Circadian control in the timing of critical periods during *Drosophila* larval neuronal development.

Sarah Doran, Adam A. Bradlaugh, Jack Corke & Richard A. Baines.

Division of Neuroscience, School of Biological Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester Academic Health Science Centre, Manchester, M13 9PL, UK.

Corresponding Author information: Sarah.Doran@postgrad.manchester.ac.uk; Richard.Baines@manchester.ac.uk

Author Contributions: SD conceived the study and carried out experimental work and data analyses. AAB provided intellectual input and training. JC provided the data for one experiment (Fig. 2A). RAB provided overall supervision and obtained funding. SD and RAB wrote the paper. All authors approved of this final version.

Classification: Biological Sciences; Neuroscience

Competing Interest Statement: The authors declare no competing interests.

Keywords: Cryptochrome, PDF, critical period, circadian clock

Significance Statement

Whilst the role of the biological clock is well understood in adult organisms, the same is not true for embryonic development. How the maternal clock impacts the mammalian fetus remains poorly understood. Given that many expectant mothers experience altered circadian rhythms, largely due to nightshift working, it is important to address these concerns. Here we identify clock-mediated periods in neural development of the embryonic Drosophila which can be manipulated by light. These findings provide an experimental opportunity to better understand the role of the circadian clock in early development.

Abstract

Critical periods (CPs) of development are temporal windows of heightened neural plasticity. Activity perturbation during CPs can produce significant, and permanent, alterations to the development of neural circuits. In this study we report a circadian mechanism underlying the timing of CPs in *Drosophila* embryonic and larval development. These CPs occur at ~24 hr intervals and are open to manipulation through blue light (BL)-activation of the circadian regulator Cryptochrome (CRY). This manipulation is sufficient to destabilize the larval CNS, evidenced by an induced seizure phenotype when tested at third instar (L3). In addition to CRY nulls, genetic ablation of the *period* gene also mitigates the BL exposure seizure phenotype and, moreover, alleles of *period* that affect circadian timing alter the timing of the CPs. Our analysis shows a clear role for the main clock neuropeptide, pigment dispersing factor (PDF), to transduce the output of these CPs. Targeted PDF receptor knockdown, in either GABAergic or CRY-positive neurons, is sufficient to prevent the CRY-mediated seizure phenotype. This study is a first demonstration of a circadian mechanism in *Drosophila* larvae, and whilst this alone is of major significance, our results highlight the potential of using *Drosophila* larvae as a model to investigate the impact of circadian rhythms on early neuronal development in higher organisms, which remains experimentally challenging.

Introduction

Developing neural circuits are maximally open to modification, including by external factors, during temporally defined periods. Such periods have been termed ‘critical’ or ‘sensitive’ based on their perceived influence. Manipulations during critical periods (CPs) often drive permanent change that becomes locked in. Similar manipulations during sensitive periods (SPs) can be equally profound but, differentially, can be modified following period closure (1–4). First

59 conceptualized in the 1960s (5), CPs are developmental windows during which changes in neural
60 activity support both anatomical and functional tuning of neural networks. This includes the
61 possible encoding of homeostatic "set points" that ensure appropriate activity levels in both
62 neurons and neural networks (1, 4, 6–8). Previous work from our group has identified a CP in the
63 *Drosophila* embryo, during 17-19 hours after egg laying (AEL), during which activity-manipulation
64 is sufficient to permanently change the stability of the locomotor network (6, 9). This change in
65 stability is measured through the induction of a seizure phenotype, indicative of significant change
66 to the larval CNS. Significantly, exposure to BL during this embryonic CP is similarly able to lead to
67 an increased seizure phenotype, an effect that requires the presence of CRY (10).

68 SPs differ from CPs in that while experiences during these timeframes still can have significant
69 influence on development and behavior, they are not so tightly bound by a specific timeframe
70 (11). SPs are, thus, believed to be required to facilitate learning at key developmental milestones
71 but, importantly, such learning can nevertheless still occur at other times. Pertinent examples of
72 SPs include early imprinting and acquisition of language or motor skills in developing animals (2,
73 12). In human, these periods include childhood adversity during SPs predicting changes in DNA
74 methylation (13), and growing evidence that there is a SP for emotional development in children
75 (14). However, the differentiation and contribution to neural development of both types of
76 plasticity-windows remains poorly understood.

77 We report that embryonic and larval development in *Drosophila* is punctuated by CPs at roughly
78 24 hr intervals, indicative of orchestration by the molecular clock. Circadian rhythms are integral
79 to all living things and serve to increase an organism's fitness. The prime purpose of the circadian
80 clock is to coordinate activity and metabolism to anticipated changes in the daily light:dark (LD)
81 cycle, using environmental factors such as light and/or temperature as zeitgebers (15). The

82 molecular clock in *Drosophila* is a negative feedback loop in which transcription factors drive
83 expression of their own repressors. This core transcription-translation feedback loop is comprised
84 of four key elements: CLOCK (CLK), CYCLE (CYC), PERIOD (PER) and TIMLESS (TIM). CLK and CYC
85 form a heterodimeric complex, which in turn promotes the transcription of the repressors *per* and
86 *tim* (16, 17). Alongside this negative feedback loop ,CRY, a BL sensitive flavoprotein, entrains this
87 molecular oscillation to the environmental LD cycle through light-mediated degradation of TIM
88 (18). The time taken for the transcription-translation feedback loop to complete a full cycle is the
89 foundation underlying the clock's ability to keep time.

90 In this study, we identify a series of CPs, occurring at ~24 hr intervals, beginning in embryonic
91 through to larval development. These CPs are open to manipulation by BL and are absent in a CRY
92 loss-of-function mutant. Moreover, genetic manipulation of *per*, or the principal clock signaling
93 neuropeptide - PDF - uncovers a novel role for the circadian clock during the larval stages of
94 *Drosophila* neural development.

95
96 **Results**
97
98 **Blue light sensitive windows occur at 24-hour intervals through larval development.**

99 Wildtype Canton-S (CS) embryos were exposed to BL for 2 hr periods prior to, during, and after
100 the characterized embryonic CP at 17-19 hrs AEL (4). Only exposure during 17-19 hrs was sufficient
101 to induce a seizure phenotype at wandering L3 (one-way ANOVA, $f(10, 224) = 1.213$, $p < 0.0001$,
102 $n = 15$, Fig. 1A-B). These results validate our previous findings by showing that BL exposure during
103 17-19 hrs AEL is sufficient to induce a seizure phenotype when compared to dark reared controls
104 (embryos that did not experience BL exposure during these hours) (6, 19). The seizure phenotype
105 is indicative of an incorrectly tuned locomotor network and provides a reliable, and relatively
106 simple assay to monitor for effects of activity-manipulation (6, 8, 19). To investigate whether the

107 17-19 hr window for BL sensitivity is a single occurrence within development, we exposed CS L1
108 larvae to BL for 6 hr intervals and measured their seizure phenotype at L3 (Fig. 1B). Where we
109 predicted that a CP might occur (based on 24 hr circadian rhythmicity) we exposed larvae to BL
110 for shorter 2 hr intervals. We found that L1 larvae exposed to BL at 20-22 hrs after larval hatching
111 (ALH) showed an increased L3 seizure phenotype compared to dark reared controls (larvae not
112 exposed to BL during this period, $p < 0.0001$). Exposure during L1 was similarly confined to this
113 single 2hr period. Thus, L1 larvae exposed to BL at 18-20 hrs ALH ($p > 0.99$) or 22-24 hrs ALH ($p >$
114 0.99) did not exhibit a seizure phenotype. With the assumption that BL-sensitive CPs are 2 hrs
115 long, we exposed larvae to BL, for 2 hr periods, at 24 hrs intervals after the effective L1 exposure
116 time: thus 44-46 hrs and again at 68-70 hrs ALH. BL exposure at both time intervals increased
117 seizure recovery time compared to dark reared controls ($p < 0.0001$). However, BL exposure either
118 before, or after, each period (42-44 hrs and 46-48 hrs; 66-68 hrs and 70-72 hrs) did not (Fig 1B,
119 Table 1). These results clearly show that there are windows of heightened neural plasticity,
120 sensitive to BL manipulation, at ~24-hour intervals throughout larval development. Moreover,
121 exposure to BL during these windows is sufficient to destabilize the larval nervous system as
122 evidenced by an induced seizure phenotype at wandering L3.

123

124

125 **Table 1. Full Descriptive Statistics of Figure 1B.**

126

BL exposure age (AEL/ALH)	Mean recovery time (s)	P value	SEM	Significance
15-17	76.87	0.310	4.149	NS
17-19	131.3	< 0.0001	7.591	****
19-21	65.93	0.992	5.218	NS

1-6	63	> 0.9999	5.765	NS
6-12	59.80	> 0.9999	6.082	NS
12-18	55.53	> 0.9999	5.306	NS
18-20	58.93	> 0.9999	6.214	NS
20-22	135.3	< 0.0001	8.734	****
22-24	65.47	0.9954	5.514	NS
42-44	62.13	> 0.9999	6.256	NS
44-46	123.6	< 0.0001	7.125	****
46-48	62.93	> 0.9999	5.200	NS
66-68	68.40	0.9313	5.645	NS
68-70	115.5	< 0.0001	9.527	****
70-72	58.80	> 0.9999	7.016	NS
CONTROL	57.93		6.771	

BL-sensitive windows differ from the embryonic CP.

