

A G Protein-Coupled Receptor, *groom-of-PDF*, Is Required for PDF Neuron Action in Circadian Behavior

Report

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Summary

The neuropeptide Pigment-Dispersing Factor (PDF) plays a critical role in mediating circadian control of behavior in *Drosophila*. Here we identify mutants (*groom-of-PDF*; *gop*) that display phase-advanced evening activity and poor free-running rhythmicity, phenocopying *pdf* mutants. In *gop* mutants, a spontaneous retrotransposon disrupts a coding exon of a G protein-coupled receptor, CG13758. Disruption of the receptor is accompanied by phase-advanced oscillations of the core clock protein PERIOD. Moreover, effects on circadian timing induced by perturbation of PDF neurons require *gop*. Yet PDF oscillations themselves remain robust in *gop* mutants, suggesting that GOP acts downstream of PDF. *gop* is expressed most strongly in the dorsal brain in regions that lie in proximity to PDF-containing nerve terminals. Taken together, these studies implicate GOP as a PDF receptor in *Drosophila*.

Introduction

Circadian pacemakers are instrumental in orchestrating daily rhythms of behavior (Hendricks and Sehgal, 2004). Genetic studies in a range of organisms have revealed transcriptional feedback loops as a shared mechanism of divergent clocks. In the fruit fly, the CLOCK (CLK) and CYCLE (CYC) transcription factors activate transcription of *period* (*per*), *timeless* (*tim*), *Par domain protein 1* (*Pdp1*), and *vri* (*vri*; Hardin, 2004). The encoded proteins PER and TIM feed back to regulate CLK/CYC activation, while PDP1 and VRI regulate *Clk* transcription. Multiple components of this system are modified posttranslationally by kinases (*doubletime* [*dbt*], *CK2*, and *shaggy* [*sgg*]) and a phosphatase (PP2A), regulating the frequency of daily oscillations (Harms et al., 2004). Circadian function is evident by the rhythmic expression and phosphorylation of multiple clock components.

Discrete neural circuits transform these molecular rhythms into behavioral rhythms (Helfrich-Forster, 2003). Circadian pacemaker neurons can be roughly divided into ventral lateral (LNV), dorsal lateral (LND), and dorsal neuron (DN) groups. Of these, the LNV, especially the small subgroup (sLNV) is especially prominent in driving circadian rhythms. *disco* mutants lack both free-running rhythms (in constant darkness [DD]) and, usually, the LNV (Dushay et al., 1989; Helfrich-Forster and Homberg, 1993). In rare *disco* flies that retain some LNVs, a single LNV can be sufficient for behavioral

rhythms (Helfrich-Forster, 1998). LNV ablation also substantially reduces free-running rhythmicity (Renn et al., 1999). Indeed, the small LNVs demonstrate robust core clock oscillations in the first 2 days of constant darkness in contrast to their larger counterparts (Shafer et al., 2002; Yang and Sehgal, 2001).

A critical mediator of LNV function is the neuropeptide Pigment-Dispersing Factor (PDF). In the adult brain, PDF is expressed exclusively in the ventral lateral neurons as well as tritocerebral neurons (Renn et al., 1999). Genetic inactivation of *pdf* (*pdf*⁰¹) results in both diurnal and circadian phenotypes. Under light:dark (LD) conditions, wild-type flies display peaks of activity in anticipation of “lights on” (morning peak) and in anticipation of “lights off” (evening peak). The sLNVs appear to be important for morning anticipation while the LNDs are essential for evening anticipation (Grima et al., 2004; Stoleru et al., 2004). In *pdf*⁰¹ mutants, morning anticipation behavior is reduced and the evening peak is phase advanced. In constant darkness (DD), *pdf*⁰¹ mutants display period shortening upon release into DD, dampening of behavioral rhythms, and ultimately arrhythmicity several days into DD (Renn et al., 1999). PDF levels in the dorsal terminals of the small LNV are also clock-regulated (Park et al., 2000). Loss of PDF results in altered PER and *tim* rhythms, indicating that PDF must feed back to regulate the core molecular oscillator in both LNVs (LNV and LND) as well as DN1s (Klarsfeld et al., 2004; Lin et al., 2004; Peng et al., 2003). Yet little is known about the target receptors/molecules important for PDF signaling (e.g., the PDF receptor) in the pacemaker neural network.

