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 – must 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.

INTRODUCTION

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

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

activity illustrates the basis by which the circadian network produces a daily series of phasic, neuronal outputs to temporally bias diverse downstream rhythmic events [19, 20]. Finally, PDF/PDFR signaling is long-lasting: its depression of basal calcium levels in target neurons persists without abatement over many hours. These observations raise fundamental questions regarding the mechanism and the time course by which PDFR signaling diminishes in anticipation of the next day's cycle of signaling.

The canonical model of GPCR phosphorylation and homologous desensitization features G protein-coupled receptor kinases (GRKs) which associate with activated GPCRs and phosphorylate cytosolic segments, thereby recruiting β -arrestins [21, 22]. β -arrestins uncouple the receptors from G proteins [23, 24], or enhance receptor endocytosis [25]; they can also serve as signal transducers by recruiting distinct signaling molecules [26]. A second major regulatory mechanism to reduce GPCR signaling is heterologous desensitization, whereby second-messenger-dependent kinases (PKA or PKC) phosphorylate GPCRs [27]. Thus, we designed experiments to modify evolutionarily-conserved residues in the C terminal tail of PDFR that could conceivably serve as substrates for phosphorylation and subsequent signal termination. Our working hypothesis is that such modifications will reveal an extension of the lifetime of PDFR activation *in vivo*.

Following activation of rhodopsin in the mammalian retina, visual arrestin is recruited with a time constant of < 80 ms [28]; for many *Drosophila* neuropeptide receptors, β -arrestin2 is recruited within a minute of exposure to ligand [29]. Does phosphorylation/de-phosphorylation also regulate the activity of a GPCR like PDFR, that signals over a much longer time base? The mammalian blue-light sensitive GPCR melanopsin (OPN4) mediates intrinsic light sensitivity in certain classes of retinal ganglion cells (RGCs): melanopsin signaling is distinguished by long latencies, graded responses and sustained RGC depolarization that can outlast the duration of the light stimulus [30]. Melanopsin-expressing RGCs produce exceptionally long timecourse integration because that GPCR behaves

differently from other opsins in two fundamental ways. Firstly, it undergoes photoequilibration between signaling and silent states: a property that maintains the availability of pigment molecules for activation, termed ‘tristability’ by Emmanuel and Do [31]. Secondly, phosphorylation and β -arrestin recruitment do contribute to the kinetics of melanopsin signaling, but over a long time course [32, 33]. Mure *et al.* [33] proposed that the distal aspect of the melanopsin C terminal tail creates a steric blockade covering a cluster of specific serine residues situated in more proximal regions. The time required for relief of that blockade therefore dictates a delayed time course of phosphorylation.

We know very little about the mechanisms that underlie termination of PDFR signaling. The *pdf* gene displays a transcript rhythm that peaks in the late day [34]. Sensitivity to PDF *in vivo* peaks in the early day and is regulated by the PER-dependent clock, via post-transcriptional mechanisms: the EC50 for PDF responses in identified neurons varies systematically 5-10 fold: as a consequence of Ral A action, as a function of time of day, and as a function of seasonality [35]. PDFR signaling is long-lasting: it persists for many hours *in vivo* [19], for as long as free peptide ligand is available in the bath [14]. In addition, β -arrestin2-GFP is not efficiently recruited to activated PDFR when the receptor is functionally-expressed in *hEK-293T* cells [35]. In contrast, each of 13 other *Drosophila* neuropeptide GPCRs (including two Family B GPCRs, CG8422 and CG17415) efficiently recruit β -arrestin2-GFP when they are activated by their cognate ligands in that cellular environment [29, 36, 37].

Here we report (i) that PDFR is normally phosphorylated *in vivo* at conserved C terminal residues; and (ii) that loss of conserved PDFR phosphorylatable sites leads to a behavioral syndrome opposite to loss-of-function *pdf* and *pdf* phenotypes. This ‘gain of function’ approach reveals a multi-hour range of potential phases for both the Morning and Evening activity peaks, within which neuropeptide PDF:PDFR signaling normally specifies rhythmic behavior, according to season.

Importantly, it shows that PDFR signaling affects the amplitude of the activity peaks and not just their phases. Finally, this work identifies specific PDFR sequence elements that are major points at which the duration of receptor activity is dictated, and through which behavior is modulated in season-specific fashion.

RESULTS

PDFR C-Terminal sequences. Based on alternative splicing, the *pdf* locus in *Drosophila melanogaster* (CG13758) encodes GPCRs with two different C terminal sequences, for which the PA and PD isoforms are representative (flybase.org/reports/FBgn0260753). The PD isoform is slightly longer and lacks the final ~20 AAs of the PA isoform. As described in the Supplemental Information (*PDFR Isoforms*), we focused on the PA protein isoform of PDFR, as it has been used in the majority of genetic studies in the field. To identify residues for mutation, we first used comparative genomic analyses to assess how well specific sequences in the C-terminal region of the PA receptor are conserved. We obtained annotated *pdf* genomic sequences from 16 additional species of *Drosophila*, in both the *Sophophora* and *Drosophila* sub-families (**Supplemental Table 1**). Together this species collection represents an estimated 40-60 MYr of *Drosophalid* evolution [38]. We defined residue V505 of the *melanogaster* protein as the start of the C terminal sequence, following the consensus 7th transmembrane TM domain (TM7) (**Supplemental Figure 1**). These 17 different C terminal sequences all display considerable length, and vary between 189 to 215 amino acids (AAs). The *melanogaster* PDFR-A C-terminal contains 28 Ser, Thr, or Tyr residues (these may be subject to post-translational phosphorylation and de-phosphorylation). To survey putative functionality among these, we chose 14 residues that are distributed across the length of the C-terminal and which display high evolutionary conservation (**Supplemental Figure 1** and **Supplemental Table 3**). For naming purposes, we grouped

them into arbitrary clusters (CL) numbered #1 to #7, with positions shown in **Figure 1**. Following a common paradigm in study of GPCR physiology [e.g., 33, 39, 40], we tested the consequences of their mutation to Alanine, a non-phosphorylatable analog [41]. Some clusters have only a single modified AA (e.g. CL4 and CL5), while in others we modified two AAs (e.g., CL1, CL6 and CL7), and in others, as many as six closely-positioned residues (e.g., CL2-3).

