Regulation of PDF receptor signaling controlling daily locomotor rhythms in *Drosophila*.

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ABSTRACT

G protein-coupled receptors (GPCRs) trigger second messenger signaling cascades following activation by cognate ligands. GPCR signaling ceases following receptor desensitization or uncoupling from G proteins. Each day and in conjunction with ambient daylight conditions, neuropeptide PDF regulates the phase and amplitude of locomotor activity rhythms in *Drosophila* through its receptor, a Family B GPCR. Its time of action – when it starts signaling and when it stops – much change every day to following changing day lengths. We studied the process by which PDF Receptor (PDFR) signaling turns off *in vivo*, by modifying as many as half of the 28 potential sites of phosphorylation in its C terminal tail. We report that many such sites are conserved evolutionarily, and that in general their conversion to a non-phosphorylatable residue (alanine) creates a specific behavioral syndrome opposite to loss of function phenotypes previously described for *pdfr*. Such "gain of function" *pdfr* phenotypes include increases in the amplitudes of both Morning and Evening behavioral peaks as well as multi-hour delays of their phases. Such effects were most clearly associated with a few specific serine residues, and were seen following alanine-conversion of as few as one or two residues. The behavioral phenotypes produced by these PDFR sequence variants are not a consequence of changes to the pharmacological properties or of changes in their surface expression, as measured *in vitro*. We conclude that the mechanisms underlying termination of PDFR signaling are complex and central to an understanding of how this critical neuropeptide modulates daily rhythmic behavior.

AUTHOR SUMMARY

In multi-cellular organisms, circadian pacemakers create output as a series of phase markers across the 24 hr day to allow other cells to pattern diverse aspects of daily rhythmic physiology and behavior. Within

circadian pacemaker circuits, neuropeptide signaling is essential to help promote coherent circadian outputs. In the fruit fly *Drosophila* 150 neurons are dedicated circadian clocks and are heavily synchronized – they all tell the same time. Yet they provide diverse phasic outputs in the form of their discrete, asynchronous neuronal activity patterns. Neuropeptide signaling breaks that symmetry and drives many pacemakers away from their preferred activity period in the morning. Each day, neuropeptide PDF is released by Morning pacemakers and delays activity by specific other pacemakers to later parts of the day. When and how PDF stops acting each day is unknown. Further, timing of signal termination is not fixed because day length changes each day, hence the modulatory delay exerted by PDF must itself be regulated. Here we consider canonical models of G protein-coupled receptor physiology to ask how PDF receptor signaling is normally de-activated. We use behavioral measures to define sequence elements of the receptor whose post-translational modifications (e.g., phosphorylation) regulate receptor signaling duration.

63 **INTRODUCTION**

64

65 In *Drosophila*, neuropeptide PDF signaling helps pattern the output of the fly circadian
66 pacemaker network in controlling rhythmic daily locomotor activity [1-3]. Its functions have been
67 compared to those of neuropeptide VIP in regulating mammalian circadian physiology [4]. Historically
68 PDF was first isolated as an active principle (Pigment Dispersing Hormone) that mediates light-
69 dependent dispersion of pigment granules in diverse chromatophores of crustacea [5, 6]. In the insect
70 circadian system, PDF acts for a specified period within each 24 hr cycle, and works in conjunction with
71 environmental light to set phase and amplitude for locomotor activity rhythms that normally occur
72 around dawn and dusk [7-9]. Each day, the precise time of dusk and of dawn change, as does the time
73 interval between them. These facts require that the time of PDF signaling, the point when it starts and
74 the point when it stops, must also be adjusted each day to appropriately follow and reflect these daily
75 variations in the light:dark transitions. PDF signaling starts following its release by specific pacemaker
76 neurons, whose period of activity *in vivo* tracks the dawn in a variety of photoperiodic conditions [10].
77 Here we address molecular mechanisms by which PDF receptor signaling normally stops.

78

79 The PDF receptor (PDFR) is a member of the Family B (secretin receptor-like) GPCR group [11-
80 13]: it is G_s-coupled and its activation elevates cAMP levels *in vivo* [14]. It regulates different adenylate
81 cyclases (AC) in diverse target pacemakers (15, 16, Duvall and Taghert, 2012; 2013), which, through PKA
82 activation, ultimately regulate the pace of the molecular clock through regulation of Timeless [17]. PDFR
83 autoreceptor signaling promotes dramatic, daily morphological changes in the axonal terminals of sLNv
84 pacemakers [18]. PDFR activation also regulates calcium dynamics in subsets of pacemaker neuron
85 groups to help dictate their group-specific, daily phases of activation (in the sLNv, in the 5th sLNv and in
86 subsets of LNd, and DN3 groups – [10]). Such target cell-specific delays of PER-dependent neuronal

87 activity illustrates the basis by which the circadian network produces a daily series of phasic, neuronal
88 outputs to temporally bias diverse downstream rhythmic events [19, 20]. Finally, PDF/PDFR signaling is
89 long-lasting: its depression of basal calcium levels in target neurons persists without abatement over
90 many hours. These observations raise fundamental questions regarding the mechanism and the time
91 course by which PDFR signaling diminishes in anticipation of the next day's cycle of signaling.
92
93 The canonical model of GPCR phosphorylation and homologous desensitization features G
94 protein-coupled receptor kinases (GRKs) which associate with activated GPCRs and phosphorylate
95 cytosolic segments, thereby recruiting β-arrestins [21, 22]. β-arrestins uncouple the receptors from G
96 proteins [23, 24]. or enhance receptor endocytosis [25]; they can also serve as signal transducers by
97 recruiting distinct signaling molecules [26]. A second major regulatory mechanism to reduce GPCR
98 signaling is heterologous desensitization, whereby second-messenger-dependent kinases (PKA or PKC)
99 phosphorylate GPCRs [27]. Thus, we designed experiments to modify evolutionarily-conserved residues
100 in the C terminal tail of PDFR that could conceivably serve as substrates for phosphorylation and
101 subsequent signal termination. Our working hypothesis is that such modifications will reveal an
102 extension of the lifetime of PDFR activation *in vivo*.
103
104 Following activation of rhodopsin in the mammalian retina, visual arrestin is recruited with a
105 time constant of < 80 ms [28]; for many *Drosophila* neuropeptide receptors, β-arrestin2 is recruited
106 within a minute of exposure to ligand [29]. Does phosphorylation/de-phopsphorylation also regulate
107 the activity of a GPCR like PDFR, that signals over a much longer time base? The mammalian blue-light
108 sensitive GPCR melanopsin (OPN4) mediates intrinsic light sensitivity in certain classes of retinal
109 ganglion cells (RGCs): melanopsin signaling is distinguished by long latencies, graded responses and
110 sustained RGC depolarization that can outlast the duration of the light stimulus [30]. Melanopsin-
111 expressing RGCs produce exceptionally long timecourse integration because that GPCR behaves

112 differently from other opsins in two fundamental ways. Firstly, it undergoes photoequilibration
113 between signaling and silent states: a property that maintains the availability of pigment molecules for
114 activation, termed 'tristability' by Emmanual and Do [31]. Secondly, phosphorylation and β-arrestin
115 recruitment do contribute to the kinetics of melanopsin signaling, but over a long time course[32, 33].
116 Mure *et al.* [33] proposed that the distal aspect of the melanopsin C terminal tail creates a steric
117 blockade covering a cluster of specific serine residues situated in more proximal regions. The time
118 required for relief of that blockade therefore dictates a delayed time course of phosphorylation.
119
120 We know very little about the mechanisms that underlie termination of PDFR signaling. The *pdfr*
121 gene displays a transcript rhythm that peaks in the late day [34]. Sensitivity to PDF *in vivo* peaks in the
122 early day and is regulated by the PER-dependent clock, via post-transcriptional mechanisms*:* the EC50
123 for PDF responses in identified neurons varies systematically 5-10 fold: as a consequence of Ral A action,
124 as a function of time of day, and as a function of seasonality [35]. PDFR signaling is long-lasting: it
125 persists for many hours *in vivo* [19], for as long as free peptide ligand is available in the bath [14]. In
126 addition, β-arrestin2-GFP is not efficiently recruited to activated PDFR when the receptor is functionally-
127 expressed in *hEK-293T* cells [35]. In contrast, each of 13 other *Drosophila* neuropeptide GPCRs (including
128 two Family B GPCRs, CG8422 and CG17415) efficiently recruit β-arrestin2-GFP when they are activated
129 by their cognate ligands in that cellular environment 29, 36, 37].
130
131 Here we report (i) that PDFR is normally phosphorylated *in vivo* at conserved C terminal
132 residues; and (ii) that loss of conserved PDFR phosphorylatable sites leads to a behavioral syndrome
133 opposite to loss-of-function *pdf* and *pdfr* phenotypes. This 'gain of function' approach reveals a multi-
134 hour range of potential phases for both the Morning and Evening activity peaks, within which
135 neuropeptide PDF:PDFR signaling normally specifies rhythmic behavior, according to season.

136 Importantly, it shows that PDFR signaling affects the amplitude of the activity peaks and not just their
137 phases. Finally, this work identifies specific PDFR sequence elements that are major points at which the
138 duration of receptor activity is dictated, and through which behavior is modulated in season-specific
139 fashion.
140
141 **RESULTS**
142
143 **PDFR C-Terminal sequences.** Based on alternative splicing, the *pdfr* locus in *Drosophila*
144 *melanogaster* (CG13758) encodes GPCRs with two different C terminal sequences, for which the PA and
145 PD isoforms are representative (flybase.org/reports/FBgn0260753). The PD isoform is slightly longer
146 and lacks the final ~20 AAs of the PA isoform. As described in the Supplemental Information (*PDFR*
147 *Isoforms*), we focused on the PA protein isoform of PDFR, as it has been used in the majority of genetic
148 studies in the field. To identify residues for mutation, we first used comparative genomic analyses to
149 assess how well specific sequences in the C-terminal region of the PA receptor are conserved. We
150 obtained annotated *pdfr* genomic sequences from 16 additional species of *Drosophila*, in both the
151 *Sophophora* and *Drosophila* sub-families (**Supplemental Table 1**). Together this species collection
152 represents an estimated 40-60 MYr of *Drosophalid* evolution [38]. We defined residue V505 of the
153 *melanogaster* protein as the start of the C terminal sequence, following the consensus 7th
154 transmembrane TM domain (TM7) (**S Figure 1**). These 17 different C terminal sequences all display
155 considerable length, and vary between 189 to 215 amino acids (AAs). The *melanogaster* PDFR-A C-
156 terminal contains 28 Ser, Thr, or Tyr residues (these may be subject to post-translational
157 phosphorylation and de-phosphorylation). To survey putative functionality among these, we chose 14
158 residues that are distributed across the length of the C-terminal and which display high evolutionary
159 conservation (**Supplemental Figure 1** and **Supplemental Table 2)**. For naming purposes, we grouped

160 them into arbitrary clusters (CL) numbered #1 to #7, with positions shown in **Figure 1.** Following a
161 common paradigm in study of GPCR physiology [e.g., 33, 39, 40], we tested the consequences of their
162 mutation to Alanine, a non-phosphorylatable analog [41]. Some clusters have only a single modified AA
163 (e.g. CL4 and CL5), while in others we modified two AAs (e.g., CL1, CL6 and CL7), and in others, as many
164 as six closely-positioned residues (e.g., CL2-3).
165
166 This series of variant receptors contains 10 different mutated versions of PDFR (not including
167 the WT 'parent' PDFR-A) and is arbitrarily divided into two broad categories as indicated in **Figure 1**. The
168 first group (termed "Simple Variants") targeted one or two clusters of conserved Ser/Thr/Tyr AAs (i.e.,
169 CL 2-3A; 4A; 5A; 6A; 7A). The second category (termed "Multiple Variants") targeted three or more of
170 the AA clusters in various combinations (i.e., CL 1-4A; 1-5A; 1-6A; 1-7A and 5-7A). The CL1-7A variant
171 mutates all 14 of the targeted residues. We studied these ten variants, as well as the WT receptor, in a
172 *pdfr* mutant strain (*han*^{5537} – [11]) for their ability to rescue and to shape the phases and periodicity of
173 rhythmic locomotor behavior. We analyzed PDFR variants in the *pdfr* mutant background to permit
174 evaluation of their properties without competition from endogenous PDFR [although we acknowledge
175 that such possible interactions warrant future study]. Further we tested this PDFR series in different
176 photoperiodic conditions reflecting different seasons, as well as in constant darkness. *pdfr* loss-of-
177 function mutants display advanced behavioral peaks under Light:Dark conditions, as well as weak and
178 shortened free-running periods under constant darkness [11]. In general, we found that several of these
179 Ala-mutant PDFRs delayed the phase of the Morning and/or Evening activity peaks (**Figures 2-7**) and that
180 some also lengthened the free-running period (**Table 1**). Such results are generally consistent with a
181 prediction whereby non-phosphorylatable PDFR variants – those having a potential to increase the
182 duration of PDFR signaling - will produce behavioral actions opposite to those seen in loss-of-function
183 *pdfr* mutant flies. To better interpret the results produced by the different sequence variants, we also