PDF has also been implicated in the circadian control of rest/activity centers in the brain. The finding of intact PER rhythms in the context of largely arrhythmic behavior suggests a role for PDF in circadian output (Lin et al., 2004). One possibility is that PDF impacts other pacemaker neurons, e.g., dorsal neurons, which, in turn, relay to activity centers (Klarsfeld et al., 2004). Alternatively, PDF may be released directly on these centers, such as the neurosecretory cells of the pars intercerebralis (PI) that lie near pacemaker nerve terminals (Jaramillo et al., 2004; Kaneko and Hall, 2000). PDF-dependent rhythmic phosphorylation of MAP kinase has also been observed in the dorsal brain (Williams et al., 2001). Consistent with this hypothesis, ectopic PDF expression in the dorsal central brain, but not elsewhere, induces behavioral alterations (Helfrich-Forster et al., 2000). Indeed, PDF may even act as a hormone influencing more distant targets in the body to control behavior. Brain transplantation experiments have implicated such humoral factors in circadian behavior (Handler and Konopka, 1979). Regardless of the anatomic locus of action, this neuropeptide most likely functions by binding a G protein-coupled receptor (Hewes and Taghert, 2001). Here, we identify a mutant in a G protein-coupled receptor, *groom-of-PDF* (*gop*), that closely mimics the diurnal and circadian phenotypes of a null allele of *pdf*. We propose that GOP encodes a receptor essential for PDF action in circadian behavior.

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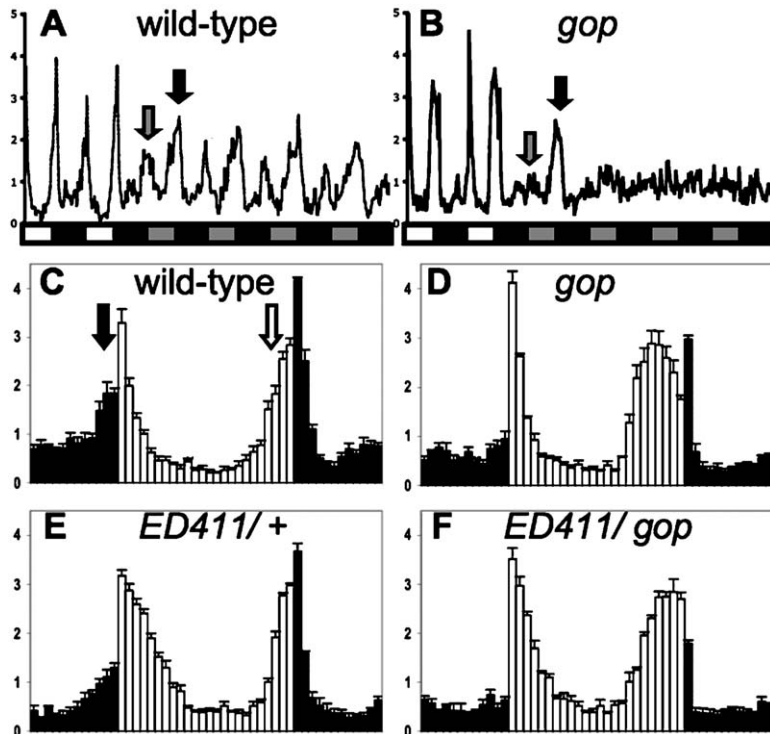


Figure 1. Circadian Behavior of the Mutant *groom-of-PDF*

(A and B) Locomotor activity profiles for (A) wild-type ($n = 26$) and (B) *gop* mutant ($n = 51$) fly populations during 12 h light:12 h dark conditions (LD) followed by constant darkness (DD). White and black boxes indicate light and dark phases, respectively, while gray boxes indicate subjective day during DD. The gray arrow indicates morning peak, and the black arrow indicates evening peak on the first day of DD.

(C–F) Normalized activity profiles for fly populations during diurnal conditions ($n = 27$ – 52). Light bars indicate activity during the light phase and dark bars indicate activity during the dark phase. The black arrow indicates morning anticipation activity, and the white arrow indicates evening anticipation activity. Error bars represent the SEM. (C and D) Wild-type and outcrossed *gop* males. (E and F) *Df(1)ED411* heterozygous females. *Df(1)ED411* deletes 3A3–3A8, including CG13758.