This series of variant receptors contains 10 different mutated versions of PDFR (not including the WT 'parent' PDFR-A) and is arbitrarily divided into two broad categories as indicated in **Figure 1**. The first group (termed "Simple Variants") targeted one or two clusters of conserved Ser/Thr/Tyr AAs (i.e., CL 2-3A; 4A; 5A; 6A; 7A). The second category (termed "Multiple Variants") targeted three or more of the AA clusters in various combinations (i.e., CL 1-4A; 1-5A; 1-6A; 1-7A and 5-7A). The CL1-7A variant mutates all 14 of the targeted residues. We studied these ten variants, as well as the WT receptor, in a *pdfr* mutant strain (*han*⁵⁵³⁷ – [11]) for their ability to rescue and to shape the phases and periodicity of rhythmic locomotor behavior. We analyzed PDFR variants in the *pdfr* mutant background to permit evaluation of their properties without competition from endogenous PDFR [although we acknowledge that such possible interactions warrant future study]. Further we tested this PDFR series in different photoperiodic conditions reflecting different seasons, as well as in constant darkness. *pdfr* loss-of-function mutants display advanced behavioral peaks under Light:Dark conditions, as well as weak and shortened free-running periods under constant darkness [11]. In general, we found that several of these Ala-mutant PDFRs delayed the phase of the Morning and/or Evening activity peaks (**Figures 2-7**) and that some also lengthened the free-running period (**Table 1**). Such results are generally consistent with a prediction whereby non-phosphorylatable PDFR variants – those having a potential to increase the duration of PDFR signaling - will produce behavioral actions opposite to those seen in loss-of-function *pdfr* mutant flies. To better interpret the results produced by the different sequence variants, we also

present experiments that test assumptions used in the experimental design. These tests consider: i) whether the C-terminal fusion of GFP (present in all variants tested) influenced the results; and ii) whether the behavioral effects of receptor variants are independent of activation by the endogenous ligand, neuropeptide PDF.

Analysis of Behavior with PDFR variant expression. Figures 2 -7 describe rhythmic locomotor behavior of adult *pdfr* mutant flies expressing diverse *pdfr* transgenes in the aggregate, during six days of light entrainment (LD), followed by activity during nine or more days of constant darkness (DD). For each genotype, we display locomotor activity during light entrainment in four ways: (i) group actograms, (ii) group educutions, (iii) derivations of behavioral phase, and (iv) direct comparisons of the amplitudes of behavioral peaks between genotypes. Thus in Figures 2, 4 and 6, a representative group actogram displays all locomotor activity double-plotted across all days in LD and DD (Panels A-L, sub-panel -1 in each Figure). Second a representative group education displays a 24 h average of the activity in the final two days in LD (days 5-6) (Panels A-L, sub-panel -2 in each Figure). Next, we define the phase onsets and offsets for Morning and Evening activity peaks during the same final two LD days across all experiments (Panels M and N in Figures 2, 4 and 6). For the comparison of peak amplitudes in these three photoperiodic conditions, we describe Bin-by-Bin analyses of locomotor activity during the last two days of entrainment (days 5-6) in Figures 3, 5 and 7. The Bin-by-Bin analyses directly compares experimental genotypes to a control one [cf. 42]. They present data averaged across 2 to 5 independent experiments, to help identify the differences that most consistently correlate with genotype. Finally, Table 1 compiles measures of rhythmic activity displayed by the different genotypes in days 3-9 of constant darkness.

Behavior under Short Day (winter-like) conditions (Figures 2 and 3). Flies lacking *pdfr* function in 8L:16D lack a prominent morning peak and their evening peak of activity begins earlier than controls;

the example shown in **Figure 2A-2** is heterozygous for the *tim*-Gal4 element. Rescue by WT-*pdf*r using *tim*-Gal4 significantly delayed (i.e., rescued) the Evening peak initiation by about 1 h (**Fig. 2B-2**). Expression of each of the Single Variants driven by *tim*-Gal4 produced also delayed the Evening peak (**Fig. 2C-2 to G-2**), with the variants 6A and 7A producing conspicuous, large amplitude peaks that occurred on average as late as ~3 h after lights-off, at ZT12 (**2F-2** and **2G-2**). Variants 2-3A, 6A and 7A also produced elevated activity during the period of ZT 18-19 (**Fig. 3C-1, 2F-1** and **2G-1**): we speculate this is promotion of a “Morning” peak of activity. Among the five Multiple Variants, we noted three main effects (**Fig. 3**). First the progressive addition of more Ala substitutions in the series 1-5A, 1-6A and 1-7A (**Figure 3D-5 through 3G-5**) produced large and delayed Evening peaks in the time period ZT11.5-12.5 (3-4 h after lights-off), with the variant 1-6A producing the most pronounced delay. The delayed Evening peak activity at ZT12 appeared often appeared at the expense of, the normal Evening peak activity that occurred prior to Lights-off (e.g., **Fig. 3G-4**), although when averaged across all days in LD, that effect was not significant (**Figure 3 – panel 2**). Second, the PDFR 1-5A and 1-7A forms in the Multiple Variant series produced significant “Morning” peaks around ZT19, (**Fig 3J-1** and **3L-1**), Finally, we noted that combining the Single 5A, 6A and 7A variants into a Multiple Variant (5-7A) did not produce the anticipated additive effects on promoting morning locomotor activity at ~ZT19 (**Fig. 3C-3**). Such anticipation was predicated on the effects seen with 1-5A, 1-6A and 1-7A (versus 1-4A) (**Fig 3J-1 and -3, K-3 and L-1 and -3**). Likewise the 5-7A did not produce the strong delay in the Evening activity peak to ~ZT12 that was expected based on effects seen with 1-5A, 1-6A and 1-7A (versus 1-4A): instead it produced only a minor delay (**Fig. 3H-3**).