184 present experiments that test assumptions used in the experimental design. These tests consider: i)
185 whether the C-terminal fusion of GFP (present in all variants tested) influenced the results; and ii)
186 whether the behavioral effects of receptor variants are independent of activation by the endogenous
187 ligand, neuropeptide PDF.
188
189 **Analysis of Behavior with PDFR variant expression**. Figures 2 -7 describe rhythmic locomotor
190 behavior of adult *pdfr* mutant flies expressing diverse *pdfr* transgenes in the aggregate, during six days
191 of light entrainment (LD), followed by activity during nine or more days of constant darkness (DD). For
192 each genotype, we display locomotor activity during light entrainment in four ways: (i) group actograms,
193 (ii) group eductions, (iii) derivations of behavioral phase, and (iv) direct comparisons of the amplitudes
194 of behavioral peaks between genotypes. Thus in Figures 2, 4 and 6, a representative group actogram
195 displays all locomotor activity double-plotted across all days in LD and DD (Panels A-L, sub-panel **-1** in
196 each Figure). Second a representative group eduction displays a 24 h average of the activity in the final
197 two days in LD (days 5-6) (Panels A-L, sub-panel **-2** in each Figure). Next, we define the phase onsets and
198 offsets for Morning and Evening activity peaks during the same final two LD days across all experiments
199 (Panels M and N in Figures 2, 4 and 6). For the comparison of peak amplitudes in these three
200 photoperiodic conditions, we describe Bin-by-Bin analyses of locomotor activity during the entire LD
201 period (days 2-6) in Figures 3, 5 and 7. The Bin-by-Bin analyses directly compares experimental
202 genotypes to a control one [cf. 42]. They present data averaged across 2 to 5 independent experiments,
203 to help identify the differences that most consistently correlate with genotype. Finally, Table 1 compiles
204 measures of rhythmic activity displayed by the different genotypes in days 3-9 of constant darkness.
205
206 **Behavior under Short Day (winter-like) conditions (Figures 2 and 3).** Flies lacking *pdfr* function
207 in 8L:16D lack a prominent morning peak and their evening peak of activity begins earlier than controls;

208 the example shown in **Figure 2A-2** is heterozygous for the *tim*-Gal4 element. Rescue by WT-*pdfr* using
209 *tim*-Gal4 significantly delayed (i.e., rescued) the Evening peak initiation by about 1 h (**Fig. 2B-2).**
210 Expression of each of the Single Variants driven by *tim*-Gal4 produced also delayed the Evening peak
211 (**Fig. 2C-2** to **G-2**), with the variants 6A and 7A producing conspicuous, large amplitude peaks that
212 occurred on average as late as ~3 h after lights-off, at ZT12 (**2F-2** and **2G-2**). Variants 2-3A, 6A and 7A
213 also produced elevated activity during the period of ZT 18-19 (**Fig. 3C-1,2F-1** and **2G-1**): we speculate
214 this is promotion of a "Morning" peak of activity. Among the five Multiple Variants, we noted three
215 main effects (**Fig. 3**). First the progressive addition of more Ala substitutions in the series 1-5A, 1-6A and
216 1-7A (**Figure 3D-5** through **3G-5**) produced large and delayed Evening peaks in the time period ZT11.5-
217 12.5 (3-4 h after lights-off), with the variant 1-6A producing the most pronounced delay. The delayed
218 Evening peak activity at ZT12 appeared often appeared at the expense of, the normal Evening peak
219 activity that occurred prior to Lights-off (e.g., **Fig.3G-4**), although when averaged across all days in LD,
220 that effect was not significant (**Figure 3 – panel 2**). Second, the PDFR 1-5A and 1-7A forms in the
221 Multiple Variant series produced significant "Morning" peaks around ZT19, (**Fig 3J-1** and **3L-1**), Finally,
222 we noted that combining the Single 5A, 6A and 7A variants into a Multiple Variant (5-7A) did not
223 produce the anticipated additive effects on promoting morning locomotor activity at ~ZT19 (**Fig. 3C-3).**
224 Such anticipation was predicated on the effects seen with 1-5A, 1-6A and 1-7A (versus 1-4A) (**Fig 3J-1**
225 **and-3, K-3 and L-1 and -3**). Likewise the 5-7A did not produce the strong delay in the Evening activity
226 peak to ~ZT12 that was expected based on effects seen with 1-5A, 1-6A and 1-7A (versus 1-4A): instead
227 it produced only a minor delay (**Fig. 3H-3**).
228
229 **Behavior under equinox conditions (Figures 4 and 5).** Under 12:12 conditions, *han* mutant flies
230 (lacking *pdfr* function) typically display elevated nocturnal activity, a lack of morning anticipation prior to
231 Lights-on, and a pronounced advance in the peak of the Evening behavior [11]. **Fig. 4A-1** to **A-2** display

232 activity patterns of *han* mutants that are heterozygous for *tim*-Gal4; they generally matched prior
233 descriptions of *pdfr* mutant behavior. Restoration of some *pdfr* function by *han*; *tim*> WT-*pdfr*,
234 produced a modest Morning peak (anticipatory activity prior to lights-on – **Fig. 4B-2**) and delayed the
235 evening peak by 1-2 hrs (**Figures 4N and 5B-2**) [7,8,9]. Among the five "Simple PDFR Variants" (**Fig 4C-G**),
236 the 6A and 7A PDFR isoforms produced significantly elevated morning activity, with a peak ~2 hr prior to
237 Lights-on in the period ZT22-23 (**Fig 5F-1** and **G-1**). This elevated pre-Lights-on activity peaked ~3-4 hr
238 later than that seen under Short Day conditions with these same PDFR variants. Separately, the 6A
239 isoform increased Morning activity after the lights-on signal (**Fig. 5F-2)** and the 4A, 6A and 7A isoforms
240 increased the amount of Evening activity, but without changing its phase (**Fig. 5D-5, F- 5** and **G-5**). In the
241 case of the five Multiple Variants, we noted three main effects. First the progressive addition of more
242 Ala substitutions in the series 1-5A, 1-6A and 1-7A all produced significant additional Morning activity in
243 the period ZT22-23 compared to that seen with 1-4A. Notably, all of that activity appeared to decline
244 prior to the Lights-on signal (**Fig 5E-3, F-3, G-3).** Those variants also produced increased activity
245 immediately after Lights-on: an effect seemingly distinct from that produced prior to Lights-on (**Figure 5**
246 **J-42 K-2** and **L-2**). Second, combining the 5A, 6A and 7A variants in PDFR 5-7A did not produce additive
247 effects on behavior in the morning, either before or after Lights-on (**Fig 5H-1** and **-2**): instead the activity
248 at ZT22-23 and ZT 0.5-3.5 appeared similar only to that displayed by rescue with a WT PDFR. Finally, the
249 1-5A variant was notable among Multiple Variants in LD12:12 conditions by displaying elevated activity
250 in mid-day between the Morning and Evening Activity peaks (**Fig 5E-4** and **E-5**).
251
252 **Behavior under Long Day (summer) conditions (Figures 6 and 7)**. Flies lacking *pdfr* function in
253 16L:8D display locomotor patterns similar to those in 12L:12D – lack of an obvious Morning peak and
254 produce a broad Evening activity period that peaks ~3hr before Lights-off: the example in **Figure 6A-2**
255 displays behavior by *han* mutants that are also heterozygous for the *tim*-Gal4 element. Rescue (*han*;

256 *tim*> WT-*pdfr*, **Figure 6B-2**) typically delayed Morning activity offset (**Fig. 6M)** and delayed the Evening
257 peak onset each by about 1 h (**Fig. 6N).** Expression of the 5A and 6A PDFR variants produced
258 significantly greater amounts of Morning activity after Lights-on (**Fig. 7C-3** through **G-3**), while all of the
259 Single Variants increased the amplitude of the Evening peak without affecting its phase (**Fig. 6 C-5** to **G-**
260 **5**). Among the five Multiple Variants, we noted three main effects (all comparable to those seen
261 previously under 12L:12D entraining conditions). First the 1-6A and 1-7A variants displayed significantly
262 increased Morning activity after Lights-on (**Fig. 7 K-2** and **L-4**). Second, the variants 1-5A, 1-6A and 1-7A
263 increased the amplitude of the evening peak without affecting its phase (**Fig. 7J-3, K-3** and **G-3**). Third,
264 as in the other photoperiodic conditions, when we combining the 5A, 6A and 7A variants into a single 5-
265 7A variant, it did not produce the expected additive effects on behavior in the morning (**Fig 7H-2**) or in
266 the evening (**Figure 7H-3**): instead these activities appeared similar to effects displayed by the 1-4 and 1-
267 5 variants.
268
269 ***Behavior under DD.*** In DD following Short Day conditions, the PDFR variants that produced 3-4 h
270 delays in the evening peak often generated periods ~ 1-2 hr longer than the controls and also lowered %
271 arrhythmicity (**Table 1**). This was especially true for the Multiple Variant series (e.g., PDFR 1-5A, 1-6A
272 and 1-7A (**Table 1**). However the correlation between delayed evening peaks and a longer tau in DD was
273 not absolute" for example, the 6A, 7A and 5-7A variants all had strong significant delaying effects on the
274 Evening phase of activity under Short Day conditions (compared to the effect of the WT PDFR), but they
275 did not lengthen tau values in subsequent DD conditions (Table 1). These observations suggest that
276 delays of activity phases produced by PDFR modulation in LD are not necessarily a simple or direct
277 consequence of its effects on the PER and TIM--dependent clock [2, 3, 17]. In addition, the dominant
278 activity periods in DD typically reflected the delayed (~ZT12) Evening peak (**Fig. 2K-1** and **L-1**). Notably,

279 the early Morning peak (~ZT18-19) promoted by PDFR 6A and 7A clearly persisted in DD (**Fig. 2F-1** and

280 **G-1**).

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Table 1.

						Average tau different from:		
Short Day Entrainment	**N**	**n**	**%AR**	**tau**	**SEM**	w[1118]	pdfrEgfp	pdf[01]
han[5304]; tim > w[1118]	5	72	22%	23.9	0.09	-	ns	nd
han[5304]; tim > pdfr	1	16	6%	23.7	0.13	ns	ns	nd
han[5304]; tim > pdfr1-7A No GFP	3	30	8%	23.6	0.07	ns	ns	nd
han[5304]; tim > pdfrEgfp	3	46	35%	24.00	0.09	ns	-	nd
han[5304]; tim > pdfrEgfp23A	2	31	13%	24.8	0.12	ns	ns	nd
han[5304]; tim > pdfrEgfp4A	2	31	26%	24.7	0.13	ns	ns	nd
han[5304]; tim > pdfrEgfp5A	2	43	26%	24.58	0.11	ns	ns	nd
han[5304]; tim > pdfrEgfp6A	3	50	38%	24.37	0.08	ns	ns	nd
han[5304]; tim > pdfrEgfp7A	3	45	27%	24.83	0.11	ns	ns	nd
han[5304]; tim > pdfrEgfp1-4A	3	40	12%	24.6	0.12	ns	ns	nd
han[5304]; tim > pdfrEgfp1-5A	3	31	16%	25	0.23	***	*	nd
han[5304]; tim > pdfrEgfp1-6A	3	42	14%	25.1	0.13	****	**	nd
han[5304]; tim > pdfrEgfp1-7A	4	59	16%	24.9	0.11	****	*	nd
han[5304]; tim > pdfrEgfp567A	2	37	16%	23.9	0.09	ns	ns	nd
tim > pdfrEgfp1-7A	1	15	0%	25.77	0.25	nd	nd	****
pdf[01]	1	12	67%	22.50	0.12	nd	nd	-
tim > pdfrEgfp1-7A; pdf[01]/pdf[01]	1	25	20%	23.63	0.12	nd	nd	ns

Equinox Entrainment	N	n	%AR	tau	SEM		
han[5304]; tim > w[1118]	6	68	34%	23.7	0.09	-	ns
han[5304]; tim > pdfr	3	16	25%	23.5	0.16	ns	ns
han[5304];; tim > pdfr1-7A No GFP	3	35	14%	23.67	0.08	ns	ns
han[5304]; tim > pdfrEgfp	4	48	13%	24.1	0.06	ns	-
han[5304]; tim > pdfrEgfp23A	2	54	48%	25.6	0.12	****	****
han[5304]; tim > pdfrEgfp4A	2	44	32%	24.1	0.07	ns	ns
han[5304]; tim > pdfrEgfp5A	2	47	34%	24.4	0.06	ns	ns
han[5304]; tim > pdfrEgfp6A	3	35	23%	24.3	0.13	ns	ns
han[5304]; tim > pdfrEgfp7A	3	47	21%	24.1	0.09	ns	ns
han[5304]; tim > pdfrEgfp1-4A	3	45	18%	24.5	0.08	**	ns
han[5304]; tim > pdfrEgfp1-5A	2	32	6%	25.00	0.13	****	***
han[5304]; tim > pdfrEgfp1-6A	2	32	19%	25.27	0.14	****	****
han[5304]; tim > pdfrEgfp1-7A	6	88	6%	25.1	0.17	****	****
han[5304]; tim > pdfrEgfp567A	2	45	24%	24.1	0.08	ns	ns

Long Day Entrainment	N	n	%AR	tau	SEM			
han[5304]; tim > w[1118]	7	###	47%	23.8	0.06	-	ns	nd
han[5304]; tim > pdfr	3	47	17%	24.88	0.15	ns	ns	nd
han[5304]; tim > pdfr1-7A No GFP	3	40	48%	24.57	0.09	ns	ns	nd
han[5304]; tim > pdfrEgfp	4	82	17%	24.87	0.08	ns	-	nd
han[5304]; tim > pdfrEgfp23A	2	48	23%	25.35	0.12	****	****	nd
han[5304]; tim > pdfrEgfp4A	1	27	22%	25.50	0.16	****	****	nd
han[5304]; tim > pdfrEgfp5A	1	36	11%	24.66	0.16	ns	ns	nd
han[5304]; tim > pdfrEgfp6A	2	51	22%	25.02	0.09	ns	ns	nd
han[5304]; tim > pdfrEgfp7A	2	54	13%	25.46	0.09	****	****	nd
han[5304]; tim > pdfrEgfp1-4A	3	40	8%	25.2	0.13	****	ns	nd
han[5304]; tim > pdfrEgfp1-5A	3	33	6%	25.18	0.14	****	*	nd
han[5304]; tim > pdfrEgfp1-6A	3	48	2%	25.59	0.09	****	****	nd
han[5304]; tim > pdfrEgfp1-7A	4	63	14%	25.3	0.08	****	***	nd
han[5304]; tim > pdfrEgfp567A	1	38	18%	24.31	0.09	ns	ns	nd
tim > pdfrEgfp1-7A	1	14	14%	25.50	0.13	nd	nd	****
pdf[01]	1	32	88%	22.75	0.05	nd	nd	-
tim > pdfrEgfp1-7A; pdf[01]/pdf[01]	1	29	21%	23.30	0.09	nd	nd	ns

Table 1. Locomotor activity measures of various genotypes during constant dark conditions.

