Synaptic Targets of Circadian Clock Neurons Influence Core Clock Parameters

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Neuronal connectivity in the circadian clock network is essential for robust endogenous timekeeping. In the *Drosophila* circadian clock network, four pairs of small ventral lateral neurons (sLN$_v$s) serve as critical pacemakers. Peptidergic communication via sLN$_v$ release of the key output neuropeptide *Pigment Dispersing Factor* (PDF) has been well characterized. In contrast, little is known about the role of the synaptic connections that sLN$_v$s form with downstream neurons. Connectomic analyses revealed that the sLN$_v$s form strong synaptic connections with a group of previously uncharacterized neurons, SLP316. Here, we show that silencing synaptic output in the SLP316 neurons via tetanus toxin (TNT) expression shortens the free-running period, whereas hyper-exciting them by expressing the Na^{+} channel NaChBac results in period lengthening. Under light-dark cycles, silencing SLP316 neurons also causes lower daytime activity and higher daytime sleep. Our results revealed that the main postsynaptic partners of the *Drosophila* pacemaker neurons are a non-clock neuronal cell type that regulates the timing of sleep and activity.

Keywords: circadian rhythms, connectomics, behavior, neuronal circuits

1

Introduction

Circadian rhythms, which allow animals to adapt to day-night changes in their environment, are generated by small groups of neurons in the brain that contain molecular clocks (1). These neurons form a network with synchronized molecular oscillations (2, 3). The maintenance of robust and coherent behavioral circadian rhythms depends on connectivity within the clock network, which controls the timing of key behavioral outputs like rhythms in feeding, mating, and sleep (3). In both mammals and flies, connectivity in the clock network is mediated by peptide and transmitter outputs (4-7). Although the molecular mechanisms driving endogenous oscillations in clock neurons are well described, the pathways through which these neurons communicate with each other and with downstream targets to propagate oscillations throughout the brain remain poorly understood.

Drosophila melanogaster is an ideal model for studying connectivity in the clock network. The fly circadian network consists of ~240 neurons ((8), reviewed in (9)) and is the functional equivalent of the mammalian suprachiasmatic nuclei, the principal clock in the vertebrate brain (10, 11). Each clock neuron within this network has an intracellular molecular timekeeping mechanism based on a transcriptional–translational feedback loop (12). Clock neurons can be organized into seven main groups based on their anatomical location (11, 13-16). Whereas the molecular oscillations of most of these groups are in phase, some major clock groups become active at specific times of day, which correlates with their roles in behavioral outputs (17). Among these cells, the small lateral ventral neurons (sLN$_v$s) are key for maintaining rhythmicity under conditions of constant darkness and temperature (DD) and are considered the dominant circadian pacemakers under these conditions (18-21). Despite the importance of these cells for sustaining physiological and behavioral rhythms, very little is known about their connectivity with target neurons. Indeed, an analysis of their synaptic connectivity revealed that they form very few monosynaptic connections with other clock neurons (16).

Peptidergic signaling is essential for clock network intercommunication and output (6). The neuropeptide *Pigment Dispersing Factor* (PDF), which is secreted by the sLN$_v$s, is required for synchronizing molecular oscillations within the network, setting the phase of neural activity in other groups of clock neurons, and maintaining activity-rest rhythms under constant conditions (18, 22-24). Expression of PDF undergoes daily and circadian oscillations in the sLN$_v$ dorsal terminals (19), and the loss of PDF or of the PDF receptor (PDFR) results in arrhythmicity under free-running conditions (25-27). In addition to PDF, the sLN$_v$s express the neuropeptide, short Neuropeptide F (sNPF) (28), which promotes sleep and is involved in feeding regulation (28-30), and the inhibitory neurotransmitter glycine (31). Interestingly, downregulation

67   of *bruchpilot* (*brp*) and of other synaptic active zone proteins expressed by the sLN$_v$s does not affect

68   rhythmicity, and dense-core vesicle release profiles of the sLN$_v$s in the Full Adult Fly Brain (FAFB) do not

69   show spatial overlap with BRP-labeled active zones (32). This finding is consistent with the observation

70   that neuropeptide release takes place from sites located all along the sLN$_v$, including the soma (33), and

71   with the persistence of robust behavioral rhythmicity upon silencing of synaptic transmission (34) in *Pdf+*

72   neurons (35, 36)

73

74   Although the circadian clock provides predictability, it must also be flexible to adapt to varying

75   environmental conditions. Distinct subsets of neurons control the morning and evening bouts of activity

76   that produce the bimodal pattern that flies show under light-dark cycles. The sLN$_v$s, which are active around

77   dawn (17), are associated with the morning peak of activity (18, 20, 21) and are called morning (M) cells.

78   The PDFR-expressing lateral neurons dorsal (LN$_d$s) and the PDF-negative 5th LN$_v$ are associated with the

79   evening peak activity (20, 21, 37) and their neural activity peaks before dusk (17). Some dorsal neurons

80   (DNs) also contribute to the timing and amount of activity and sleep via the modulation of M and E cells

81   (38-41). The phase of the morning and evening activity bouts adjusts to environmental light and temperature

82   (42-47), allowing flies to adjust their activity patterns according to the season.

83

84   The four sLN$_v$s form few direct synaptic connections with other clock neurons or with other neurons

85   outside the clock network, yet they form strong connections with a previously uncharacterized group of

86   neurons named SLP316. The SLP316 cells synapse onto the DN1pA class of dorsal clock neurons, which

87   are associated with the reorganization of sleep/wake cycles (reviewed in (40)). Here, we show that despite

88   not being clock neurons themselves, silencing SLP316 neuronal activity resulted in a shortening of the free-

89   running period of activity, whereas their excitation resulted in the opposite phenotype. Neither manipulation

90   led to the loss of rhythmicity. Under light-dark cycles (LD), manipulations of the SLP316 activity led to a

91   reduction in evening activity and an increase in sleep, both under ambient temperature of 25°C, and under

92   cold, winter-like days. Under both LD and DD, the behavioral phenotypes observed were different from

93   those caused by the loss of PDF. In conclusion, our data reveal a role for a previously unknown neuronal

94   population that connects two classes of clock neurons but is not itself part of the circadian clock network,

95   in regulating the timing of sleep/wake cycles.

96

97   **Results**

98

99   **1-Connectomics datasets identify SLP316 neurons as primary post-synaptic partners of sLN$_v$s**

100

101   Based on the *Drosophila* hemibrain connectome (48), the sLN$_v$s form much stronger connections with an
102   uncharacterized neuronal cell type than with any other neurons, including other clock neurons (16) (Fig.
103   1A). This cell type, SLP316, consists of three neurons with somas in the dorsal-most region of the Superior
104   Lateral Protocerebrum (SLP) (48). Interestingly, the sLN$_v$s are in turn the main presynaptic inputs to
105   SLP316 neurons (Fig 1A, B). Most inputs to SLP316 cells from their top ten presynaptic partners are located
106   along the ventromedial projections, whereas their output synapses are concentrated along the dorsal
107   arborizations (Fig. 1C).

108

109   The synapses formed by the sLN$_v$s onto the SLP316 neurons represent 70% of the synapses by all groups
110   that form strong connections with these cells (weight >10) (Fig 1A, B). Many of the synapses that the sLN$_v$s
111   form onto the SLP316s (~20%) are located below the point at which the sLN$_v$s extend and ramify their
112   dorsal termini (Suppl. Fig. 1A), the region long thought to be the location of sLN$_v$ output synapses. The
113   other main inputs to SLP316 are from two neurons that belong to the SLP364 cell type and from
114   ventroposterior (VP) antennal lobe glomeruli neurons. The SLP316s are innervated largely by neurons in
115   the SLP neuropil but also from the superior medial (SMP) and posterior lateral (SLP) protocerebrum (Suppl.
116   Fig. 1B).

117

118   The SLP316 neurons are largely homogeneous in their connectivity patterns with upstream neurons, but
119   their outputs are more varied (Fig 1B'). These cells form some of the strongest connections with another
120   group of clock neurons, posterior dorsal neurons (DN1p). The DN1pA class of clock neurons is the top
121   output of the SLP316 cells. The main neuropil innervated by SLP316s is the SLP, followed by the SMP
122   and PLP with a much smaller number of connections (Suppl. Fig. 1B). We next examined the connectivity
123   patterns of the sLN$_v$s in the FlyWire *Drosophila* connectome (49) to determine whether cells similar in
124   morphology to the SLP316s were among their top post-synaptic partners. We found that in both
125   hemispheres, the sLN$_v$s form most of their output synapses with cells highly similar in location and
126   morphology (Suppl. Fig. 1C). Computational predictions based on synapse architecture for both the
127   hemibrain and FAFB-Flywire datasets (50) indicate that SLP316 cells are likely glutamatergic.