Behavior under equinox conditions (Figures 4 and 5). Under 12:12 conditions, *han* mutant flies (lacking *pdf*r function) typically display elevated nocturnal activity, a lack of morning anticipation prior to Lights-on, and a pronounced advance in the peak of the Evening behavior [11]. **Fig. 4A-1 to A-2** display

activity patterns of *han* mutants that are heterozygous for *tim*-Gal4; they generally matched prior descriptions of *pdf* mutant behavior. Restoration of some *pdf* function by *han*; *tim*> WT-*pdf*, produced a modest Morning peak (anticipatory activity prior to lights-on – **Fig. 4B-2**) and delayed the evening peak by 1-2 hrs (**Figures 4N and 5B-2**) [7,8,9]. Among the five “Simple PDFR Variants” (**Fig 4C-G**), the 6A and 7A PDFR isoforms produced significantly elevated morning activity, with a peak ~2 hr prior to Lights-on in the period ZT22-23 (**Fig 5F-1 and G-1**). This elevated pre-Lights-on activity peaked ~3-4 hr later than that seen under Short Day conditions with these same PDFR variants. Separately, the 6A isoform increased Morning activity after the lights-on signal (**Fig. 5F-2**) and the 4A, 6A and 7A isoforms increased the amount of Evening activity, but without changing its phase (**Fig. 5D-5, F- 5 and G-5**). In the case of the five Multiple Variants, we noted three main effects. First the progressive addition of more Ala substitutions in the series 1-5A, 1-6A and 1-7A all produced significant additional Morning activity in the period ZT22-23 compared to that seen with 1-4A. Notably, all of that activity appeared to decline prior to the Lights-on signal (**Fig 5E-3, F-3, G-3**). Those variants also produced increased activity immediately after Lights-on: an effect seemingly distinct from that produced prior to Lights-on (**Figure 5 J-42 K-2 and L-2**). Second, combining the 5A, 6A and 7A variants in PDFR 5-7A did not produce additive effects on behavior in the morning, either before or after Lights-on (**Fig 5H-1 and -2**): instead the activity at ZT22-23 and ZT 0.5-3.5 appeared similar only to that displayed by rescue with a WT PDFR. Finally, the 1-5A variant was notable among Multiple Variants in LD12:12 conditions by displaying elevated activity in mid-day between the Morning and Evening Activity peaks (**Fig 5E-4 and E-5**).

Behavior under Long Day (summer) conditions (Figures 6 and 7). Flies lacking *pdf* function in 16L:8D display locomotor patterns similar to those in 12L:12D – lack of an obvious Morning peak and produce a broad Evening activity period that peaks ~3hr before Lights-off: the example in **Figure 6A-2** displays behavior by *han* mutants that are also heterozygous for the *tim*-Gal4 element. Rescue (*han*;

tim> WT-pdf^r, **Figure 6B-2**) typically delayed Morning activity offset (**Fig. 6M**) and delayed the Evening peak onset each by about 1 h (**Fig. 6N**). Expression of the 5A and 6A PDFR variants produced significantly greater amounts of Morning activity after Lights-on (**Fig. 7C-3** through **G-3**), while all of the Single Variants increased the amplitude of the Evening peak without affecting its phase (**Fig. 6 C-5 to G-5**). Among the five Multiple Variants, we noted three main effects (all comparable to those seen previously under 12L:12D entraining conditions). First the 1-6A and 1-7A variants displayed significantly increased Morning activity after Lights-on (**Fig. 7 K-2 and L-4**). Second, the variants 1-5A, 1-6A and 1-7A increased the amplitude of the evening peak without affecting its phase (**Fig. 7J-3, K-3 and G-3**). Third, as in the other photoperiodic conditions, when we combining the 5A, 6A and 7A variants into a single 5-7A variant, it did not produce the expected additive effects on behavior in the morning (**Fig 7H-2**) or in the evening (**Figure 7H-3**): instead these activities appeared similar to effects displayed by the 1-4 and 1-5 variants.

Behavior under DD. In DD following Short Day conditions, the PDFR variants that produced 3-4 h delays in the evening peak often generated periods ~ 1-2 hr longer than the controls and also lowered % arrhythmicity (**Table 1**). This was especially true for the Multiple Variant series (e.g., PDFR 1-5A, 1-6A and 1-7A (**Table 1**). However the correlation between delayed evening peaks and a longer tau in DD was not absolute” for example, the 6A, 7A and 5-7A variants all had strong significant delaying effects on the Evening phase of activity under Short Day conditions (compared to the effect of the WT PDFR), but they did not lengthen tau values in subsequent DD conditions (Table 1). These observations suggest that delays of activity phases produced by PDFR modulation in LD are not necessarily a simple or direct consequence of its effects on the PER and TIM--dependent clock [2, 3, 17]. In addition, the dominant activity periods in DD typically reflected the delayed (~ZT12) Evening peak (**Fig. 2K-1 and L-1**). Notably,

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

Table 1.

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

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

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

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

Behavioral activity records from days 3-9 in constant conditions following entrainment in the three indicated photoperiods (Short Day, Equinox and Long Day). N = number of independent experiments performed for each genotype::photoperiod combination. n = total number of flies tested for each genotype::photoperiod combination. % AR: the percentage of flies judged arrhythmic by criteria; tau = average circadian periods calculated according to χ^2 -periodogram analysis. Statistical analysis compared tau's using Tukey's multiple comparisons post hoc test following a one-way ANOVA: ns: not significant; * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.; nd: not determined.

In DD following Equinox or Long Day entrainment, we noted that several variants produced dominant activity phases which were phase-delayed relative to the Evening peak displayed in LD (e.g., **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 appear to repress expression of the delayed, post Lights-off Evening activity observed under SD conditions: this was suggested by the emergence of a delayed phase activity peak once flies only after release to DD. Once again, several of the variants significantly increased Tau in DD (**Table 1**), yet had only slight effects on phase in LD.

Control experiments. The design of the *PDFR* variants contains numerous assumptions and genotypic constraints, distinct from simply substituting Alanine at potential sites of phosphorylation in the receptor C terminal tail. To determine their potential to affect the results we report, we performed the following two sets of experiments as controls. (i) - ***The influence of epitope fusions.*** The *PDFR* variants we have tested include epitope fusions (4xFLAG and eGFP), which in principle could confer some of the properties observed. In part, the concern regarding fused sequences is mitigated by the fact that all UAS-receptor isoforms described above (WT and sequence-variant) contained both epitopes

(in addition – all were genetically transduced to the same integration site). Hence comparisons across different variants (e.g., **Figures 2 through 7**), should largely normalize for the effects of the non-receptor sequences, and instead highlight variant-specific properties. However, we wished to test this assumption explicitly to define the extent to which a bulky GFP fused to the C Terminal tail might confer some of the behavioral properties we might otherwise ascribe to PDFR C terminal sequences. We therefore compared a 1-7A version of PDFR to a wild type PDFR, both of which lacked GFP fusions. The results in both Short Day and Long Day conditions, followed by release into DD, support the hypothesis that alterations in daily locomotor rhythmicity were largely derived from sequence variation of the PDFR, and not from the properties of the fused GFP (**Supplemental Figures 2 and 3** and tabulated in **Table 1**).