291 Behavioral activity records from days 3-9 in constant conditions following entrainment in the three
292 indicated photoperiods (Short Day, Equinox and Long Day). N = number of independent experiments
293 performed for each genotype::photoperiod combination. n = total number of flies tested for each
294 genotype::photoperiod combination. % AR: the percentage of flies judged arrhythmic by criteria; tau =
295 average circadian periods calculated according to χ2-periodogram analysis. Statistical analysis
296 compared tau's using Tukey's multiple comparisons post hoc test following a one-way ANOVA: ns: not
297 significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.; nd: not determined.
298
299
300 In DD following Equinox or Long Day entrainment, we noted that several variants produced
301 dominant activity phases which were phase-delayed relative to the Evening peak displayed in LD (e.g.,
302 **Fig.4F-1, G-1**, and **J-, K-** and **L-1; Fig.6F-1, G-1,** and **J-, K-** and **L-1**). Thus, photophases > 8 hr in duration
303 appear to repress expression of the delayed, post Lights-off Evening activity observed under SD
304 conditions: this was suggested by the emergence of a delayed phase activity peak once flies only after
305 release to DD. Once again, several of the variants significantly increased Tau in DD (**Table 1**), yet had
306 only slight effects on phase in LD.
307
308 ***Control experiments.*** The design of the *PDF*R variants contains numerous assumptions and
309 genotypic constraints, distinct from simply substituting Alanine at potential sites of phosphorylation in
310 the receptor C terminal tail. To determine their potential to affect the results we report, we performed
311 the following two sets of experiments as controls. (i) - ***The influence of epitope fusions.*** The PDFR
312 variants we have tested include epitope fusions (4xFLAG and eGFP), which in principle could confer
313 some of the properties observed. In part, the concern regarding fused sequences is mitigated by the
314 fact that all UAS-receptor isoforms described above (WT and sequence-variant) contained both epitopes

315 (in addition – all were genetically transduced to the same integration site). Hence comparisons across
316 different variants (e.g., **Figures 2 through 7**), should largely normalize for the effects of the non-receptor
317 sequences, and instead highlight variant-specific properties. However, we wished to test this
318 assumption explicitly to define the extent to which a bulky GFP fused to the C Terminal tail might confer
319 some of the behavioral properties we might otherwise ascribe to PDFR C terminal sequences. We
320 therefore compared a 1-7A version of PDFR to a wild type PDFR, both of which lacked GFP fusions. The
321 results in both Short Day and Long Day conditions, followed by release into DD, support the hypothesis
322 that alterations in daily locomotor rhythmicity were largely derived from sequence variation of the
323 PDFR, and not from the properties of the fused GFP (**Supplemental Figure 4** and tabulated in **Table 1**).
324
325 ***Control experiments (ii) - dependence of GPCR PDFR variant effects on the presence of PDF ligand***.
326 Second, we assumed that the altered behavioral phenotypes produced by certain PDFR variants
327 depended on activation by their endogenous cognate ligand, the neuropeptide PDF. However, we could
328 not *a priori* exclude the possibility that PDFR variants may in fact produce novel constitutive activity
329 (neomorphic properties). We therefore placed the 1-7A PDFR variant in the pdf^{01} (null) background and
330 re-tested its activity when driven by *tim*(UAS)-Gal4 under both short day and long day conditions, and
331 then in constant darkness. In all conditions, the behavioral phenotypes largely resembled those of the
332 pdf^{01} background (lack of a morning activity peak, advanced evening peak, and shorter tau/s in DD). The
333 results are shown in **Supplemental Figure 5** and tabulated in **Table 1**. The 1-7A PDFR variant did reduce
334 the % arrhythmicity present in the *pdf* mutant background to nearly the same value as found in a WT
335 *pdf* background, which suggests some degree of constitutive (ligand-independent) activity. Apart from
336 that one aspect, we conclude that constitutive activity explains at best a small proportion of the
337 behavioral effects of the PDFR variants here tested.
338

339 **PDFR expression and signaling *in vitro*.** The PDFR is a G_s-coupled receptor and several reports
340 have documented the importance of the downstream cAMP pathway to mediate PDF behavioral
341 regulation [15, 17, 43]. In that context, we asked whether the *in vivo* properties of those PDFR variants
342 that affected behavioral phase and amplitude could be correlated with *in vitro* properties when
343 expressed in *hEK-293T* calls. We found no strong correlation. Significant differences in basal signaling
344 levels, in EC50 or in maximum values of signal transduction were rare (**Supplemental Figures 6-8**).
345 When noted, they were poorly correlated with behavioral effects (**Supplemental Table 5**). We also
346 measured surface expression of the PFDR variants in *hEK* cells using β-lactamase N terminal fusions [44]
347 to determine if PDFR variants tended to display longer surface lifetimes. The 1-4 and 1-7 variants
348 displayed higher basal levels, but no others were different from the WT levels (**Supplemental Figure 9**).
349 Following 20 min exposure to PDF, neither the WT not any of the variants displayed a change in surface
350 expression levels (**Supplemental Figure 10**).
351
352
353 **Measuring the phosphorylation state of over-expressed PDFR *in vivo*.** To obtain direct
354 evidence that PDFR sequences are phosphorylated *in vivo*, we first over-expressed an epitope-tagged-
355 PDFR WT construct using *tim*-Gal4, which directs expression broadly in cells that feature the PER-
356 dependent molecular oscillator. We immunoprecipitated the receptor from head extracts, then
357 employed tandem mass spectroscopy to determine which if any specific PDFR peptide fragments are
358 phosphorylated. We performed eight biological replicates, with two collections in the morning (ZT2-3)
359 and six in the evening hours (ZT11). **Table S3** reports the phosphorylated peptides detected from the
360 PDFR-GFP fusion protein. Among the 28 conserved Ser/Thr and Tyr residues in the PDFR C terminal tail,
361 five were phosphorylated in one or more of these samples. In the two Mornings samples S563 was
362 phosphorylated in one sample; in the Evening samples, S531 (in CL2) was phosphorylated in four of

363 samples, S534 (in CL2) in one, T543 (in CL3) in one, and S560 in two of six samples. The spectra
364 documenting detection of these phosphopeptides are presented in **Supplemental Figure 2**.
365
366 **Testing GRKs and β-arrestin2 contributions to locomotor rhythmicity.** Regarding which
367 potential PDFR phosphorylation sites might be β-arrestin binding sites, we considered a recent report by
368 Zhou *et al.* [45] who proposed a conserved GPCR sequence motif that promotes high affinity
369 interactions with β-arrestin. The motif includes three phosphorylation sites that align with three
370 conserved, positively-charged pockets in the arrestin N-domain. In *D. melanogaster* PDFR, the CL1
371 region contains a match with the proposed motif beginning with S512 (SLATQLS) and shows moderate
372 sequence conservation: of the 17 species we considered, 9 others retained this precise motif (**S Figure**
373 **1**). In addition, the *D. melanogaster* PDFR sequence beginning with S629 (SRTRGS) also displays a match
374 for the motif, but is retained in only 6 of the other 16 species. The evidence that these two sites
375 represent high affinity β-arrestin2 binding domains is therefore equivocal. We previously reported that
376 β-arrestin2-GFP is not efficiently recruited to activated PDFR when functionally-expressed in *hEK-293T*
377 cells [35]. In contrast, each of 13 other *Drosophila* neuropeptide GPCRs do efficiently recruit β-
378 arrestin2-GFP when expressed and activated in that cellular environment [29, 36, 37].
379
380 We tested the potential to detect *in vivo* involvement by *Drosophila* orthologues of the
381 canonical desensitization effectors - mammalian G-protein coupled receptor kinases (GRKs – GPRK1
382 (CG40129) and GPRK2 (CG17998)) and of mammalian β-arrestin2 (βarr2 – *kurtz* (CG1487)) - in the
383 control of locomotor rhythms We drove specific over-expression of WT cDNAs and RNAi constructs
384 using *tim*-Gal4, to broadly affect signaling in the circadian pacemaker system. The PDFR-PA protein is
385 highly restricted in its expression to subsets of the pacemaker neural network [46]. We predicted that
386 elimination of a desensitizing component for PDFR signaling should produce behavioral phenotypes

387 opposite to that of *pdfr* loss of function phenotypes: these would potentially include (for example) a
388 delayed evening activity phase in LD conditions and a longer tau under DD. GPRK1- and *krz*-specific
389 RNAi's produced normal average locomotor profiles under 12:12, while one of two GPRK2-specific RNAi
390 constructs tested slightly advanced the evening peak (**Supplemental Figure 5**). Over-expressing GPRK1
391 cDNAs did not affect morning or evening phase; over-expressing GPRK2 broadened the evening peak.
392 Under DD, *krz* RNAi flies were uniformly arrhythmic, while GPRK RNAi's were normal or slightly
393 lengthened the circadian period (**Supplemental Table 10**). These results do not support the hypothesis
394 and suggest that the kinetics by which PDFR signaling terminates do not depend exclusively on the
395 activities of either dGPRK-1 or -2, or on that of the *Drosophila* β-arrestin2 ortholog, *krz*.
396
397
398 ***DISCUSSION***
399
400 We found that several PDF receptor variants, containing Ala-substitutions of phosphorylatable
401 residues, modulated the amplitudes of the Morning and/or or Evening activity peaks, or delayed their
402 phases and/ or lengthened the free-running period. Such results are generally consistent with a
403 prediction whereby non-phosphorylatable PDFR variants – those with a potential to increase the
404 duration of PDFR signaling - would produce behavioral actions opposite to those seen in loss-of-function
405 *pdf* [1], or *pdfr* [11] mutant stocks. The loss of function phenotypes include advanced behavioral peaks,
406 as well as weaker and shortened free-running periods. In this context, we acknowledge that GPCR
407 modification by phosphorylation does not exclusively lead to signal termination. GRK2-dependent
408 phosphorylation of the smoothened (smo) GPCR (which helps mediate Hh signaling) follows reception of
409 the Hh signal: that leads to smo <u>activation</u> in both *Drosophila* and mammalian systems [47]. Thus
410 modification of putative phosphorylation sites on GPCRs (to preclude phosphorylation) will not

411 exclusively promote extended signaling and therefore our interpretations must correspondingly
412 consider outcomes without *a priori* assumption of mechanisms.
413
414 ***GPCR signal termination.*** PDFR belongs to the Secretin Receptor Family (Family B) of
415 neuropeptide receptors [11-13]: there is no clear consensus regarding mechanisms of desensitization
416 and internalization for this receptor family. For VPAC2 receptors, phosphorylation and internalization is
417 mediated exclusively by GRK2 (48. Murthy, 2008;). Likewise, *Drosophila* orthologues of Cortocotrophin
418 Releasing Factor receptors (CG8422, DH44-R1) and Calcitonin receptors (CG17415, DH31-R1) are
419 internalized in *hEK* cells following recruitment of β-arrestin2 [29]. In contrast, PDFR, which is also
420 related to the mammalian Calcitonin receptor, is not internalized following exposure to PDF [35]. VPAC2
421 receptors are also regulated by heterologous receptor signaling: M3 cholinergic receptors via PKC
422 signaling can block VPAC2 phosphorylation, desensitization and internalization [49]. Furthermore,
423 secretin receptors and VPAC1 receptors undergo phosphorylation by GRKs and β-arrestin2-dependent
424 desensitization, but these are not sufficient to facilitate or mediate internalization [50-52]. Finally, GLP2-
425 Receptor associates with β-arrestin2 via its distal C terminal sequences, but that receptor domain is
426 required neither for GLP2-R desensitization nor its internalization [53]. Thus kinases other than GRKs
427 and effectors other than β-arrestin2 may regulate internalization of diverse Family B receptors.
428
429 ***Validating the behavioral phenotypes produced by PDFR variants.*** The actions of PDFR
430 variants we have described, following substitutions of Ala for various Ser/Thr or Tyr residues, are not
431 easily explained by a hypothesis invoking neomorphic or constitutive GPCR properties. The evidence for
432 this conclusion is three-fold. First, the effects on both amplitude and phase of activity peaks by these
433 variants are not of a random assortment: rather they are all strictly opposite to that of loss-of-function
434 models for *pdf* and *pdfr*. Second, the actions of PDFR variants display strong dependence on wild type