128

2-The SLP316 neurons are non-clock neurons that extend along the sLN$_v$ projection tract

130

131   To further study the SLP316 neurons, we generated a Split-Gal4 line that targets these cells (#SS76489, see
132   Methods) (Suppl. Fig. 2A). Since both the hemibrain and FAFB/FlyWire connectomes correspond to an
133   adult female (48), we dissected brains from both *SLP316>mCD8-GFP* males and females to determine if
134   there were sex differences in the number of cells or their morphology. The projections from the SLP316

135 neurons extend toward the ventral brain along the same tract as the projections of the sLN$_v$s, which extend

136 from the ventral to the dorsal brain (Fig. 2A-B). We did not find expression in additional neurons in the

137 central brain or the ventral nerve cord, or sex differences in the number of cells (Fig. 2C). The number of

138 cells remains similar in older flies (Suppl. Fig. 2B). Therefore, we conclude that the driver is likely

139 specifically expressed in SLP316 neurons in adults and that the expression pattern is similar in males and

140 females.

141

142 The SLP316 neurons are in the dorsoposterior area of the brain, where the DN1ps are located. To determine

143 if these cells were yet unidentified clock neurons, we co-stained with a PER antibody. Adult

144 *SLP316>mCD8-GFP* flies were entrained to a 12h:12h light-dark cycle (LD) for 5 days, and brains were

145 dissected at the end of the night (ZT23), when PER nuclear signal is strongest (51). We did not detect PER

146 nuclear signal in the somas of any of the SLP316 cells (Fig. 2D). Therefore, we conclude that the SLP316

147 neurons are not circadian clock neurons. To determine if there were daily changes in excitability in these

148 cells, we used a transcriptional reporter in which the *Hr38* regulatory DNA is upstream of a nuclear-

149 localized fluorescent Tomato reporter (52). In the sLN$_v$s, the *Hr38-Tomato* reporter gene shows higher

150 expression around ZT2, consistent with a previous study (53). In contrast, SLP316 cells showed consistently

151 low signal, and the presence of physiological rhythms cannot be excluded based on these data (Suppl. Fig.

152 2C-D).

153

154 Next, we assessed connectivity between the cells labeled by the SLP316 split-GAL4 line and the sLN$_v$s via

155 BAcTrace. This is a retrograde synaptic tracing technique in which *Clostridium botulinum* neurotoxin A1

156 (BoNT/A) is transferred from a postsynaptic donor cell (driven by Gal4 components) to a presynaptic

157 receiver cell (driven by LexA components) by targeting the interior of a synaptic vesicle (54). In the receiver

158 cell, the toxin releases a membrane-localized transcription factor, inducing Tomato expression. When a

159 vesicle is recycled, the toxin is internalized into the cell. We combined flies expressing the BAcTrace

160 transgenes (see the methods section) with flies carrying both the SLP316 split Gal4 and the *Pdf-LexA*

161 transgenes and stained brains with antibodies against PDF, RFP, and GFP (Fig 2E-H). Experimental flies

162 showed RFP signal in the sLN$_v$s but not the lLN$_v$s, whereas PDF-immunoreactivity was detected in both

163 groups of LN$_v$s (Fig. 2E). Control flies without the SLP316 split Gal4 driver did not express RFP (Fig. 2G).

164 As expected, GFP signal could be detected in both the sLN$_v$s and lLN$_v$s in control flies, reflecting the

165 expression pattern of the *Pdf-LexA* driver present in both lines (Fig. 2F and H). We did not observe sex

166 differences in expression pattern in experimental or control flies. These results support the notion that the

167 SLP316 neurons are post-synaptic partners of the sLN$_v$s, which is consistent with connectomics data.

168

169 **3- Altering the activity of SLP316 neurons changes the free-running period.**

170

171 Elimination of the LN_vs via expression of the pro-apoptotic gene *hid* leads to severe loss of rhythmicity and
172 shortening of the free running period, similar to the behavioral phenotypes of the *Pdf* null mutation,
173 *Pdf*[01](18). Whereas inhibiting synaptic transmission in *Pdf* neurons via tetanus toxin (TNT) does not affect
174 the ability of the flies to maintain rhythmicity in DD, it leads to changes in free-running period, as *Pdf*>TNT
175 flies show longer periods than their parental controls (35, 36). Since the SLP316s are the main cell type
176 downstream of the clock pacemaker neurons, we asked how manipulations of these cells would affect
177 behavioral rhythms.

178

179 The bacterial voltage-gated sodium channel *NaChBac* is used to increase neuronal excitability (reviewed
180 in (55)). Expression of *NaChBac* in *Pdf*+ expressing neurons results in complex rhythms, with multiple
181 superimposed period components under free-running conditions (56). *NaChBac* expression in the SLP316
182 cells resulted in lengthening of the free-running period without affecting overall rhythmicity (Fig. 3A-C
183 and Table 1), similar to the phenotype observed upon silencing activity of the *Pdf*+ neurons via TNT. After
184 eight days in DD, experimental flies showed a 3.3-hour phase delay in their activity peak (Fig. 3D-E).
185 Under a light dark-dark cycle, *SLP316>NaChBac* flies showed a significant reduction in daytime activity
186 (Suppl. Fig. 3A-D).

187

188 Next, we expressed *hid* under the SLP316 driver. Under DD conditions, *SLP316>hid* flies had a short free
189 running period (Fig 3F-G). The overall number of rhythmic flies and their rhythmic power were not affected
190 (Fig 3H and Table 1). A phase advance in activity can be detected on day 8 under DD conditions (Suppl.
191 Fig. 3P-Q). *SLP316>hid* females also show a shortening in free-running period and no effect on the overall
192 number of rhythmic flies (Suppl. Fig. 4A-B and E). Therefore, elimination of most SLP316 cells and
193 *NaChBac*-mediated hyperexcitation of these cells led to opposite phenotypes in free-running period of
194 activity rhythms.

195

196 To determine whether synaptic activity of the SLP316 cells mediates the effects on period we expressed
197 TNT (34). This manipulation led to a shortened period (Fig 3F, I and Table 1). We also observed a small
198 but significant reduction in the percentage of rhythmic flies and a phase advance at DD 8 of ~3.2 hours
199 (Fig. 3J-L). In *SLP316>TNT* females, the reduction in free-running period was not significantly different
200 from that of controls (Suppl. Fig. 4C-E). Under LD, both *hid* and *TNT* expression in SLP neurons resulted
201 in a reduction in daytime activity (Suppl. Fig. 3G, L). Shortening of free-running period is typically
202 associated with an early phase of the evening peak of activity under a light-dark cycle (LD), such as in *Pdf*[01]

203 mutants or flies in which *Shaggy* or *Doubletime* (DBT^S) are expressed in the sLN$_v$s (57, 58). However, LD

204 activity profiles of *SLP316>hid* and *SLP316>TNT* flies indicate that these flies could still anticipate the

205 lights-on transition and did not have an early phase of the evening peak (Suppl. Fig. 3F,I,K and N). The

206 evening peak area in experimental *SLP316>hid* and *SLP316>TNT* males was significantly lower than the

207 controls, indicating lower activity at this time of day (Suppl. Fig.3J,O).

208

209 The only behavioral rhythm that is mediated by the LN$_v$s but does not rely on PDF is adult emergence from

210 the pupal case (eclosion) (59, 60). The larval LNs, which survive metamorphosis and become the adult

211 sLN$_v$s (61), communicate with the PTTH neurons to regulate eclosion rhythms (59). In pharate adults, the

212 sLN$_v$s inhibit PTTH neuron Ca^{2+} activity via short neuropeptide F (sNPF), not PDF, and the PTTH neurons

213 appear to be post-synaptic targets of the sLN$_v$s based on transsynaptic tracing experiments (59). In addition,

214 PTTH neurons express sNPF and not PDF receptors (59). To determine if manipulations of SLP316 neurons

215 affected eclosion rhythms, we conducted population experiments in which these cells were eliminated via

216 expression of *hid*. We did not detect eclosion rhythm phenotypes in experimental *SLP316>hid* flies (Fig.

217 4A-B). Taken together, these results show that manipulations of the SLP316 neurons affect free-running

218 rhythms in activity but not eclosion rhythms.

219

220 **4.- Organization of activity under cold days is disrupted upon SLP316 silencing**

221

222 Since a subset of the DN1p class of clock neurons are major postsynaptic partners of the SLP316 cells, we

223 investigated the effect of manipulating these cells under low temperature light-dark (LD) conditions, where

224 the DN1ps appear to play a more prominent role in shaping activity (45). Only under conditions with low

225 light intensity, low temperature, or low or absent PDF can rescuing the molecular clock in a subset of

226 glutamatergic DN1ps promote an evening peak of activity (44, 45).