Results

A Mutant *groom-of-PDF* Phenocopies *pdf⁰¹* in Diurnal and Circadian Rhythms

While testing circadian behavior of various *Drosophila* ion channel mutants, we identified an inversion allele of the *ether-a-go-go* potassium channel (*eag^{sc29}*) with a phenotype very similar to that of *pdf⁰¹* mutants (Drysdale et al., 1991). Based on this similarity, we refer to this mutant as *groom-of-PDF* (*gop*). In DD conditions, these flies display reduced rhythmicity (Figure S1, and Table S1 in the Supplemental Data available with this article online). A day-by-day analysis reveals a dampening of rhythmicity upon release into constant darkness, with a reduced morning peak and an evening activity peak (black arrow, Figures 1A and 1B) clearly evident only on the first day of DD. Like *pdf⁰¹* mutants, *gop* mutants display a loss of morning anticipation (black arrow) as well as a phase advance of the evening activity peak in LD (open arrow, Figures 1C and 1D).

The mutant phenotype is recessive and maps to the X chromosome, as the male progeny of out-crossed virgin *gop eag^{sc29}* females retain LD and DD phenotypes, but heterozygous females are essentially wild-type (Table S1 and data not shown). Deletions that fail to complement *eag* (*Df(1)RK2*, *Df(1)RK3*, and *Df(1)RK4*) complement *gop eag^{sc29}* (Table S1, Figure S2B, and data not shown). The inversion allele of *eag* also disrupts *scute* (*sc*). *gop eag^{sc29}* is also complemented by a *sc* allele (*In(1)sc⁷*), suggesting that the phenotype is due to a disruption of another gene (Table S1 and Figure S2F).

A G Protein-Coupled Receptor Is Disrupted in *gop*

To map *gop*, we focused on deficiencies that uncover G protein-coupled receptors on the X chromosome. One

deficiency *Df(1)ED411* (breakpoints 3A3; 3A8) that deletes the peptide GPCR, CG13758, failed to complement *gop* (Table S1, Figures 1E and 1F; Hewes and Taghert, 2001; Ryder et al., 2004). Of note, this region includes the circadian rhythm gene *sgg*. A second deletion, *Df(1)TEM7* (3A3; 3B3) that removes both *sgg* and *per* also fails to complement *gop*, although it had poor rhythmicity as a heterozygote, perhaps due to the compound loss of clock genes (Table S1; Figures S2C and S2G). Importantly, a deficiency *Df(1)Sp2f-3A* (2F2; 3A6) that complements *sgg* also fails to complement the LD and DD phenotypes of *gop* (Table S1; Figures S2D and S2H; FlyBase Consortium, 2002). Based on complementation analysis in combination with published molecular characterization and complementation data, the critical interval to which the *gop* mutant maps includes only six genes (in genomic order): CG32796, *trol*, CG13758, CG8310, CG13759, and CG13760 (FlyBase Consortium, 2002). Given the similarity of *gop* to the *pdf* phenotype, we focused on the peptide GPCR gene CG13758.

To assess whether CG13758 transcripts were disrupted in *gop* mutants, we performed RT-PCR. Amplifying overlapping regions of CG13758, RT-PCR produced a doublet in *gop* mutants, but only a singlet in wild-type (Figure 2A). To determine the molecular nature of the aberration, we sequenced cloned PCR products and identified two main transcripts among seven clones. Product A contained an 8 bp deletion and a substitution of T for A at the 3' end of exon 3 (Figure 2B). Product B contained two products with 205 (one clone; B) or 210 (three clones; B') bp insertions at this same site of exon 3 and splices into exon 4. Conceptual translation of these mutant products indicated that they would translate in-frame stops soon after these

