

# PDF Receptor Signaling in *Drosophila* Contributes to Both Circadian and Geotactic Behaviors

## Report

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### Summary

The neuropeptide Pigment-Dispersing Factor (PDF) is a principle transmitter regulating circadian locomotor rhythms in *Drosophila*. We have identified a Class II (secretin-related) G protein-coupled receptor (GPCR) that is specifically responsive to PDF and also to calcitonin-like peptides and to PACAP. In response to PDF, the PDF receptor (PDFR) elevates cAMP levels when expressed in HEK293 cells. As predicted by *in vivo* studies, cotransfection of *Neurofibromatosis Factor 1* significantly improves coupling of PDFR to adenylate cyclase. *pdf* mutant flies display increased circadian arrhythmicity, and also display altered geotaxis that is epistatic to that of *pdf* mutants. PDFR immunosignals are expressed by diverse neurons, but only by a small subset of circadian pacemakers. These data establish the first synapse within the *Drosophila* circadian neural circuit and underscore the importance of Class II peptide GPCR signaling in circadian neural systems.

### Introduction

Molecular genetic studies have provided a detailed model of the cell-autonomous molecular oscillator that generates circadian rhythms in RNA and protein levels (Van Gelder et al., 2003). However within the nervous system, cellular clocks must interact with other clock cells, as well as with nonclock cells, as elements of neuronal circuits. Cellular and synaptic interactions appear to play critical roles in the maintenance and synchronization of circadian rhythms across pacemaker networks (Nitabach et al., 2002; Yamaguchi et al., 2003; Lin et al., 2004; Stoleru et al., 2004). Thus, there is growing interest in the neuronal properties of critical pacemaker cells and in the principles by which circadian oscillator neurons operate within larger networks. Neurons of the mammalian suprachiasmatic nucleus (SCN) exhibit

diverse transmitter phenotypes, and certain of these transmitter systems appear to be critical for normal SCN functions. As evidenced by synthetic *VIP* mutant mice, *VIP* signaling is required to maintain or consolidate molecular and behavioral rhythms (Harmar et al., 2002; Colwell et al., 2003; Aton et al., 2005).

Many of the fundamental components of the circadian molecular oscillator were first identified in *Drosophila*, which exhibits a morning and evening peak of activity under light dark (LD) conditions. There are six defined pacemaker cell groups in the fly brain (termed DN1, DN2, DN3, LNd, large LNV, and small LNV). Both the large and small LNV neuronal groups express the neuropeptide PDF (Helfrich-Forster, 1995). Under LD conditions, *pdf* mutant animals are rhythmic, although they lack a morning peak of activity; under constant dark conditions, a majority of the mutant flies display arrhythmicity, while those still rhythmic display short periods (Renn et al., 1999). The non-LNV pacemaker neurons do not express PDF, and their transmitter phenotype(s) is currently unknown. Recent mosaic analyses suggest that the morning peak of locomotor activity is associated with activity in the PDF-secreting LNV group (Grima et al., 2004; Stoleru et al., 2004). Similarly, the evening peak appears to be associated with activity in one or more of the non-PDF pacemaker cells. PDF also modifies geotactic behavior (Toma et al., 2002). PDF is primarily expressed by circadian pacemaker neurons, and the relationship between circadian and geotactic behaviors (if any) is presently uncertain.

To pursue mechanisms of PDF signaling *in vivo*, we conducted an extensive screen of GPCRs for those specifically sensitive to PDF. There are estimated to be 44 peptide GPCRs encoded by the *Drosophila* genome (Hewes and Taghert, 2001), and many share ancestry with mammalian receptor families. Thirty-nine are rhodopsin (class I) GPCRs, while five are secretin (class II) GPCRs. Here, we provide evidence that CG13758, a class II peptide GPCR most related to receptors for calcitonin and CGRP, represents the *Drosophila* PDF receptor.