435 *pdf* gene function: this strongly argues that the actions of PDFR variants reflect responses to normal,
436 endogenous PDF signaling. Third, the action of PDFR variants on the phases and amplitudes of
437 locomotor activity reflected PDFR sequence variation, and not the properties of the GFP C-terminal
438 fusion. Together these control observations support the hypothesis that phosphorylation of some or all
439 of the C terminal residues we studied are normally modified to attenuate the strength and duration of
440 PDFR signaling.
441
442 ***PDF Signaling and seasonal adaptation.*** PDF neuropeptide actions were discovered in the
443 context of physiological adaptions to daylight in crustacea [5,6]. Helfrich-Förster [54] discovered PDF
444 expression within a defined subset of the insect *Drosophila* circadian pacemaker network. Renn *et al.*
445 [1] demonstrated that *pdf* makes a fundamental contribution by setting the normal behavioral phase of
446 Evening activity in *Drosophila* during light:dark entrainment and promoting normal rhythmicity during
447 constant darkness. PDF signaling coordinates with that driven by light: It works in parallel to direct
448 photosensitivity along with the CRY blue light photoreceptor. Flies doubly mutant for *cry* and *pdf* display
449 severe deficits of locomotor rhythmicity [7, 8], and such effects can be as severe as measured in clock-
450 deficient flies [9]. PDF and environmental light also work coordinately at the level of neuronal activity
451 patterns: in the case of the evening pacemakers (LNd and the 5th s-LNv), both light and PDF signaling
452 promote a delay in their PER-dependent activation period, and help align it to a phase just prior to dusk
453 [19].
454
455 Based on the close association of PDF signaling and photoperiodic signaling, we considered it
456 essential to test the different variant PDF Receptors in a variety of photoperiodic conditions. The
457 consensus of many prior observations indicates that PDF neuropeptide signaling is necessary for the
458 display of a Morning peak (its amplitude) and for proper adjustment of phase of the Evening activity

459 peak [e.g., 1, 7, 8, 55-57]. Our present observations, concerning likely gain-of-function *pdfr* variants,
460 support a revised hypothesis regarding regulation of the Morning peak: we propose PDF/PDFR can
461 affect - and normally does affect - both the phase and amplitude of the both Morning and Evening
462 activity peaks. Thus the consensus of many prior descriptions – concluding there is "no Morning activity
463 peak" in *Pdf* and *Pdfr* mutants – beginning with the description by Renn *et al.* [1], may have failed to
464 discern the presence of a greatly-diminished Morning peak that was also advanced. There is some
465 precedent for this contrary conclusion: Yoshii *et al.* [58] described behavior of pdf^{01} mutants and noted
466 advanced morning peaks especially under longer photoperiods. Likewise, the duration of the
467 photoperiod strongly influenced the effects we observed on the Morning versus the Evening activity
468 peaks. We only observed delayed Evening activity peak, produced by PDFR gain-of-function variants,
469 under Short Day conditions: the delayed evening peak appears strongly inhibited by light durations > 8
470 hr: and it remains unclear at what molecular level light exerts this inhibitory effect. We note a striking
471 similarity of these observations with effects recently described in loss-of-function states for the
472 phosphatase *PRL-1* [51]. Like the most active PDFR variants we herein describe (e.g., 6A, 7A, 1-5A 1-6A
473 and 1-7A), *PRL-1* mutants produce a 3-4 h delay in the phase of the evening activity peak, but only under
474 winter-like (short day) conditions, not under equinox or summer-like (long day) conditions. Kula-
475 Eversole *et al.* [59] have shown that TIM phosphorylation is affected by PRL-1 activity and suggest the
476 seasonal action of PRL-1 to advance the evening locomotor activity phase is mediated by modification of
477 TIM levels within the transcription-translation feedback loop. The extensive similarity of these two sets
478 of behavioral phenotypes reveals either serial or parallel pathways to effect comparable outcomes on
479 locomotor activity. If serial, then according to the simplest model, PRL1 acts downstream of PDFR, and
480 the combined results to-date suggest PRL-1 is inhibited by PDFR activation. Further genetic and
481 biochemical experiments are needed to evaluate if and how these pathways converge.
482

483 ***PDFR signaling effects on phase and on period.*** The behavioral effects of the PDFR variants
484 tested were most robust in modulating the phase of evening activity bouts. Evening locomotor activity
485 derives in large part from the activity of the primary Evening oscillators, termed E cells, the LNd and the
486 5th small LNv [10, 20, 51, 60-63]. However, numerous network interactions are also known to provide
487 critical contributions to the accuracy, precision and adaptibility of that timing system [e.g., 64 -67]. The
488 Evening oscillator group itself is known to be heterogeneous and to constitute at least three separate,
489 functionally distinct oscillators [68], only some of which express PDFR and respond to PDF [57, 68]. Vaze
490 and Helfrich-Förster [57] reported that the phase of Period protein accumulation in E neurons is phase-
491 locked to the previous lights-off transition and also sensitive to PDF signaling. They conclude that the
492 peak of PER accumulation is key to determining the phase of the evening activity peak, but also note
493 that the correlation between the Period-clock timing cue and the Evening activity peak is not perfect.
494 They suggest the Evening behavioral phase is better described by also factoring in the delay in E cell
495 neuronal activity driven by PDF neuromodulation [10, 19]. In that context, we interpret the many
496 examples of delayed Evening peaks resulting from PDFR variants (e.g., Figure 2M) as products at least
497 partly derived from PDF modulation (delay) of the cell intrinsic molecular clocks in E cells: that
498 conclusion is supported by the larger tau values in DD evident in the behavior of many PDFR variants
499 (Table 1). However, among the different variants, the association between delayed Evening activity
500 peaks in LD and longer tau values in DD was not perfect. For example, under short days, the 5A, 6A, 7A
501 and 5-7A variants all significantly delayed the offsets of Evening activity peaks versus that seen with WT
502 PDFR, but these variants did not significantly lengthen circadian period. Likewise under long day, 4A and
503 7A produced significant increases in tau versus that seen with WT PDFR, but they did not significantly
504 delay the onset of the Evening activity peak. Our working hypothesis is therefore that the duration of
505 PDF>PDFR signaling to target pacemakers in the *Drosophila* brain is modulated each day to
506 independently effect (i) entrainment of clock period, and (ii) delays of pacemaker neuronal activity. It is

507 the combination of these two PDFR signaling effects that is essential for proper rhythmic behavioral
508 outcomes across seasons. The signaling pathways downstream of PDFR, that independently regulate
509 diurnal phase and circadian period within E pacemaker neurons, are largely unknown.
510
511 ***Relations between in vitro and in vivo PDFR signaling measures.*** Using an *in vitro* assay for
512 cyclic AMP generation, we found that modifying phosphorylation properties of PDFR does not affect the
513 strength of signaling. That conclusion suggests that *in vivo* other GPCR features are normally affected to
514 regulate the extent/duration of PDFR signaling according to season, which corresponds to findings
515 described with other Family B GPCRs like VPAC1 [40]. Mutation of all the Ser and Thr residues of the C
516 terminal tail and of Ser250 to Ala led to a receptor with binding properties and adenylate cyclase activity
517 not different from that of the wild type receptor; however that variant receptor was neither
518 phosphorylated nor internalized. We propose that the PDFR variants we have described do not modify
519 locomotor behavior by virtue of greater second messenger signaling. Rather they do so by generating
520 cAMP (and perhaps other second messengers) over time periods longer than normal sustained by a WT
521 receptor over a portion of a 24-hr cycle.
522
523 **Specific versus non-specific phosphorylation regulating GPCR activity.** There are two broadly
524 divergent hypotheses to describe the mechanisms by which GPCR phosphorylation promotes
525 desensitization. The first posits that modification of specific residues have greatest significance for
526 downstream desensitizing mechanisms. The second invokes the triggering of termination processes by
527 the aggregate negative charge accumulated with phosphorylation, regardless of where it might occur.
528 For some GPCRs (e.g., OPN4), the evidence supports both models [33, 39]. Our data concerning PDFR
529 desensitization also support both viewpoints and we present a model in **Figure 8**. The evidence for the
530 importance of PDFR sequence specificity rests on the data that modification of certain single Clusters is,

531 among all variants tested, the most effective in affecting locomotor activity rhythms. In particular we
532 regard CL6 and CL7 to be especially critical in terminating the time course of PDFR signaling. Notably,
533 converting just the single pair of CL6 AAs, or the single pair of CL7 AAs, is enough to generate hours-long
534 delays in the peaks of locomotor rhythms under Light:Dark conditions (**Figure 2 F-G, 4F-G; 6 F-G, 7 F-G**),
535 as well as generating a significant increase in tau under Constant Dark conditions (**Table 1**). We note
536 that the splicing event that distinguishes the PDFR-A and PDFR-D isoforms occurs just prior to the
537 position of sequences encoding CL6 and 7, such that the D form lacks these two highly conserved
538 domains. Other clusters (like CL2-3) also appear to have potential for 'specific' contributions to PDFR
539 regulation. Together, these results speak to the potency of the inferred specific termination
540 mechanisms for PDFR GPCR signaling. In contrast, the evidence for the bulk phosphorylation hypothesis
541 comes from comparison of the 1-4A, versus the 1-5A, 1-6A and 1-7A variants. By inactivating increased
542 numbers of phosphorylatable residues, we observed a graded series increasingly delayed evening
543 phases in short day conditions (e.g., **Figure 2I-2 though 2L-2**). The tandem mass-spectroscopy results,
544 indicating endogenous phosphorylation of certain PDFR residues (**Supplemental Table 3**) demonstrates
545 such post-translational modifications can occur, but likely does not provide a complete accounting of
546 them.
547
548 Finally, our results point to what we propose as "context-dependent" effects of modifying
549 specific GPCR residues – ones that may produce opposite behavioral effects, depending on the
550 phosphorylation status of neighboring sites. In particular we point to the contradictory results we
551 observed with Ala-variants of the CL5 site. In comparing results from the series 1-4A, 1-5A, 1-6A and 1-
552 7A, we found that the 1-4A had only mild effects on the evening peak compared to over-expressing WT
553 PDFR (e.g., Figure **2B-2** vs. **2I-2**), whereas the others produced significant delays (**Figures 2M and 3I-3**
554 **through 3L-3)** under Short Day conditions. Those observations suggest modification of the CL5 residue

555 (S6333) promotes desensitization of PDFR and termination of PDFR signaling. However, the PDFR
556 variant 5-7A was constructed with the expectation that it would be as effective as 1-5A, 1-6A or 1-7A in
557 delaying the evening phase. Instead it proved only weakly effective. This same mis-match of expected
558 behavioral effects for 5-7A was also seen in other photoperiodic conditions (**Figure 6N, and 7H-3 vs 7J-3**
559 **through 7L-3).** Likewise, Langlet *et al*. [40] reported that effects on mutating Serine residues in the
560 carboxy terminus of VPAC1 did not produce additive effects. We propose that phosphorylation of CL5
561 will have either positive or negative consequences on PDFR desensitization depending on which other
562 neighboring residues are also modified. Thus we speculate that CL5 takes on outsize importance in
563 determining desensitization rates for PDFR and so may itself be subject to exceptional regulation. Such
564 complex interactions between phosphorylation sites is reminiscent of interactions documented between
565 diverse phosphorylation sites in the circadian clock protein PERIOD [69, 70]. Better resolution of these
566 apparent conflicting mechanisms (specific phosphorylation versus bulk negative charge, and paradoxical
567 effects of the CL5 site) awaits precise molecular definition of where and when PDFR is modified *in vivo*,
568 and by which modifying pathways.
569
570 ***MATERIALS and METHODS***
571
572 **Fly Rearing and Stocks.** *Drosophila* were raised on a cornmeal agar diet supplemented with
573 yeast at 25°C in 12 hr:12 hr LD cycles. The UAS-*pdfr* mutant transgenic series was created by injecting
574 *yw P{nos phiC31\int.NLS}X;P{CaryP}attP40* embryos (Rainbow Transgenic Flies, Inc, Camarillo, CA). The
575 UAS-*pdfr*-tandem construct was injected into *y[1] w[*] P{y[+t7.7]=nos-phiC31\int.NLS}X;*
576 *P{y[+t7.7]=CaryIP}su(Hw)attP6* embryos. For PDFR-tandem fusion protein expression, we made a stable
577 *yw*; *tim*(UAS)-Gal4; UAS-*pdfr*-tandem stock. *pdfr* mutant flies are described in [11]; *tim*(UAS)-Gal4 flies

