-LNVs have a cell soma
148 diameter of approximately 10-11 μm and the s-LNVs have a diameter of approximately 7 μm
149 (Schubert et al. 2018). Whole-cell recordings were performed using glass electrodes with 8-18
150 MΩ resistance filled with intracellular solution (in mM: 102 K-gluconate, 17 NaCl, 0.94 EGTA,
151 8.5 HEPES, 0.085 CaCl₂, 1.7 MgCl₂ or 4 Mg-ATP and 0.5 Na-GTP, pH 7.2) and an Axon
152 MultiClamp 700B amplifier, digitized with an Axon DigiData 1440A (sampling rate: 20 kHz; filter:
153 Bessel 10 kHz) and recorded using pClamp 10 (Molecular Devices, Sunnyvale, CA, USA). A
154 cell was included in the analysis if the access resistance was less than 50 MΩ as in other
155 studies (Chen et al. 2015; Buhl et al. 2016) and was compensated by 70% on average.
156 Dynamic clamp recordings were performed using the CED Power 1401 system (Cambridge
157 Electronic Design) and recorded using Signal (Version 6.05, Cambridge Electronic Design) at
158 a sampling frequency of 10 kHz. The equations and parameters used are as shown in the
159 'Computational Modeling' section. Maximal conductance of channel models was adjusted as
160 per Tables 2 and 3.

161

162 ***Circadian locomotor behavior***

163 Analysis of locomotor activity of male flies was performed using the *Drosophila* Activity Monitor
164 System (DAM2, Trikinetics Inc., Waltham, MA, USA) with individual flies in recording tubes
165 containing standard food. The DAM monitors, were located inside a light- and temperature-
166 controlled incubator (Percival Scientific Inc., Perry, IA, USA) where the fly's activity was
167 monitored for 5 days in 12 h:12 h LD followed by 10 days in constant darkness (DD) at 25°C.

168 Plotting of behavioral activity and period calculations were performed using a signal-processing
169 tool-box (Levine et al. 2002) implemented in Matlab (MathWorks, Natick, MA, USA). All activity
170 counts were recorded in 1-minute bins. Measurements of rhythmicity were performed by
171 autocorrelation analysis (Levine et al. 2002); in short, each data-point is compared to other
172 data-points at differing time intervals. The process is repeated for a range of intervals and this
173 forms an autocorrelogram, in which the third peak gives a rhythmicity index from which the
174 rhythmicity statistic (RS) is calculated.

175

176 **Computational modeling**

177 Computational modeling of ion channels and whole-cell neuronal electrical activity was carried
178 out in Matlab (MATLAB Release 2015a, The Mathworks Inc., Natick, Massachusetts, US) using
179 the following Hodgkin-Huxley formalism (Hodgkin and Huxley 1952). In particular, we use the
180 following formulation for the ion-channel currents:

$$181 \quad I = g_{max} * (m^P * h^Q) * (V - E)$$

182

$$183 \quad \frac{dx}{dt} = \frac{x_{\infty} - x}{\tau_x}$$

184

$$185 \quad x_{\infty} = \frac{1}{1 + e^{-\frac{V-V_h}{k}}}$$

186

$$187 \quad \tau_x = Amp * e^{-\frac{V-V_{max}}{\sigma}}$$

188 where g_{max} denotes the maximum conductance, and x the state of the activation m and
189 inactivation h gates respectively. The above formulation also includes the driving force, which
190 is the difference between the membrane voltage, V , and the reversal potential, E . The reversal
191 potentials were calculated using the Nernst equation based on the electrophysiological
192 solutions used as 52 mV for sodium, -90 mV for potassium, and 132 mV for calcium. The
193 steady-state of the gating variable x_{∞} and the time-constant τ_x are calculated using parameters

194 unique to each channel (see Table 1). These parameters have been determined by fitting to
 195 voltage-clamp experimental data in the same manner as originally done in (Hodgkin and Huxley
 196 1952). The resulting current-balance equation describing the evolution of the whole cell
 197 membrane potential is:

$$198 \quad C \frac{dV}{dt} = I_{app} - g_{Na}m^3h(V - E_{Na}) - g_{Ca}mh(V - E_{Ca}) - g_{Kv1}m^4h(V - E_K) - g_{Kv2}m^4(V - E_K)$$

$$199 \quad - g_{Kv3}m^4h(V - E_K) - g_{Kv4}m^4h(V - E_K) - g_{leak}(V - E_{leak})$$

200 This equation includes the capacitance of the cell body, C (set at 3.7 pF in accordance with
 201 our recordings), along with the applied current, I_{app} , and the currents of the sodium, calcium,
 202 potassium, and leak channels to calculate the change in the membrane voltage, V . The
 203 sodium, calcium, and leak channel models are used unaltered from the previous Sim-Forger
 204 model (Sim and Forger 2007). Whole cell activity simulations are performed using the
 205 stochastic Euler-Maruyama method to iteratively calculate the membrane voltage
 206 incorporating white noise as follows:

$$207 \quad V_{n+1} = V_n + \frac{dV_n}{dt} \Delta t + (\sigma W \sqrt{\Delta t})$$

208 Where V_n is the membrane voltage, dV_n/dt is the change in membrane voltage as calculated
 209 by the current-balance equation, $\Delta t = T/N$ is the time-step, T is the total simulation time, $n =$
 210 $1, \dots, N$ denotes the number of iterations, W is the white noise component, and σ is a scaling
 211 factor for the noise magnitude.