### Results

#### Identification of the *pdf* r

We employed transient functional expression of receptor cDNAs in mammalian HEK293 cells to test their potential sensitivity to candidate neuropeptide ligands. Initial measures using a promiscuous G protein subunit indicated slight sensitivity of CG13758 to PDF, and not to any of 67 vertebrate peptides or 37 insect peptides (see Supplemental Data available with this article online). To further evaluate this sensitivity, we cotransfected a *CRE*-luciferase construct that reports on cAMP levels or a *SRE*-luciferase that reports on intracellular calcium levels. Neurofibromatosis1 (NF1) helps to mediate PDF actions *in vivo* (Williams et al., 2001); thus, we hypothesized that NF1 may augment PDF signaling at its receptor in these cell-based assays. Therefore, we cotransfected *Drosophila* NF1 cDNA with

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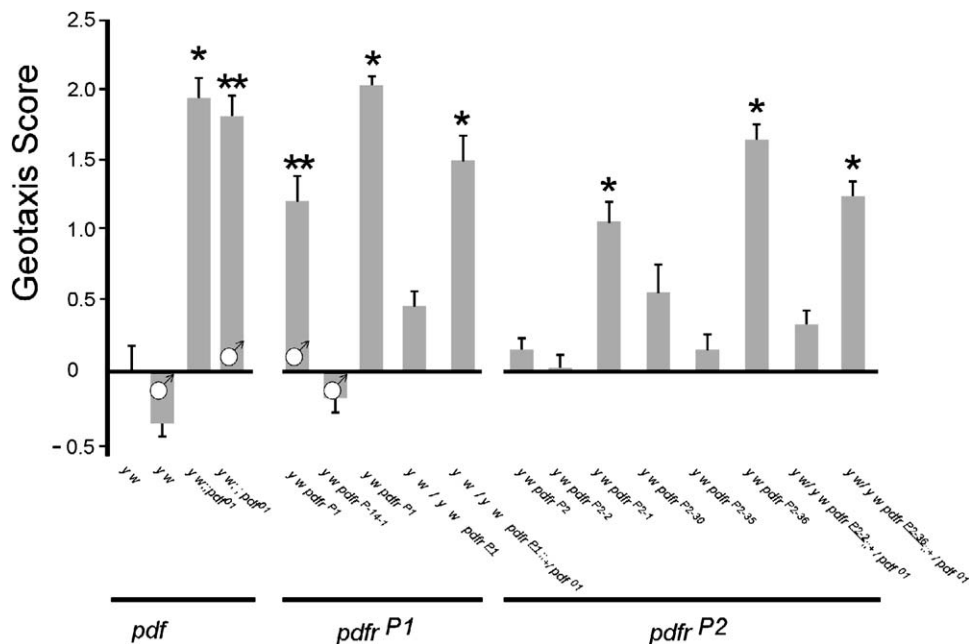


Figure 2. *pdfr* Alleles Display Geotactic Defects Comparable and Epistatic to Those in *pdf*

The score reports the difference in average tube number (height) between a test genotype and the *yw* control. Females were used primarily, except in those cases denoted by inclusion of the male symbol. The number of flies tested and the number of tests performed for each genotype are reported in Table S2. The values are grouped into three categories: “*pdf*,” “*pdfr*<sup>P1</sup>,” and “*pdfr*<sup>P2</sup>.” Single asterisks denote average values different from *yw* females at a level of  $p < 0.01$  (one-way ANOVA followed by Dunnett’s post hoc test). *P1-14* flies were tested as males and so were compared to *yw*, *yw*; *pdf*<sup>01</sup> and *P1* males; double asterisks denote average values different from *yw* males at a level of  $p < 0.01$  (one-way ANOVA followed by Dunnett’s post hoc test). Error bars represent  $\pm$ SEM.

analysis in the 3’ UTR deletion *P2-36* versus the control *P2-2* stock, but found no significant difference.