The discovery of larval BL-sensitive CPs was unexpected. For clarity, we hereafter term these CPs as 'BL-CPs' to distinguish them from the previously characterized embryonic activity-dependent CP (hereafter termed 'activity-CP'). An immediate question is whether BL-CPs are equivalent to the well-characterized embryonic activity-CP. In addition to BL, the embryonic activity-CP can be manipulated by genetics or pharmacology (6, 8). However, neither approach easily lends itself to temporal control. It has, however, recently been shown that the embryonic activity-CP can be manipulated by elevating ambient temperature (to 32°C, per comm. Matthias Landgraf, Cambridge). We similarly find that exposing CS embryos to 32°C, during 17-19 hrs AEL, is sufficient to induce a seizure phenotype at L3 (one-way ANOVA $f(3, 56) = 4.76$, $p = 0.0001$, $n = 15$. Fig 2A). However, identical 32°C exposure is not sufficient to produce a seizure phenotype at 15-17 hrs

139 AEL ($p = 0.86$) or at 19-21 hrs AEL ($p = 0.57$). Surprisingly, CS larvae exposed to 32°C, at late stage
140 L1 during the BL-CP of 20-22 ALH, did not exhibit a seizure phenotype at L3 (unpaired t-test, $t(28)$
141 $= 1.88$, $p = 0.071$, $n = 15$, Fig. 2B). This indicates that the BL-CPs cannot be activity-manipulated in
142 the same manner as the embryonic activity-CP.

143 To further investigate whether BL-CPs identified above are equivalent to the embryonic activity-
144 CP, we utilized a characterized seizure mutant, para^{bangsenseless} *(para^{bss}),* which we have previously
145 shown to lose its characteristic seizure phenotype when reared at lower temperatures during the
146 embryonic activity-CP (6). We validated this observation by showing that *para^{bss}* embryos reared
147 at 18°C, between 17-19 hrs AEL, have a decreased seizure recovery time at L3, compared to 25°C
148 reared controls (unpaired t-test, $t(28) = 3.52$, $p = 0.0015$, $n = 15$, Fig 2C). To investigate whether
149 this same seizure rescue occurs during a larval BL-CP, *para^{bss}* larvae were reared at 18°C between
150 18-24 hrs ALH (spanning the L1 BL-CP). We found that the *para^{bss}* larvae reared at 18°C did not
151 show a decreased recovery time (unpaired t-test, $t(28) = 0.82$, $p = 0.421$, $n = 15$, Fig. 2D). This
152 demonstrates, again, that larval BL-CPs differ from the previously defined embryonic activity-CP.

153

154 The opening of a canonical CP is dictated by a given neural network reaching a key developmental
155 milestone, often coinciding with the maturation of inhibitory GABAergic interneurons (4, 20–22).
156 In this instance, the embryonic activity-CP and BL-CP occur simultaneously between 17-19hrs AEL.
157 Thus, we asked whether the activity-CP was locked to a specific developmental checkpoint, whilst
158 we predicted that the BL-CP was temperature compensated as predicted for the circadian clock
159 (23, 24). Embryogenesis lasts ~21 hrs at 25°C, but is extended to ~30 hrs at 21°C (6). We first
160 exposed 21°C reared embryos to BL at either 17-19 hrs AEL or the developmentally adjusted time
161 of 24-27 hrs AEL. We found that the timing of BL sensitivity did not change, remaining at the

162 earlier 17-19 hr period (one-way ANOVA, $f(2, 42) = 0.46$, $p = 0.0017$, $n = 15$, Fig 2E). BL-exposure
163 between 24-27 hrs AEL was without effect ($p = 0.33$). This suggests that, despite the changes in
164 temperature causing development to lengthen, the BL-CP is temperature compensated.

165 If, on the other hand, the embryonic activity-CP occurs at a specific developmental checkpoint,
166 then its timing should shift accordingly from 17-19 hrs to 24-27 hrs AEL to compensate for the
167 elongated development time. Intriguingly, exposure to 32°C between 17-19 hrs AEL, when
168 development was delayed, was embryonic lethal. However, higher temperature exposure (32°C)
169 between 24-27 hrs AEL was sufficient to result in L3 larvae with a clear seizure phenotype
170 (unpaired t-test, $t(28) = 5.45$, $p < 0.0001$, $n = 15$, Fig. 2F). These results, in combination, suggest
171 that there are two plasticity windows that overlap during embryogenesis when rearing occurs at
172 25°C: a BL-CP time locked at 17-19 hrs, and an activity-CP governed by development time and
173 sensitive to a range of activity manipulations (in this instance 32°C).

174

175 **Circadian mechanism underlying the timing of the BL-CPs.**

176 Two observations are individually suggestive of a possible circadian-based mechanism. First, the
177 BL-CPs are ~24hrs apart and second, like the clock, they are temperature-compensated (23–25).
178 To investigate this further, we first focused on the potential requirement for CRY, a blue light
179 sensitive clock protein (26). Thus, *cry* null mutant (cry^{02}) embryos were exposed to BL during either
180 the embryonic or L1 BL-CP. We found that *cry* null embryos that were exposed to BL between 17-
181 19 hrs (one-way ANOVA, $f(2, 42) = 1.61$, $p = 0.97$, $n = 15$) or during L1 at 20-22 hrs ALH did not
182 exhibit a characteristic seizure phenotype at L3 (one-way ANOVA, $f(2, 42) = 1.87$, $p = 0.93$, $n = 15$,
183 Fig 3A-B). The involvement of CRY implicates the circadian clock. To validate this, we tested a
184 *period* null mutant (per^{01}). We found that the BL exposure, during the same two time windows,

185 also did not induce a seizure phenotype (17-19hrs one-way ANOVA, $f(2, 42) = 10.44$, $p = 0.82$, n
186 $= 15$), (20-22 hrs ALH one-way ANOVA, $f(2, 42) = 3.87$, $p = 0.98$, $n = 15$, Fig. 3C-D). In contrast to
187 this, we found that exposure to 32°C, in cry^{02}, during 17-19 hrs AEL was sufficient to induce a clear
188 seizure phenotype at L3 (one-way ANOVA, $f(2, 42) = 2.58$, $p < 0.0001$, $n = 15$). Interestingly,
189 exposure to 32°C in per^{01} during 17-19 hrs AEL was lethal.

190 We next measured effect of BL exposure in two different *per* alleles: per^{Short} (per^{S}), with a 5-hr
191 shorter circadian period of 19 hrs, and per^{Long} (per^{L}), with a 4-hr longer period length of 28 hrs (27).
192 Interestingly, per^{S} exhibited an equivalent increased seizure recovery time when dark reared
193 (control) compared to when exposed to BL at the L1 sensitive period. Whilst this points to a
194 potentially interesting genotype-specific effect on neuronal excitability, further investigation is
195 beyond the scope of the current study. By contrast, we observed that per^{L} embryos exposed to
196 BL at 21-23 hrs AEL (i.e. 4hrs after the normal embryonic period) exhibited an increased seizure
197 recovery time compared to dark reared control (one-way ANOVA, $f(2, 42) = 2.038$, $p < 0.0001$, n
198 $= 15$, Fig 3E). Significantly, per^{L} embryos exposed to BL between 17-19 hrs AEL did not show a
199 seizure phenotype ($p = 0.95$), indicative of a failure to correctly time the opening of the BL-CP.

200 To take this investigation one step further, we investigated the potential impact of the ZT
201 exposure time. Thus, we altered the ZT time at which 17-19 hrs AEL occurred. Throughout this
202 study, 17-19 hrs AEL occurs at ZT 18-20, due to laying occurring in the morning. For this specific
203 experiment we adjusted our embryo collection times so that 17-19 hrs AEL occurred at ZT 0-2.
204 This allowed us to separate the potential effect of circadian timing from developmental timing, as
205 17-19 hrs AEL now occurred at lights on, rather than in the middle of the night when low light is
206 anticipated. First, we confirmed that exposure to 32°C during 17-19 hrs AEL (ZT 0-2) still caused a
207 seizure phenotype (unpaired t-test, $t(28) = 4.57$, $p < 0.0001$, $n = 15$, Fig. 3F). This demonstrated

208 that the change in the ZT did not influence the characterized embryonic activity-CP. By contrast,
209 we found that BL exposure during the same ZT-shifted period had no effect on seizure recovery
210 time (Mann-Whitney U, $U = 99.5$, $p = 0.60$, $n = 15$, Fig 3G). A potential explanation for this change
211 may, conceivably, be the clock's anticipation of light at ZT 0, demonstrating that the clock is an
212 underlying factor in the BL exposure response. Taken together, our data greatly supports the
213 premise that the BL-CPs have a clear link to, and are possibly regulated by, the circadian clock.

214

215 **Clock mediated *cry* mRNA abundance coincides with BL-CPs.**

216 To further link the BL-CPs to the circadian clock, we measured mRNA levels for selected clock
217 proteins (*cry*, *tim* and *per*) during both embryonic and larval development. All mRNA expression
218 levels were analyzed using the $2^{-\Delta\Delta Ct}$ method with the first collection, at 12 hrs AEL, being
219 normalized to 1 and used as the reference point. The mRNA expression of *cry* was observed to
220 cycle throughout development, with significant peaks at 18 hrs AEL, 24 hrs ALH, and at 48 hrs ALH
221 (all equivalent to ZT 19). Further larval stages were not examined. We also observed a significant
222 decrease in expression at 0, 12 and 36 hrs ALH. This is clearly indicative of cyclical expression on
223 an ~24-hour cycle. Importantly, the peaks in *cry* expression coincide with the timing of the BL-CPs
224 that we identify (Fig 4A). By comparison, both *per* and *tim* mRNA levels were found to decrease
225 significantly after 12 hrs AEL and remain low until late L2, thereafter increasing back to initial
226 levels (Fig 4B-C). This suggests that BL-CPs may not require cycling of all clock proteins. However,
227 the clear cycling of *cry* highlights a potential key role for this blue light-sensitive protein.