578 are described in [71] – *BL80941*). The recombinant fly stock - *han*^{5304}; *tim* (UAS)-Gal4 - was confirmed by
579 PCR and sequencing.
580
581 ***hEK-293* Cell Culture**. *hEK-293* cells were maintained in DMEM, 10% FBS and 100U/mL penicillin
582 and streptomycin in 5% CO_2 atmosphere at 37°C. For all transient transfections, 1.5 x 10^6 cells were used
583 to inoculate T25 flasks, incubated overnight, then transfected with 10ug plasmid DNA and 20 uL
584 lipofectamine 2000 reagent (Invitrogen Life Technologies). Five hours after transfection, cells were split
585 4 x 10^4 cells/well into a 96-well assay plate. We created a series of stably-transfected *hEK-293* cells
586 expressing WT and sequence-variant PDFRs, using the Flp-In System (Invitrogen Life Technologies,
587 Waltham, Massachusetts) per manufacturers recommendations, and maintained them in DMEM
588 supplemented with 150 µg/ml hygromycin B.
589
590 **cAMP Assays**. We measured PDF Receptor signaling activity using a CRE-*Luciferase* reporter
591 gene, following methods described by Johnson *et al.* [29]. The reporter gene construct was transiently
592 transfected to each stable cell line and luminescence measured using Firefly Luciferase Assay Kit
593 (Biotium, Inc., Fremont, California) and a Wallac 1420 VICTOR2 microplate reader (PerkinElmer, Inc.,
594 Waltham, Massachusetts). Concentration-effect curves, EC_{50}, top values and p-values were calculated
595 using the dose response, in a nonlinear regression using GraphPad Prism 8.0 software (San Diego,
596 California).
597
598 **Locomotor activity Measures.** All locomotor activity experiments were conducted with 2-5
599 days-old male flies at 25°C using Trikinetics Activity Monitors as previously described [9]. We crossed
600 Gal4 lines to *w*^{1118} to create control progeny. Locomotor activities were monitored for 6 days under
601 different photoperiodic conditions, and then for 9 days under constant dark (DD). To analyze rhythmicity

602 under constant conditions, we normalized the activity of flies from DD day 3 to day 9 and used χ2-
603 periodogram analysis with a 95% confidence cut-off, as well as SNR analysis [72] Arrhythmic flies were
604 defined by a power value $\leq$ 10 and width value $\leq$ 2, and period outside the range, 18 to 30 hours. To
605 analyze periods, we used Graphpad Instat (v 8) software to run one-way ANOVA measures followed by
606 the Tukey-Kramer Multiple Comparisons Test. We used Clocklab (Actimetrics) software to produce
607 actograms and the Brandeis Rhythms Package [73] to produce average activity plots (group eductions).
608 To analyze onset and offset phases of Morning and Evening activity bouts on the final two days of
609 light:dark entrainment (LD 5&6), we followed the method of Kula-Eversole *et al.* (55, 2021): [$(A_{n+2} + A_{n+1})$
610 $- (A_{n-1} + A_{n-2}) = \Delta$Activity]. Experimental genotypes were tested in the *han* mutant background*, pdfr*^{5304}
611 [11]*,* or in the *pdf*^{01} (null) mutant background [1], or in the *w*^{1118} background, as noted.
612
613 Additional details on Methods and procedures are found in **Supplemental Information**.
614
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623 ***AUTHOR CONTRIBUTIONS***
624 W.L., J.T. and P.H.T designed experiments. W.L. and J.T. performed experiments. W.L., J.T. and P.H.T
625 analyzed experiments. P.H.T wrote the manuscript.

626

627

628 ***LITERATURE CITED***

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837 ***FIGURE LEGENDS***

838

839 **Figure 1. A map illustrating the series of PDFR sequence variants used to test a role for PDFR**
840 **phosphorylaytion in regulating the locomotor rhythms.** The C Terminal of the PDFR-A isoform is
841 diagrammed (top) immediately following the position of the 7th transmembrane domain (TM7, blue).
842 Asterisks indicate the positions of the 28 serine, threonine or tyrosine residues present within the PDFR
843 C Terminal tail. The residues marked by the white asterisks were those chosen for study by sequence
844 alteration to encode Alanine; they were given arbitrary designations as Clusters (CL) 1 through 7. Each
845 cluster contains a single residue (e.g., CL4 or CL5), or as many as four residues (e.g., CL2). The ten
846 sequence variants are named by the Cluster(s) that was altered followed by the letter 'A". Simple
847 Variants include those wherein residues in one or two Clusters were altered. Multiple Variants include
848 those wherein residues within three or more Clusters were altered. All 14 targeted residues were
849 altered in the PDFR 1-7A variant.

850

851 **Figure 2. Locomotor Rhythms exhibited by WT PDFR and by Simple PDFR Variants under**
852 **Winter-like (Short Day) conditions.** All behavioral records were recorded from *han* (*pdfr* mutant) flies
853 that expressed either no UAS transgene (A), or a UAS-WT *pdfr* transgene (B) or a variety of Simple *PDFR*
854 Variants, including 2-3A (C), 4A (D), 5A (E), 6A (F) or 7A (G), or Multiple PDFR variants, including 5-7A
855 (H), 1-4A (I), 1-5A (J), 1-6A (K) and 1-7A (L). Each Panel (A)-(L) contains sub-panels (1) and (2). Sub-Panel
856 (1) displays a double-plotted group actogram throughout the 6 days of Light: dark entrainment, followed

857 by ~9 days of constant darkness (DD, grey background). Sub-panel (2) displays a daily plot of locomotor
858 activity (a group eduction) averaged over the last two days of entrainment (LD 5-6). Open bars indicate
859 the periods of Lights-on and filled bars indicate periods of Lights-off. Panel M displays the average
860 Phase Offset timepoint for the Evening activity phase (marked by a Blue Arrow) for each genotype over
861 the last two days of entrainment (LD 5-6). Panel N displays the average Phase Onset timepoint for the
862 Morning activity phase (marked by a Red Arrow) for each genotype over the last two days of
863 entrainment (LD 5-6). The positions of the Blue and Red arrow in panels A-L are representative; panels
864 M and N presents their true values. Analyses represent ANOVA followed by Dunnett's post hoc multiple
865 comparisons of all compared to WT: ns = not significant; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.005$; **** =
866 $p<0.001$.
867
868 **Figure 3. Amplitude measures of Locomotor activity exhibited by WT PDFR and by diverse**
869 **PDFR Variants under Winter-like (Short Day) conditions.** All behavioral records were recorded from
870 *han* (*pdfr* mutant) flies that expressed either no UAS transgene (A – marked in YELLOW), or a WT *pdfr*
871 cDNA (B – marked in BLACK) or a variety of Simple *pdfr* Variants (marked in RED) including 2-3A (C), 4A
872 (D), 5A (E), 6A (F), 7A (G), 5-7A (H), 1-4A (I), 1-5A (J), 1-6A (K) or 1-7A (L). These measures were
873 averaged across all experiments run for each individual genotype (see Table 1 for N and n values). Each
874 Panel (A)-(L) contains sub-panels (1) through (3), each of which displays Bin-by-Bin analyses of activity
875 levels sorted by 30 min bins, for three different time periods: (1) ZT17-23.5; (2) ZT 0.5 – 8; (3) ZT 8.5-16.
876 The missing bin at timepoint 0 contains the startle response that accompanies the sudden lights-on
877 signal. Asterisks indicate significantly-different activity levels according to a Student's T-test ($p< 0.05$).
878 Red arrows indicate elevated Morning activity levels displayed by different PDFR variants. Blue arrows
879 indicate elevated Evening activity levels produced by different PDFR variants.
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882 **Figure 4. Locomotor Rhythms exhibited by WT PDFR and by Simple PDFR Variants under**
883 **Equinox (12:12) conditions.** All behavioral records were recorded from *han* (*pdfr* mutant) flies that
884 expressed either no UAS transgene (A), or a UAS-WT *pdfr* transgene (B) or a variety of Simple *pdfr*
885 Variants, including 2-3A (C), 4A (D), 5A (E), 6A (F) or 7A (G), or Multiple pdfr variants, including 5-7A (H),
886 1-4A (I), 1-5A (J), 1-6A (K) and 1-7A (L). Each Panel (A)-(L) contains sub-panels (1) and (2). Sub-Panel (1)
887 displays a double-plotted group actogram throughout the 6 days of Light: dark entrainment, followed by
888 ~9 days of constant darkness (DD, grey background). Sub-panel (2) displays a daily plot of locomotor
889 activity (a group eduction) averaged over the last two days of entrainment (LD 5-6). Open bars indicate
890 the periods of Lights-on and filled bars indicate periods of Lights-off. Panel M displays the average
891 Phase Offset timepoint for the Evening activity phase (marked by a Blue Arrow) for each genotype over
892 the last two days of entrainment (LD 5-6). Panel N displays the average Phase Onset timepoint for the
893 Morning activity phase (marked by a Red Arrow) for each genotype over the last two days of
894 entrainment (LD 5-6). The positions of the Blue and Red arrow in panels A-L are representative; panels
895 M and N presents their true values. Analyses represent ANOVA followed by Dunnett's post hoc multiple
896 comparisons of all compared to WT: ns = not significant; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.005$; **** =
897 $p<0.001$.

898

899 **Figure 5. Locomotor Rhythms exhibited by WT PDFR and by Multiple PDFR Variants under**
900 **Equinox (12:12) conditions.** All behavioral records were recorded from *han* (*pdfr* mutant) flies that
901 expressed either no UAS transgene (A – marked in YELLOW), or a WT *pdfr* cdNA (B – marked in BLACK)
902 or a variety of Simple *pdfr* Variants (marked in RED) including 2-3A (C), 4A (D), 5A (E), 6A (F), 7A (G), 5-7A
903 (H), 1-4A (I), 1-5A (J), 1-6A (K) or 1-7A (L). These measures were averaged across all experiments run for
904 each individual genotype (see Table 1 for N and n values). Each Panel (A)-(L) contains sub-panels (1)

905 through (3), each of which displays Bin-by-Bin analyses of activity levels sorted by 30 min bins, for three
906 different time periods: (1) ZT17-23.5; (2) ZT 0.5 – 8; (3) ZT 8.5-16. The missing bin at timepoint 0
907 contains the startle response that accompanies the sudden lights-on signal. Asterisks indicate
908 significantly-different activity levels according to a Student's T-test ($p < 0.05$). Red arrows indicate
909 elevated Morning activity levels displayed by different PDFR variants. Blue arrows indicate elevated
910 Evening activity levels produced by different PDFR variants.
911
912 **Figure 6. Locomotor Rhythms exhibited by WT PDFR and by Simple PDFR Variants under**
913 **Summer-like (Long Day) conditions.** All behavioral records were recorded from *han* (*pdfr* mutant) flies
914 that expressed either no UAS transgene (A), or a UAS-WT *pdfr* transgene (B) or a variety of Simple *pdfr*
915 Variants, including 2-3A (C), 4A (D), 5A (E), 6A (F) or 7A (G), or Multiple *pdfr* variants, including 5-7A (H),
916 1-4A (I), 1-5A (J), 1-6A (K) and 1-7A (L). Each Panel (A)-(L) contains sub-panels (1) and (2). Sub-Panel (1)
917 displays a double-plotted group actogram throughout the 6 days of Light: dark entrainment, followed by
918 ~9 days of constant darkness (DD, grey background). Sub-panel (2) displays a daily plot of locomotor
919 activity (a group eduction) averaged over the last two days of entrainment (LD 5-6). Open bars indicate
920 the periods of Lights-on and filled bars indicate periods of Lights-off. Panel M displays the average
921 Phase Offset timepoints for the Evening activity periods (marked by a Blue Arrow) for each genotype
922 over the last two days of entrainment (LD 5-6). Panel N displays the average Phase Onset timepoints for
923 the Morning activity periods (marked by a Red Arrow) for each genotype over the last two days of
924 entrainment (LD 5-6). The positions of the Blue and Red arrows in panels A-L are representative; panels
925 M and N presents their true values. . Analyses represent ANOVA followed by Dunnett's post hoc
926 multiple comparisons of all compared to WT: ns = not significant; * = $p<0.05$; ** = $p<0.01$; *** =
927 $p<0.005$; **** = $p<0.001$.
928

929 **Figure 7. Locomotor Rhythms exhibited by WT PDFR and by Multiple PDFR Variants under**
930 **Summer-like (Long Day) conditions.** All behavioral records were recorded from *han* (*pdfr* mutant) flies
931 that expressed either no UAS transgene (A – marked in YELLOW), or a WT *pdfr* cdNA (B – marked in
932 BLACK) or a variety of Simple *pdfr* Variants (marked in RED) including 2-3A (C), 4A (D), 5A (E), 6A (F), 7A
933 (G), 5-7A (H), 1-4A (I), 1-5A (J), 1-6A (K) or 1-7A (L). These measures were averaged across all
934 experiments run for each individual genotype (see Table 1 for N and n values). Each Panel (A)-(L)
935 contains sub-panels (1) through (3), each of which displays Bin-by-Bin analyses of activity levels sorted
936 by 30 min bins, for three different time periods: (1) ZT17-23.5; (2) ZT 0.5 – 8; (3) ZT 8.5-16. The missing
937 bin at timepoint 0 contains the startle response that accompanies the sudden lights-on signal. Asterisks
938 indicate significantly-different activity levels according to a Student's T-test ($p < 0.05$). Red arrows
939 indicate elevated Morning activity levels displayed by different PDFR variants. Blue arrows indicate
940 elevated Evening activity levels produced by different PDFR variants.
941
942 **Figure 8. A model predicting the effects of phosphorylating different sites on the PDFR CT on**
943 **downstream signaling.** Asterisks indicate the positions Ser/ Thr/ and Tyr residues as described in Figure
944 1 and Supplemental Figure 1: white asterisks were targets of mutational analysis in this study. Mutation
945 of CL6 and CL7 consistently demonstrated the greatest delaying effects on the phases of locomotor
946 peaks, suggesting their phosphorylation normally will have the greatest effect to slow or terminate PDFR
947 signaling. Other sites are also effective, although to lesser degrees, including CL2-3, CL4 and CL5.
948 Primarily, such phosphorylation will decrease the extent of PDFR signaling (red bars) and so reduce the
949 delay that PDF imposes on the period of neuronal activation displayed by PDFR-responsive pacemaker
950 groups (like the Evening cells and the DN3: Liang *et al.*, 2016, 2017). The model also predicts that the
951 effects of phosphorylating CL5 will depend on the phosphorylation status of neighboring sites. In some
952 contexts (e.g., PDFR 1-5A), it will help terminate PDFR signaling, but in others (e.g., PDFR 567A) it may

953 promote the duration or extent of PDFR signaling, perhaps by blocking the effects of phosphorylating

954 CL6 and CL7 (green bar).

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Figure 1.