### Locomotor Assays

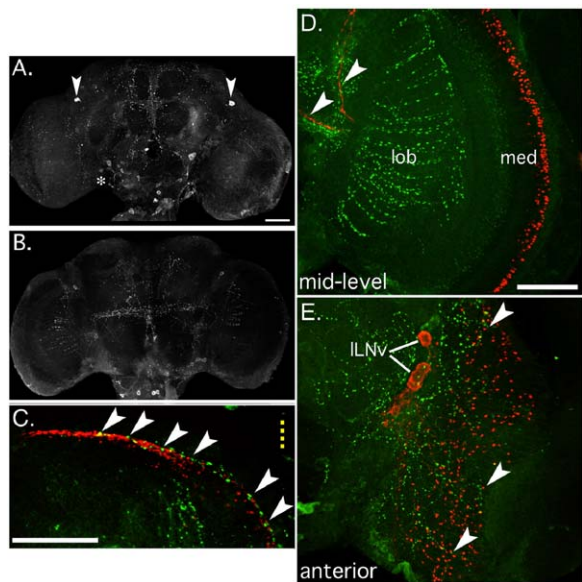
All mutant stocks tested could entrain to a 12:12 LD regimen (Figure S2). The LD phenotype of *pdfr* mutants in this study did not resemble the *pdf* null allele in severity. Under constant conditions, various mutant stocks of the *P1* and *P2* insertion groups displayed slight to moderate increases in the percentage of arrhythmic (AR) flies as measured by periodogram analysis of locomotor assays (Table S1). *P1* homozygotes produced approximately 37% AR, which by deficiency mapping could be attributed to the *CG13758* gene locus. Because the relevant deficiency stock (*Df(X)5990-3A1-3C3-4*) also uncovers the *period* gene, we observed a tendency for increases in period during constant conditions in flies heterozygous for the deficiency. Likewise, the *P2* insertion produced increased AR as a homozygote and also in *trans* to the *Df(X)5990* chromosome. The control revertant *P2-2* displayed low power, but a normal rhythmic percentage. Notably, two of the four *P2* deletion lines displayed high AR percentages (Table S1). Among the four *P2* deletion stocks, there was excellent correspondence in the severity of phenotypes: *P2-1* and *P2-36*, which were clearly aberrant in geotaxis assays (Table S1; Figure 2), also displayed the largest AR percentages in locomotor assays. Changes in period length were not correlated with mutation of the *pdfr* locus. Thus, diverse mutations associated within the *pdfr* locus produced consistent phenotypes in each of the two relevant behavioral assays.

### Geotaxis

*P1* homozygous flies displayed a strong negative geotaxis phenotype, as severe as that of *pdf* mutant flies (Figure 2). *P1* heterozygous flies were normal, but transheterozygotes with a *pdf* null allele (i.e., heterozygous for each locus) displayed aberrant negative geotaxis. The *P1-14* control line produced normal behavior (Figure 2). *P2* homozygous flies displayed normal geotactic responses, as did the *P2* derivative control line (*P2-2*) (Figure 2). However, derivative fly stocks bearing small deletions of the *P2* insertion site mutated to display similar aberrant geotactic behavior. As with *P1* homozygotes, testing *P2* mutant alleles as transheterozygotes with *pdf* null alleles revealed significant genetic interaction: While the control revertant allele (*P2-2*) in *trans* to *pdf*<sup>01</sup> displayed normal behavior, the deletion revertant (*P2-36*) in *trans* to *pdf*<sup>01</sup> displayed severe negative geotaxis (Figure 2).

### Expression of the *pdfr*

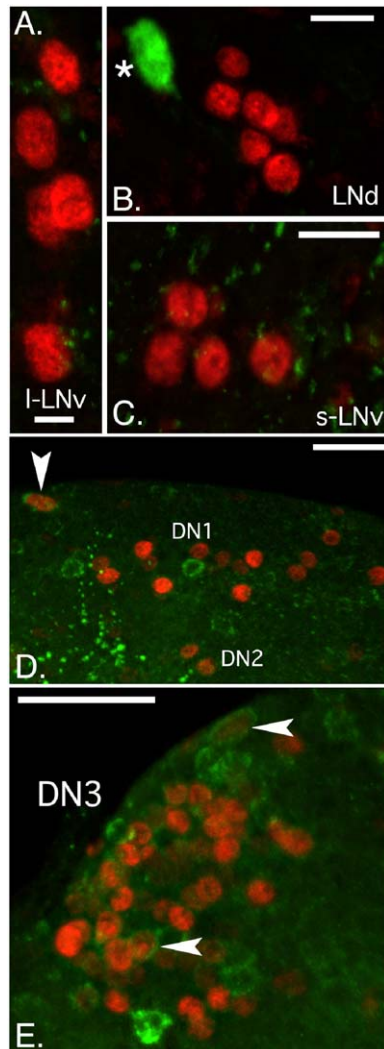
An antiserum against the final 20 amino acids of the predicted C terminus was used to establish sites of PDFR expression within the adult brain. The specificity of the antiserum was tested both in vitro and in vivo, as described in the Supplemental Data. In the wild-type adult brain, the most prominent PDFR immunosignals revealed a large cell body in the dorsal-lateral protocerebrum (Figures 3A, 3B, and 4B). Roughly 20 neuronal cell bodies were stained in the anterior and medial subesophageal ganglion (SEG) (Figure 3A). In addition, scores of more weakly stained soma were detected in all regions of the brain, especially along the superficial