Control experiments (ii) - dependence of GPCR PDFR variant effects on the presence of PDF ligand.

Second, we assumed that the altered behavioral phenotypes produced by certain PDFR variants depended on activation by their endogenous cognate ligand, the neuropeptide PDF. However, we could not *a priori* exclude the possibility that PDFR variants may in fact produce novel constitutive activity (neomorphic properties). We therefore placed the 1-7A PDFR variant in the *pdf⁰¹* (null) background and re-tested its activity when driven by *tim*(UAS)-Gal4 under both short day and long day conditions, and then in constant darkness. In all conditions, the behavioral phenotypes largely resembled those of the *pdf⁰¹* background (lack of a morning activity peak, advanced evening peak, and shorter tau/s in DD). The results are shown in **Supplemental Figures 4 and 5** and tabulated in **Table 1**. The 1-7A PDFR variant did reduce the % arrhythmicity present in the *pdf* mutant background to nearly the same value as found in a WT *pdf* background, which suggests some degree of constitutive (ligand-independent) activity. Apart from that one aspect, we conclude that constitutive activity explains at best a small proportion of the behavioral effects of the PDFR variants here tested.

PDFR expression and signaling *in vitro*. The PDFR is a G_s-coupled receptor and several reports have documented the importance of the downstream cAMP pathway to mediate PDF behavioral regulation [15, 17, 43]. In that context, we asked whether the *in vivo* properties of those PDFR variants that affected behavioral phase and amplitude could be correlated with *in vitro* properties when expressed in *hEK-293T* cells. We found no strong correlation. Significant differences in basal signaling levels, in EC₅₀ or in maximum values of signal transduction were rare (**Supplemental Figures 6-8**). When noted, they were poorly correlated with behavioral effects (**Supplemental Table 5**). We also measured surface expression of the PDFR variants in *hEK* cells using β -lactamase N terminal fusions [44] to determine if PDFR variants tended to display longer surface lifetimes. The 1-4 and 1-7 variants displayed higher basal levels, but no others were different from the WT levels (**Supplemental Figure 9**). Following 20 min exposure to PDF, neither the WT nor any of the variants displayed a change in surface expression levels (**Supplemental Figure 10**).

Measuring the phosphorylation state of over-expressed PDFR *in vivo*. To obtain direct evidence that PDFR sequences are phosphorylated *in vivo*, we first over-expressed an epitope-tagged-PDFR WT construct using *tim*-Gal4, which directs expression broadly in cells that feature the PER-dependent molecular oscillator. We immunoprecipitated the receptor from head extracts, then employed tandem mass spectroscopy to determine which if any specific PDFR peptide fragments are phosphorylated. We performed eight biological replicates, with two collections in the morning (ZT2-3) and six in the evening hours (ZT11). **Supplemental Table 4** reports the phosphorylated peptides detected from the PDFR-GFP fusion protein. Among the 28 conserved Ser/Thr and Tyr residues in the PDFR C terminal tail, five were phosphorylated in one or more of these samples. In the two Mornings samples S563 was phosphorylated in one sample; in the Evening samples, S531 (in CL2) was

phosphorylated in four of samples, S534 (in CL2) in one, T543 (in CL3) in one, and S560 in two of six samples. The spectra documenting detection of these phosphopeptides are presented in **Supplemental Figure 11**.

Testing GRKs and β -arrestin2 contributions to locomotor rhythmicity. Regarding which potential PDFR phosphorylation sites might be β -arrestin binding sites, we considered a recent report by Zhou *et al.* [45] who proposed a conserved GPCR sequence motif that promotes high affinity interactions with β -arrestin. The motif includes three phosphorylation sites that align with three conserved, positively-charged pockets in the arrestin N-domain. In *D. melanogaster* PDFR, the CL1 region contains a match with the proposed motif beginning with S512 (SLATQLS) and shows moderate sequence conservation: of the 17 species we considered, 9 others retained this precise motif (**Supplemental Figure 1**). In addition, the *D. melanogaster* PDFR sequence beginning with S629 (SRTRGS) also displays a match for the motif, but is retained in only 6 of the other 16 species. The evidence that these two sites represent high affinity β -arrestin2 binding domains is therefore equivocal. We previously reported that β -arrestin2-GFP is not efficiently recruited to activated PDFR when functionally-expressed in *hEK-293T* cells [35]. In contrast, each of 13 other *Drosophila* neuropeptide GPCRs do efficiently recruit β -arrestin2-GFP when expressed and activated in that cellular environment [29, 36, 37].

We tested the potential to detect *in vivo* involvement by *Drosophila* orthologues of the canonical desensitization effectors - mammalian G-protein coupled receptor kinases (GRKs – GPRK1 (CG40129) and GPRK2 (CG17998)) and of mammalian β -arrestin2 (β arr2 – *kurtz* (CG1487)) - in the control of locomotor rhythms We drove specific over-expression of WT cDNAs and RNAi constructs using *tim*-Gal4, to broadly affect signaling in the circadian pacemaker system. The PDFR-PA protein is

highly restricted in its expression to subsets of the pacemaker neural network [46]. We predicted that elimination of a desensitizing component for PDFR signaling should produce behavioral phenotypes opposite to that of *pdfr* loss of function phenotypes: these would potentially include (for example) a delayed evening activity phase in LD conditions and a longer tau under DD. GPRK1- and *krz*-specific RNAi's produced normal average locomotor profiles under 12:12, while one of two GPRK2-specific RNAi constructs tested slightly advanced the evening peak (**Supplemental Figure 12**). Over-expressing GPRK1 cDNAs did not affect morning or evening phase; over-expressing GPRK2 broadened the evening peak. Under DD, *krz* RNAi flies were uniformly arrhythmic, while GPRK RNAi's were normal or slightly lengthened the circadian period (**Supplemental Table 5**). These results do not support the hypothesis and suggest that the kinetics by which PDFR signaling terminates do not depend exclusively on the activities of either dGPRK-1 or -2, or on that of the *Drosophila* β -arrestin2 ortholog, *krz*.