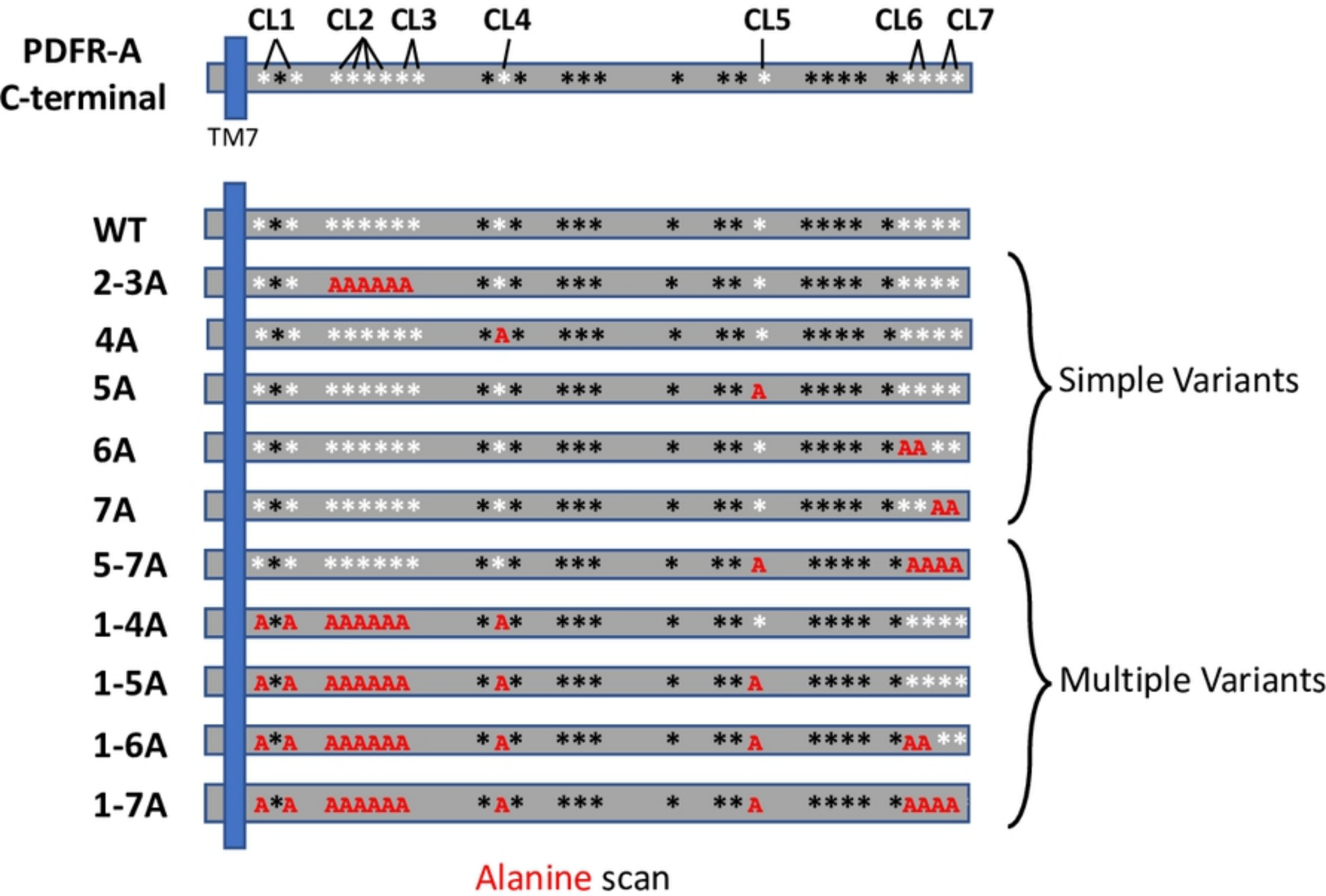
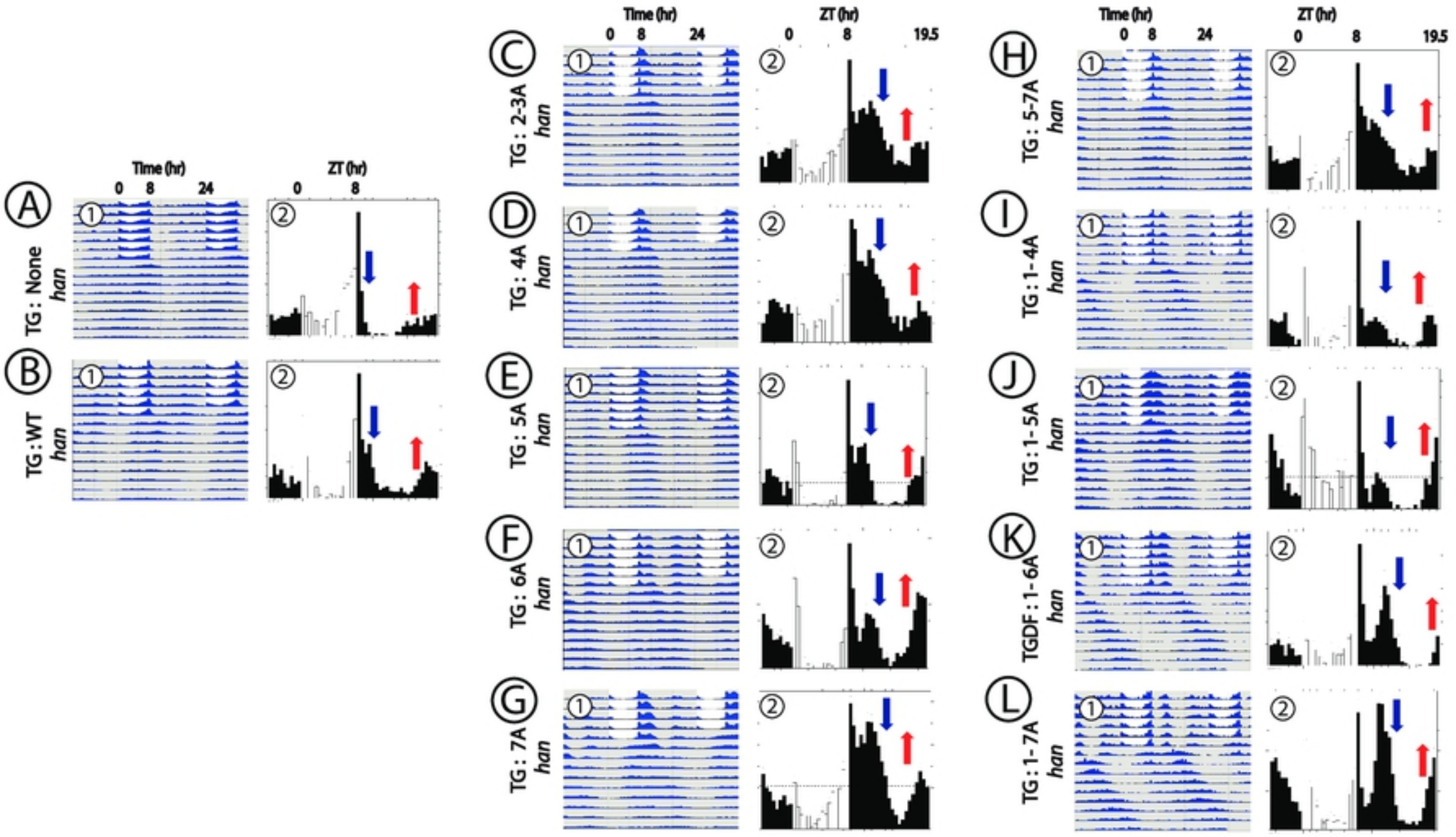
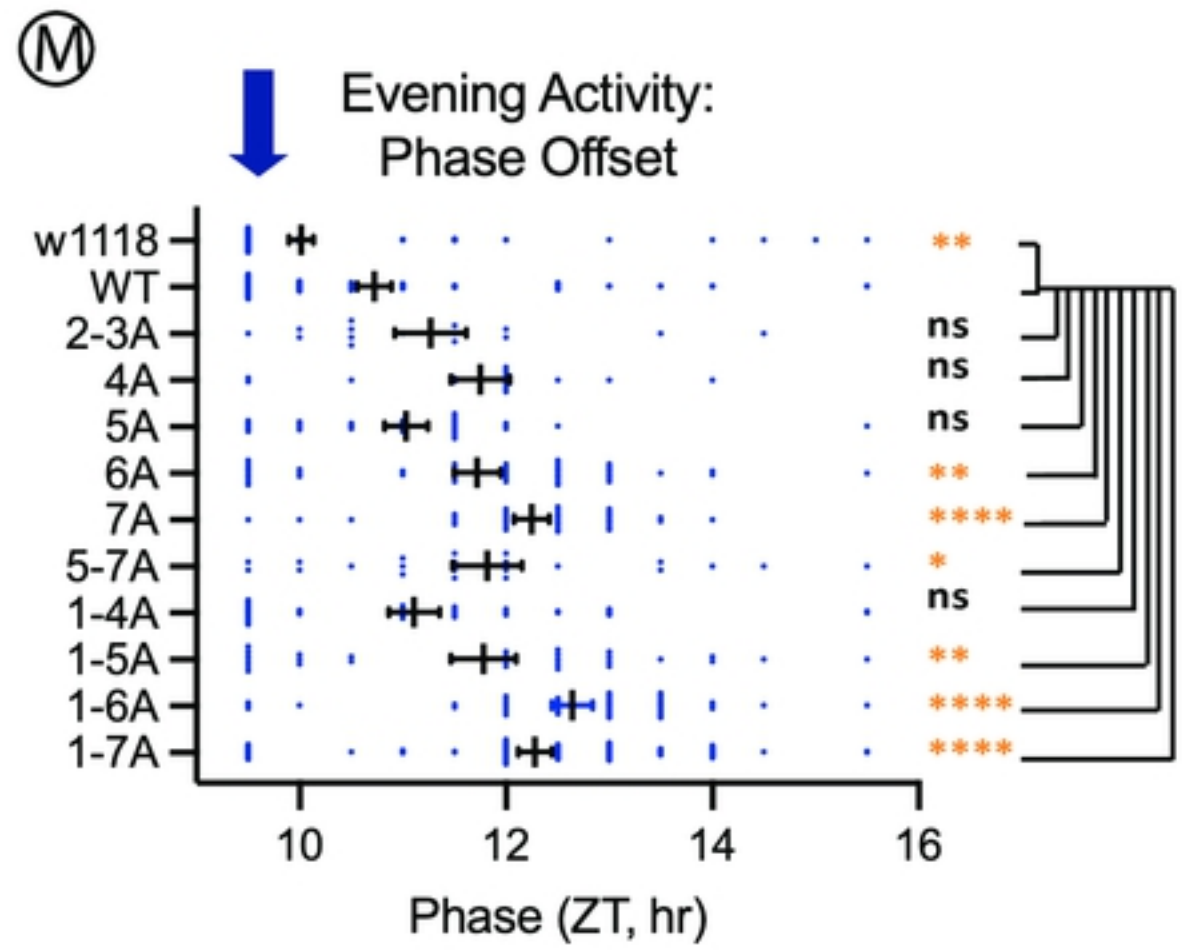