**Figure 3.** An Overview of PDFR Immunosignals in the Adult Brain (A) Montage of PDFR immunosignals in a whole brain imaged through the anterior surface. A single cell body is intensely stained in the anterior lateral protocerebrum (arrowheads) as are cell bodies in the tritocerebrum and SEG (asterisk). Z depth = 100  $\mu\text{m}$ . (B) Montage of PDFR immunosignals in a whole-mount section of brain imaged through the posterior surface. Z depth = 100  $\mu\text{m}$ . (C) The dorsalmost segment of the small LNV projection, (anti-proPDF, red), is intertwined (arrowheads) with PDFR-positive puncta (green). (D) A Z-stack within the optic lobe of an adult brain costained for PDF (red) and PDFR (green). Note the prominent PDFR expression in the lobula (lob) and within a proximal tangential layer of the medulla (med). PDF expression is limited to the lateralmost aspect of the medulla. Note the proximity of PDFR puncta to the PDF projections emanating from accessory medulla (arrowheads). (E) A Z-stack encompassing the anterior aspect of the optic lobe shown in (D). Arrowheads indicate closely apposed PDF and PDFR puncta. ILNV = large ventrolateral neurons. All scale bars, 50  $\mu\text{m}$ .

aspects of the medulla. Strong staining of neuronal processes was evident throughout the central brain and optic lobes (Figures 3). These immunosignals were lost when the antibody was preincubated with the immunizing peptide, but were not altered in either the *pdfR P1* or *P2-36* mutant stocks (data not shown).

Throughout the brain, PDF-positive processes were always associated with PDFR-positive processes. For example, the projection of the small LNV neurons was closely associated with PDFR-positive puncta in the dorsal protocerebral neuropil (Figure 3C). Single optical sections revealed that the PDF-positive terminals were closely apposed to PDFR-labeled processes. PDF-positive projections within the median bundle were likewise in proximity to abundant PDFR-positive processes (data not shown). The large PDF-expressing LNV neurons make a broad tangential projection along the distal medulla (Figures 3D and 3E). Notably, numerous PDFR-stained cells and processes were evident in areas of the medulla and lobula that lacked PDF-stained processes (Figure 3D). Along the lateral aspect of the medulla, we saw little evidence of receptor processes immediately adjacent to the tangential PDF projection (Figure 3D) except in the anterior aspect (Figure 3E).



**Figure 4.** Expression of PDFR among Defined Circadian Pacemaker Neurons of the Adult Brain

Pacemakers are identified by anti-PERIOD immunostaining (red), and PDFR is shown in green.

- (A) The large LNVs do not express PDFR but reside near PDFR puncta (11  $\mu\text{m}$  Z-series; scale bar, 5  $\mu\text{m}$ ).  
 (B) The LNDs do not express PDFR, but were always situated near the large PDFR-positive cell body (asterisk) of the lateral protocerebrum. (12  $\mu\text{m}$  Z-series; scale bar, 10  $\mu\text{m}$ ).  
 (C) The small LNVs do not express PDFR, but reside near PDFR puncta (7  $\mu\text{m}$  Z-series; scale bar, 10  $\mu\text{m}$ ).  
 (D) Among the DN1 and DN2 cell groups there is reliable PDFR co-expression in a pair of DN1 neurons (arrowhead) that are adjacent to the dorsal surface of the brain. Note the presence of PER-negative PDFR soma and puncta near the DN1s (27  $\mu\text{m}$  Z-series; scale bar, 20  $\mu\text{m}$ ).  
 (E) Within the DN3 group, arrowheads mark weak but reliable PDFR expression in two or three cells per hemisphere. Note the presence of PER-negative PDFR soma (a 23  $\mu\text{m}$  Z-series; scale bar, 20  $\mu\text{m}$ ).