227

228 Low temperatures lead to a reorganization of activity in wild-type flies as they adapt to the shorter days of

229 winter. Under low temperature conditions (18°C) and light-dark cycles, the distance between the morning

230 and evening peaks is reduced, primarily due to a substantially earlier phase of the evening peak, which

231 decreases the phase angle of entrainment (42). We exposed flies in which we eliminated or silenced SLP316

232 cells to LD cycles under cold temperatures. *SLP316>hid* flies show significantly reduced daytime activity

233 and increased daytime sleep compared to controls (Fig. 5A-D). Similar phenotypes, both in terms of

234 daytime activity and daytime sleep, were observed in experimental *SLP316>TNT* flies (Fig. 5E-H). Neither

235 nocturnal activity nor sleep were affected (Suppl. Fig. 5A-D).

236

Under free-running conditions, manipulations of SLP316 neuron activity and the loss of *Pdf* result in distinct phenotypes. The *Pdf*[01] mutant is predominantly arrhythmic (18). However, the *Pdf*[01] flies that remained rhythmic exhibited a shortened free-running period, similar to the effect observed after silencing SLP316 neurons (18). Under light-dark (LD) conditions, manipulations of SLP316 neurons did not cause the expression of the classic phenotypes associated with the loss of *Pdf*, such as a lack of morning anticipation or an advanced evening peak (Suppl. Fig. 3). The advanced evening peak is thought to result from a phase-delay effect of PDF on evening cells: in the absence of PDF, neuronal activity in the evening cells show a large phase advance (24). PDF levels are temperature-sensitive, increasing during hot, summer-like days and decreasing under colder conditions (62).

We exposed *Pdf*[01] flies to the same LD 18°C cycles and observed a significant advance in the evening peak and a 1.5-fold increase in daytime activity (Fig. 5I-J). This indicates that the early peak observed in wild-type flies under LD (42) is not solely attributable to the reduction in PDF under lower temperatures, since flies that lack PDF show a pronounced shift in evening activity. *Pdf* mutants show higher levels of daytime sleep in 12:12 LD cycles at 25°C (63); however, the pronounced increase in evening activity under LD 18°C is accompanied by an overall decrease in daytime sleep (Fig. 5K-L). The onset of activity was earlier, and activity levels remained high until the lights-off transition. After the lights-off transition, activity and sleep levels between control and *Pdf*[01] flies converge (Fig. 5I,K and Suppl. Fig. 6E-F). These findings indicate that under cold-day conditions, the loss of *Pdf* and the silencing or elimination of SLP316 neurons results in the expression of opposite behavioral phenotypes.

5.- Silencing SLP316 neurons increases daytime sleep

Under standard light-dark conditions at 25°C, *Pdf* mutants show increased sleep during the day, as well as during the subjective day under constant conditions (63). However, the effect of *Pdf* loss on sleep appears to be dependent on temperature: indeed, these flies show increased sleep at 25°C and decreased sleep at 18°C (Fig. 5L). We analyzed the sleep profiles of flies expressing *hid* in the SLP316 cells under a LD cycle at 25°C and observed a significant increase in daytime sleep (Fig. 6A-B). (These results were obtained using the standard quantification based on periods of 5 minutes of inactivity (64, 65)). A similar phenotype was observed in *SLP316>TNT* flies (Fig. 6E-F). Neither of those manipulations affected nighttime sleep (Suppl. Fig 6). These findings allow us to conclude that the effects on sleep are not unique to low temperature cycles but, in contrast to PDF's temperature dependent roles, reflect a feature of the SLP316 neurons at both low and high temperature.

271   To determine the underlying cause for this increase in sleep, we analyzed P(doze) and P(wake), the

272   probabilities that an inactive fly will start moving or an active fly will stop moving, respectively, between

273   1-minute activity bins. P(doze) corresponds to sleep pressure, and P(wake) corresponds to sleep depth; thus,

274   these conditional probabilities can offer insight into the biological processes driving changes in sleep (66).

275   P(doze) was higher throughout most of the day and night for both *SLP316>hid* and *SLP316>TNT* animals,

276   especially during the evening (ZT6-12) and morning (ZT18-3), when flies are most active (Fig. 6 C,D and

277   G,H). The average P(wake) was not significantly affected in either manipulation, although *SLP316>hid*

278   flies showed a minor reduction in P(wake) from ZT9-12 (Suppl. Fig. 7A-D). This increase in P(doze)

279   suggests that the increased sleep observed when the SLP316s are silenced was due to increased sleep

280   pressure, especially at times when flies should be most active. We conclude that silencing SLP316 neurons

281   leads to an increase in daytime sleep under both 25°C and low temperature conditions, and that this

282   phenotype is not dependent on PDF since *Pdf* manipulations lead to opposite effects.

283

284   **Discussion**

285

286   The recent availability of connectomics data allows the identification of neuronal connections that have not

287   been described using other approaches. We used openly available *Drosophila* connectomes to identify the

288   SLP316 neurons, which are the main post-synaptic partners of the sLN$_v$s, the key pacemaker neurons in the

289   *Drosophila* clock network. We found that genetic manipulations of the SLP316 neurons led to changes in

290   free-running rhythms: silencing their activity via TNT or eliminating them via *hid* leads to a period

291   shortening, whereas hyperexciting them via *NaChBac* led to a period lengthening. Another important

292   behavioral output of the sLN$_v$s, eclosion rhythm, was not affected by genetic elimination of SLP316

293   neurons. Under light dark cycles, silencing SLP316s resulted in a reduction in daytime activity and an

294   increase in sleep, under both 25°C and low temperature conditions. Neither the free-running nor the light-

295   dark (LD) phenotypes observed following SLP316 manipulations resembled those caused by the loss of

296   PDF, a well-known output of the sLN$_v$s, which facilitates communication with the rest of the circadian

297   clock network.

298

299   Our study identified a non-clock group of neurons that affects circadian rhythms and contributes to our

300   understanding of neuronal connectivity within the clock network. To date, the best characterized output of

301   the sLN$_v$s is PDF (18, 67), a neuropeptide that accumulates--and is also released (33)- rhythmically from

302   their dorsal termini and soma, likely independently of classical transmitter release from these cells (32).

303   The sLN$_v$s also express sNPF (28), and there is evidence that they also use glycine to communicate with

304   postsynaptic targets (31). Based on the availability of neurotransmitter use information, the flywire EM

305 prediction for the sLN$_v$s is acetylcholine (50), but the likelihood is relatively low. If the sLN$_v$s contact the

306 SLPs via glycine, they would likely inhibit them, which is consistent with the observation that inhibiting

307 synaptic release from *Pdf* neurons via TNT and exciting the SLP316 neurons via *NaChBac* both lead to

308 period lengthening. Functional connectivity experiments between the sLN$_v$s and the SLPs would be needed

309 to determine the nature of this connection.

310

311 Given their prominent role in the clock network, several studies have examined neuronal targets of the

312 sLN$_v$s that would contribute to the propagation of rhythmic signals throughout the brain. PDF receptors

313 (*PdfR*) are expressed by a wide range of neurons distributed throughout the brain, including much of the

314 clock network (6), and PDFR-expressing cells respond to sLN$_v$ stimulation (68). PTTH neurons, which are

315 downstream of sLN$_v$s, express sNPF receptors, are inhibited by the sLN$_v$s via sNPF to regulate eclosion

316 rhythms (59). Connections between *Pdf*+ cells and non-clock cells that regulate rhythm amplitude were

317 detected via GRASP (69). More recently, the Blau laboratory used the transsynaptic tracer trans-tango to

318 identify neuronal populations downstream of the sLN$_v$s and reported that they form connections with a

319 subset of DN1p clock neurons that express the neuropeptide CNMa and with mushroom body Kenyon cells

320 (70). Strong monosynaptic connections between the sLN$_v$s and the DN1ps cannot be detected in the

321 connectomes (16, 49), but it is possible that those synapses were missed because the sLN$_v$s show daily

322 structural plasticity rhythms (71), as do the VIP expressing neurons in the SCN (72). Truncation of the

323 dorsal termini of the sLN$_v$s, which are the sites of synaptic output and undergo clock-controlled plasticity,

324 does not affect free-running rhythms (38, 73), suggesting that PDF release is independent of the arborization

325 rhythms. In addition, silencing synaptic transmission in *Pdf*+ neurons via TNT does not lead to the loss of

326 behavioral rhythms (36), nor does downregulation of *bruchpilot* (*brp*) or other active zone proteins (32).

327 Taken together, these results suggest that unlike PDF release, the synaptic connections that the sLN$_v$s form

328 with downstream neurons are not required for maintaining activity-rest rhythms under free running

329 conditions. However, those connections play a role in regulating the free-running period of those rhythms.