212 Electrophysiological data for the Kv channels Shaker, Shab, Shaw, and Shal were fit to
 213 Hodgkin-Huxley equations by standard Hill climbing (or direct search) search-based
 214 optimization algorithms (Kolda et al. 2003) to generate parameters describing kinetics of each
 215 individual ion channel. The parameters estimated for the four potassium channels and used in
 216 the dynamic clamp experiments are shown in Table 1.

217

218 ***Experimental design and statistical analysis***

219 The liquid junction potential of the recordings was calculated as 13 mV and was subtracted
220 from all the membrane voltages presented here. All values are given as mean and standard
221 deviation (SD). Statistical tests were performed in Matlab (MATLAB Release 2015a, The
222 MathWorks Inc., Natick, Massachusetts, US) or Prism (GraphPad Software Inc., La Jolla, CA,
223 USA). A one-way ANOVA followed by Tukey's *post-hoc* test was used for circadian current data
224 (Figures 1 and 3), Mantel-Cox log-rank test (Clark et al. 2003) for longevity data (Figure 4E)
225 and a two-way ANOVA for the model-experimental firing rate comparisons (Figure 5C). For
226 dynamic clamp channel experiments (Figures 6) equal variance is not assumed. A standard t-
227 test was used for all other data after testing for a standard distribution with the Kolmogorov-
228 Smirnov test.

229

230 ***Code accessibility***

231 Matlab codes utilized in the modelling and Signal scripts used for dynamic clamp are available
232 upon request.

233 Results

234 *Circadian changes in Shaw and Shal channel function*

235 To determine the functional properties of Shaker, Shab, Shaw and Shal across the day, we
236 examined the electrophysiological properties of the LNvs by whole-cell patch-clamp. Each
237 channel was isolated by the use of ion channel specific pharmacological blockers: α -
238 dendrotoxin (DTX, 100 nM) to block Shaker (Grissmer et al. 1994), guangxitoxin-1E (GxTX, 20
239 nM) to block Shab (Herrington et al. 2006), blood depressing substance (BDS-I, 300 nM) to
240 block Shaw (Yeung 2005), and phrixotoxin-1 (PaTX, 100 nM) to target Shal (Gasque et al.
241 2005). Whole-cell current densities in response to a voltage-clamp protocol were recorded
242 before and after application of the appropriate channel toxin (Figure 1). The respective ion
243 channel currents were then estimated by subtracting the currents recorded in these two
244 conditions (Figure 1B). Importantly, each toxin was unable to affect whole-cell currents when
245 the corresponding channel was functionally removed by either dominant negative (DN) or *RNAi*
246 transgenes specific for each of the individual Kv channels expressed in the LNvs (Figure 2A).
247 This significant reduction in drug-sensitive current for each of Shaker ($t(22)=8.206$, $p<0.0001$,
248 t-test), Shab ($t(22)=13.511$, $p<0.0001$, t-test), Shaw ($t(22)=2.765$, $p=0.0113$, t-test), and Shal
249 ($t(22)=3.8764$, $p=0.0008$, t-test) demonstrates selectivity at their respective concentrations. The
250 corresponding currents of each channel were also mostly restored by wash-out of the toxins
251 (Figures 1D and 2B).

252 Each of the assayed Kv channels displayed characteristic kinetics in response to a voltage-
253 clamp protocol (Tsunoda and Salkoff 1995; Gasque et al. 2005) (Figure 1C). Analysing the
254 peak current density evoked by the highest depolarizing step at different times-of-day (Figure
255 1A) showed a consistent response for Shaker and Shab, whereas Shaw ($F(3,8)=16.43$,
256 $p=0.0009$, one-way ANOVA) and Shal ($F(3,8)=9.36$, $p=0.0054$, one-way ANOVA) varied across
257 the day. Shaw displayed a higher peak current density in the morning (Zeitgeber time (ZT)2)
258 compared with the night (ZT14) ($t(4)=11.406$, $p=0.0003$, t-test). Conversely, Shal conducted a

259 higher peak current density in the evening (ZT8) than at late night near dawn (ZT20)
260 ($t(4)=11.429$, $p=0.0003$, t-test).