DISCUSSION

We found that several PDF receptor variants, containing Ala-substitutions of phosphorylatable residues, modulated the amplitudes of the Morning and/or Evening activity peaks, or delayed their phases and/ or lengthened the free-running period. Such results are generally consistent with a prediction whereby non-phosphorylatable PDFR variants – those with a potential to increase the duration of PDFR signaling - would produce behavioral actions opposite to those seen in loss-of-function *pdf* [1], or *pdfr* [11] mutant stocks. The loss of function phenotypes include advanced behavioral peaks, as well as weaker and shortened free-running periods. In this context, we acknowledge that GPCR modification by phosphorylation does not exclusively lead to signal termination. GRK2-dependent phosphorylation of the smoothened (*smo*) GPCR (which helps mediate Hh signaling) follows reception of

the Hh signal: that leads to smo activation in both *Drosophila* and mammalian systems [47]. Thus modification of putative phosphorylation sites on GPCRs (to preclude phosphorylation) will not exclusively promote extended signaling and therefore our interpretations must correspondingly consider outcomes without *a priori* assumption of mechanisms.

GPCR signal termination. PDFR belongs to the Secretin Receptor Family (Family B) of neuropeptide receptors [11-13]: there is no clear consensus regarding mechanisms of desensitization and internalization for this receptor family. For VPAC2 receptors, phosphorylation and internalization is mediated exclusively by GRK2 (48. Murthy, 2008;). Likewise, *Drosophila* orthologues of Corticotrophin Releasing Factor receptors (CG8422, DH44-R1) and Calcitonin receptors (CG17415, DH31-R1) are internalized in *hEK* cells following recruitment of β -arrestin2 [29]. In contrast, PDFR, which is also related to the mammalian Calcitonin receptor, is not internalized following exposure to PDF [35]. VPAC2 receptors are also regulated by heterologous receptor signaling: M3 cholinergic receptors via PKC signaling can block VPAC2 phosphorylation, desensitization and internalization [49]. Furthermore, secretin receptors and VPAC1 receptors undergo phosphorylation by GRKs and β -arrestin2-dependent desensitization, but these are not sufficient to facilitate or mediate internalization [50-52]. Finally, GLP2-Receptor associates with β -arrestin2 via its distal C terminal sequences, but that receptor domain is required neither for GLP2-R desensitization nor its internalization [53]. Thus kinases other than GRKs and effectors other than β -arrestin2 may regulate internalization of diverse Family B receptors.

Validating the behavioral phenotypes produced by PDFR variants. The actions of PDFR variants we have described, following substitutions of Ala for various Ser/Thr or Tyr residues, are not easily explained by a hypothesis invoking neomorphic or constitutive GPCR properties. The evidence for this conclusion is three-fold. First, the effects on both amplitude and phase of activity peaks by these

variants are not of a random assortment: rather they are all strictly opposite to that of loss-of-function models for *pdf* and *pdf^r*. Second, the actions of PDFR variants display strong dependence on wild type *pdf* gene function: this strongly argues that the actions of PDFR variants reflect responses to normal, endogenous PDF signaling. Third, the action of PDFR variants on the phases and amplitudes of locomotor activity reflected PDFR sequence variation, and not the properties of the GFP C-terminal fusion. Together these control observations support the hypothesis that phosphorylation of some or all of the C terminal residues we studied are normally modified to attenuate the strength and duration of PDFR signaling.

PDF Signaling and seasonal adaptation. PDF neuropeptide actions were discovered in the context of physiological adaptations to daylight in crustacea [5,6]. Helfrich-Förster [54] discovered PDF expression within a defined subset of the insect *Drosophila* circadian pacemaker network. Renn *et al.* [1] demonstrated that *pdf* makes a fundamental contribution by setting the normal behavioral phase of Evening activity in *Drosophila* during light:dark entrainment and promoting normal rhythmicity during constant darkness. PDF signaling coordinates with that driven by light: It works in parallel to direct photosensitivity along with the CRY blue light photoreceptor. Flies doubly mutant for *cry* and *pdf* display severe deficits of locomotor rhythmicity [7, 8], and such effects can be as severe as measured in clock-deficient flies [9]. PDF and environmental light also work coordinately at the level of neuronal activity patterns: in the case of the evening pacemakers (LNd and the 5th s-LNv), both light and PDF signaling promote a delay in their PER-dependent activation period, and help align it to a phase just prior to dusk [19].

Based on the close association of PDF signaling and photoperiodic signaling, we considered it essential to test the different variant PDF Receptors in a variety of photoperiodic conditions. The

consensus of many prior observations indicates that PDF neuropeptide signaling is necessary for the display of a Morning peak (its amplitude) and for proper adjustment of phase of the Evening activity peak [e.g., 1, 7, 8, 55-57]. Our present observations, concerning likely gain-of-function *pdf* variants, support a revised hypothesis regarding regulation of the Morning peak: we propose PDF/PDFR can affect - and normally does affect - both the phase and amplitude of the both Morning and Evening activity peaks. Thus the consensus of many prior descriptions – concluding there is “no Morning activity peak” in *Pdf* and *Pdfr* mutants – beginning with the description by Renn *et al.* [1], may have failed to discern the presence of a greatly-diminished Morning peak that was also advanced. There is some precedent for this contrary conclusion: Yoshii *et al.* [58] described behavior of *pdf⁰¹* mutants and noted advanced morning peaks especially under longer photoperiods. Likewise, the duration of the photoperiod strongly influenced the effects we observed on the Morning versus the Evening activity peaks. We only observed delayed Evening activity peak, produced by PDFR gain-of-function variants, under Short Day conditions: the delayed evening peak appears strongly inhibited by light durations > 8 hr: and it remains unclear at what molecular level light exerts this inhibitory effect. We note a striking similarity of these observations with effects recently described in loss-of-function states for the phosphatase *PRL-1* [51]. Like the most active PDFR variants we herein describe (e.g., 6A, 7A, 1-5A 1-6A and 1-7A), *PRL-1* mutants produce a 3-4 h delay in the phase of the evening activity peak, but only under winter-like (short day) conditions, not under equinox or summer-like (long day) conditions. Kula-Eversole *et al.* [59] have shown that TIM phosphorylation is affected by PRL-1 activity and suggest the seasonal action of PRL-1 to advance the evening locomotor activity phase is mediated by modification of TIM levels within the transcription-translation feedback loop. The extensive similarity of these two sets of behavioral phenotypes reveals either serial or parallel pathways to effect comparable outcomes on locomotor activity. If serial, then according to the simplest model, PRL1 acts downstream of PDFR, and

the combined results to-date suggest PRL-1 is inhibited by PDFR activation. Further genetic and biochemical experiments are needed to evaluate if and how these pathways converge.