We asked whether any circadian pacemaker neurons (as assayed through PER immunostaining) coexpressed PDFR. We found receptor expression in only a subset of defined circadian pacemaker neurons (Figure 4). A prominent pair of DN1 neurons was positive for both PER and for PDFR; these two cells were closely abutted to the dorsal surface of the brain and placed anterior to

the other DN1s (Figure 4A;  $n = 8$ ). Two to three DN3 neurons were weakly PDFR immunopositive (Figure 4B;  $n = 8$ ). The DN2 neurons (Figure 4A,  $n = 5$ ), large LNV neurons (Figure 4C;  $n = 5$ ), LND neurons (Figure 4D;  $n = 5$ ) and small LNV neurons (Figure 4E;  $n = 5$ ) all lacked PDFR immunosignals. PDFR immunosignals did not vary diurnally (data not shown). *pdfr* mRNA did not exhibit diurnal or circadian variation according to previous results of RNA profiling (Lin et al., 2002); instead, it was regulated at a steady-state level by *per*.

## Discussion

Our evidence indicates that, in *Drosophila*, PDF signals via a Class II GPCR that is most closely related to the calcitonin-CGRP receptor family. The  $EC_{50}$  of  $\sim 25$  nM measured in this study is likely an overestimate that reflects the heterologous expression system that we employed. PDFR signaling properties in vitro parallel published accounts of PDF actions in vivo. PDF elicits increases in cAMP in vivo (Nery and Castrucci, 1997), and we found that the PDFR appears coupled to  $G_s$  in HEK293 cells. Also, genetic analysis showed that the NF1 protein operates downstream of PDF to support circadian output (Williams et al., 2001). Similarly, we found that cotransfection of *dNF1* in HEK293 cells greatly increases the efficacy of PDF in producing high-amplitude signaling through the PDFR. This effect is reminiscent of NF1 coupling another Class II peptide GPCR, the PACAP receptor (PAC 1), to adenylate cyclase (Dasgupta et al., 2003). While Class II peptide GPCRs typically couple to  $G_s$ , many Class II receptors also signal via calcium (reviewed by Mayo et al., 2003), including CGRP receptors (Burns et al., 2004) and VPAC receptors (DeHaven and Cuevas, 2004). Similarly, we have found that PDFR signaling also increased calcium levels, albeit with a much higher  $EC_{50}$  value in comparison with the effect on cAMP levels (cf. Johnson et al., 2004). In all, these data provide a basis for future evaluation of PDF receptor properties in situ.

While PDF-related peptide and DNA sequences appear to be restricted to invertebrate lineages (Taghert, 2001), its receptor (CG13758) is clearly related to certain mammalian receptors (Hewes and Taghert, 2001). These observations suggest that there is conservation of the PDF signaling pathway between arthropods and chordates. PDFR is a Class II peptide GPCR, and other members of this category (e.g., PACAP and VIP receptors) exert profound influences in the mammalian circadian system (e.g., Aton et al., 2005; Harmar et al., 2002). In some respects, the functional roles of PDF in the fly circadian system and VIP in the mouse are parallel. Both peptides are required for the normal display of behavioral rhythms in constant conditions—producing short period rhythms or arrhythmicity—and both affect the rhythmicity of cellular pacemaking (VIP: Harmar et al., 2002; Aton et al., 2005; PDF: Peng et al., 2003; Klarsfeld et al., 2004; Lin et al., 2004). Among the mammalian Class II GPCRs, PDFR is more closely related to calcitonin and CGRP receptors than to either PACAP or VIP receptors (Hewes and Taghert, 2001). CGRP immunosignals (Park et al., 1993) and CGRP binding sites have been measured in the SCN (Skofitsch and Jacobowitz, 1985). Nevertheless, the functional analogies of