261 To examine whether these changes in peak currents are of circadian origin or a consequence
262 of light input, we recorded Shaw and Shal currents on the third day of DD in both l-LNvs and s-
263 LNvs (Figure 3), as molecular oscillations in the l-LNvs dampens by then in DD, while remaining
264 sustained in the s-LNvs (Yang and Sehgal 2001). Recordings in the s-LNvs exhibited
265 oscillations in Shaw ($F(1,4)=12.89$, $p=0.0229$, one-way ANOVA) and Shal ($F(1,4)=96.43$,
266 $p=0.0006$, one-way ANOVA) current density (Figure 3B) that were consistent with those
267 obtained in LD (Figure 3A). However, corresponding recordings in the l-LNvs in DD (Figure 3B)
268 showed reduced currents in both Shaw ($F(1,4)=0.07$, $p=0.7982$, one-way ANOVA) and Shal
269 ($F(1,4)=0.83$, $p=0.4149$, one-way ANOVA) and abolished this rhythm. This is likely due to the
270 disruption of the molecular clock observed after a transition from LD to DD. In *Drosophila* the
271 molecular clock is dependent on the heterodimeric bHLH-PAS transcription factors CLOCK
272 (CLK) or CYCLE (CYC), therefore clock neuron expression of DN transgenes to CLK and CYC
273 results in completely arrhythmic flies under DD conditions and loss of rhythmic expression of
274 timeless (tim) driven luciferase expression and rhythmic expression of tim mRNA at ZT9, ZT15
275 and ZT21 (Tanoue et al., 2004). Therefore, we recorded Shaw and Shal currents in flies
276 expressing CLOCK (CLK) or CYCLE (CYC) DN transgenes in the LNvs (Figure 3C), which
277 block the function of these core clock proteins and thus stops the molecular clock. Interestingly,
278 when CLK or CYC DN transgenes are expressed only in the LNvs the rhythm of Shaw and Shal
279 is abolished even in LD conditions (Figure 3C). These results showed that the observed
280 variations of Shaw and Shal currents were driven by the molecular clock.

281

282 ***Shaw and Shal currents affected circadian locomotor behavior and longevity***

283 The behavioral consequences of disrupting the rhythm of Shaw and Shal currents were
284 examined by assaying circadian behavior and longevity of flies expressing either Shaw or Shal

285 DN transgenes in the LNVs (Figure 4). Locomotor behavior showed significant increases in
286 overall activity (Figures 4A and D), particularly at night-time, when either Shaw ($t(27)=-6.5263$,
287 $p<0.0001$, t-test) or Shal ($t(39)=-4.4307$, $p<0.0001$, t-test) channels are blocked. However,
288 despite the increase in activity, the period (Figure 4C, Shaw: $t(27)=-5.878$, $p<0.0001$, Shal:
289 $t(39)=1.163$, $p=0.2518$) and strength of the rhythms (Figure 4B, Shaw: $t(27)=2.003$, $p=0.0554$,
290 Shal: $t(39)=0.1251$, $p=0.9011$) are not generally affected other than a small period lengthening
291 seen with Shaw DN in DD. Interestingly, the longevity of flies expressing either Shaw
292 ($p<0.0001$, Mantel-Cox) or Shal ($p<0.0001$, Mantel-Cox) DN in only the LNVs was significantly
293 reduced compared to controls (Figure 4E).

294

295 ***Computational modeling of the circadian neurons***

296 In order to determine the contribution of the observed oscillations in Shaw and Shal to the day-
297 night rhythms of activity seen in clock neurons, we developed a computational model describing
298 the kinetics of each Kv channel utilizing the LNV electrophysiological data, and tested it using
299 dynamic clamp. The individual currents' models were parameterized by fitting the experimental
300 data to Hodgkin-Huxley equations (Hodgkin and Huxley 1952), one of the most commonly used
301 biophysical frameworks for studying ion channel activity that have already been used to model
302 the SCN (Sim and Forger 2007) and *Drosophila* DN1s (Flourakis et al. 2015) (Table 1). These
303 Kv channel models were combined with a previously existing model of mammalian SCN sodium
304 and calcium channels (Sim and Forger 2007) to produce a whole-cell neuronal model that
305 described the LNV electrical activity.

306 Simulations of whole-cell electrical activity in this model reproduced the experimental
307 observations of the LNVs (Figures 5A). Interestingly, the model only incorporated a time-of-day
308 variation in Shaw and Shal conductance, while keeping Shaker and Shab constant, indicating
309 that the former channels accounted for the day-night difference in electrical activity. During the
310 simulation of individual action potentials, the current of the channels can also be observed. This

311 revealed that Shaw and Shal were also preferentially active during the action potential in the
312 morning and evening respectively (Figure 5B). We further analyzed our model in terms of
313 spontaneous action potential firing frequency and found that it faithfully reproduced the
314 difference in morning/evening firing rate (Figure 5C).

315 The availability of electrophysiological data and the construction of channel models allowed us
316 to employ a dynamic clamp approach to study the behavior of the LNvs facilitated by real-time
317 modulation of their electrical activity. First, to validate the channel models in an experimental
318 setting, each voltage-gated potassium channel was separately blocked by their respective
319 drugs (Figure 6). In current clamp, this produced a noticeable change in action potential firing
320 rate (Figures 6A middle traces). The electrical activity was then rescued by injecting the
321 calculated current (Figures 6A right traces), based on the respective channel model, and in real
322 time using dynamic clamp (conductances used in Table 2). The firing rate (Figure 6B) changed
323 on application of the drug (Shaker: $t(4.8)=-6.5493$, $p=0.0014$, Shab: $t(5.5)=-3.2578$, $p=0.0197$,
324 Shaw: $t(5.7)=3.6298$, $p=0.012$, Shal: $t(7.8)=-7.3673$, $p<0.0001$) and was rescued in all cases
325 by application of the model (Shaker: $t(6.8)=5.6572$, $p=0.0008$, Shab: $t(4.1)=3.9641$, $p=0.0159$,
326 Shaw: $t(6.7)=-4.5437$, $p=0.003$, Shal: $t(6.5)=9.1094$, $p<0.0001$). This affirms that the channel
327 models described sufficiently well the channel kinetics and can substitute for the function of the
328 actual channels in regulating electrical activity.