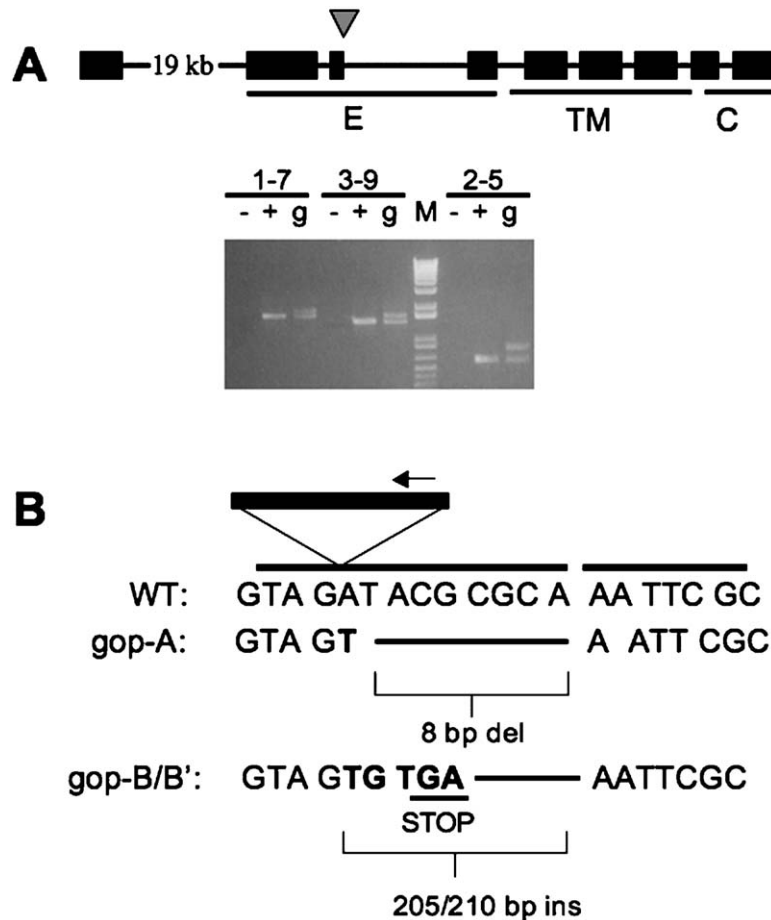


Figure 2. CG13758 Is Disrupted in *gop* Mutants

(A) RT-PCR analysis of CG13758 transcripts. (Top panel) Exon/intron structure of CG13758, with exons indicated in boxes. The triangle indicates locus of retrotransposon insertion in exon 3. E, exons encoding putative extracellular domain; TM, seven transmembrane domains; C, cytoplasmic domain. (Bottom panel) Exon numbers of forward and reverse primers indicated at top of each section. The minus symbol (–) indicates *yw* RNA, no reverse transcriptase control; the plus symbol (+), *yw* RNA; g, *gop* mutant RNA; M, 1 Kb plus ladder (Invitrogen).

(B) Sequence analysis of *gop* mutants. WT: the wild-type transcript sequence at exon 3/exon 4 junction is indicated by black lines above the sequence. Triplets correspond to coding frame. *gop*-A corresponds to one *gop* transcript with 8 bp del. *gop*-B/B' refers to transcripts with ~200 bp insertion. The in-frame stop codon is indicated by TGA/STOP. Bases in bold indicate inserted sequences. Black bar above wild-type indicates the genomic insertion site of retrotransposon 412, with the transcriptional orientation indicated by the arrow.

sequence alterations (Figure 3B). The insertion sequence closely matches that of the long terminal repeat of the 412 retrotransposon (Will et al., 1981). We confirmed insertion of the retrotransposon in antisense orientation by sequencing the insertion breakpoints, identifying a direct target site repeat GTAG at each end (data not shown). Alignment of the retrotransposon sequence to *gop* mutant transcripts indicates that the transcripts are derived from differential splicing (data not shown).

This genomic insertion disrupts the sequence coding for the extracellular domain of this neuropeptide receptor. CG13758 is a class B (or class II) peptide GPCR consisting of an extracellular domain, a seven transmembrane domain, and a cytoplasmic domain (denoted by E, TM, and C, respectively in Figure 2A). This insertion disrupts transcripts from this locus such that few or no wild-type receptors are produced. Based on the complementation data and the strong molecular lesion, we conclude that this disruption is responsible for the behavioral phenotype of *gop* mutants and hereafter refer to the CG13758 gene as *gop*.