228 A lack of cycling in *per*, yet its necessity for BL-effect is interesting. Possibilities include a
229 requirement of Per to allow *cry* to cycle. Thus, we measured *cry* mRNA expression in the per^{01}
230 mutant and observed just that, a lack of *cry* cycling in this genetic background (Fig 4D). To

231 determine whether high *cry* expression levels alone are sufficient to support BL-CPs, we pan-
232 neuronally overexpressed *cry* throughout embryonic and larval development (elav-Gal4;;cry^{03}>w-
233 ;UAS-DmCRY;cry^{01}).

234 We specifically used a *cry* null background to eliminate endogenous *cry* cycling. We found that
235 overexpression of transgenic *cry* throughout development was not sufficient to open additional
236 CPs (Fig. 4E). Embryos exposed to BL at 17-19 hrs AEL showed an expected increased seizure
237 recovery time (one-way ANOVA, $f(2, 42) = 1.389$, $p = 0.0035$, $n = 15$). However, embryos exposed
238 to BL outside of this timing, whilst *cry* expression was equally high, did not ($p = 0.806$).

239

240 **PDF highlighted as a potential signaling pathway.**

241 Our data support CRY being essential for BL-sensitivity. This would suggest that CRY-expressing
242 neurons are integral to the signaling pathway underlying the induced seizure phenotype. Because
243 CRY is only expressed in few neurons during larval development (28), there is an assumption that
244 these neurons are coordinating a wider network wide effect. To show this, we inhibited activity
245 of CRY-positive neurons by expressing an inwardly-rectifying potassium channel (Cry39-
246 Gal4>UAS-Kir) (29), and then exposed embryos to BL at 17-19 hrs AEL. Inhibiting activity of CRY
247 neurons was sufficient to prevent BL-induction of a seizure phenotype (one way ANOVA, $f(2, 42)$
248 $= 2.887$, $p = 0.25$, $n = 15$, Fig. 5A).

249 It is important to establish how CRY-expressing neurons transduce the effect of BL-stimulation.
250 Thus, we expressed tetanus toxin (Cry39-Gal4>UAS-TeT, (30)) to abolish classical synaptic
251 transmission in CRY neurons. We found that suppressing classical synaptic transmission in CRY
252 neurons was not sufficient to prevent the seizure phenotype induced by BL exposure during the

253 embryonic CP (one way ANOVA, $f(2, 42) = 1.577$, $p = 0.024$, $n = 15$, Fig. 5B). Our results are
254 confounded, however, by the fact that expression of tetanus was alone sufficient (i.e. without BL
255 stimulation) to induce a seizure phenotype. Thus, this result remains inconclusive. Studies have
256 shown that tetanus is not always effective to prevent neuronal release of neuropeptides (31).
257 CRY-containing neurons express pigment dispersing factor (PDF, (28)). We used a *pdf* null mutant
258 (pdf^{01}) and exposed embryos to BL during the 17-19 hrs AEL. The pdf^{01} embryos exposed to BL did
259 not show an expected increase in seizure recovery time compared to dark reared controls
260 (unpaired t-test, $t(28) = 0.729$, $p = 0.47$, $n = 15$, Fig. 5C). Taken together, these observations are
261 consistent with CRY neurons, through the balanced release of both a classical neurotransmitter(s)
262 and a neuropeptide(s), being required to stabilize neural circuit functionality.

263 Having established a requirement for PDF, we initiated a limited screen to identify target neurons.
264 To do this, we first knocked down PDF receptor expression pan-neuronally using elav-Gal4>UAS-
265 PDFR-RNAi. We found that BL exposure at 17-19 hrs AEL, under these conditions, did not induce
266 a seizure phenotype (unpaired t-test, $t(28) = 0.738$, $p = 0.47$, $n = 15$, Fig. 5D). To localize PDF-target
267 neurons, we knocked down PDFRs in excitatory (cholinergic B19-Gal4) or inhibitory (GABAergic,
268 gad1-T2A-Gal4; TM6b) neurons. BL exposure, at 17-19 hrs AEL, in embryos where PDF receptors
269 were knocked down in cholinergic neurons, was still sufficient to increase seizure recovery time
270 compared to dark reared controls (unpaired t-test, $t(28) = 7.15$, $p < 0.0001$, $n = 15$, Fig. 5E). By
271 contrast, PDFR knockdown in GABAergic neurons prevented the induced seizure phenotype
272 (unpaired t-test, $t(28) = 1.97$, $p = 0.06$, $n = 15$, Fig. 5F).

273 PDF-containing neurons partake in autocrine signaling (28). We investigated the impact of
274 knocking down PDF-receptors in CRY positive neurons (Cry39-Gal4>UAS-PDFR-RNAi), to assess
275 whether autocrine signaling is integral to the mechanism of this study. Significantly, BL exposure

276 (17-19hrs AEL) in this genotype resulted in no seizure phenotype (unpaired t-test, $t(28) = 0.299, = 0.77, n = 15$, Fig. 5G).

278 **Discussion**
279
280 It is established that circadian rhythms regulate most, if not all, aspects of physiology and behavior
281 (15, 32, 33). However, the specific influence of the biological clock on embryonic development
282 remains less well understood. Moreover, clock-mediated effects in developing model systems
283 that are amenable to study are lacking, due to constraints such as *in utero* development. In this
284 regard, our study is significant in that it clearly identifies clock-controlled CPs at 24 hr intervals,
285 from embryo through to larval stages, in the experimentally tractable *Drosophila* model system.
286 Manipulation of these CPs, by exposure to BL, results in a larval seizure phenotype that is a proxy
287 for an altered excitation-inhibition balance within the developing larval locomotor circuitry (34).
288 Thus, our results show a clear clock-mediated effect to neural network development in an
289 embryonic system. In this regard it is interesting to note that the larval stages of holometabolous
290 insects (e.g. *Drosophila*) have been argued to represent free-living embryos: an advantageous
291 strategy that may favor survival of the species (35). This highlights that this study is not only the
292 first instance of a circadian influence on larval development, but also as a potentially more
293 accessible model for further investigation into circadian influence on developing organisms.

294 Whilst evidence exists to demonstrate the influence of the maternal biological clock on the
295 developing fetus in mammals (for review see (36)), specific effects on the fetus remain
296 speculative. However, disturbance of the fetal circadian system has been linked to increased risk
297 of cardiovascular and other diseases in later life (37, 38). Our observation of CPs in the
298 development of the embryonic and larval nervous system, that are regulated by the clock,
299 provides convincing evidence for circadian influence on a developing organism. In keeping with

300 this hypothesis, it is notable that clock genes, in mice, have been shown to time the postnatal
301 development of neurons pivotal to CP plasticity (39). This demonstrates that the molecular clock,
302 and the plethora of genes under its control have a role in the timing of CPs, providing support for
303 our results. Interestingly, in the same study, it was demonstrated that the pharmacological
304 enhancement of GABA, via exposure to diazepam, was able to overcome delayed neuron
305 development, due to clock gene manipulation, and restore the timing of CP onset (39). This
306 suggests that clock genes may directly control neuronal activity, which in turn is mediated by
307 neurotransmitter release, an area that is explored in the current study.

308 Clock-based modulation of neuronal activity is well established, in both mammals and *Drosophila*
309 (40, 41). Our results are consistent with manipulation of CPs mediating effects through alterations
310 in release of neurotransmitters. Thus, we show that hyperpolarization of clock neurons, achieved
311 by expression of Kir, is sufficient to prevent effects of BL exposure. The simplest explanation is
312 that hyperpolarization is sufficient to prevent SNARE-mediated neurotransmitter release (29).
313 However, developmental changes in these neurons, that for example might include altered
314 synaptic targeting, remain a possibility. The paradoxical effect of expression of tetanus in clock
315 neurons, which is alone sufficient to induce a seizure phenotype, is intriguing. Coupled with our
316 demonstration that the neuropeptide PDF is required for BL-mediated perturbation of the CPs,
317 our results are consistent with clock neuron activity being tightly regulated to maintain an
318 appropriate balance in transmitter release, or possibly that neurotransmitter release is
319 appropriately timed. Preventing release of a classical transmitter (most likely GABA, which is a key
320 neurotransmitter in the adult clock (42)) achieved by tetanus expression, is sufficient to
321 destabilize the circuit, a similar effect also resulting from BL-mediated release of PDF. How these
322 respective transmitters mediate their effects, and the identity of downstream targets remains to
323 be determined. Downregulating clock genes in PDF-expressing clock neurons, in developing adult

324 *Drosophila*, is sufficient to prevent these cells from fasciculating to form typical axonal bundles
325 (43). As PDF is one of the main signaling outputs of clock neurons (28), it is perhaps not surprising
326 that we implicate this neuropeptide to be a key mediator of BL-exposure of the CPs we identify.
327 Previous studies have demonstrated that small lateral ventral neurons (sLNv) first show robust
328 PDF staining (visible by antibody), along with a small number of other cells, from the first few hrs
329 ALH (44). Therefore, it is logical to propose the sLNvs are likely mediating the effects we observe.

330 In summary, we identify a series of CPs, regulated by the molecular clock, the manipulation of
331 which by BL significantly impacts neuronal development in the embryo and larva of *Drosophila*.
332 Whilst there is evidence to link the molecular clock with neuronal development in other species
333 and in adult *Drosophila*, this report is the first example in *Drosophila* larvae. This is of significance
334 because this discovery might offer a tractable animal model with which to investigate the role of
335 the molecular clock and circadian activity in the development of an embryonic CNS; something
336 that remains difficult to achieve in the experimentally inaccessible mammalian fetus.