329 Since the computational model can also recapitulate the change in electrical activity between
330 morning and evening states, we used the dynamic clamp set-up to modulate the activity of the
331 LNvs (Figure 7 and Table 3). Addition of Shaw current and removal of Shal current using
332 dynamic clamp caused a reduction in firing rate of the LNvs at ZT0 from the morning level of
333 ~ 2 Hz to an evening level of ~ 1 Hz ($t(6)=8.9324$, $p<0.0001$) (Figures 7A left panel). Removal of
334 Shaw current and addition of Shal current in contrast caused the converse effect on the LNvs
335 at ZT12 shifting them from an evening level to a morning level of activity ($t(4)=-10.6904$,
336 $p<0.0001$) (Figures 7A right panel). In other words, we were able to effectively switch a morning

337 cell to an evening cell and vice versa (Figure 7B). Taken together our modeling confirmed that
338 Shaw and Shal currents are sufficient to account for the day-night variation in electrical activity.
339

340 **Discussion**

341 In this study we identified the voltage-gated potassium channels Shaw and Shal as major
342 contributors to the circadian changes in electrical activity of LNV clock neurons. Previous work
343 has shown that the spontaneous action potential firing rate (e.g. ZT1-3: 2Hz and ZT13-15:
344 0.5Hz) and resting membrane potential (e.g. ZT1-3: -50mV and ZT13-15: -58 mV) of the LNVs
345 changed over the day (Cao and Nitabach 2008; Sheeba et al. 2008; Buhl et al. 2016). However,
346 the specific ion currents that underpinned rhythms of neuronal excitability were not known. Here
347 we showed that the currents of individual voltage-gated potassium channels, as revealed by
348 selective blockers, displayed circadian variation (Figure 1). This variation persisted in DD but
349 was abolished when the molecular clock was stopped (Figure 3) indicating that the variation in
350 ion channel currents required a functional core molecular clock. Conversely, the disruption of
351 the channels just in the LNV resulted in changes in circadian locomotor activity and decreased
352 lifespan (Figure 4), showing the importance of circadian changes in clock neuron excitability
353 and the behavioral rhythms they drive to the health span of an individual. Taken together, this
354 positions the oscillation of Shaw and Shal currents as critical components of the molecular
355 machinery in the membrane that regulates rhythmic changes in clock neuronal activity. We also
356 implemented a dynamic clamp methodology, in *Drosophila* and for clock neurons for the first
357 time and showed how the above findings can be utilized by computational modeling and
358 dynamic clamp to modulate neuronal activity in real-time in order to switch between morning
359 and evening states (Figure 7).

360 The circadian rhythm of Shaw and Shal currents observed here may be generated by a number
361 of processes. A previous transcriptomic study (Kula-Eversole et al. 2010) indicated that the
362 mRNA levels of both channels cycle in a circadian manner in the LNVs with Shaw being highest
363 in the morning and Shal being highest in the evening, and so the changes in current could be
364 underpinned by changes in transcription. Indeed, studies in the SCN also report circadian
365 variation in the Shal ortholog Kv4.1, its knock-down had a greater effect at night (Hermansteyne

366 et al. 2017) with similar effects reported in *Drosophila* (Feng et al. 2018). While neither Shaw
367 nor Shal genes contain a canonical E-box sequence (Nakahata et al. 2008), many genes found
368 to have a cycling mRNA level do not contain known circadian transcription elements (Claridge-
369 Chang et al. 2001; McDonald and Rosbash 2001). Furthermore, many rhythmic proteins found
370 in the mouse liver and SCN have been shown not to be encoded by rhythmic mRNAs
371 (Mauvoisin et al. 2014; Robles et al. 2014). Therefore, studies at the protein and functional level
372 are more reliable, in this case the functional assay for ion channels is electrophysiology. In the
373 SCN, Kv3 showed higher expression during the day (Itri et al. 2005). It is, however, interesting
374 that disruption of the molecular clock led to, not only an abolition of the Shaw and Shal current
375 cycling, but also an abolition of Shaw and Shal current in general (Figure 3C). This suggests
376 that, while circadian transcription of specific channels maybe unclear, the molecular clock
377 seems necessary for the rhythmic timing of membrane properties of clock neurons. Indeed,
378 circadian expression and degradation of clock neuron ion channels may not be the most
379 parsimonious means to circadianly regulate membrane properties, for instance post-
380 transcriptional modifications have also been found to be important (Ko et al. 2009). In addition,
381 alternative splicing is important for changing ion channel function, especially for voltage-gated
382 potassium channels (Jan and Jan 2012), with *Drosophila* ion channels such as Shaker and
383 Shab that are expressed in clock neurons can also undergo RNA editing (Ingleby et al. 2009;
384 Ryan et al. 2008). Furthermore, a recent study showed that potassium channels were the most
385 alternatively spliced gene family in the genome, with alternative splicing transcripts (especially
386 Shaker and Shab) being particularly enriched in LNV and DN1 compared to non-circadian
387 neurons. It is not known if or how this massive alternative splicing regulation of clock neurons
388 contributes to circadian rhythms (Wang et al. 2018).