330

331 The sLN$_v$s appear to contact the DN1p clock neurons both via PDF release (58) and synaptically through

332 the SLP316s (Fig. 1). A functional clock in CRY-positive DN1ps, along with the presence of PDF, is

333 sufficient for morning anticipation (44, 45, 74). Neither inhibition of synaptic transmission from sLN$_v$ nor

334 manipulation of the SLP316 cells appeared to affect morning anticipation, suggesting that this feature

335 depends on only PDF signaling from the sLN$_v$s. Rescuing the molecular clock in a glutamatergic subset of

336 DN1ps can promote an evening peak of activity when PDF is reduced or absent (44, 62). The pronounced

337 and very early peak of activity we observed in *Pdf^{01}* flies under cold temperatures may at least in part be

338 due to increased prominence of the activity of evening DN1ps in the absence of PDF (75). Another clock

339 subset, DN1a, which is inhibited by cold temperature, is connected reciprocally to the sLN$_v$s through the

340 neuropeptides CCHamide1 and PDF (76, 77). Thus, a cold-temperature induced change in sLN$_v$ activity

341 could alter evening DN1p activity via the SLP316s, while simultaneous loss of PDF eliminates suppression

342 of the DN1p-induced effects. These glutamatergic DN1ps are also sleep-promoting; activating them

343 increases daytime sleep and arousal threshold at ZT6 and 10, whereas inhibiting them decreases both

344 daytime and nighttime sleep (78). If the SLP316 neurons are glutamatergic as predicted in Flywire (49) and

345 inhibitory, silencing them would be expected to increase the neuronal activity of the DN1ps and thus

346 daytime sleep levels, in agreement with our results.

347

348 Studying how neurons use different signals to communicate with downstream targets is essential for

349 understanding how networks of neurons produce behavior. The *Drosophila* sLN$_v$s are an excellent model

350 for studying neuronal basis of behavior given their well characterized role as key circadian pacemakers.

351 This role relies on their release of the neuropeptide PDF as a critical output signal for synchrony within the

352 network. Our study is the first to examine the role on rhythmic behavior of direct synaptic targets of the

353 sLN$_v$s based on connectomics data, a connection that is likely independent of PDF. Our results reveal that

354 manipulations of the SLP316 cells, the primary postsynaptic partners of the sLNvs in the connectome, can

355 affect a core circadian parameter, free-running period. This finding indicates that a non-clock neuronal cell

356 type can modulate circadian timekeeping. We show that manipulations of the SLP316 cells and loss of PDF

357 lead to different behavioral phenotypes, both under free running conditions and under standard and low

358 temperature light-dark cycles. These two pathways of clock neuron output may add plasticity to the system

359 and allow the circadian network to adjust to changing environmental conditions.

360 **Materials and Methods**

361 **Fly lines and husbandry:**

362 Flies were reared on cornmeal-sucrose-yeast media under a 12hr:12hr Light:Dark (LD) cycle at 25 °C. The

363 fly lines used in this study were: *SLP316 split-Gal4* (SS76489: w; 38E08-p65ADZp in attP40; 36B07-

364 ZpGdbd in attP2), w; *Pdf-Red, Pdf-Gal4; w;;UAS-NaChBac, yw;UAS-hid/cyo;UAS-mCD8-GFP, w;UAS-*

365 *TeTxLC;,* *w^{1118},* *w;;Pdf^{01},* and *w;* LexAop2-Syb.GFPN146I.TEVT173V LexAop2-

366 QF2.V5.hSNAP25.HIVNES.Syx1A/CyO,20XUAS-B3R.PEST-UAS(B3RT.B2)BoNTA QUAS-

367 mtdTomato-3xHA/TM6B, Tb1 (see Fly Lines and Reagents table for details).

368

369 We identified the SLP316 split-GAL4 line (SS76489) by screening candidate hemidriver combinations

370 selected by comparing GAL4 driver expression patterns and the hemibrain reconstructions of SLP316. This

371 work was done in collaboration with the Janelia Fly Light Project Team using established methods and the

372 driver line is included in a recently published split-GAL4 collection (79).

373

374 **Immunohistochemistry:**

375 Flies were entrained to 12:12 LD cycles at 25°C and dissected at the specified times in cold Schneider's

376 *Drosophila* Medium. For all experiments, dissections were completed within 30 minutes. For anti-PER

377 staining (Fig. 2D), brains were dissected at ZT23 on the fifth day under LD. Brains were fixed in 2%

378 paraformaldehyde prepared in Schneider's *Drosophila* Medium for 30 minutes at room temperature.

379 Blocking was performed using 5% normal goat serum in PBS containing 0.3% Triton (PBS-TX) for 1 hour

380 at room temperature. Samples were incubated with primary antibodies for 24 to 48 hours at 4°C, followed

381 by several washes with PBS-TX under agitation. Secondary antibody cocktails were applied for 24 to 48

382 hours at 4°C, after which samples were washed again with PBS-TX. Antibodies (see table) were diluted in

383 PBS-TX supplemented with 5% normal goat serum. Brains were mounted between coverslips and imaged

384 at the Advanced Science Research Center (GC-CUNY) using an Olympus Fluoview 3000 laser-scanning

385 confocal microscope (Olympus, Center Valley, PA). Brains for the BacTrace experiments were imaged

386 using an Olympus/Evident Fluoview 4000 laser-scanning confocal microscope (Fernández lab, Indiana

387 University Bloomington).

388

389 The LM images in Figure S2 are based on confocal stacks previously reported (79) and the original images

390 are available online (https://splitgal4.janelia.org/cgi-bin/view_splitgal4_imagery.cgi?line=SS76489). The

391 EM-LM overlay was generated using VVDviewer (https://zenodo.org/records/14468001). The other

392 images show maximum intensity projections produced using Fiji (https://imagej.net/software/fiji/).

393

394 **Connectome analysis:**

395 The *Drosophila* Hemibrain (release v2.1) was accessed via neuprint (https://neuprint.janelia.org/). FlyWire

396 data are based on FAFB version 783 (https://flywire.ai/)(49, 80). Hemibrain reconstructions are identified

397 by their instance names in neuprint (e.g. 'SLP316_R') with individual cells distinguished here by an

398 additional number ('SLP316_R_1').

399

400 **Behavior recording and analysis:**

401 Locomotor activity rhythms of adult male and virgin female flies were recorded using DAM2 *Drosophila*

402 Activity Monitors. Three- to six-day old flies were placed individually in TriKinetics capillary tubes

403 containing 2% agar- 4% sucrose food at one end sealed with a cap, plugged with a small length of yarn,

404 and loaded into the DAM2 monitors for locomotor activity recording. Flies were entrained to 12:12 LD

12

405 cycles for at least five days and then released into constant darkness (DD) for at least nine days at a constant

406 temperature of 18 or 25°C (as indicated in the figure legend for each experiment).

407

408 Free-running activity rhythms were analyzed with ClockLab software. ClockLab's Chi-square periodogram

409 function was used to analyze rhythmicity and rhythmic power in individual flies using 30-minute bins for

410 activity and a confidence level of 0.01. The "Rhythmic Power" for each designated rhythmic fly was

411 determined by subtracting the "Significance" value from the "Power" value associated with the predominant

412 peak. Flies that did not exhibit a Rhythmic Power of 10 were categorized as "arrhythmic," and their period

413 and rhythmic power were not included in the analysis (81). ClockLab periodogram analysis was repeated

414 for rhythmic flies using 1-minute bins to quantify free-running period. For each of the genotypes tested,

415 only periodicities falling within the 14 to 34-hour range were taken into consideration. Phases on the eighth

416 day of free-running were calculated using 1-minute bins and applying a Least Squares sine-wave fit in the

417 ClockLab activity profile, excluding any phases with P>0.05. All relevant information about statistical

418 analyses is included in Table S1.

419

420 Activity counts were collected in 1-minute bins, and averaged across the population and profiles of specific

421 genotypes under LD were generated using PHASE (82). First, individual fly activity levels were normalized

422 by establishing the average activity across all 30-minute bins over days 2-4 under LD. Population averages

423 of this normalized activity were computed for each 30-minute bin and averaged into a single representative

424 24-hour day. Total daytime (ZT0-12) and nighttime (ZT12-24) activity counts from days 2-4 of LD were

425 recorded. Representative sleep plots and amounts were similarly generated for LD days 2-4 by classifying

426 5-minute periods of inactivity as sleep, unless otherwise specified. Total daytime and nighttime sleep over

427 the three-day period were also recorded. Morning and evening phase were quantified in PHASE using a

428 filter frame length of 481 minutes and filter order of 2. P(wake) and P(doze) values (66). were averaged for

429 the entire duration of LD days 2-4, or in three-hour windows and averaged across the same days.

430

431 **Adult emergence monitoring**

432

433 Flies were raised at 25°C for 5 days, then transferred to 20°C and entrained under a LD 12:12 light:dark

434 cycle. Resulting pupae were collected and fixed on eclosion plates with Elmer's glue and mounted on

435 Trikinetics eclosion monitors (Trikinetics, MA, USA). Emergence was then monitored for 7 days at 20°C

436 under DD conditions and analyzed as previously described (83). Rhythmicity of eclosion profiles was

437 analyzed using MATLAB (MathWorks, Inc., Natick) and the appropriate MATLAB toolbox (84). Using

438 autocorrelation analyses, records were considered rhythmic if the rhythmicity index (RI) was 0.3, weakly

439 rhythmic if 0.1<0.3, and arrhythmic if RI<0.1 (20).