337
338 **Materials and Methods**
339
340 **Fly Stocks**: Flies were reared on standard corn meal medium at 25°C under a 12hr-12hr light-dark
341 cycle except for when embryos/larvae were kept in darkness before BL or 32°C exposure, after
342 which they were placed back on this light-dark cycle. Embryos were collected at ZT 1-5 and
343 exposed to stimulation at ZT 18-20, except for experimental circumstances in which the change is
344 noted. Fly strains used are listed in Table 2.

345

346

347

348 **Table 2. Fly Stock List**

Fly Line	Obtained from	Bloomington Stock Number
Canton-Special	Bloomington *Drosophila* Stock Centre	BL64349
para^{bangsenseless}	Baines group	
Dmcry^{02}		BL86267
per^{01}		BL80928
per^{S}	Ezio Rosato (University of Leicester)	
per^{L}	Ezio Rosato	
y^{1}*w*;UAS-CRY24B*	Ezio Rosato	
y^{1}*w*;cry39-Gal4;*	Ezio Rosato	
ElaV^{C155}*-GAL4;;cry*^{03}	Ezio Rosato	
w-;UAS-DmCRY;*cry*^{01}	Ezio Rosato	
y^{1}*w*;UAS-KIR;*	Ralph Stanewsky (University of Münster)	
;*UAS-TeT*;	Baines group	
y^{1}*w*;Pdf*^{01}		BL26654
;UAS-PDFR^{RNAi} *;*		BL38347
;;*gad1-T2A-Gal4* / TM6b	Matthias Landgraf (University of Cambridge)	
;B19-Gal4;	Baines group	

349

350 **Blue light manipulation of neuronal activity**: Mated adult females were allowed to lay eggs on
351 grape agar (Dutscher, Essex, UK) plates supplemented with yeast paste at 25°C. Embryos were
352 collected and transferred to a fresh grape agar plate which was subsequently transferred to a
353 25°C incubator. L1 larvae, when used for manipulation, were collected at 0-1 hours after larval

354 hatching (ALH), by first removing any hatched larvae at lights on (ZT0) and then collecting any
355 newly hatched larvae at ZT1-2. Embryos or larvae were exposed to collimated BL from an
356 overhead LED (8 Wcm^2). The LED had a peak emission at 470 nm (bandwidth 25 nm, OptoLED,
357 Cairn Instruments, Kent, UK). Light was pulsed for 100msec duration at 2 Hz. Manipulated
358 embryos/larvae were transferred to standard corn meal medium containing vials and reared at
359 25°C under a 12hr-12hr light-dark cycle until wandering L3.

360 **Temperature manipulation**: Mated adult females were allowed to lay eggs on grape agar plates
361 supplemented with yeast paste at 25°C. Embryos were collected and transferred to an empty PCR
362 tube (0.2ml Eppendorf) which was subsequently placed into a PCR machine (Eppendorf
363 Mastercycler Gradient). When larvae were used in the protocol, these were collected as described
364 above. Embryos/larvae were exposed to 32°C at the appropriate time by programming the PCR
365 machine to warm to this temperature. For embryos exposed to high temperatures at 17-19 hours
366 AEL, the PCR thermal profile was 25°C for 13 hours, 32°C for 6 hours and hold at 25°C. For larvae
367 exposed to high temperatures at 20-22 hours ALH, larvae were placed into PCR tubes at a later
368 stage (6 hours ALH) to minimize lethality due to starvation and desiccation, prior to this they were
369 maintained at 25°C on a grape agar plate supplemented with yeast paste. The larval PCR thermal
370 profile was 25°C for 13 hours, 32°C for 3 hours, hold at 25°C. A small amount of water was added
371 to each sample tube to increase survival rates. Manipulated embryos/larvae were transferred to
372 standard corn meal medium containing vials and reared at 25°C under a 12hr-12hr light-dark cycle
373 until wandering L3.

374 **Low temperature rescue of neuronal activity**: Embryos or larvae were collected and transferred
375 to a fresh grape agar plate as previously described. Embryos were placed in an 18°C incubator for
376 the duration of embryogenesis (determined by the hatching of larvae) or for the duration of L1

377 (full development at this lower temperature being 44 hours). The embryos/larvae were
378 transferred to standard corn meal medium containing vials and reared at 25°C under a 12hr-12hr
379 light-dark cycle until wandering L3.

380 **Seizure induction**: Wandering L3 larvae were washed in ddH_2O to remove food residue and
381 subsequently placed onto a clean plastic dish and gently dried with a paper towel. Once normal
382 crawling behavior was observed, a conductive probe composed of two tungsten wires (0.1 mm
383 diameter, ~1-2 mm apart) was positioned over the approximate position of the CNS. A 3 V DC
384 pulse for 2 s, generated by an isolated constant voltage stimulator (Digitimer Ltd. Uk) was applied.
385 In response to the stimulus, larvae initiated a transitory paralysis with occasional spasms and
386 rolling behavior. The time to resume normal crawling behavior was measured as recovery time
387 (RT). Normal crawling behavior was defined as three consecutive whole body peristaltic waves
388 resulting in forward movement (45). Videos of this phenotype are available in (19).

389 **RTqPCR:** Embryos/larvae were collected as previously described. The embryos were placed into
390 2 ml Eppendorf tubes and left to mature at 25°C in a normal LD cycle. Once the embryos/larvae
391 had reached the required age, they were flash frozen using liquid nitrogen and stored at -80°C. It
392 is important to note that freezing took place under the appropriate light conditions consistent
393 with the rearing LD cycle. The Qiagen RNeasy Mini Kit was used to extract RNA following the
394 standard protocol (#74004, Qiagen, Germany). RNA was converted to cDNA using the
395 ThermoScientific RevertAid First Strand cDNA Synthesis Kit (#K1622, Thermo Fisher Scientific, UK)
396 following the standard protocol: 10 µl total RNA was incubated with 1µl random hexamers and 1
397 µl Oligo(dt) at 65°C for 5 minutes then stored on ice. To this mixture 4µl 5x Reaction Buffer, 2µl
398 10mM dNTP, 1µl RNase inhibitor, and 1µl reverse transcriptase was added. This was incubated at
399 25°C for 10 minutes, 42°C for 60 minutes, then 70°C for 10 minutes and subsequently stored at -

400 20°C. The 20µl resultant cDNA product was diluted to 40µl using RNA-free water, prior to RT-
401 qPCR. RT-qPCR was carried out using LightCycler® 480 (Roche Diagnostics, Germany) with 10µl
402 LightCycler® 480 SYBR Green I Master mix (#04707516001) 0.5µl forward primer (5pmol/µl), 0.5
403 µl reverse primer (5pmol/µl), 1µl single stranded cDNA, and 8µl ddH_2O in each well of a 96-well
404 plate (#04729692001, Roche). The same thermal profile was used for all primers: 10 minutes at
405 95°C followed by 55 cycles of [10 seconds at 95°C, 10 seconds at 60°C and 10 seconds at 72°C]
406 (46). All primer pairs used are listed in Table 2. N-syb was used as the reference gene. Data were
407 normalized using the $2^{-\Delta\Delta CT}$ method, such that the relative expression of each genotype = 1. For
408 each genotype, *n = 3* independent biological replicates, comprising 2-3 technical repeats per
409 replicate.

410 **Table 3. Full primer list.**

Gene	Primer 1 (5' – 3')	Primer 2 (3' – 5')	Source
n-syb	GGACATCATGCGCACGAAC	TAATGCCCACGACAACCAGG	https://www.flyrnai.org/cgi-bin/DRSC_primerbank.pl (Primer pair PD41952)
cry	CCATGATAATCCCGCTCTA	ATGTCCTGCAACGAGTC	Designed by Adam Bradlaugh
per	GACTCGGCCTACTCGAACAG	CGCGAGTTATCCTTGTTGCG	https://www.flyrnai.org/cgi-bin/DRSC_primerbank.pl (Primer pair PP10581)
tim	ATGGACTGGTTACTAGCAACTCC	GGTCCTCATAGGTGAGCTTGT	https://www.flyrnai.org/cgi-bin/DRSC_primerbank.pl (Primer pair PP11542)

411
412
413

Acknowledgments

We thank Ezio Rosato, Ralf Stanewsky and Matthias Landgraf for their generous provision of *Drosophila* strains. We thank Matthew Cobb and Mino Belle and Nara Muraro for comments on a draft of this paper. SD would like to thank Wei-Hsiang Lin (University of Manchester) for training in qRT-PCR. We are also grateful to the members of the Baines group for support and ideas. SD was funded through a BBSRC DTP award provided by the University of Manchester. AAB and RAB were funded through an award from BBSRC to RAB (BB/V005987/1). Work on this project benefited from the Manchester Fly Facility, established through funds from the University and the Wellcome Trust (087742/Z/08/Z).

Data Availability Statement

Figures show all raw data values in addition to means and sem. Fly stocks used are available on request.

References

1. B. Coulson, *et al.*, Critical periods in Drosophila neural network development: Importance to network tuning and therapeutic potential. *Front Physiol* **13**, 1073307 (2022).

2. E. I. Knudsen, Sensitive periods in the development of the brain and behavior. *J Cogn Neurosci* **16**, 1412–1425 (2004).

3. D. H. Hubel, T. N. Wiesel, The period of susceptibility to the physiological effects of unilateral eye closure in kittens. *J Physiol* **206**, 419–436 (1970).