389 A study of the *Drosophila* dorsal clock neurons identified that localization of the sodium channel
390 NA is important for circadian changes in electrical activity (Flourakis et al. 2015). Here, RNA-
391 seq analysis showed no cycling of NA transcripts, but instead showed rhythms in the

392 localization factor *NLF-1*. The mammalian ortholog of NA, termed NALCN, showed similar
393 rhythms suggesting conservation between flies and mammals. Voltage-gated potassium
394 channels, such as Shaker, have also been shown to interact with accessory subunits such as
395 Hyperkinetic with both being shown to be important for *Drosophila* sleep and longevity, with null
396 mutants sleeping a third as long as normal flies resulting in flies with significant reductions in
397 lifespan (Bushey et al. 2007; Cirelli et al. 2005; Kempf et al. 2019). Phosphorylation may also
398 be an important post-translational means of circadian regulation of ion channel activity, with
399 mammalian slo or BK, being under phosphorylation control in the SCN (Shelley et al. 2013).
400 Circadian phosphorylation by clock kinases maybe a more general bi-directional link between
401 the molecular clock and membrane ion channels that regulate the rhythmic firing of clock
402 neurons (Ko et al. 2009; Allada and Chung 2010; Allen et al. 2017).

403 Membrane activity of the LNvs has been previously suggested to be particularly important for
404 self-sustained oscillations as electrical silencing of the LNvs stopped the molecular clock
405 (Nitabach et al. 2002; Depetris-chauvin et al. 2011). Additionally, hyperexcitation of the LNvs
406 has been reported to create a shift in the transcriptome towards a morning-like state whereas
407 hyperpolarization shifts towards an evening-like state (Mizrak et al. 2012; Emery 2012). This
408 link between the molecular clock and the rhythmic changes in the membrane excitability could
409 also relate (at least in part) to the ion channels Shaw and Shal themselves as proposed above
410 and via membrane potential control of neurotransmitter and peptide release (Choi et al. 2012).
411 PDF is known to rhythmically accumulate in the dorsal terminals of the s-LNvs with more PDF
412 during the day than at night (Park et al. 2000). Previous reports (Hodge and Stanewsky 2008)
413 indicate that expression of the *Shaw DN* transgene throughout the *Drosophila* clock network
414 results in lower PDF levels in the terminals across the day with a reduced strength of oscillation,
415 presumably due to reduced Shaw causing depolarization of terminals and PDF release. The
416 opposite effect is seen with over-expression of Shaw resulting in increased Shaw-mediated
417 hyperpolarization and block of calcium-mediated exocytosis and therefore an increase in overall

418 PDF levels in the terminals and a loss of rhythm. Taken together this indicates that reduction
419 of Shaw function leads to a lower level of PDF, and hence changes in PDF signaling, possibly
420 underlying effects observed here in altering circadian locomotor activity (Figure 4).

421 The increased activity and reduced sleep associated with altering Shaw and Shal currents
422 (Figure 4A-D) indicate a hyperactive behavioral phenotype. The increase in locomotor activity
423 is particularly pronounced in night-time activity, consistent with the role of the l-LNvs as wake-
424 promoting neurons (Parisky et al. 2008) and with previous reports of the role of Shaw (Hodge
425 and Stanewsky 2008) and Shal (Feng et al. 2018) in circadian behavior. The decrease in
426 lifespan seen by disrupting either Shaw or Shal (Figure 4E) also mirrors other studies showing
427 that loss of Shaker or Shal throughout the brain leads to a shortening of lifespan (Ping et al.
428 2011; Bushey et al. 2007). However, our study extends these findings, by demonstrating that
429 the mechanism by which the reduction in lifespan can occur is by disruption of circadian rhythms
430 caused by the loss of specific voltage-gated potassium channels just in the LNvs. In humans,
431 a related disease is Morvan's syndrome whereby antibodies are produced against the Kv1
432 channel and result in loss of sleep and increased mortality (Irani et al. 2010). Mutation in human
433 Kv9, which co-assembles with Kv2, has similarly been shown to be associated with essential
434 tremor and when expressed in *Drosophila* clock neurons results in hyperexcitability, loss of
435 night-time sleep, shaking and increased mortality (Smith et al. 2018).