440

441 **Fly lines and reagents**

442

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
SLP316 split Gal4 *(w; 38E08-p65ADZp in attP40; 36B07-ZpGdbd in attP2)*	Gerry Rubin and Aljoscha Nern, HHMI Janelia Research Campus	SS76489
w;Pdf-Red,Pdf-Gal4/cyo;+	Justin Blau, New York University	(85)
w;;UAS-NaChBac	Bloomington *Drosophila* Stock Center	RRID:BDSC_9468
yw;*UAS-hid/CyO*;*+*	Bloomington *Drosophila* Stock Center	RRID:BDSC_1557
w; UAS TeTxLC; +	Cahir O'Kane, University of Cambridge	RRID:BDSC_28838
w^{1118};+;+	Bloomington *Drosophila* Stock Center	RRID:BDSC_3605
w;;Pdf^{01}	Paul Taghert, Washington Univ. Med. School	(18)
LexAop2-Syb.GFPN146I.TEVT173V LexAop2QF2.V5.hSNAP25.HIVNES.Syx1A/CyO, 20XUAS-B3R.PEST UAS(B3RT.B2)BoNTA QUAS-mtdTomato-3xHA/TM6B, Tb1	Bloomington *Drosophila* Stock Center	RRID:BDSC 90825
Antibodies		
Mouse anti-PDF (1:3000 or 1:2000)	Developmental Studies Hybridoma Bank	
Rabbit anti-GFP (1:2000)	Thermo Fisher	A-11122
Rat anti-PER (1:500)	Orie Shafer, Indiana University Bloomington	
Rabbit anti-RFP (1:2000)	Rockland	#600-401-379-RTU
Chicken anti-GFP (1:2000)	Rockland	#600-901-215
Anti-mouse Alexa-488 (1:3000)	Thermo Fisher	A11029
Anti-rabbit Alexa-488 (1:3000)	Thermo Fisher	A48282
Anti-rat Alexa-568 (1:3000)	Thermo Fisher	A78946

Anti-rabbit Alexa-568 (1:3000)	Thermo Fisher	A11036
Anti-chicken Alexa-488 (1:3000)	Thermo Fisher	A11039
Software		
Fiji	http://fiji.sc	RRID: SCR_002285
PHASE	Open-source software: https://github.com/ajlopatkin/PHASE	(82)
MATLAB R2022b	MathWorks, Natick	RRID: SCR_001622
GraphPad Prism 9.0	GraphPad Software	RRID: SCR_002798
DAM FileScan	Trikinetics	
ClockLab	Actimetrics	RRID:SCR_014309
Chemicals		
Vectashield Mounting Medium	Vector Laboratories	#H-1000-10

443

Acknowledgments

Conflict of interest statement

Authors declare that they have no competing interests.

457

458

15

Figure legends

Fig. 1: SLP316 neurons are the main postsynaptic partner of the sLN$_v$s.

(A-B) Connectomic mapping from the hemibrain dataset. (A) Map of top input/output neuronal groups to/from the SLP316s and from the sLN$_v$s. The number of neurons in each group are indicated in parenthesis if greater than one and reflected by circle size. Connection strength between cell types is represented by arrow width and decreases in a clockwise direction for each connection type (input/output). SLP232 likely corresponds to DN3s. (B) Strong shared inputs to, and (B') outputs of the SLP316s (>10 synapses to/from at least two SLP316 neurons) with neuronal morphology shown above or below. (C) Input synapses to, and output synapses of, the three SLP316 neurons (SLP316_R_1, SLP316_R_2 and SLP316_R_3) shown in green, excluding synapses from neuron segments. Synapse color corresponds to neuron color in (A) and (B) and all other synapses are shown in grey.

Fig. 2: The SLP316 driver labels non-clock neurons that share functional synapses with the sLN$_v$s.

(A-B) Representative confocal images of a brain from a 7-day old male fly (*SLP316>GFP)* stained with antibodies against GFP (green) and PDF (magenta). (A) Whole brain, (B) Superior Lateral Protocerebrum z-stack, and anterior (B') and posterior (B") single optical slices (step size 0.9 µm). (C) Quantification of cell body count on the left hemisphere labelled by the *SLP316* driver in 7-day and 1-day old male and female flies from three replicate experiments (n > 21 for each age/sex). (D) Representative confocal images of a brain hemisphere of 6–7-day old male brains (*SLP316>GFP*) stained with antibodies against GFP (green) and PER (magenta). (E-F) Representative confocal images of *SLP316-Gal4* and *Pdf-LexA* driving expression of BAcTrace components. RFP signal can only be detected in the sLNvs. (E') sLN$_v$ dorsal termini show PDF and RFP expression. (E'') Cell bodies of sLN$_v$s and lLN$_v$s. (E''') sLN$_v$ cell bodies plus dorsal projections show RFP signal, whereas the lLN$_v$s do not. (F) syb-GFP expression in the small and large LN$_v$s in experimental brains. (G-H) Representative confocal images of *Pdf-LexA* driving expression of BATrace components, without the SLP316-Gal4 driver. All scale bars are 50 µm.

Fig. 3: Manipulation of the SLP316s modulates free-running period length

(A) Representative double-plotted actograms, (B) free-running period calculated using the Chi-square periodogram, and (C) percentage of rhythmic flies for control (*SLP316 split Gal4* and *UAS-NaChBac*) and experimental (*SLP316>NaChBac*) flies. (D) Activity plot on the eighth day of free running, indicated in (A) with yellow box. (E) Phase of activity from (D), calculated using a sine-fit of the waveform of activity in CLOCKLAB. (F) Representative actograms, (G) free-running period and (H) percentage of rhythmic flies for control (w*;UAS-hid;UAS-GFP* and w*;pUAS-tetx-LC-TNT;*) and experimental (*SLP316 >hid+GFP* or *SLP316>tetx-LC-TNT*) flies. (I) Free-running period, and (J) percentage of rhythmic flies

16

493   for control (*SLP316 split Gal4* and *;pUAS-tetx-LC-TNT;*) and experimental *(SLP316>tetx-LC-TNT)* flies.

494   (K) Activity plot and (L) phase of activity on the eighth day of free-running for control (*SLP316 split*

495   *Gal4* and *;pUAS-tetx-LC-TNT;*) and experimental *(SLP316>tetx-LC-TNT)* flies. Actograms show 5 days

496   of LD followed by 9 days of DD. Flies were raised and activity-rest behavior recorded at 25°C. Rhythmic

497   power was calculated using the Chi-square periodogram in ClockLab using 30-minute activity bins, and

498   flies with a rhythmic power of 10 or greater were classified as rhythmic. Free-running period was

499   calculated for rhythmic flies using the Chi-square periodogram routine of ClockLab using 1-minute

500   activity bins. Statistical comparisons were conducted using the Kruskal-Wallis test followed by Dunn's

501   multiple comparisons test for free-running period and phase of activity, and using Fischer's exact test for

502   the number of rhythmic and arrhythmic flies. Data are plotted from three replicate experiments for each

503   genotype.

504

505   **Fig. 4: Silencing or eliminating SLP316 neurons does not affect the eclosion rhythm.** (A) Records

506   showing the time course of emergence of flies under DD conditions (left), their corresponding

507   autocorrelation analysis (middle), and periodicities (right). (B) Period of eclosion of populations of flies of

508   the indicated genotypes.

509

510   **Fig. 5: *Pdf* and SLP316 disruption have opposite effects on daytime activity and sleep quantities**

511   **under cold temperature**. (A-D) Activity and sleep phenotypes of *SLP316>hid+GFP flies* during three

512   days of 12:12 LD at 18°C*.* Representative activity (A) and sleep (C) plots averaged for (*SLP316 split*

513   *Gal4* and *w;UAS-hid;UAS-GFP*) and experimental (*SLP316>hid+GFP*) flies. Daytime (ZT0-12) (B)

514   activity counts and total sleep amount (D). (E-H) Activity and sleep phenotypes of *SLP316>;tetx-LC-*

515   *TNT* flies and parental controls. Representative activity (E) and sleep (G) plots, and their respective

516   quantifications (F, H). (I-l) Activity and sleep phenotypes of for wild-type *w^{1118}* and *pdf^{01}* flies.