4. T. K. Hensch, Critical period plasticity in local cortical circuits. *Nat Rev Neurosci* **6**, 877–888 (2005).

5. T. N. Wiesel, D. H. Hubel, SINGLE-CELL RESPONSES IN STRIATE CORTEX OF KITTENS DEPRIVED OF VISION IN ONE EYE. *J Neurophysiol* **26**, 1003–1017 (1963).

443 6. C. N. G. Giachello, R. A. Baines, Inappropriate Neural Activity during a Sensitive Period in
444 Embryogenesis Results in Persistent Seizure-like Behavior. *Current Biology* **25**, 2964–2968
445 (2015).

446 7. R. K. Reh, *et al.*, Critical period regulation across multiple timescales. *Proc Natl Acad Sci U S*
447 *A* **117**, 23242–23251 (2020).

448 8. I. Hunter, *et al.*, Balance of activity during a critical period tunes a developing network.
449 *eLife* **12**, RP91599 (2024).

450 9. R. Marley, R. A. Baines, Increased persistent Na+ current contributes to seizure in the
451 slamdance bang-sensitive Drosophila mutant. *Journal of Neurophysiology* **106**, 18–29
452 (2011).

453 10. R. Marley, C. N. G. Giachello, N. S. Scrutton, R. A. Baines, A. R. Jones, Cryptochrome-
454 dependent magnetic field effect on seizure response in Drosophila larvae | Scientific
455 Reports. *Scientific Reports* **4**, 5799 (2014).

456 11. P. Voss, Sensitive and critical periods in visual sensory deprivation. *Frontiers in Psychology*
457 **4** (2013).

458 12. J. Gilkerson, *et al.*, Language Experience in the Second Year of Life and Language Outcomes
459 in Late Childhood. *Pediatrics* **142**, e20174276 (2018).

460 13. E. C. Dunn, *et al.*, Sensitive Periods for the Effect of Childhood Adversity on DNA
461 Methylation: Results From a Prospective, Longitudinal Study. *Biological Psychiatry* **85**, 838–
462 849 (2019).

463 14. K. Woodard, S. D. Pollak, Is there evidence for sensitive periods in emotional
464 development? *Current Opinion in Behavioral Sciences* **36**, 1–6 (2020).

465 15. C. Dubowy, A. Sehgal, Circadian Rhythms and Sleep in Drosophila melanogaster. *Genetics*
466 **205**, 1373–1397 (2017).

467 16. R. Allada, N. E. White, W. V. So, J. C. Hall, M. Rosbash, A Mutant Drosophila Homolog of
468 Mammalian Clock Disrupts Circadian Rhythms and Transcription of period and timeless.
469 *Cell* **93**, 791–804 (1998).

470 17. T. K. Darlington, *et al.*, Closing the Circadian Loop: CLOCK-Induced Transcription of Its Own
471 Inhibitors per and tim. *Science* **280**, 1599–1603 (1998).

472 18. P. Emery, W. V. So, M. Kaneko, J. C. Hall, M. Rosbash, CRY, a Drosophila Clock and Light-
473 Regulated Cryptochrome, Is a Major Contributor to Circadian Rhythm Resetting and
474 Photosensitivity. *Cell* **95**, 669–679 (1998).

475 19. R. Marley, R. A. Baines, Increased persistent Na+ current contributes to seizure in the
476 slamdance bang-sensitive Drosophila mutant. *Journal of Neurophysiology* **106**, 18–29
477 (2011).

478 20. T. K. HENSCH, E. M. QUINLAN, Critical periods in amblyopia. *Vis Neurosci* **35**, E014 (2018).

479 21. M. Fagiolini, T. K. Hensch, Inhibitory threshold for critical-period activation in primary
480 visual cortex. *Nature* **404**, 183–186 (2000).

481 22. N. Aoki, *et al.*, GABA-A and GABA-B Receptors in Filial Imprinting Linked With Opening and
482 Closing of the Sensitive Period in Domestic Chicks (Gallus gallus domesticus). *Frontiers in*
483 *Physiology* **9**, 1837 (2018).

484 23. R. Narasimamurthy, D. M. Virshup, Molecular Mechanisms Regulating Temperature
485 Compensation of the Circadian Clock. *Frontiers in Neurology* **8** (2017).

486 24. W. F. Zimmerman, C. S. Pittendrigh, T. Pavlidis, Temperature compensation of the
487 circadian oscillation in Drosophila pseudoobscura and its entrainment by temperature
488 cycles. *Journal of Insect Physiology* **14**, 669–684 (1968).

489 25. M. Versteven, K.-M. Ernst, R. Stanewsky, A Robust and Self-Sustained Peripheral Circadian
490 Oscillator Reveals Differences in Temperature Compensation Properties with Central Brain
491 Clocks. *iScience* **23**, 101388 (2020).

492 26. M. Damulewicz, G. M. Mazzotta, One Actor, Multiple Roles: The Performances of
493 Cryptochrome in Drosophila. *Front Physiol* **11**, 99 (2020).

494 27. R. J. Konopka, S. Benzer, Clock Mutants of Drosophila melanogaster. *Proceedings of the*
495 *National Academy of Sciences* **68**, 2112–2116 (1971).

496 28. S. Dissel, *et al.*, The Logic of Circadian Organization in Drosophila. *Curr Biol* **24**, 2257–2266
497 (2014).

498 29. R. A. Baines, J. P. Uhler, A. Thompson, S. T. Sweeney, M. Bate, Altered Electrical Properties
499 in DrosophilaNeurons Developing without Synaptic Transmission. *J. Neurosci.* **21**, 1523–
500 1531 (2001).

501 30. S. T. Sweeney, K. Broadie, J. Keane, H. Niemann, C. J. O'Kane, Targeted expression of
502 tetanus toxin light chain in Drosophila specifically eliminates synaptic transmission and
503 causes behavioral defects. *Neuron* **14**, 341–351 (1995).

504 31. D. Pauls, *et al.*, Potency of Transgenic Effectors for Neurogenetic Manipulation in
505 Drosophila Larvae. *Genetics* **199**, 25–37 (2015).

506 32. P. Ma, M. A. Woelfle, C. H. Johnson, An Evolutionary Fitness Enhancement Conferred by
507 the Circadian System in Cyanobacteria. *Chaos Solitons Fractals* **50**, 65–74 (2013).

508 33. T. Roenneberg, M. Merrow, The Circadian Clock and Human Health. *Current Biology* **26**,
509 R432–R443 (2016).

510 34. I. Hunter, B. Coulson, A. A. Zarin, R. A. Baines, The Drosophila Larval Locomotor Circuit
511 Provides a Model to Understand Neural Circuit Development and Function. *Frontiers in*
512 *Neural Circuits* **15** (2021).

513 35. J. W. Truman, L. M. Riddiford, The origins of insect metamorphosis. *Nature* **401**, 447–452
514 (1999).

515 36. K. Bates, E. D. Herzog, Maternal-Fetal Circadian Communication During Pregnancy. *Front*
516 *Endocrinol (Lausanne)* **11**, 198 (2020).

517 37. M. Serón-Ferré, *et al.*, Circadian rhythms in the fetus. *Molecular and Cellular Endocrinology*
518 **349**, 68–75 (2012).

519 38. T. J. Varcoe, K. L. Gatford, D. J. Kennaway, Maternal circadian rhythms and the
520 programming of adult health and disease. *American Journal of Physiology-Regulatory,*
521 *Integrative and Comparative Physiology* **314**, R231–R241 (2018).

522 39. Y. Kobayashi, Z. Ye, T. K. Hensch, Clock Genes Control Cortical Critical Period Timing.
523 *Neuron* **86**, 264–275 (2015).

524 40. M. D. C. Belle, C. O. Diekman, D. B. Forger, H. D. Piggins, Daily Electrical Silencing in the
525 Mammalian Circadian Clock. *Science* **326**, 281–284 (2009).

526 41. M. N. Nitabach, V. Sheeba, D. A. Vera, J. Blau, T. C. Holmes, Membrane electrical
527 excitability is necessary for the free-running larval Drosophila circadian clock. *Journal of*
528 *Neurobiology* **62**, 1–13 (2005).

529 42. Y. Hamasaka, C. Wegener, D. R. Nässel, GABA modulates Drosophila circadian clock
530 neurons via GABAB receptors and decreases in calcium. *Journal of Neurobiology* **65**, 225–
531 240 (2005).

532 43. G. Biondi, G. McCormick, M. P. Fernandez, The Drosophila Circadian Clock Gene Cycle
533 Controls Development of Clock Neurons. [Preprint] (2023). Available at:
534 https://www.biorxiv.org/content/10.1101/2023.10.29.564626v1 [Accessed 17 January
535 2024].

536 44. C. Helfrich-Förster, Development of pigment-dispersing hormone-immunoreactive
537 neurons in the nervous system of Drosophila melanogaster. *Journal of Comparative*
538 *Neurology* **380**, 335–354 (1997).

539 46. W.-H. Lin, M. He, R. A. Baines, Seizure suppression through manipulating splicing of a
540 voltage-gated sodium channel. *Brain* **138**, 891–901 (2015).