436 The combination of electrophysiology and computational modeling enabled us to employ
437 dynamic clamp in order to directly test whether changes in Shaw and Shal currents are sufficient
438 to recapitulate changes in electrical activity (Figure 7). The dynamic clamp technique allows us
439 to simulate the presence or absence of a channel, by injecting appropriate current based on its
440 mathematical model in real time (Prinz et al. 2004; Goillard and Marder 2006). This approach
441 reinforces the capability of these ion channels to generate the specific patterns of electrical
442 activity observed, such as action potential firing frequency, by showing that changes only in
443 these ion channels recreate this phenomenon.

444 The kinetics of Shaw (Kv3) and Shal (Kv4) channels seem particularly well suited for modulating
445 the firing rate with Shal mediating the fast transient I_A current necessary for rapid repolarization
446 of action potentials required for high frequency firing and the delay to the first spike in a burst
447 of action potentials of a range of *Drosophila* neurons including clock neurons (Feng et al., 2018;
448 Hodge et al., 2005; Kulik et al., 2019; Ping et al., 2011). Kv4 also mediates the I_A current in a
449 range of mammalian neurons including in the SCN thereby regulating spike timing, repetitive
450 firing, spontaneous firing, dendritic integration and plasticity and has been shown to be able to
451 tune pacemaker frequency of action potentials (Amarillo et al., 2008; Hermanstynne et al., 2017;
452 Liss et al., 2001). On the other hand, Shaw regulates the firing rate in a range of *Drosophila*
453 neurons including clock neurons probably by a separate mechanism to Shal, via controlling the
454 resting membrane potential (Buhl et al., 2016; Hodge et al., 2005; Tsunoda and Salkoff, 1995).
455 Interestingly, the duplication of the Shaw gene in mammals has created a diversity of Kv3
456 channels, some of which have particularly fast kinetics that can adjust and maintain firing rates
457 of neurons such as in the auditory system up to around 200 Hz (Rudy and McBain, 2001; Wang
458 et al., 1998). Kv3 channels also control spike timing, spontaneous firing and firing patterns in a
459 range of other neurons including in the SCN (Akemann and Knopfel, 2006; Joho et al., 2006;
460 Kudo et al., 2011; Kudo et al., 2013). In future, the model we developed could, alongside other
461 similar models (Flourakis et al. 2015), contribute to a larger model encapsulating how different
462 orthologues of the same channel in clock neurons of different species result in their respective
463 firing behavior or how the different ion channels contribute to the firing properties of different
464 types of neurons in the same species, for instance neurons from different clock groups or even
465 describe the clock network at large and the role of rhythmic changes in membrane properties
466 to the clock and its function. Investigating the interaction of Shaw and Shal potassium with the
467 NA sodium currents and the rescue of neuronal activity rhythms using dynamic clamp, will help
468 us to understand the possible cooperation between different ion channels in regulating rhythmic
469 activity. In addition, this model reiterates the conservation between *Drosophila* and mammalian

470 clock neuron excitability and circadian rhythms and their importance in health and disease and
471 potential of applications to chronotherapy (Allen et al. 2017; Julienne et al. 2017; Zwarts et al.
472 2017).

473 In conclusion, we demonstrate that the voltage-gated potassium channels Shaw and Shal
474 exhibit circadian changes in their current profile within the LN_v clock neurons and that these
475 rhythmic changes in membrane excitability have real consequences for the animal. We built
476 computational models that describe how these changes affect whole-cell electrophysiology and
477 indicate the importance of these channel oscillation for functional day-night differences. Finally,
478 by using dynamic clamp we could verify our models and were able to switch the neurons
479 between morning- and evening-like electrical states.

480 **Author contributions:** P.S. conducted the experiments and wrote the paper, E.B. developed
481 novel technology for the project, supervised project and edited the paper, K.A.-T. and J.H.
482 secured funding, designed experiments, supervised project and edited the paper.

483

484 **Competing interest:** Authors report no competing interests.

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735

736 **Figure legends**

737 **Figure 1:** Daily changes in current profiles of voltage-gated potassium channels in I-LNvs.

738 **A,** Peak current density of each of the channels: Shaker (N=12), Shab (N=12), Shaw (N=13),
739 and Shal (N=12) in the I-LNvs at different times of day in a 12 h:12 h light/dark cycle (LD).
740 Shaker and Shab showed no significant changes over the day, whereas Shaw was highest in
741 the morning ($p=0.0003$) and Shal was highest in the evening ($p=0.0003$). Asterisks indicate
742 statistical significance throughout ($*p<0.05$, $**p<0.01$, $***p<0.001$) in a one-way ANOVA,
743 Tukey's *post-hoc* test. Box and whiskers are median, inter-quantile range (IQR) and minimum-
744 maximum (min-max) throughout over four trials per time-point. **B,** Average current density
745 profile of each individual channel in response to a voltage protocol shown in **C,** The presented
746 profiles are the difference between control condition and after application of a selective blocker
747 (Shaker: α -Dendrotoxin (DTX) 100 nM, Shab: Guangxitoxin-1E (GxTX) 20 nM, Shaw: blood-
748 dispersing substance (BDS) 300 nM and Shal: Phrixotoxin-1 (PaTX) 100 nM). Shaker and Shab
749 profiles were averages over the day, the Shaw profile was an average at ZT1-3, the Shal profile
750 was an average at ZT7-9. **D,** A representative response to depolarization to -3 mV before
751 (control) and after drug application (+DTX) as well as after a subsequent 5 minutes wash-out
752 (after wash-out) that restored the blocked current. Similar wash-out experiments were
753 conducted for each of the channels Shaker, Shab, Shaw, and Shal and their respective
754 blockers.