PDFR-like signaling to CGRP-R-like signaling may be limited, as RCP did not affect PDFR coupling in our experiments. It is notable that both *Drosophila* PDFR and mammalian VPAC receptors respond to PACAP peptides. In fact, PDFR was activated by PDF and PACAP-38, also by the peptides calcitonin, adrenomedullin, and a *Drosophila* ortholog of calcitonin called  $DH_{31}$ . Among these, PDF is clearly the most potent ligand and produces the strongest secondary signals.  $DH_{31}$  activates a separate *Drosophila* Class II GPCR called CG17415, a receptor that is not sensitive to PDF (Johnson et al., 2005). We propose that the PDF receptor displays partial agonism by diverse ligands, which is a common feature among Class II peptide GPCRs (reviewed by Hay et al., 2004). For example, VPAC receptors demonstrate high-affinity interactions with VIP and PACAP and, to lesser extents, with other naturally occurring peptides such as GRF and secretin (e.g., Usdin et al., 1994). PACAP-38 has several physiological effects in *Drosophila* tissues (e.g., Zhong and Pena, 1995). Whether PDFR also represents an endogenous PACAP receptor in vivo is now open to investigation.

To what extent can the properties of PDFR explain the in vivo behavioral signaling controlled by PDF? We tested four of the five *Drosophila* Class II GPCRs and found that CG13758 alone displays sensitivity to PDF. The one untested Class II GPCR is CG12370, and, on the basis of its strong sequence similarity to CG8422 (Hewes and Taghert, 2001), it likely encodes a CRF receptor-related receptor that is sensitive to the peptide  $DH_{44}$  (cf. Johnson et al., 2004). The genetic analysis to date does not allow us to exclude the contribution of other (potential) PDFRs to the regulation of circadian rhythmic behavior. However, the results clearly indicate that PDFR is primarily responsible for PDF signaling underlying the modulation of the *Drosophila* geotactic behavioral response. Two results underscore this point. *pdfr* alleles produced a geotactic phenotype as severe as that displayed by *pdf* mutant flies. Also, flies transheterozygous for *pdf* and either of two distinct *pdfr* mutations displayed a strong mutant phenotype, while individual heterozygotes were not distinguished from controls. Together, these data clearly link the actions of *pdf* and *pdfr* within the same physiological pathway. The simplest hypothesis to explain our results is that PDFR is the primary receptor for PDF in the context of geotactic behavior. Why do certain alleles of *pdfr* display a strong geotactic phenotype, but not a strong locomotor phenotype? Those results are consistent with published properties of the *pdf* mutant flies indicating that the geotaxis assay is sensitive to small increments of PDF signaling (Toma et al., 2002). We propose that such sensitivity may underlie the differential effects that we have measured with receptor mutants. It follows that definition of the complete locomotor phenotype of *pdfr* mutant flies awaits recovery of stronger mutant alleles. Indeed, this prediction is fully met by analysis of a naturally-occurring mutation of the *pdfr* (CG13758) locus that produces a circadian locomotor defect which closely matches that of *pdf* (Lear et al., 2005 [this issue of *Neuron*]). That independent genetic data strongly supports the contention that CG13758 encodes the principle PDF receptor in *Drosophila*.

In general, we found excellent correspondence between PDFR-positive processes and PDF-positive processes in diverse brain regions. In the dorsal brain, the trajectory and extent of processes from the small LNv neurons were closely matched by receptor-positive processes. Likewise, receptor processes were closely intermingled with PDF-positive varicosities in the anterior medulla and the median bundle. By contrast, many receptor-positive processes were tens of microns away from the closest PDF-positive processes. These distances do not necessarily preclude physiological interactions between PDF and PDFR, as indicated by previous studies of “receptor mismatches” (e.g., Ruocco et al., 2001). These and other examples support the concept of volume transmission (Nicholson and Sykova, 1998), which refers to the diffusion of bioactive substances across considerable distances via the extracellular space. Given these antecedents and based on the proximity of receptor immunosignals to PDF signals in several areas, we propose that the pattern of PDFR expression is consistent with a role in mediating PDF signaling throughout the brain and optic lobes. An additional and nonexclusive hypothesis is that PDFR displays high-affinity interactions with more than one ligand (analogous to mammalian VPAC receptors), and this possibility is supported by its partial agonism that we observed in vitro.