541

542

543

544

545

546

547 **Figure legends**
548
549 **Figure 1. Blue light sensitive CPs occur at 24-hour intervals.** (**A**) Schematic diagram of the
550 electroshock protocol following BL exposure. Pink brackets identify the 2-hour time windows
551 during which BL exposure occurred. Blue light exposure did not occur sequentially but separately
552 during different experiments. Wandering L3 were challenged by electroshock to evoke a seizure-
553 like phenotype. The recovery time for normal crawling behavior to resume was measured. (**B**)
554 Seizure phenotypes were observed when BL exposure occurred at ~24-hour intervals throughout
555 development. CS embryos were exposed to BL at 2-hour intervals where a sensitive window was
556 expected or at 6-hour intervals during L1 when not predicted. CS embryos exposed to BL at 17-19
557 hrs AEL exhibited a seizure phenotype (one-way ANOVA, $f(10,224) = 1.213$, $p < 0.0001$, $n = 15$,
558 dark reared = red line, dark reared SEM = grey box). CS late-stage L1 larvae exposed to BL at 20-
559 22 hrs ALH ($p < 0.0001$, $n = 15$), and L2 larvae exposed to BL for 2 hrs at 44-46 hrs ALH ($p < 0.0001$,
560 $n = 15$), and 24 hrs after again for mid-stage L3 larvae exposed to BL at 68-70 hrs ALH ($p < 0.0001$,
561 $n = 15$) all show an induced seizure phenotype. The grey data points depict the BL exposure
562 groups that did not have an increased seizure recovery time at wandering L3 (full descriptive
563 statistics and p values are reported in Table 1).
564
565
566 **Figure 2. BL-sensitive CPs differ to a previously characterized embryonic activity-CP.** (A) CS
567 embryos exposed to 32°C, between 17-19 hrs AEL, show a higher seizure recovery time when
568 electroshocked at wandering L3 (One-way ANOVA $f(3, 56) = 4.715$, $p = 0.0001$, $n = 15$). Exposure
569 to 32°C at 15-17 hrs AEL ($p = 0.858$) or 19-21 hrs AEL ($p = 0.575$) was without effect. Control
570 range (± sem) are dark-reared (gray horizontal bar). (B) CS late-stage L1 larvae exposed to 32°C
571 at 20-22 hrs ALH did not show an increased recovery time (unpaired t-test, $t(28) = 1.88$, $p =$
572 0.071, $n = 15$). (C) parabss, reared at 18°C between 17-19 hrs AEL, show a significantly reduced
573 seizure recovery time compared to a 25°C reared control (unpaired t-test, $t(28) = 3.523$, $p =$
574 0.0015, $n = 15$). (D). parabss L1 larvae, reared at 18°C between 20-22 hrs ALH, did not show a
575 lower recovery time than a 25°C reared control (unpaired t-test, $t(28) = 0.817$, $p = 0.421$, $n = 15$).
576 (E) The embryonic BL-CP is time locked at 17-19 hrs, regardless of developmental temperature.
577 There was an increase in recovery time in 21°C reared embryos exposed to BL at 17-19 hrs AEL
578 (one-way ANOVA, $f(2, 42) = 0.462$, $p = 0.0017$, $n = 15$) but not at 24-27 hrs AEL ($p = 0.333$). (F)
579 The embryonic activity-CP is developmentally locked. The increase in seizure recovery time, due

580 to 32°C exposure, occurred later at 24-27 hrs AEL in embryos developing at 21°C (unpaired t-
581 test, $t(28) = 5.45$, $p < 0.0001$, $n = 15$). 32°C exposure between 17-19 hrs AEL was lethal.

582

583 **Figure 3. The circadian clock regulates BL-sensitivity.** (A) cry02 embryos exposed to BL between
584 17-19 hrs AEL did not show an increased seizure recovery time compared to dark reared
585 controls (one-way ANOVA, $f(2, 42) = 1.607$, $p = 0.970$, $n = 15$). (B) cry02 late-stage L1 exposed to
586 BL at 20-22 hrs ALH did not have a higher recovery time compared to dark reared controls (one-
587 way ANOVA, $f(2, 42) = 1.871$, $p = 0.931$, $n = 15$). (C) per01 embryos exposed to BL between 17-
588 19 hrs AEL did not exhibit a higher recovery time compared to dark reared controls (one-way
589 ANOVA, $f(2, 42) = 10.44$, $p = 0.825$, $n = 15$). (D) per01 late-stage L1 exposed to BL at 20-22 hrs
590 ALH did not have a higher recovery time compared to dark reared controls (one-way ANOVA,
591 $f(2, 42) = 3.867$, $p = 0.982$, $n = 15$). (E) perL embryos exposed to BL between 21-23 hrs AEL had a
592 higher recovery time compared to dark reared controls (one-way ANOVA, $f(2, 42) = 2.038$, $p <$
593 0.0001, $n = 15$). (F) CS embryos exposed to 32°C between 17-19 hrs AEL (ZT 0-2), showed an
594 increased recovery time compared to 25°C reared control (Unpaired t-test, $t(28) = 4.57$, $p <$
595 0.0001, $n =15$). (G) CS embryos exposed to BL between 17-19 hrs AEL (ZT 0-2), did not have an
596 increased recovery time compared to dark reared controls (Mann-Whitney U, $U = 99.5$, $p =$
597 0.602, $n = 15$).

598

599 **Figure 4. Cry expression coincides with BL-CPs.** (A) cry mRNA levels cycle throughout
600 development, with peaks at 18 hrs AEL, 21 hrs ALH and 45 hrs ALH, which coincide with the
601 opening of BL-CPs. (B) per mRNA levels reduce after 12 hrs AEL (set at 1) and remain low until
602 late stage L2, without cycling. (C) tim mRNA levels also reduce after 12 hrs AEL levels and remain
603 low until late stage L2, without cycling. (D) cry mRNA levels did not cycle in a per null mutant. (E)
604 Embryos overexpressing cry and exposed to BL between 17-19 hrs AEL show an increased
605 recovery time at L3, compared to the dark reared control (one-way ANOVA, $f(2, 42) = 1.389$, $p =$
606 0.0035, $n = 15$). Exposure to BL either side of 17-19 hrs AEL did not, however, induce an
607 increased recovery time ($p = 0.806$).

608

609 **Figure 5. PDF is required for transduction of effect of BL exposure.** (A) Silencing activity in CRY-
610 positive neurons, using Kir2.1, is sufficient to prevent induction of a seizure phenotype following
611 BL exposure between 17-19 hrs AEL (one way ANOVA, $f(2, 42) = 2.887$, $p = 0.256$, $n = 15$) (B)
612 Expression of tetanus (TeT) in CRY-positive neurons did not prevent induction of a seizure
613 phenotype following exposure to BL between 17-19 hrs AEL (unpaired t-test, $t(28) = 1.577$, $p =$
614 0.024, $n = 15$,). However, the presence of TeT alone was sufficient to induce a seizure phenotype
615 in dark-reared animals. (C) pdf01 embryos exposed to BL at 17-19 hrs AEL did not have an
616 increased recovery time compared to dark reared controls (unpaired t-test, $t(28) = 0.472$, $p =$
617 0.472, $n = 15$). (D) Pan-neuronal PDFR knockdown was sufficient to prevent induction of a
618 seizure phenotype (unpaired t-test, $t(28) = 0.738$, $p = 0.467$, $n = 15$). (E) PDFR knockdown in
619 cholinergic neurons did not prevent induction of a seizure phenotype following exposure to BL
620 at 17-19 hrs AEL (unpaired t-test, $t(28) = 7.15$, $p < 0.0001$, $n = 15$). (F) PDFR knockdown in
621 GABAergic neurons was sufficient to prevent a seizure induction in embryos exposed to BL at
622 17-19 hrs AEL (unpaired t-test, $t(28) = 1.97$, $p = 0.06$, $n = 15$). (G) PDFR knockdown in CRY
623 positive neurons is sufficient to prevent induction of a seizure phenotype (unpaired t-test, $t(28)$
624 $= 0.299$, $p = 0.767$, $n = 15$).