755

756 **Figure 2:** Validation of individual voltage-gated potassium channel blocker specificity in I-LNvs.

757 **A,** Peak current density blocked by ion channel blockers (Shaker: DTX, Shab: GxTX, Shaw:
758 BDS, Shal: PaTX) on depolarization to -3 mV from -133 mV in I-LNvs of control flies (left colored
759 boxes) or flies deficient for the corresponding channel (right black boxes). Shaker and Shab
760 currents were obtained at ZT7-9 in control flies ($n=12$ and 12) and in *PDF-Gal4* driven
761 expression of *Shaker RNAi* ($n=9$) or *Shab RNAi* ($n=9$). Shaw currents were obtained at ZT1-3

762 in controls (n=9) and I-LNvs expressing Shaw DN (n=9). Shal currents were obtained at ZT13-
763 15 in controls (n=9) and I-LNvs expressing Shal DN (n=9). **B**, Percentage of blocked current
764 that was recovered upon wash-out of the drug over 5 minutes for each ion channel. Box and
765 whiskers are median, IQR and min-max over three trials per drug (n=3 per drug).

766

767 **Figure 3:** Effects of the molecular clock on membrane clock oscillations in I- and s-LNvs.

768 **A**, Peak channel current density at -3 mV at different times of day in LD (grey, lights off; white,
769 lights on) for each of the channels Shaw (left panel, s-LNv: n=12, I-LNv: n=13) and Shal (right
770 panel, s-LNv: n=12, I-LNv: n=12) or **B**, on day 3 of constant darkness (DD) in either small LNvs
771 (s-LNv) or in large LNvs (I-LNv); a similar pattern was seen at other voltages. In LD, Shaw (left
772 panels) and Shal (right panels) rhythms was seen in both s- and I-LNvs. In DD, rhythms persist
773 in Shaw for the s-LNvs (p=0.0229, n=12) but were abolished in the I-LNvs (p=0.7982, n=13).
774 Similarly, rhythms persist in Shal for the s-LNvs (p=0.0006, n=12) but were abolished in the I-
775 LNvs (p=0.4149, n=12). Mean \pm SD over three trials per time-point and condition; one-way
776 ANOVA, Tukey's *post-hoc* test. **C**, Peak channel current density in I-LNvs at different times of
777 day in a 12 h:12 h light/dark cycle (LD) (grey, lights off; white, lights on) in control conditions
778 (same as in A), or in LNvs that expressed either CLOCK (CLK Δ 5, n=12) or CYCLE DN
779 transgenes (CYC Δ 103, n=12). Rhythms were abolished in I-LNvs in the absence of a functional
780 molecular clock even in a standard LD cycle.

781

782 **Figure 4:** Circadian locomotor activity and longevity effects of reduced Shaw and Shal current.

783 **A**, Average activity profiles of flies that expressed Shaw (n=14) and Shal (n=21) DN transgenes
784 in the LNvs compared to control (PDF/+; n=16) over 5 days of LD. Shaded areas represent
785 lights-off and white areas represent lights-on, dots indicate SD. **B**, Rhythmic statistics over days
786 5-7 of constant darkness (DD). There is no significant effect of DN expression on rhythm
787 strength with either Shaw (p=0.0554) or Shal (p=0.9011) DN expression. Bars represent the

788 mean and error bars represent SD. **C**, Length of rhythm period in DD. There was no significant
789 change with expression of Shal DN ($p=0.2518$), but there was a small period lengthening with
790 expression of Shaw DN ($p<0.0001$). **D**, Activity counts of day (left bars) and night (right bars)
791 activity in control flies (PDF/+ $n=16$, Shaw DN/+ $n=15$, Shal DN/+ $n=20$) and flies that expressed
792 the Shaw (Shaw DN, $n=14$) or Shal (Shal DN, $n=21$) DN transgenes in the LNvs. In particular,
793 night activity is increased in both Shaw ($p<0.0001$) and Shal ($p<0.0001$) DN expression flies
794 compared with undriven controls; similar differences are seen when compared with PDF/+
795 controls (#). **E**, Longevity of flies. Compared with PDF control flies (median age 75, $n=165$),
796 expression of either Shaw (median age 64, $p<0.0001$, $n=77$) or Shal (median age 61, $p<0.0001$,
797 $n=202$) DN significantly reduced lifespan in a Mantel-Cox log-rank test. Undriven Shaw (median
798 age 74, $p=0.5166$, $n=100$) and Shal (median age 75, $p=0.9805$, $n=100$) controls did not differ
799 from PDF/+ controls.

800

801 **Figure 5:** Computational modeling of I-LNv electrophysiological data.