517   Representative activity (I) and sleep (K) plots, and total daytime activity counts (J) and sleep amount (L).

518   Flies were raised at 25°C and activity-rest behavior was recorded at 18°C. Statistical comparisons were

519   conducted using the Kruskal-Wallis test followed by Dunn's multiple comparisons test for comparisons

520   between three genotypes in (B), (D), (F) and (H), and using the Mann-Whitney test for comparisons

521   between two genotypes in (J) and (L). Data plotted are from two replicate experiments for (B), (D), (F)

522   and (H), and three replicate experiments for each genotype in (J) and (L).

523

524   **Fig. 6: Silencing or eliminating SLP316 neurons increases the amount of daytime sleep**

525   (A) Representative sleep plot averaged from three days of 12:12 LD at 25°C for control (*SLP316 split*

526   *Gal4* and *;UAS-hid;UAS-GFP*) and experimental (*SLP316>;hid;GFP*) flies. (B) Total daytime sleep over

17

527   three days for each genotype. (C) Representative sleep plot and (D) total daytime sleep for control

528   (*SLP316-Gal4* and *;pUAS-tetx-LC-TNT;*) and experimental (*SLP316>tetx-LC-TNT*) flies. (E) P(doze)

529   from the same three days of LD, plotted in three-hour for either control shown for controls, *SLP316 split*

530   *Gal4* and *;UAS-hid;UAS-GFP*), and experimental (*SLP316>;hid;GFP*) flies. (F) Average P(doze)

531   throughout entire 3-day period. (G) P(doze) plot and (H) average P(doze) for control (*SLP316-Gal4* and

532   *;pUAS-tetx-LC-TNT;*) and experimental (*SLP316>tetx-LC-TNT*) flies under same conditions. Sleep was

533   quantified in PHASE based on periods of inactivity of 5 minutes or more and plotted in (A) and (C) as

534   minutes of sleep per 30-minute bin. P(doze) was quantified in R using routines from the Griffith

535   Laboratory (66). Statistical comparisons were conducted using the Kruskal-Wallis test followed by

536   Dunn's multiple comparisons test. Data is plotted from four replicate experiments.

537

538   **Table 1. Summary of free running activity rhythms.** Activity analysis of the above genotypes at either

539   25˚C or 18˚C. Light conditions for each experiment were 12:12 LD-DD. For each genotype the number of

540   flies (n) is listed above. All flies were raised at 25˚C. ClockLab's χ-square periodogram analysis was used

541   to analyze rhythmicity, rhythmic power, and free-running period for each genotype. The percent

542   rhythmicity, along with the number of rhythmic flies (nR), the period in hours with the SEM, and the

543   rhythmic power with the SEM are indicated. Arrhythmic flies were not considered in the analysis.

544   Statistical analysis is included in Table S1.

545

546   **Supplementary Fig. 1:**

547   (A) Synapses from the four sLN$_v$s (red) onto the three SLP316 neurons above and below the main

548   branching point of the dorsal termini arborization in the hemibrain connectome. Synapses were counted

549   between each sLN$_v$ and all their SLP316 targets. (B) Number of input synapses from, and output synapses

550   to, neurons classified by neuropil. (C) Connectomic mapping from FlyWire. Neuronal morphology of the

551   eight sLN$_v$s shown in red and their top post synaptic target cell type shown in green.

552

553   **Supplementary Fig. 2:**

554   (A) Overlay of the reconstructed SLP316 neuronal morphology from the hemibrain connectome

555   (magenta) and an LM image of SLP316 cells labeled using the SLP316 split-GAL4 line (SS76489). The

556   neuropil marker is anti-Brp (mAb NC82). (B) SLP316 cell body count in 7-day old vs 5-week-old male

557   flies. Statistical comparison was conducted using the Mann-Whitney test. (C) Representative confocal

558   image of an adult brain of *SLP316>mCD8GFP* flies stained with PDF (white), GFP (green), and RFP

559   (magenta) antibodies. Flies also carry a Hr38-Tomato transgene. Scale bar = 50 μm. (D) Quantification of

560   Hr38-Tomato reporter intensity in the sLN$_v$s (left) and SLP316 neurons (right). Scatterplots of RFP

561   staining intensities plotted at different time points over a 24-hr cycle on LD. Each dot represents the mean

562   RFP intensity value averaged over both hemispheres of one brain. The black lines are the best fit

563   cosine curves from the parameters that were extracted via COSINOR analysis.

564

565   **Supplementary Fig. 3:**

566   (A) Representative activity plot and (B) daytime activity counts from three days of 12:12 LD at 25°C for

567   male control (*SLP316-Gal4* and *w;;UAS-NaChBac*) and experimental (*SLP316>NaChBac*) flies. (C)

568   Morning and (D) evening phase and (E) evening peak area from the same days and genotypes, calculated

569   in PHASE using a 481-minute frame length and filter order of 2 to detect peaks. (F) Representative

570   activity plot, (G) daytime activity counts, (H) morning and (I) evening phase of activity, and (J) evening

571   peak area for male control (*SLP316-Gal4* and w*;UAS-hid;UAS-GFP*) and experimental

572   (*SLP316>hid+GFP*) flies. (K) Representative activity plot (L) daytime activity counts, (M) morning and

573   (N) evening phase of activity, and (O) evening peak area for male control (*SLP316-Gal4* and *w;pUAS-*

574   *tetx-LC-TNT;*) and experimental (*SLP316>tetx-LC-TNT*) flies. (P) Activity plot and (Q) phase of activity

575   from the eight day of free-running for control (*SLP316-Gal4* and *w;UAS-hid;UAS-GFP*) and

576   experimental (*SLP316>hid+GFP*) male flies. Statistical comparisons were conducted using the Kruskal-

577   Wallis test followed by Dunn's multiple comparisons test. Data from three replicate experiments for (B-E

578   and P-Q) and four replicate experiments for (G-J) and (L-O).

579

580   **Supplementary Fig. 4:**

581   (A) Free-running period for female control (*SLP316-Gal4* and *w;UAS-hid;UAS-GFP*) and experimental

582   (*SLP316 >hid+GFP*) flies. (B) Difference between male and female experimental (*SLP316 >hid+GFP*)

583   free-running periods, corrected by subtracting the average control free-running period from each

584   experimental value. (C) Free-running period for control (*SLP316 split Gal4* and *w;pUAS-tetx-LC-TNT;*)

585   and experimental (*SLP316 >tetx-LC-TNT*) female flies. (D) Difference between male and female

586   experimental (*SLP316>tetx-LC-TNT*) free-running periods, corrected as in (B). (E) Percent rhythmicity

587   for control (*SLP316-Gal4, w;UAS-hid;UAS-GFP,* and *w;pUAS-tetx-LC-TNT;*) and experimental

588   (*SLP316>hid+GFP* and *SLP316>tetx-LC-TNT*) female flies, with all relevant significance indicated. (F)

589   Representative sleep plot from three days of 12:12 LD at 25°C for control (*SLP316-Gal4* and *w;UAS-*

590   *hid;UAS-GFP*) and experimental (*SLP316 >hid+GFP*) female flies. (G) Total daytime sleep and (H)

591   nighttime sleep from plotted days for each genotype. (I) Representative sleep plot, (J) daytime sleep and

592   (K) nighttime sleep for control (*SLP316-Gal4* and *;pUAS-tetx-LC-TNT;*) and experimental (*SLP316*

593   *>tetx-LC-TNT*) flies under same conditions. Statistical comparisons were conducted using the Kruskal-

594 Wallis test followed by Dunn's multiple comparisons test. Data from one experiment for (A-E) and two

595 replicate experiments for (G-H) and (J-K).

596

Supplementary Fig. 5:

598 (A) Records showing the time course of emergence of flies under DD conditions (left), their corresponding

599 autocorrelation analysis (middle), and periodicities (right). (B) Period of eclosion of populations of flies of

600 the indicated genotypes.

601

Supplementary Fig. 6:

603 (A) Nighttime activity and (B) nighttime sleep for control (*SLP316-Gal4* and *w;UAS-hid;UAS-GFP*) and

604 experimental (*SLP316 >hid+GFP*) flies for three days of 12:12 LD at 18°C. (C) Nighttime activity and

605 (D) nighttime sleep for control (*SLP316-Gal4* and *w;pUAS-tetx-LC-TNT;*) and experimental (*SLP316*

606 *>tetx-LC-TNT*) flies. (E) Nighttime activity and (F) nighttime sleep for wild-type w^{1118} and pdf^{01} flies.

607

Supplementary Fig. 7:

609 (A-B) P(wake) profiles (A) and values (B) plotted for three days of LD in three-hour bins shown for

610 controls, *SLP316 split Gal4* and *w;UAS-hid;UAS-GFP*), and experimental (*SLP316 >hid + GFP*) flies.

611 (C-D) P(wake) profiles (C) and values (D) plotted for three days of LD in three-hour bins shown for

612 controls, *SLP316 split Gal4* and *w;pUAS-tetx-LC-TNT;*), and experimental (*SLP316 >TNT*) flies. P(wake)

613 was quantified in R using routines from the Griffith Laboratory (66). Statistical comparisons were

614 conducted using the Kruskal-Wallis test followed by Dunn's multiple comparisons test.