Figure 1

Figure 2





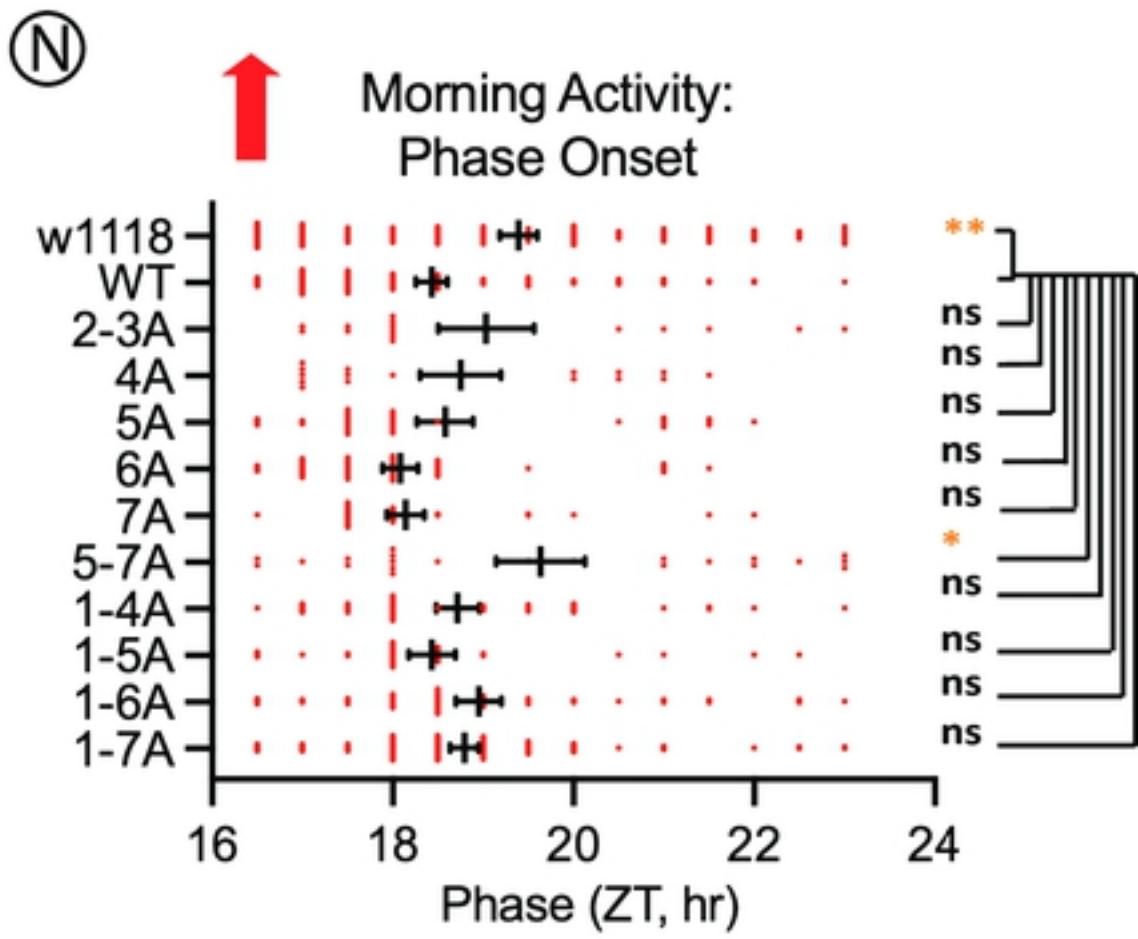


Figure 2

Figure 3 .