PDFR signaling effects on phase and on period. The behavioral effects of the PDFR variants tested were most robust in modulating the phase of evening activity bouts. Evening locomotor activity derives in large part from the activity of the primary Evening oscillators, termed E cells, the LNd and the 5th small LNV [10, 20, 51, 60-63]. However, numerous network interactions are also known to provide critical contributions to the accuracy, precision and adaptability of that timing system [e.g., 64 -67]. The Evening oscillator group itself is known to be heterogeneous and to constitute at least three separate, functionally distinct oscillators [68], only some of which express PDFR and respond to PDF [57, 68]. Vaze and Helfrich-Förster [57] reported that the phase of Period protein accumulation in E neurons is phase-locked to the previous lights-off transition and also sensitive to PDF signaling. They conclude that the peak of PER accumulation is key to determining the phase of the evening activity peak, but also note that the correlation between the Period-clock timing cue and the Evening activity peak is not perfect. They suggest the Evening behavioral phase is better described by also factoring in the delay in E cell neuronal activity driven by PDF neuromodulation [10, 19]. In that context, we interpret the many examples of delayed Evening peaks resulting from PDFR variants (e.g., Figure 2M) as products at least partly derived from PDF modulation (delay) of the cell intrinsic molecular clocks in E cells: that conclusion is supported by the larger tau values in DD evident in the behavior of many PDFR variants (Table 1). However, among the different variants, the association between delayed Evening activity peaks in LD and longer tau values in DD was not perfect. For example, under short days, the 5A, 6A, 7A and 5-7A variants all significantly delayed the offsets of Evening activity peaks versus that seen with WT PDFR, but these variants did not significantly lengthen circadian period. Likewise under long day, 4A and 7A produced significant increases in tau versus that seen with WT PDFR, but they did not significantly

delay the onset of the Evening activity peak. Our working hypothesis is therefore that the duration of PDF>PDFR signaling to target pacemakers in the *Drosophila* brain is modulated each day to independently effect (i) entrainment of clock period, and (ii) delays of pacemaker neuronal activity. It is the combination of these two PDFR signaling effects that is essential for proper rhythmic behavioral outcomes across seasons. The signaling pathways downstream of PDFR, that independently regulate diurnal phase and circadian period within E pacemaker neurons, are largely unknown.

Relations between in vitro and in vivo PDFR signaling measures. Using an *in vitro* assay for cyclic AMP generation, we found that modifying phosphorylation properties of PDFR does not affect the strength of signaling. That conclusion suggests that *in vivo* other GPCR features are normally affected to regulate the extent/duration of PDFR signaling according to season, which corresponds to findings described with other Family B GPCRs like VPAC1 [40]. Mutation of all the Ser and Thr residues of the C terminal tail and of Ser250 to Ala led to a receptor with binding properties and adenylate cyclase activity not different from that of the wild type receptor; however that variant receptor was neither phosphorylated nor internalized. We propose that the PDFR variants we have described do not modify locomotor behavior by virtue of greater second messenger signaling. Rather they do so by generating cAMP (and perhaps other second messengers) over time periods longer than normal sustained by a WT receptor over a portion of a 24-hr cycle.

Specific versus non-specific phosphorylation regulating GPCR activity. There are two broadly divergent hypotheses to describe the mechanisms by which GPCR phosphorylation promotes desensitization. The first posits that modification of specific residues have greatest significance for downstream desensitizing mechanisms. The second invokes the triggering of termination processes by the aggregate negative charge accumulated with phosphorylation, regardless of where it might occur.

For some GPCRs (e.g., OPN4), the evidence supports both models [33, 39]. Our data concerning PDFR desensitization also support both viewpoints and we present a model in **Figure 8**. The evidence for the importance of PDFR sequence specificity rests on the data that modification of certain single Clusters is, among all variants tested, the most effective in affecting locomotor activity rhythms. In particular we regard CL6 and CL7 to be especially critical in terminating the time course of PDFR signaling. Notably, converting just the single pair of CL6 AAs, or the single pair of CL7 AAs, is enough to generate hours-long delays in the peaks of locomotor rhythms under Light:Dark conditions (**Figure 2 F-G, 4F-G; 6 F-G, 7 F-G**), as well as generating a significant increase in tau under Constant Dark conditions (**Table 1**). We note that the splicing event that distinguishes the PDFR-A and PDFR-D isoforms occurs just prior to the position of sequences encoding CL6 and 7, such that the D form lacks these two highly conserved domains. Other clusters (like CL2-3) also appear to have potential for ‘specific’ contributions to PDFR regulation. Together, these results speak to the potency of the inferred specific termination mechanisms for PDFR GPCR signaling. In contrast, the evidence for the bulk phosphorylation hypothesis comes from comparison of the 1-4A, versus the 1-5A, 1-6A and 1-7A variants. By inactivating increased numbers of phosphorylatable residues, we observed a graded series increasingly delayed evening phases in short day conditions (e.g., **Figure 2I-2 though 2L-2**). The tandem mass-spectroscopy results, indicating endogenous phosphorylation of certain PDFR residues (**Supplemental Table 4**) demonstrates such post-translational modifications can occur, but likely does not provide a complete accounting of them.