Figure 1

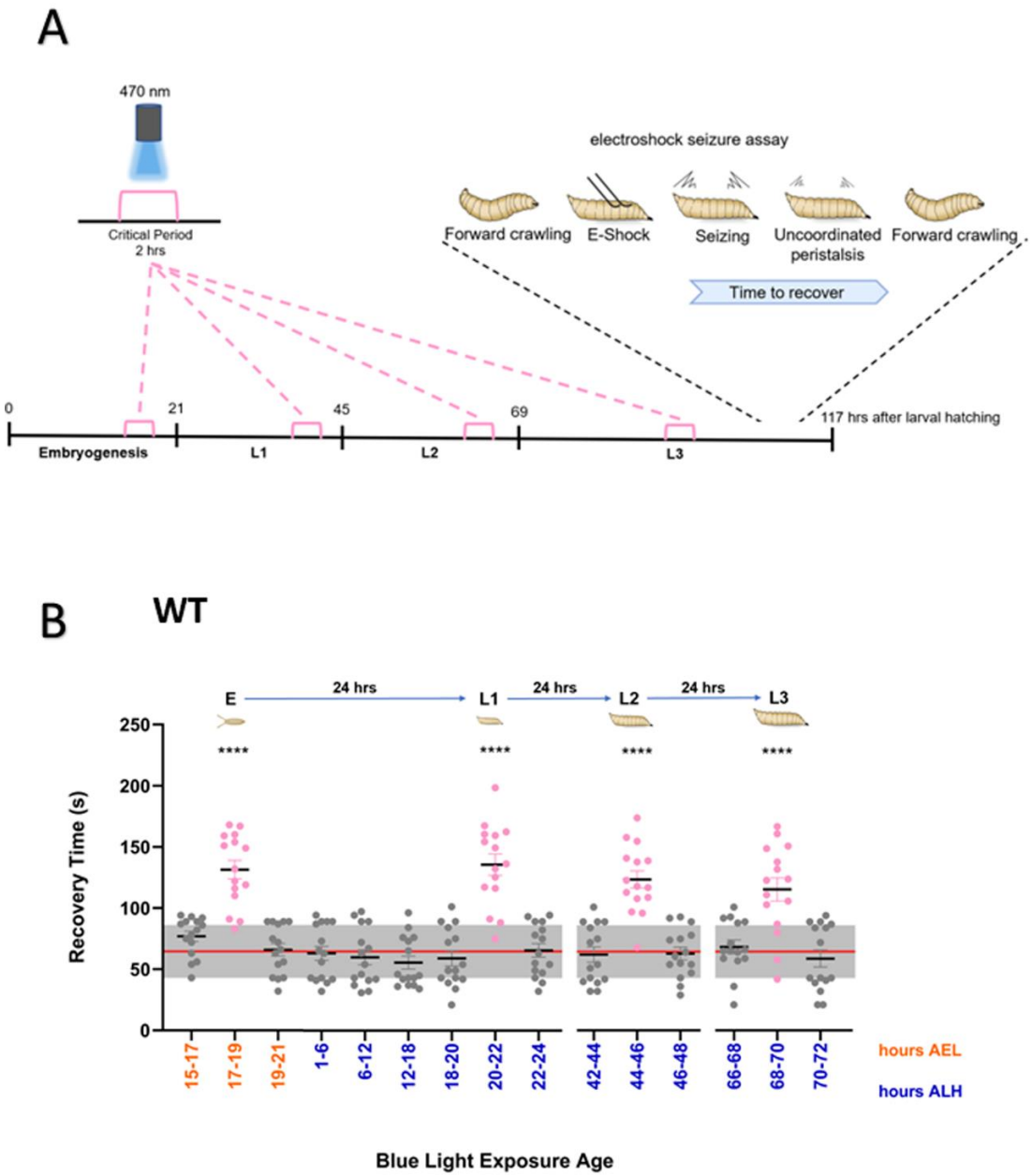

A
470 nm
Critical Period
2 hrs
electroshock seizure assay
Forward crawling
E-Shock
Seizing
Uncoordinated peristalsis
Forward crawling
Time to recover
0
21
45
69
117 hrs after larval hatching
Embryogenesis
L1
L2
L3
B
WT
E
24 hrs
L1
24 hrs
L2
24 hrs
L3
Recovery Time (s)
250
200
150
100
50
0
****
15-17
17-19
19-21
1-6
6-12
12-18
18-20
20-22
22-24
42-44
44-46
46-48
66-68
68-70
70-72
hours AEL
hours ALH
Blue Light Exposure Age

Figure 2

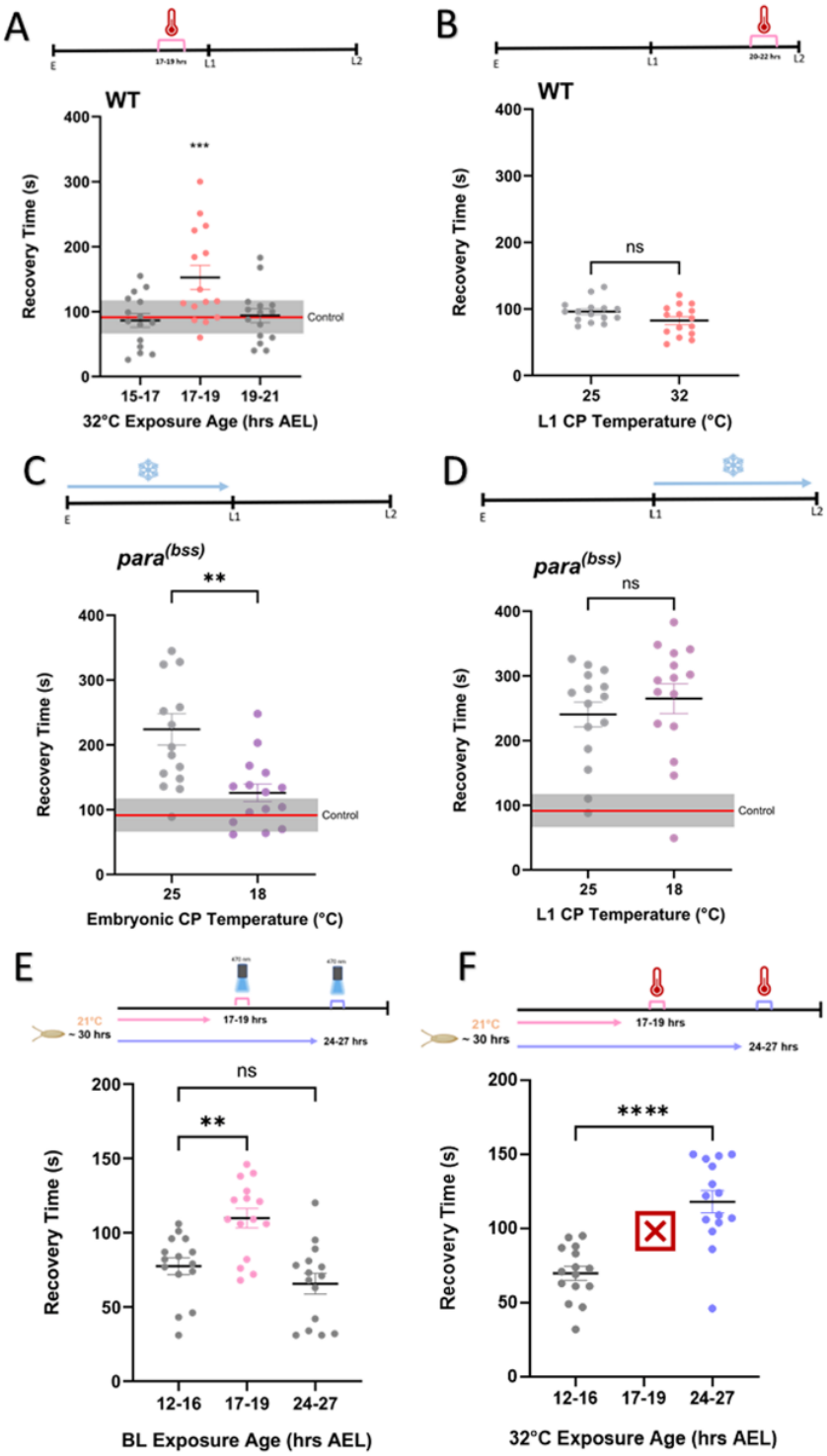

A
E
17-19 hrs
L1
L2
WT
Recovery Time (s)
400
300
200
100
0
***
Control
15-17
17-19
19-21
32°C Exposure Age (hrs AEL)
B
E
L1
20-22 hrs
L2
WT
Recovery Time (s)
400
300
200
100
0
ns
25
32
L1 CP Temperature (°C)
C
E
L1
L2
para(bss)
**
Recovery Time (s)
400
300
200
100
0
Control
25
18
Embryonic CP Temperature (°C)
D
E
L1
L2
para(bss)
ns
Recovery Time (s)
400
300
200
100
0
Control
25
18
L1 CP Temperature (°C)
E
21°C
~ 30 hrs
17-19 hrs
24-27 hrs
ns
**
Recovery Time (s)
200
150
100
50
0
12-16
17-19
24-27
BL Exposure Age (hrs AEL)
F
21°C
~ 30 hrs
17-19 hrs
24-27 hrs
****
Recovery Time (s)
200
150
100
50
0
12-16
17-19
24-27
32°C Exposure Age (hrs AEL)