615

616 **Table S1. Statistical analyses**. Summary of statistical analyses used throughout the manuscript. All

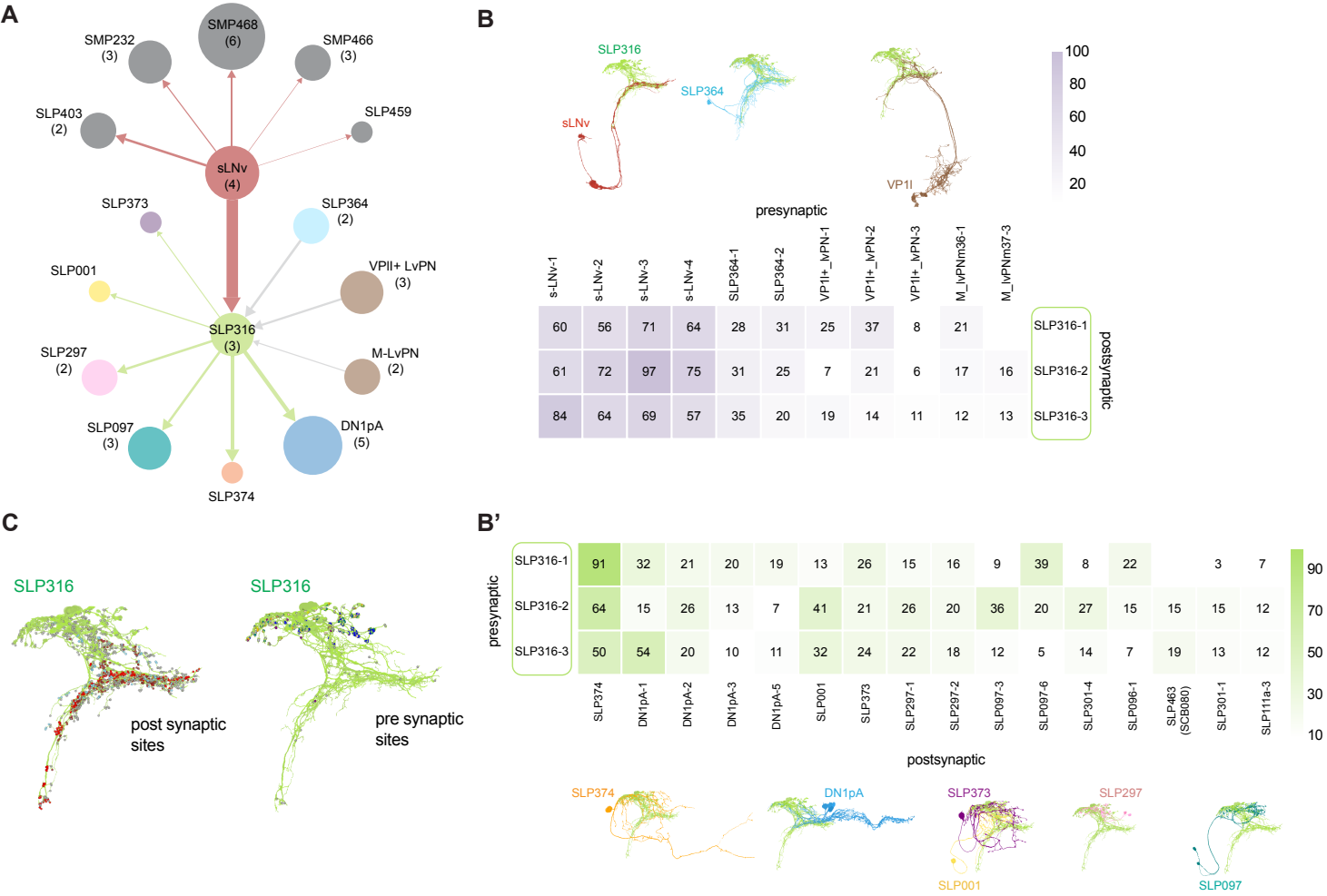617 comparisons were conducted in GraphPad Prism, version 10.4.0

618

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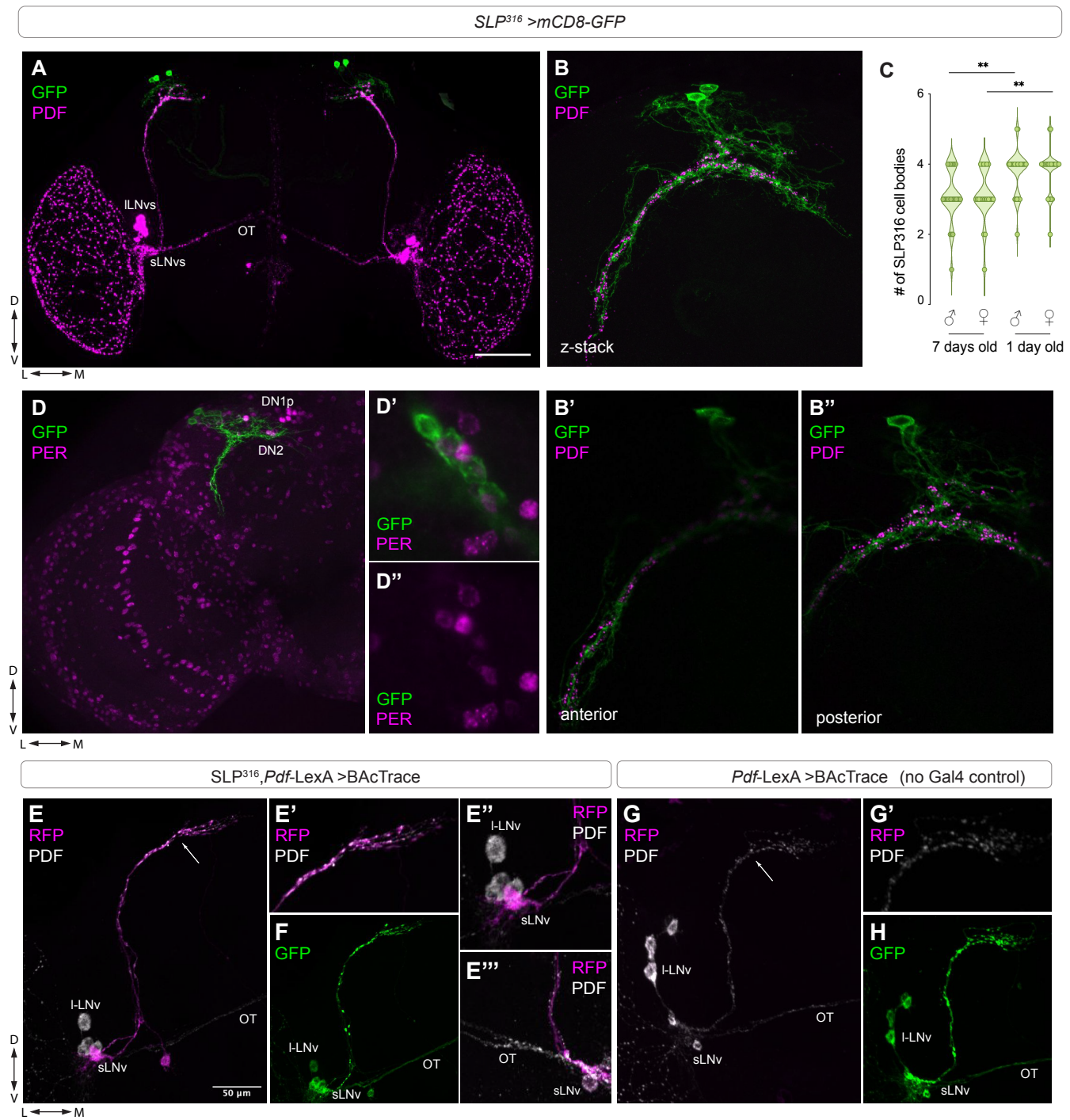718   17.2018, 2259-2217 (2018).