802 **A**, 5-second epoch of a representative current-clamp recording of I-LNv electrical activity (upper
803 trace) in the morning (ZT0-2) and the evening (ZT12-14) and a corresponding 5-second epoch
804 of model simulation of electrical activity (lower trace) in the morning and the evening. **B**,
805 Magnification of one action potential in the model simulation (top) in both morning and evening
806 and the underlying voltage-gated potassium channel currents (bottom). The Shaw current was
807 a large contributor to action potential current in the morning, whereas Shal was a large
808 contributor to the action potential in the evening. **C**, Comparison of modelled and recorded
809 action potential firing rate in the morning and evening showed a clear difference for time of day
810 ($p<0.001$, two-way ANOVA) that was consistent between the model and experimental data. Box
811 and whiskers are median, IQR and min-max, $n=20$ for each.

812

813 **Figure 6:** Dynamic clamp models rescue pharmacological block of specific voltage-gated
814 potassium channels.

815 **A,** Representative current-clamp recording of I-LNv electrical activity (top row) recorded at ZT6
816 showing control activity (left panel) application of the Shaker blocker DTX (100 nM) (middle
817 panel) and subsequent reintroduction of Shaker current using dynamic clamp to rescue action
818 potential firing rate (right panel). Dynamic clamp current output during the current-clamp
819 recordings are also shown (bottom row). **B,** Quantification showed the action potential firing
820 frequency before (left) and after (middle) application of the respective channel blockers (DTX
821 n=5, GxTX n=5, BDS n=6, PaTX n=5), and then with the blocker plus the dynamic clamp model
822 (right). Application of all channel blockers can be rescued by use of the dynamic clamp model.
823 Conductances used for dynamic clamp models are given in Table 2.

824

825 **Figure 7:** Switching between morning- and evening-like electrical states of the I-LNvs using
826 dynamic clamp.

827 **A,** Representative current-clamp recording (upper trace) and dynamic clamp output (lower
828 trace) of I-LNv electrical activity in the morning showed the characteristic morning action
829 potential firing rate of ~2 Hz. Removal of Shaw current and introduction of Shal current using
830 dynamic clamp switched the firing rate to the evening level of ~1 Hz (left panel). In the evening,
831 introduction of Shaw and removal of Shal switched the firing rate from ~1 Hz to the morning
832 state of ~2 Hz (right panel). **B,** Quantification showed the switch from a morning to an evening
833 state (left panel, $p < 0.0001$, $n=4$) and from an evening to a morning state (right panel, $p < 0.0001$,
834 $n=3$). Conductances used for dynamic clamp models are given in Table 3.

835

836 **Table 1: Hodgkin-Huxley equation parameters for ion channel models.** For each of the
837 voltage-gated potassium channel models describing Shaker, Shab, Shaw and Shal, the

838 activation and inactivation parameters and conductances are given as determined by fit to
 839 Hodgkin-Huxley equations (see Methods for details).

<u>Activation (m)</u>	<u>Shaker (Kv1)</u>	<u>Shab (Kv2)</u>	<u>Shaw (Kv3)</u>	<u>Shal (Kv4)</u>
Vh (mV)	-34.18	-29.69	-57.30	-48.63
K (mV ⁻¹)	9.75	10.49	14.63	7.65
Amp (s ⁻¹)	1.79	30.72	0.03	0.05
Vmax (mV)	-4.07	-63.69	20.03	-74.86
σ (mV ⁻¹)	73.88	28.54	238.58	61.54
<u>Inactivation (h)</u>				
Vh (mV)	-93.19	NA	-25.82	-43.05
K (mV ⁻¹)	54.49	NA	2.5	1.73
Amp (s ⁻¹)	22.49	NA	154.83	44.52
Vmax (mV)	35.81	NA	159.82	-66.24
σ (mV ⁻¹)	52.04	NA	167.10	180.11
<u>Conductance (nS)</u>				
P	4	4	4	4
Q	1	0	1	1

840

841

842 **Table 2: Dynamic clamp rescue experiment conductances.** For each of the voltage-gated
 843 potassium channel models describing Shaker, Shab, Shaw, and Shal, the conductances used
 844 to rescue pharmacological block of the channel are given (see Figure 6).

<u>Conductance (nS)</u>	<u>Shaker (Kv1)</u>	<u>Shab (Kv2)</u>	<u>Shaw (Kv3)</u>	<u>Shal (Kv4)</u>
	2.35	0.86	1.40	0.8
	2.05	0.66	1.40	0.8
	1.90	0.66	1.30	0.8
	1.60	0.44	1.30	0.8
	1.55	0.44	1.30	0.8
			1.30	

845

846

847 **Table 3: Dynamic clamp day/night switching experiment conductances.** For the voltage-
 848 gated potassium channel models describing Shaw and Shal, the conductances used to switch
 849 the I-LNvs between morning and evening firing states are given (see Figure 7).

<u>Conductance (nS)</u>	<u>Shaw (Kv3)</u>	<u>Shal (Kv4)</u>
Day to Night	-1.4	1.25
	-1.2	1.15
	-1.1	1.00
	-0.8	0.90
Night to Day	1.4	-1.25
	1.2	-1.1
	1.2	-1.05

850