Figure 3

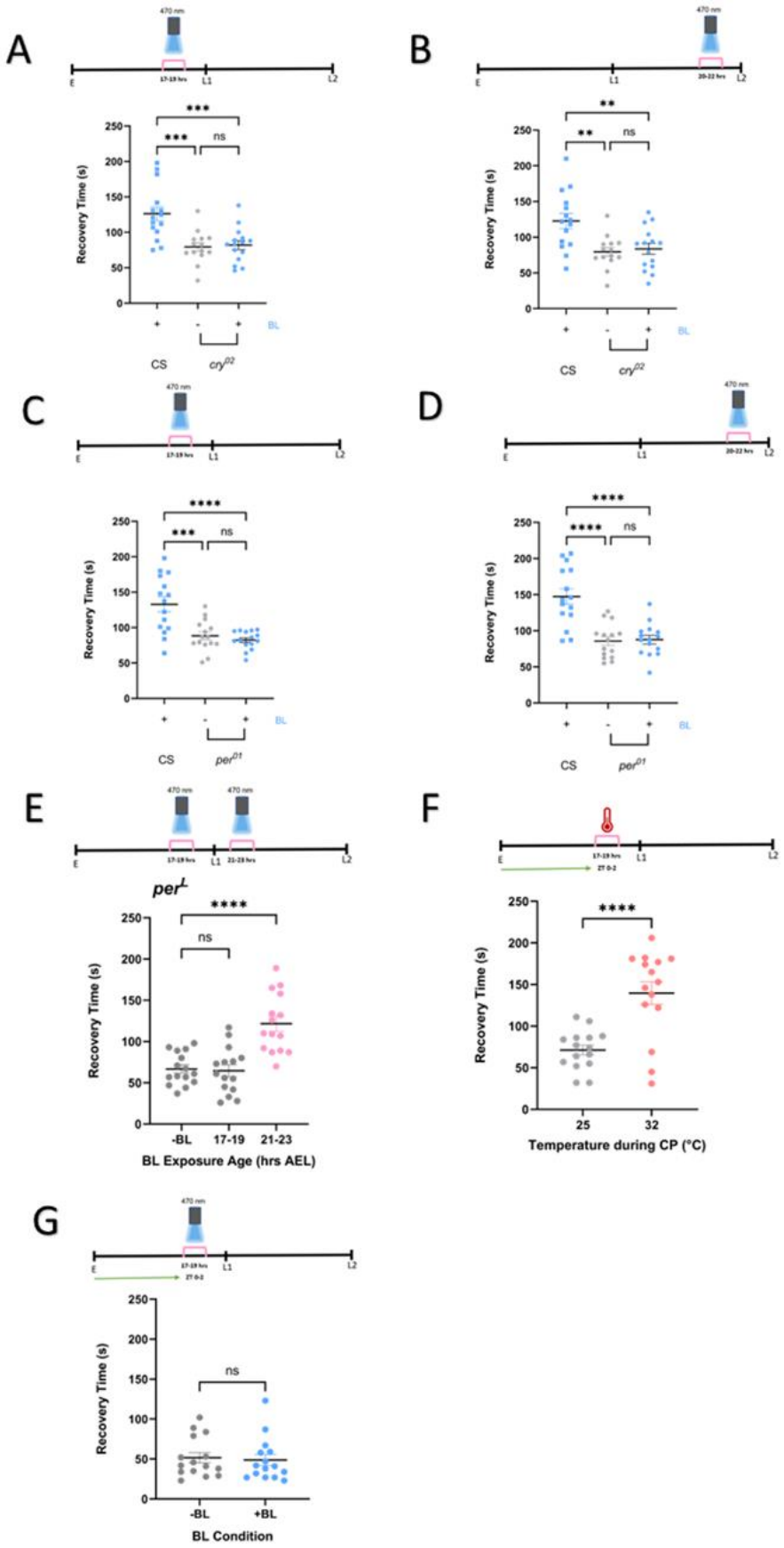

A
B
C
D
E
F
G
470 nm
E
L1
L2
17-19 hrs
20-22 hrs
21-23 hrs
27 0-2
Recovery Time (s)
***
**
****
ns
BL
CS
cry02
per01
perL
-BL
+BL
17-19
21-23
25
32
Temperature during CP (°C)
BL Exposure Age (hrs AEL)
BL Condition

Figure 4

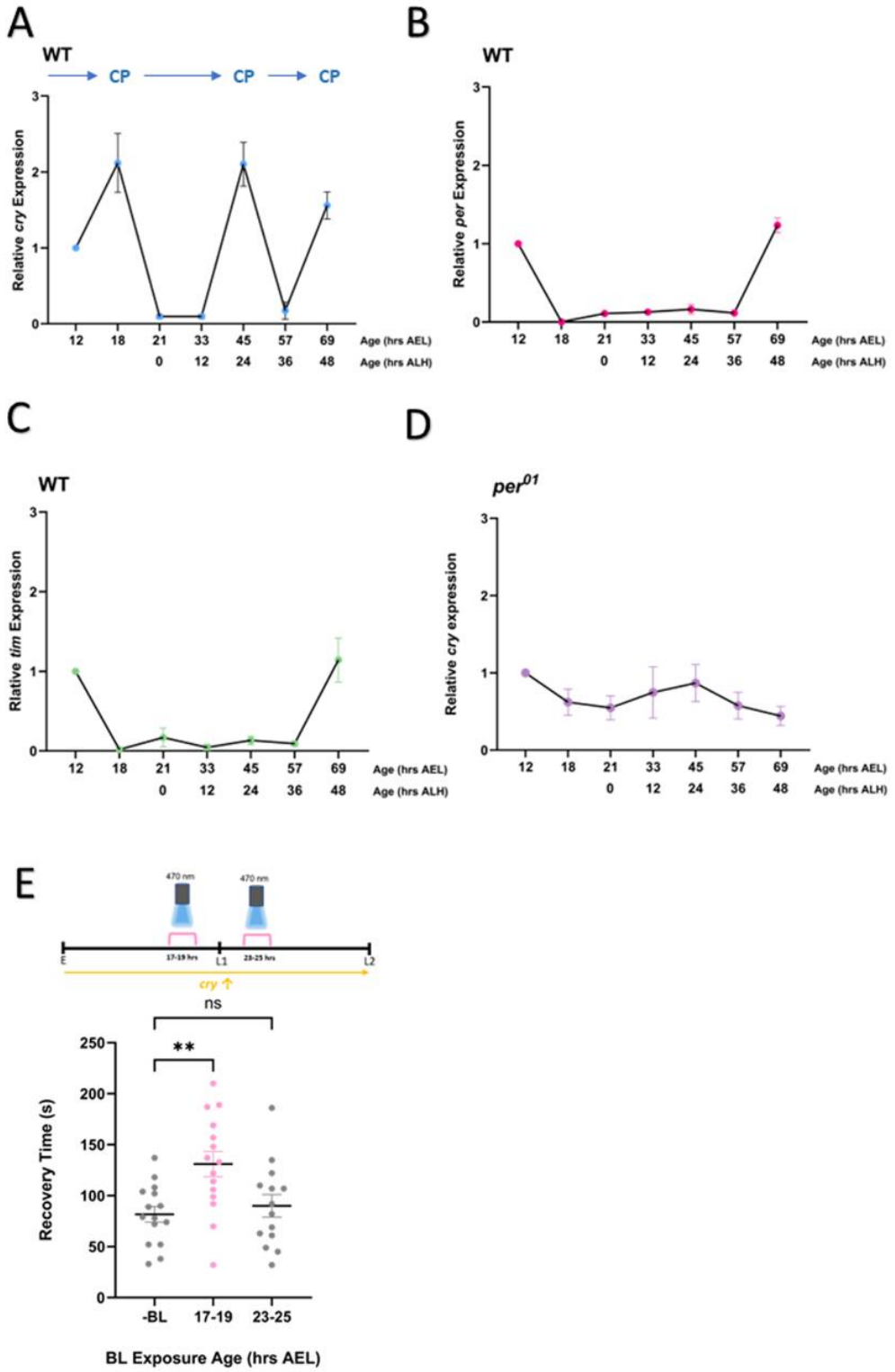
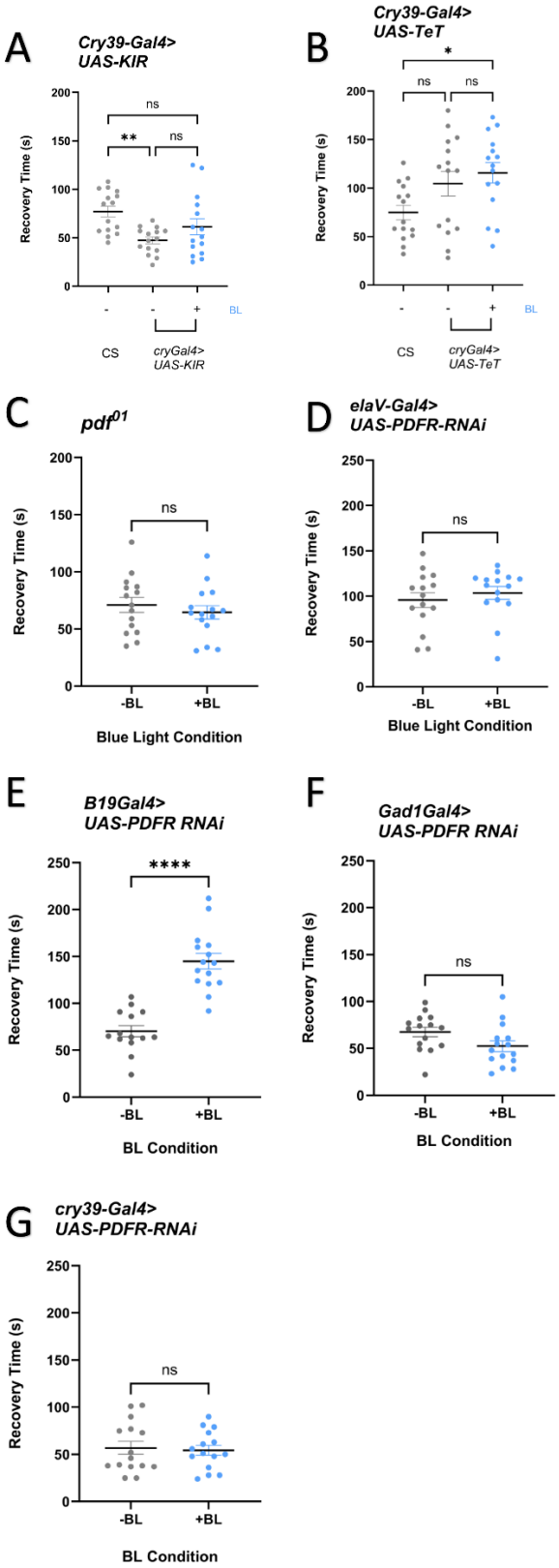

A
WT
CP
CP
CP
Relative cry Expression
Age (hrs AEL)
Age (hrs ALH)
B
WT
Relative per Expression
C
WT
Rlative tim Expression
D
per01
Relative cry expression
E
470 nm
470 nm
E
17-19 hrs
L1
23-25 hrs
L2
cry
ns
**
Recovery Time (s)
-BL
17-19
23-25
BL Exposure Age (hrs AEL)

Figure 5


A
Cry39-Gal4>
UAS-KIR
Recovery Time (s)
ns
**
ns
BL
CS
cryGal4>
UAS-KIR
B
Cry39-Gal4>
UAS-TeT
*
ns
ns
BL
CS
cryGal4>
UAS-TeT
C
pdf01
ns
-BL
+BL
Blue Light Condition
D
elaV-Gal4>
UAS-PDFR-RNAi
ns
-BL
+BL
Blue Light Condition
E
B19Gal4>
UAS-PDFR RNAi
****
-BL
+BL
BL Condition
F
Gad1Gal4>
UAS-PDFR RNAi
ns
-BL
+BL
BL Condition
G
cry39-Gal4>
UAS-PDFR-RNAi
ns
-BL
+BL
BL Condition