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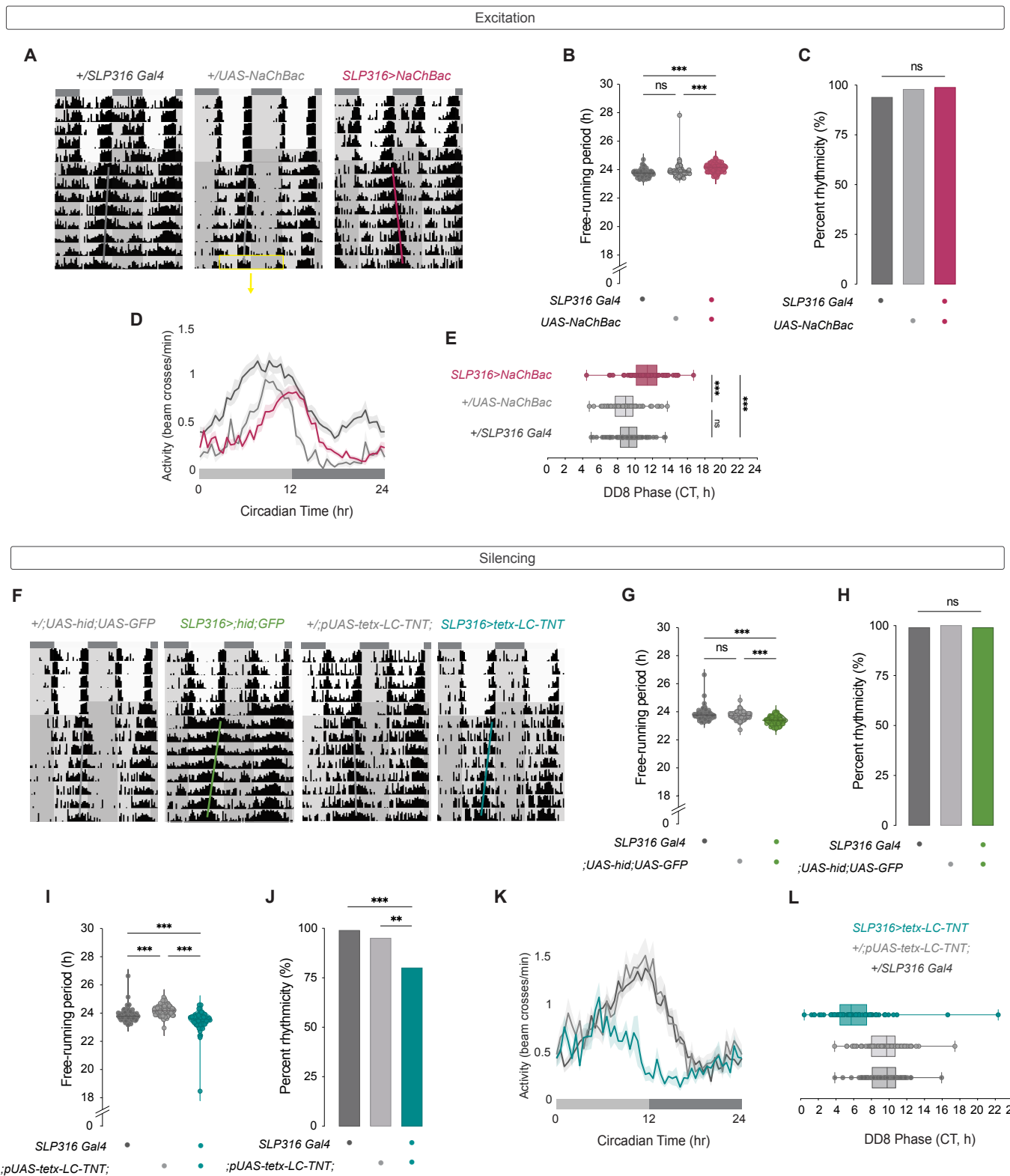769   analysis in Drosophila. *Proc Natl Acad Sci U S A* **117**, 10024-10034 (2020).

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Fig. 1

A

B

presynaptic												postsynaptic
s-LNv-1	s-LNv-2	s-LNv-3	s-LNv-4	SLP364-1	SLP364-2	VP1l+_lvPN-1	VP1l+_lvPN-2	VP1l+_lvPN-3	M_lvPNm36-1	M_lvPNm37-3		
60	56	71	64	28	31	25	37	8	21		SLP316-1
61	72	97	75	31	25	7	21	6	17	16	SLP316-2
84	64	69	57	35	20	19	14	11	12	13	SLP316-3

C

post synaptic sites

pre synaptic sites

B'

presynaptic	SLP374	DN1pA-1	DN1pA-2	DN1pA-3	DN1pA-5	SLP001	SLP373	SLP297-1	SLP297-2	SLP097-3	SLP097-6	SLP301-4	SLP096-1	SLP463 (SCB080)	SLP301-1	SLP111a-3
SLP316-1	91	32	21	20	19	13	26	15	16	9	39	8	22		3	7
SLP316-2	64	15	26	13	7	41	21	26	20	36	20	27	15	15	15	12
SLP316-3	50	54	20	10	11	32	24	22	18	12	5	14	7	19	13	12

postsynaptic

Fig. 2

Fig. 3

A +/SLP316 Gal4 +/UAS-NaChBac SLP316>NaChBac

B Free-running period (h) — ns, ***, ***

C Percent rhythmicity (%) — ns

D Activity (beam crosses/min) vs Circadian Time (hr)

E DD8 Phase (CT, h)

F +/;UAS-hid;UAS-GFP SLP316>;hid;GFP +/;pUAS-tetx-LC-TNT; SLP316>tetx-LC-TNT

G Free-running period (h) — ns, ***, ***

H Percent rhythmicity (%) — ns

I Free-running period (h) — ***, ***, ***

J Percent rhythmicity (%) — ***, **

K Activity (beam crosses/min) vs Circadian Time (hr)

L DD8 Phase (CT, h)

25°C

Excitation

Silencing

Fig. 4

Fig. 5

Fig. 6

Table 1. Summary of free running activity rhythms at 25°C and 18°C.

Temperature: 25°C					
Genotype	Sex	Number of Flies (n)	% Rhythmicity (nR)	Period (h) ± SEM	Rhythmic Power ± SEM
;Pdf-Red,Pdf-Gal4;	M	90	100 (90)	24.57 ± 0.04	148.3 ± 6.06
;pUAS-tetx-LC-TNT;	M	113	96.46 (109)	24.11 ± 0.04	91.38 ± 4.75
;Pdf-Red,Pdf-Gal4; > ;tetx-LC-TNT;	M	76	78.95 (60)	24.99 ± 0.11	64.58 ± 5.45
SLP316 split Gal4	M	170	95.88 (163)	23.82 ± 0.03	117.4 ± 4.14
NaChBac	M	73	98.63 (72)	23.91 ± 0.06	172.0 ± 5.82
SLP316 > NaChBac	M	69	98.55 (68)	24.13 ± 0.04	171.0 ± 5.30
;UAS-hid;UAS-GFP	M	76	100 (76)	23.73 ± 0.03	167.9 ± 5.39
SLP316 > ;hid;GFP	M	68	98.80 (82)	23.38 ± 0.04	144.5 ± 6.98
SLP316 > ;tetx-LC-TNT;	M	85	80.0 (68)	23.47 ± 0.09	41.02 ± 3.46
SLP316 split Gal4	F	31	80.65 (25)	24.41 ± 0.12	89.78 ± 11.06
;UAS-hid;UAS-GFP	F	32	100 (32)	24.39 ± 0.06	174.5 ± 8.34
SLP316 > ;hid;GFP	F	32	100 (32)	24.26 ± 0.26	169.1 ± 11.20
;pUAS-tetx-LC-TNT;	F	29	93.10 (27)	25.14 ± 0.34	117.4 ± 10.96
SLP316 > ;tetx-LC-TNT;	F	31	96.77 (30)	24.25 ± 0.07	131.1 ± 7.69

Temperature: 18°C					
Genotype	Sex	Number of Flies (n)	% Rhythmicity (nR)	Period (h) ± SEM	Rhythmic Power ± SEM
SLP316 split Gal4	M	160	97.50 (156)	23.86 ± 0.11	81.45 ± 2.87
NaChBac	M	32	100 (32)	23.72 ± 0.06	100.7 ± 5.74
SLP316 > NaChBac	M	32	100 (32)	23.70 ± 0.07	106.5 ± 6.93
;UAS-hid;UAS-GFP	M	94	100 (94)	23.78 ± 0.03	121.5 ± 4.91
SLP316 > ;hid;GFP	M	92	89.13 (82)	23.53 ± 0.18	67.05 ± 4.41
;pUAS-tetx-LC-TNT;	M	94	79.79 (75)	23.83 ± 0.18	49.49 ± 3.57
SLP316 > ;tetx-LC-TNT;	M	95	68.42 (65)	23.45 ± 0.17	36.00 ± 2.84
Pdf01	M	31	93.55 (29)	23.88 ± 0.04	63.02 ± 6.584
SLP316 split Gal4	F	63	84.13 (53)	24.12 ± 0.18	79.16 ± 6.03
;UAS-hid;UAS-GFP	F	25	84.00(21)	24.15 ± 0.13	66.93 ± 7.93
SLP316 > ;hid;GFP	F	26	92.31 (24)	23.54 ± 0.56	84.16 ± 9.95
;pUAS-tetx-LC-TNT;	F	64	93.75 (60)	24.29 ± 0.06	103.9 ± 6.31
SLP316 > ;tetx-LC-TNT;	F	58	91.38 (53)	23.75 ± 0.09	82.40 ± 5.64