Finally, our results point to what we propose as “context-dependent” effects of modifying specific GPCR residues – ones that may produce opposite behavioral effects, depending on the phosphorylation status of neighboring sites. In particular we point to the contradictory results we observed with Ala-variants of the CL5 site. In comparing results from the series 1-4A, 1-5A, 1-6A and 1-

7A, we found that the 1-4A had only mild effects on the evening peak compared to over-expressing WT PDFR (e.g., Figure **2B-2** vs. **2I-2**), whereas the others produced significant delays (**Figures 2M and 3I-3 through 3L-3**) under Short Day conditions. Those observations suggest modification of the CL5 residue (S6333) promotes desensitization of PDFR and termination of PDFR signaling. However, the PDFR variant 5-7A was constructed with the expectation that it would be as effective as 1-5A, 1-6A or 1-7A in delaying the evening phase. Instead it proved only weakly effective. This same mis-match of expected behavioral effects for 5-7A was also seen in other photoperiodic conditions (**Figure 6N, and 7H-3 vs 7J-3 through 7L-3**). Likewise, Langlet *et al.* [40] reported that effects on mutating Serine residues in the carboxy terminus of VPAC1 did not produce additive effects. We propose that phosphorylation of CL5 will have either positive or negative consequences on PDFR desensitization depending on which other neighboring residues are also modified. Thus we speculate that CL5 takes on outsize importance in determining desensitization rates for PDFR and so may itself be subject to exceptional regulation. Such complex interactions between phosphorylation sites is reminiscent of interactions documented between diverse phosphorylation sites in the circadian clock protein PERIOD [69, 70]. Better resolution of these apparent conflicting mechanisms (specific phosphorylation versus bulk negative charge, and paradoxical effects of the CL5 site) awaits precise molecular definition of where and when PDFR is modified *in vivo*, and by which modifying pathways.

MATERIALS and METHODS

Fly Rearing and Stocks. *Drosophila* were raised on a cornmeal agar diet supplemented with yeast at 25°C in 12 hr:12 hr LD cycles. The UAS-*pdf*r mutant transgenic series was created by injecting *yw P{nos phiC31\int.NLS}X;P{CaryP}attP40* embryos (Rainbow Transgenic Flies, Inc, Camarillo, CA). The UAS-*pdf*r-tandem construct was injected into *y[1] w[*] P{y[+t7.7]=nos-phiC31\int.NLS}X;*

P{y[+t7.7]=CaryIP}su(Hw)attP6 embryos. For PDFR-tandem fusion protein expression, we made a stable *yw; tim(UAS)-Gal4; UAS-pdfr*-tandem stock. *pdfr* mutant flies are described in [11]; *tim(UAS)-Gal4* flies are described in [71] – *BL80941*). The recombinant fly stock - *han⁵³⁰⁴; tim(UAS)-Gal4* - was confirmed by PCR and sequencing.

hEK-293 Cell Culture. *hEK-293* cells were maintained in DMEM, 10% FBS and 100U/mL penicillin and streptomycin in 5% CO₂ atmosphere at 37°C. For all transient transfections, 1.5 x 10⁶ cells were used to inoculate T25 flasks, incubated overnight, then transfected with 10ug plasmid DNA and 20 uL lipofectamine 2000 reagent (Invitrogen Life Technologies). Five hours after transfection, cells were split 4 x 10⁴ cells/well into a 96-well assay plate. We created a series of stably-transfected *hEK-293* cells expressing WT and sequence-variant PDFRs, using the Flp-In System (Invitrogen Life Technologies, Waltham, Massachusetts) per manufacturers recommendations, and maintained them in DMEM supplemented with 150 µg/ml hygromycin B.

cAMP Assays. We measured PDF Receptor signaling activity using a CRE-*Luciferase* reporter gene, following methods described by Johnson *et al.* [29]. The reporter gene construct was transiently transfected to each stable cell line and luminescence measured using Firefly Luciferase Assay Kit (Biotium, Inc., Fremont, California) and a Wallac 1420 VICTOR2 microplate reader (PerkinElmer, Inc., Waltham, Massachusetts). Concentration-effect curves, EC₅₀, top values and p-values were calculated using the dose response, in a nonlinear regression using GraphPad Prism 8.0 software (San Diego, California).

Locomotor activity Measures. All locomotor activity experiments were conducted with 2-5 days-old male flies at 25°C using Trikinetics Activity Monitors as previously described [9]. We crossed

Gal4 lines to w^{1118} to create control progeny. Locomotor activities were monitored for 6 days under different photoperiodic conditions, and then for 9 days under constant dark (DD). To analyze rhythmicity under constant conditions, we normalized the activity of flies from DD day 3 to day 9 and used χ^2 -periodogram analysis with a 95% confidence cut-off, as well as SNR analysis [72]. Arrhythmic flies were defined by a power value ≤ 10 and width value ≤ 2 , and period outside the range, 18 to 30 hours. To analyze periods, we used Graphpad InStat (v 8) software to run one-way ANOVA measures followed by the Tukey-Kramer Multiple Comparisons Test. We used Clocklab (Actimetrics) software to produce actograms and the Brandeis Rhythms Package [73] to produce average activity plots (group reductions). To analyze onset and offset phases of Morning and Evening activity bouts on the final two days of light:dark entrainment (LD 5&6), we followed the method of Kula-Eversole *et al.* (55, 2021): $[(A_{n+2} + A_{n+1}) - (A_{n-1} + A_{n-2}) = \Delta \text{Activity}]$. Experimental genotypes were tested in the *han* mutant background, *pdf^{r5304}* [11], or in the *pdf⁰¹* (null) mutant background [1], or in the w^{1118} background, as noted.

Additional details on Methods and procedures are found in **Supplemental Information**.

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AUTHOR CONTRIBUTIONS

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 analyzed experiments. P.H.T wrote the manuscript.

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FIGURE LEGENDS

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

Figure 2. Locomotor Rhythms exhibited by WT PDFR and by Simple PDFR Variants under Winter-like (Short Day) conditions. All behavioral records were recorded from *han* (*pdf* mutant) flies that expressed either no UAS transgene (A), or a UAS-WT *pdf* transgene (B) or a variety of Simple PDFR Variants, including 2-3A (C), 4A (D), 5A (E), 6A (F) or 7A (G), or Multiple PDFR variants, including 5-7A (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 (1) displays a double-plotted group actogram throughout the 6 days of Light: dark entrainment, followed

by ~9 days of constant darkness (DD, grey background). Sub-panel (2) displays a daily plot of locomotor activity (a group reduction) averaged over the last two days of entrainment (LD 5-6). Open bars indicate the periods of Lights-on and filled bars indicate periods of Lights-off. Panel M displays the average Phase Offset timepoint for the Evening activity phase (marked by a Blue Arrow) for each genotype over the last two days of entrainment (LD 5-6). Panel N displays the average Phase Onset timepoint for the Morning activity phase (marked by a Red Arrow) for each genotype over the last two days of entrainment (LD 5-6). The positions of the Blue and Red arrow in panels A-L are representative; panels M and N presents their true values. Analyses represent ANOVA followed by Dunnett's post hoc multiple comparisons of all compared to WT: ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$; **** = $p < 0.001$.