PDF is a synchronizing factor that sustains or delays molecular oscillations within pacemaker neurons, including oscillations within the pacemakers that release PDF (e.g., Peng et al., 2003; Lin et al., 2004; Schneider and Stengl, 2005). However, the degree to which PDF acts directly or indirectly on pacemaker cells remains uncertain. Our results of studying PDFR-like immunoreactivity do not support the hypothesis of broad, direct PDF action on pacemaker neurons. Among the ~150 brain pacemakers, PDFR immunosignals were only expressed by a pair of DN1s and more weakly by two to three scattered DN3s. The antibody could detect PDFR in most pacemaker cells when the protein was overexpressed (Figure S4), but not in native tissue. These results argue that, normally, most pacemaker neurons contain very low amounts of the receptor. Hence, we suggest that the predominant influence of PDF on the synchronization of circadian pacemaker neurons proceeds via indirect neuronal connections. Specifically, these results focus attention on the prominent pair of PDFR-positive DN1 cells as potentially critical relay neurons within the circadian pacemaker network.

In summary, the identification of a PDF receptor provides the basis for addressing PDF functions in a cellular context. Its sites of expression define potential sites of PDF actions. Its signaling properties will illuminate the mechanisms by which PDF modifies geotactic behavior and helps organize daily locomotor rhythms.

## Experimental Procedures

### Receptor Assays

We generated full-length constructs of *CG13758* for functional expression in HEK293 cells and measured changes in calcium levels and cAMP levels as previously described (Meeusen et al., 2002; Johnson et al., 2003; Supplemental Data).

### Flies

*Drosophila* were raised on a cornmeal agar diet supplemented with yeast at 24.5°C. The two *CG13758* single insertion P element stocks were: *P{EY11851}* (here called *P1*), inserted upstream of the transcription start site and *P{BG00979}* (here called *P2*), located ~2 kB downstream of the *CG13758* termination codon. See the Supplemental Data for specifics of P element mobilization and generation of *UAS-pdfR* transgenic flies.

### Quantitative PCR

Adult head mRNA (1 µg) from the stocks reported in the Results sections were used to generate cDNA with reagents according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). qPCR reactions were performed according to kit instructions (Applied Biosystems, Foster City, CA) using *RP49* as a control. The experiment was repeated three times with separate RNA purifications (Qiagen, Valencia, CA).

### Western Blot Analysis

Adults were heat-shocked, and blots were prepared and probed as described in the Supplemental Data.

### Behavioral Analyses

Assays of geotactic bias were performed following procedures in Toma et al. (2002). Males or virgin females were collected over CO<sub>2</sub> within 2 to 6 days of eclosion, left to recover overnight, and then tested in groups of 75 without further anesthesia in nine-position mazes. Flies were placed in the entry port to the maze for 10 min and then allowed to enter the maze under dim light at room temperature with a bright white-light source placed within 2 ft of the collection tubes. Vertical position was scored within 30 min. Each genotype was tested at least five times. The mazes were cleaned with dH<sub>2</sub>O and air-dried between runs. Locomotor activity was analyzed as previously described (Renn et al., 1999; Lin et al., 2004). Flies were entrained for 6 to 7 days in 12 hr L:D cycles at ~3 × 10<sup>14</sup> W/cm<sup>2</sup>; light was measured using a calibrated photodetector (LI-COR, LI-250 with Quantum sensor). Behavior was then analyzed for 9 days under constant dark conditions. Arrhythmic flies were scored according to periodogram analysis. Periods were compared by one-way analysis of variance (ANOVA), using InStat software (GraphPad, San Diego).

### Immunocytochemistry

Immunocytochemistry was performed as previously described (Lin et al., 2004). Specific details and details of antibody generation are found in the Supplemental Data.

### Supplemental Data

Supplemental Data include four figures, two tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at <http://www.neuron.org/cgi/content/full/48/2/213/DC1/>.

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