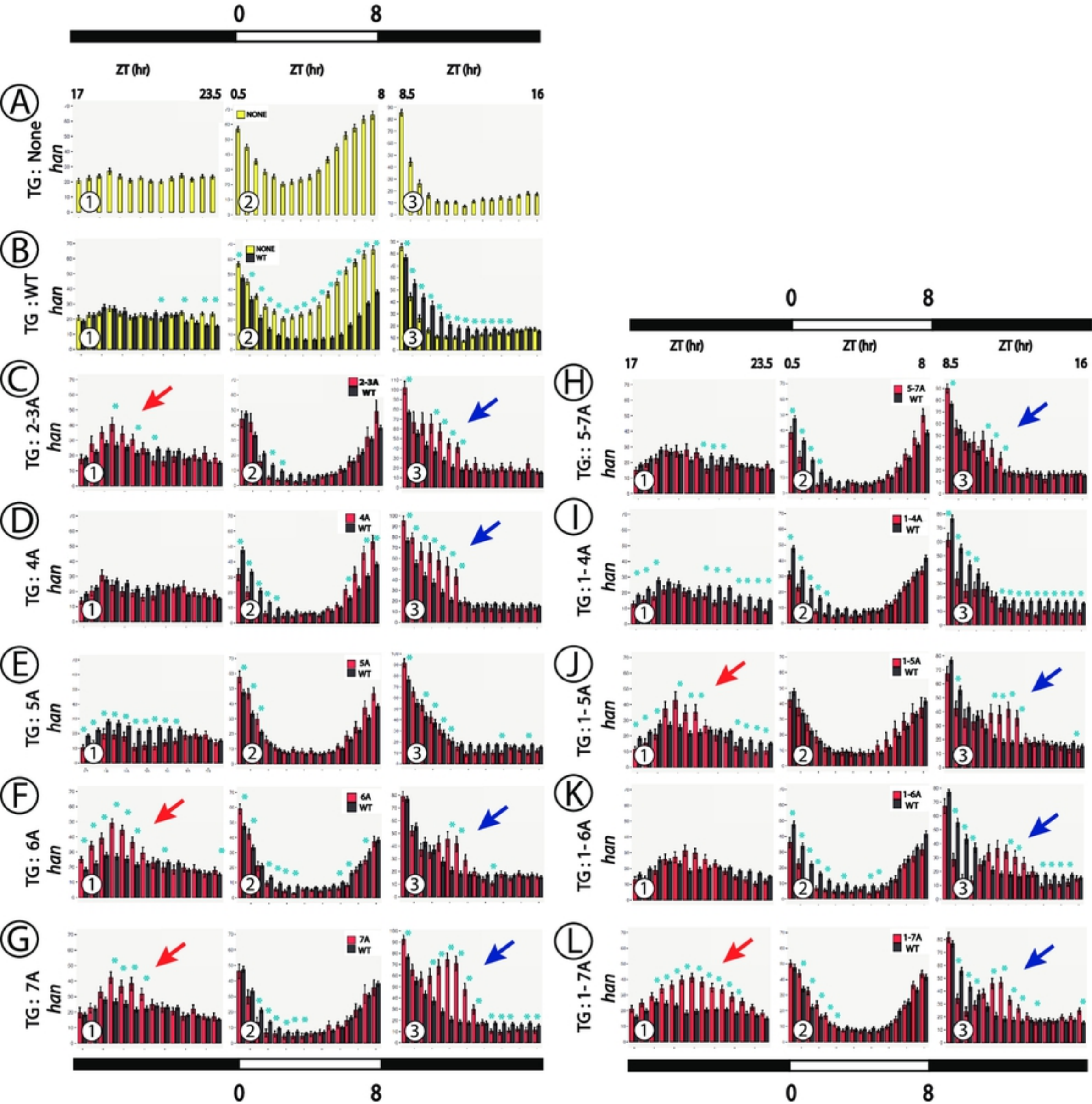


Figure 3

Figure 4

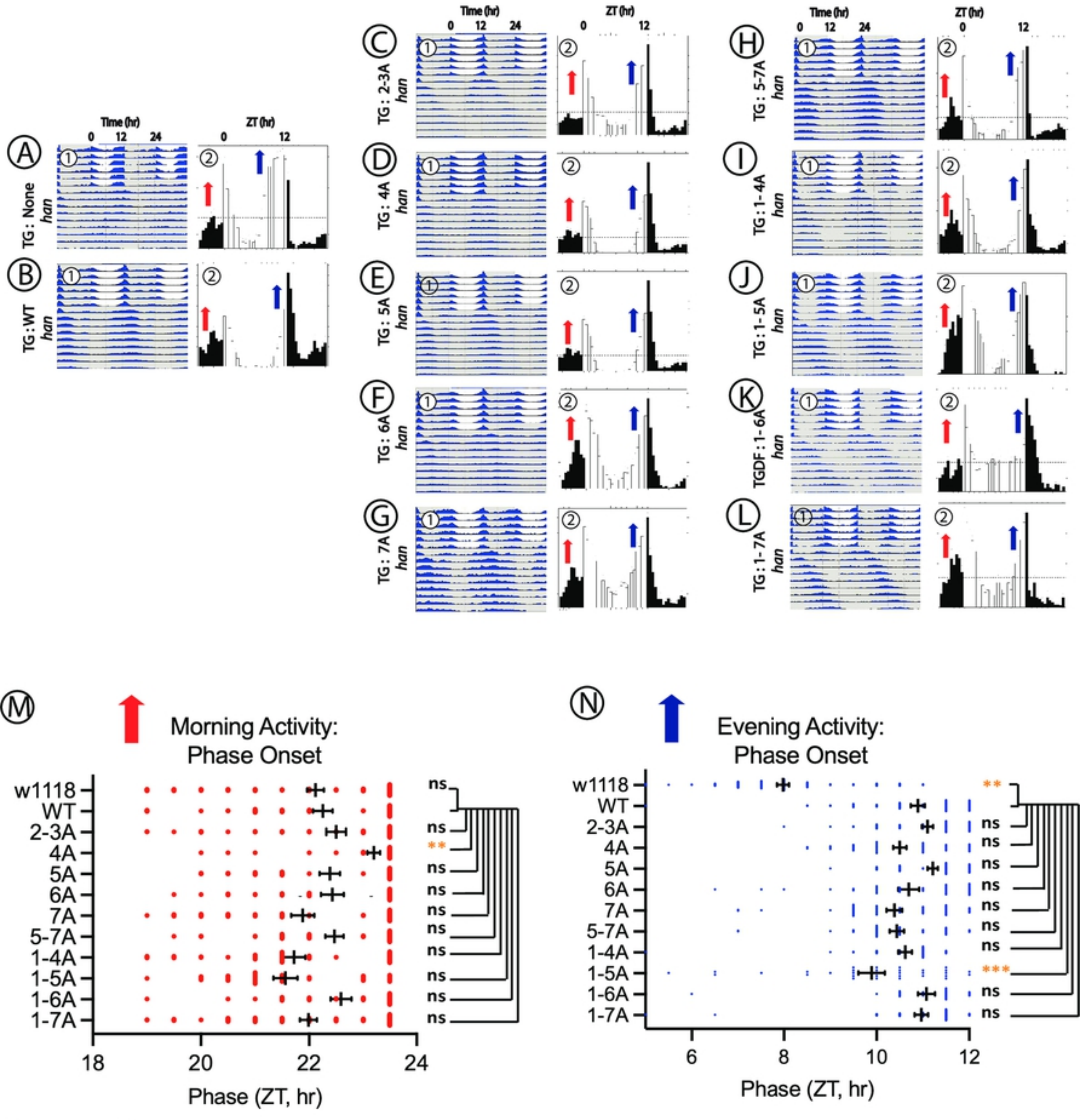


Figure 4

Figure 5

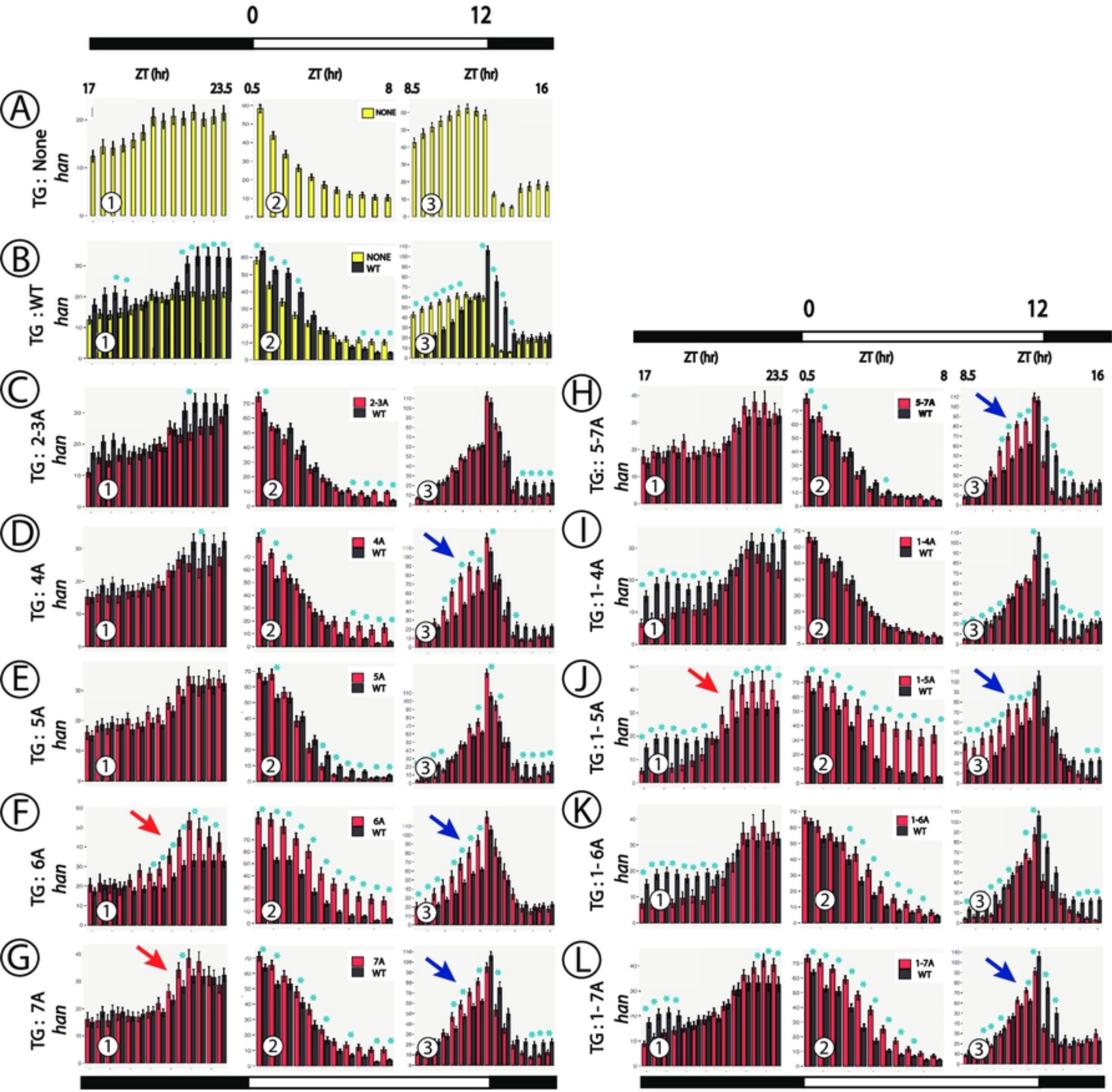


Figure 5

Figure 6

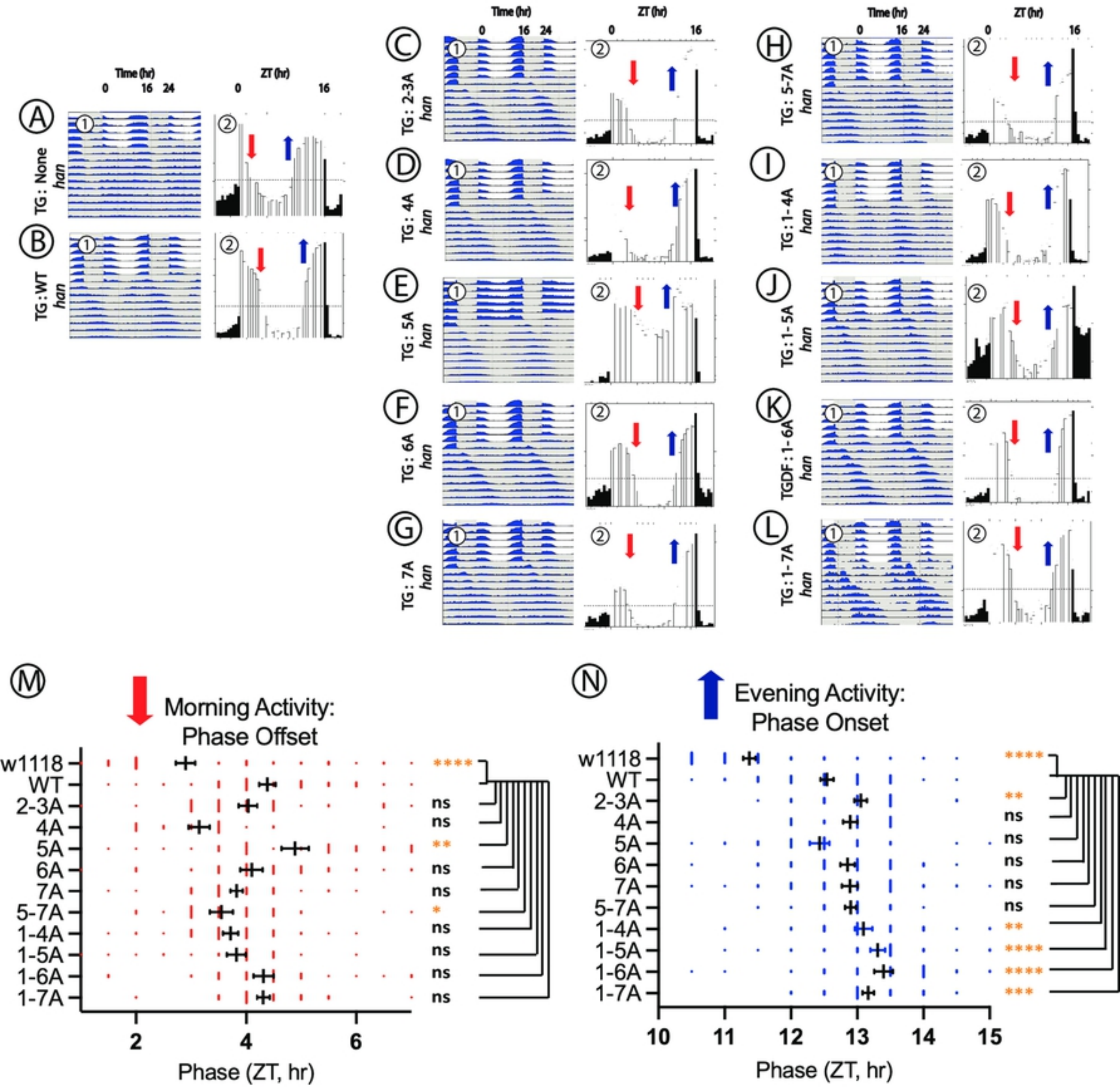


Figure 6

Figure 7.

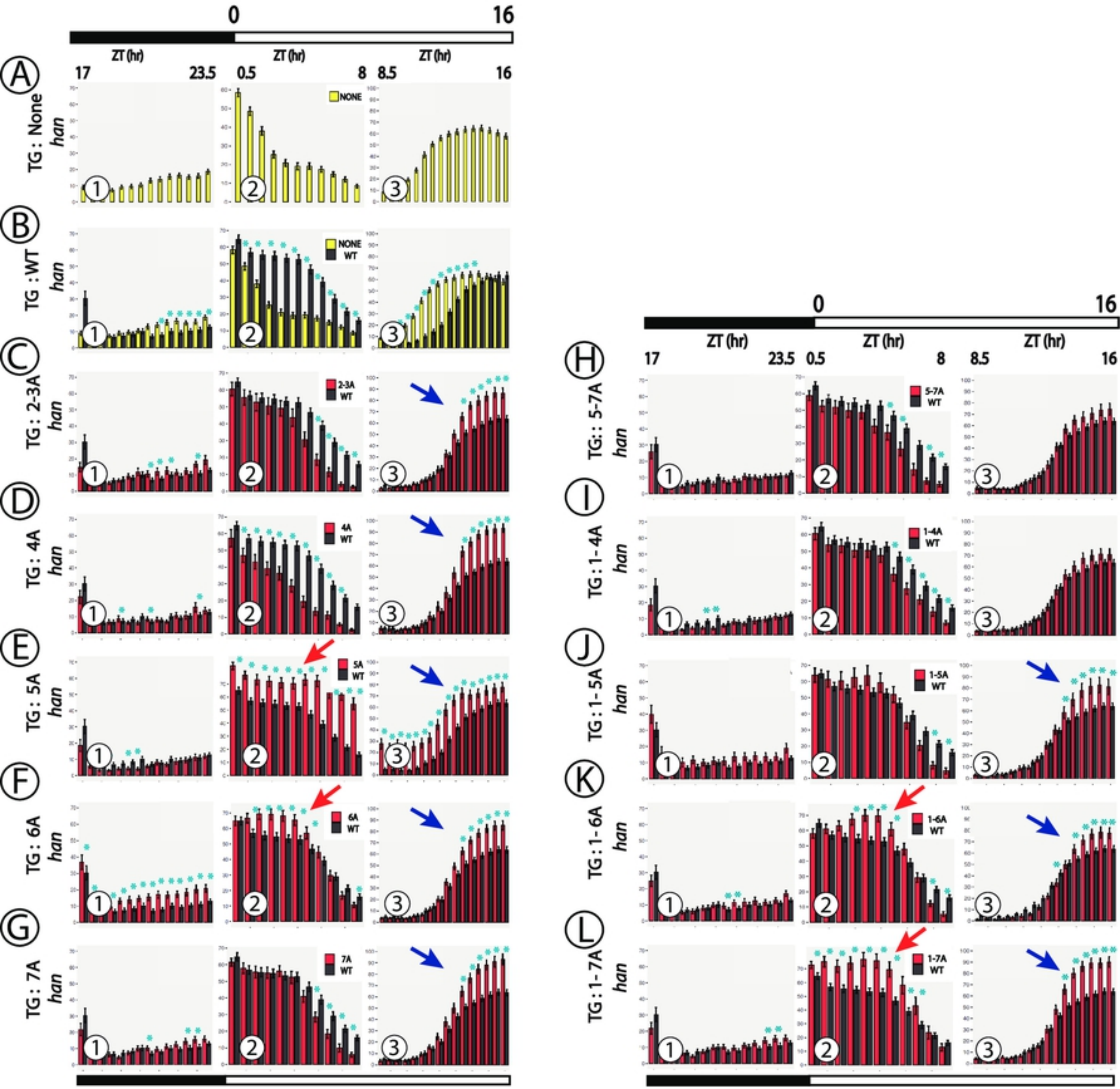


Figure 7

Figure 8.

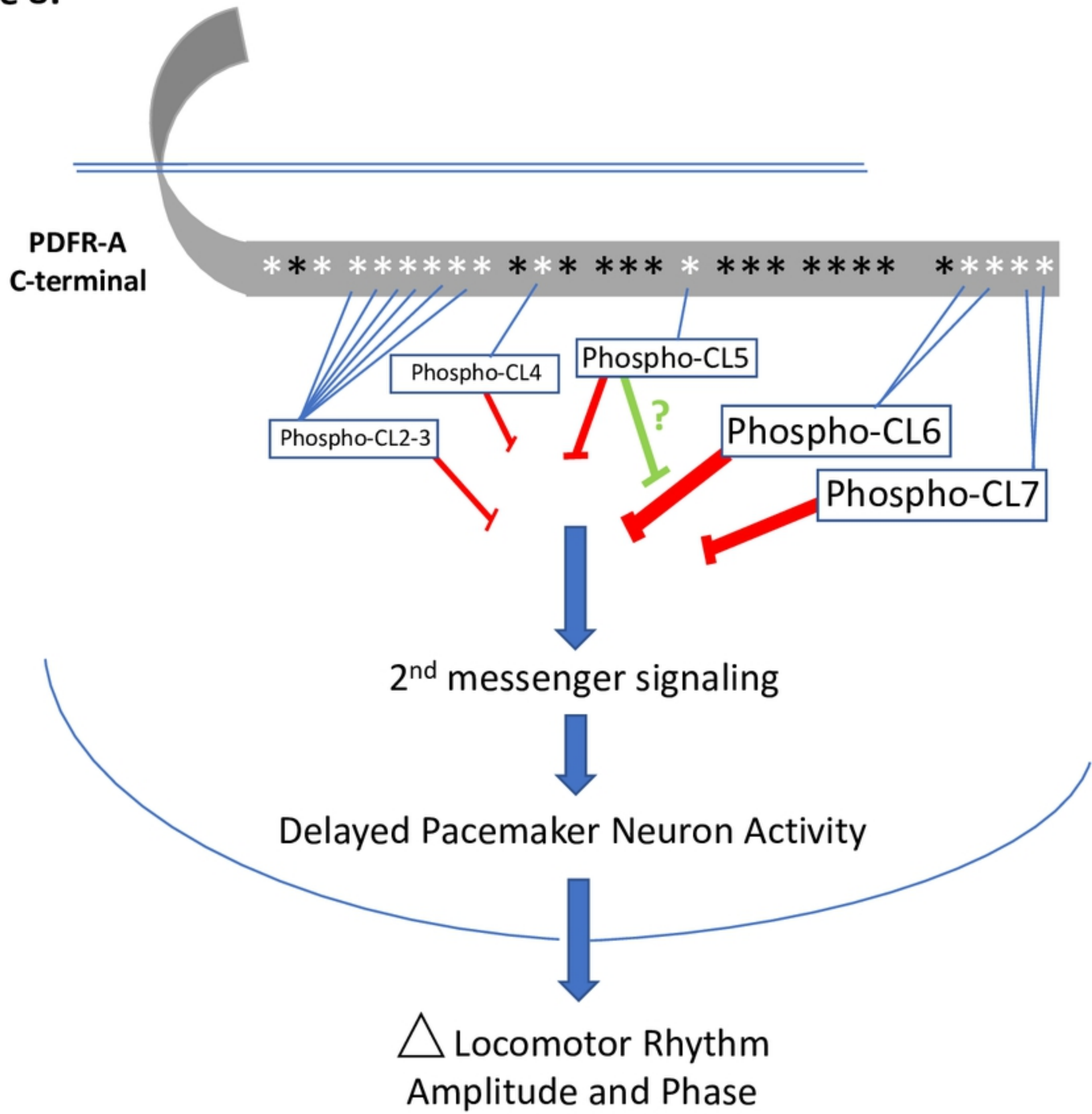


Figure 8