Figure 3. Amplitude measures of Locomotor activity exhibited by WT PDFR and by diverse PDFR Variants under Winter-like (Short Day) conditions. All behavioral records were recorded from *han* (*pdfR* mutant) flies that expressed either no UAS transgene (A – marked in YELLOW), or a WT *pdfR* cDNA (B – marked in BLACK) or a variety of Simple *pdfR* Variants (marked in RED) including 2-3A (C), 4A (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 averaged across all experiments run for each individual genotype (see Table 1 for N and n values). Each Panel (A)-(L) contains sub-panels (1) through (3), each of which displays Bin-by-Bin analyses of activity 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. The missing bin at timepoint 0 contains the startle response that accompanies the sudden lights-on signal. Asterisks indicate significantly-different activity levels according to a Student's T-test ($p < 0.05$). Red arrows indicate elevated Morning activity levels displayed by different PDFR variants. Blue arrows indicate elevated Evening activity levels produced by different PDFR variants.

Figure 4. Locomotor Rhythms exhibited by WT PDFR and by Simple PDFR Variants under Equinox (12:12) conditions. All behavioral records were recorded from *han* (*pdfr* mutant) flies that expressed either no UAS transgene (A), or a UAS-WT *pdfr* transgene (B) or a variety of Simple *pdfr* Variants, including 2-3A (C), 4A (D), 5A (E), 6A (F) or 7A (G), or Multiple *pdfr* variants, including 5-7A (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 (1) displays a double-plotted group actogram throughout the 6 days of Light: dark entrainment, followed by ~9 days of constant darkness (DD, grey background). Sub-panel (2) displays a daily plot of locomotor activity (a group education) averaged over the last two days of entrainment (LD 5-6). Open bars indicate the periods of Lights-on and filled bars indicate periods of Lights-off. Panel M displays the average Phase Offset timepoint for the Evening activity phase (marked by a Blue Arrow) for each genotype over the last two days of entrainment (LD 5-6). Panel N displays the average Phase Onset timepoint for the Morning activity phase (marked by a Red Arrow) for each genotype over the last two days of entrainment (LD 5-6). The positions of the Blue and Red arrow in panels A-L are representative; panels M and N presents their true values. Analyses represent ANOVA followed by Dunnett's post hoc multiple comparisons of all compared to WT: ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$; **** = $p < 0.001$.

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

through (3), each of which displays Bin-by-Bin analyses of activity 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. The missing bin at timepoint 0 contains the startle response that accompanies the sudden lights-on signal. Asterisks indicate significantly-different activity levels according to a Student's T-test ($p < 0.05$). Red arrows indicate elevated Morning activity levels displayed by different PDFR variants. Blue arrows indicate elevated Evening activity levels produced by different PDFR variants.

Figure 6. Locomotor Rhythms exhibited by WT PDFR and by Simple PDFR Variants under Summer-like (Long Day) conditions. All behavioral records were recorded from *han* (*pdfR* mutant) flies that expressed either no UAS transgene (A), or a UAS-WT *pdfR* transgene (B) or a variety of Simple *pdfR* Variants, including 2-3A (C), 4A (D), 5A (E), 6A (F) or 7A (G), or Multiple *pdfR* variants, including 5-7A (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 (1) displays a double-plotted group actogram throughout the 6 days of Light: dark entrainment, followed by ~9 days of constant darkness (DD, grey background). Sub-panel (2) displays a daily plot of locomotor activity (a group reduction) averaged over the last two days of entrainment (LD 5-6). Open bars indicate the periods of Lights-on and filled bars indicate periods of Lights-off. Panel M displays the average Phase Offset timepoints for the Evening activity periods (marked by a Blue Arrow) for each genotype over the last two days of entrainment (LD 5-6). Panel N displays the average Phase Onset timepoints for the Morning activity periods (marked by a Red Arrow) for each genotype over the last two days of entrainment (LD 5-6). The positions of the Blue and Red arrows in panels A-L are representative; panels M and N presents their true values. . Analyses represent ANOVA followed by Dunnett's post hoc multiple comparisons of all compared to WT: ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$; **** = $p < 0.001$.

Figure 7. Locomotor Rhythms exhibited by WT PDFR and by Multiple PDFR Variants under Summer-like (Long Day) conditions. All behavioral records were recorded from *han* (*pdfR* mutant) flies that expressed either no UAS transgene (A – marked in YELLOW), or a WT *pdfR* cdNA (B – marked in BLACK) or a variety of Simple *pdfR* Variants (marked in RED) including 2-3A (C), 4A (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 averaged across all experiments run for each individual genotype (see Table 1 for N and n values). Each Panel (A)-(L) contains sub-panels (1) through (3), each of which displays Bin-by-Bin analyses of activity 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. The missing bin at timepoint 0 contains the startle response that accompanies the sudden lights-on signal. Asterisks indicate significantly-different activity levels according to a Student's T-test ($p < 0.05$). Red arrows indicate elevated Morning activity levels displayed by different PDFR variants. Blue arrows indicate elevated Evening activity levels produced by different PDFR variants.

Figure 8. A model predicting the effects of phosphorylating different sites on the PDFR CT on downstream signaling. Asterisks indicate the positions Ser/ Thr/ and Tyr residues as described in Figure 1 and Supplemental Figure 1: white asterisks were targets of mutational analysis in this study. Mutation of CL6 and CL7 consistently demonstrated the greatest delaying effects on the phases of locomotor peaks, suggesting their phosphorylation normally will have the greatest effect to slow or terminate PDFR signaling. Other sites are also effective, although to lesser degrees, including CL2-3, CL4 and CL5. Primarily, such phosphorylation will decrease the extent of PDFR signaling (red bars) and so reduce the delay that PDF imposes on the period of neuronal activation displayed by PDFR-responsive pacemaker groups (like the Evening cells and the DN3: Liang *et al.*, 2016, 2017). The model also predicts that the effects of phosphorylating CL5 will depend on the phosphorylation status of neighboring sites. In some contexts (e.g., PDFR 1-5A), it will help terminate PDFR signaling, but in others (e.g., PDFR 567A) it may

955 promote the duration or extent of PDFR signaling, perhaps by blocking the effects of phosphorylating
956 CL6 and CL7 (green bar).
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Figure 1.

Figure 2

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