Core Clock Function Is Altered in *gop* Mutants

Previous studies of PDF function have identified a key role for this neuropeptide in regulating core clock function (Klarsfeld et al., 2004; Lin et al., 2004; Peng et al., 2003). Phase-advanced evening activity rhythms in LD,

as well as short period rhythms in DD, are accompanied by phase-advanced PER oscillations in core clock neurons, suggesting PDF-mediated feedback (Lin et al., 2004). To address the role of *gop*, we assessed PER oscillations in pacemaker neurons after 6 days of constant darkness, a time period when PDF feedback is evident (Lin et al., 2004). To reduce potential genetic background issues, we compared *Df(1)ED411/+* with *Df(1)ED411/gop* females. We find that PER oscillations in levels are phase advanced for the *gop* flies in two key groups of pacemaker neurons, the small LNvs and the LNds (Figures S3A and S3B). We also observed phase dispersal of PER nuclear localization in the small LNvs of *gop* mutants (Figures S3C and S3D). These changes in PER oscillations are reminiscent of those in *pdf⁰¹* (Lin et al., 2004). We also observed a reduction in staining in the DN1s, putative targets of PDF-expressing neurons, due to a few strongly staining cells in wild-type that are not observed in the *gop* mutants (Figures S3E and S3F; Klarsfeld et al., 2004). These data indicate that, similar to PDF, *gop* plays an important role in regulating the core oscillator.

gop Is Required for PDF Neuron Action in Circadian Timing

To assess the role of *gop* in PDF neuronal function, we also selectively manipulated the core clock in PDF neurons and assessed the consequences for circadian

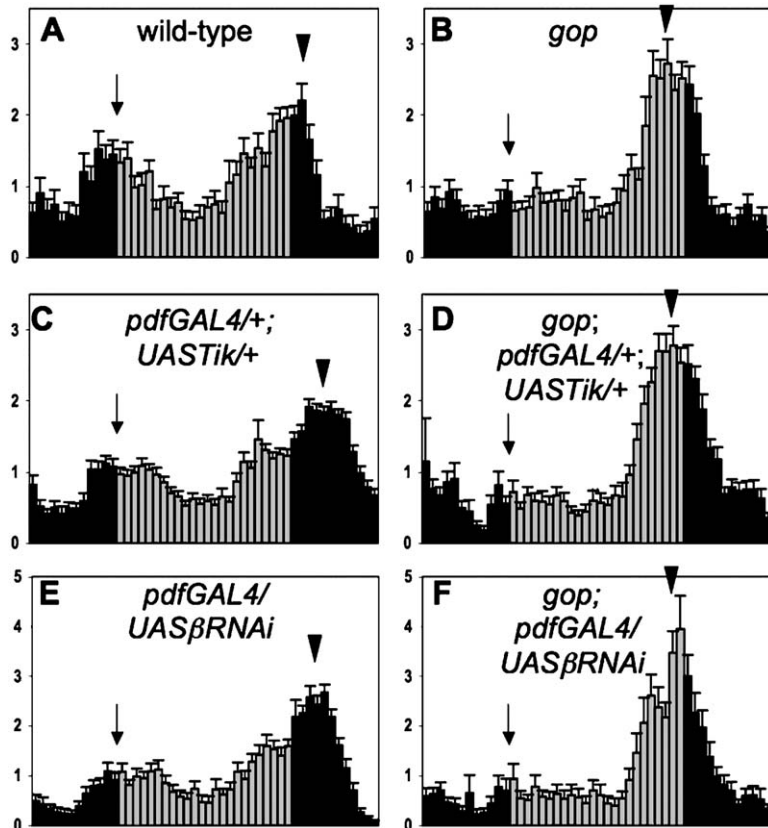


Figure 3. *gop* Is Required for PDF Neuronal Effects on Circadian Phase

(A–F) Normalized activity profiles for fly populations on the first day of DD ($n = 27–72$). Gray bars indicate subjective day and dark bars indicate subjective night. The black arrow indicates location of morning peak, and the black triangle indicates evening peak. (E and F) UAS- β RNAi refers to a UAS-RNAi construct targeting CK2- β . Error bars represent the SEM.

phase. We then determined whether the circadian effects of perturbing PDF neuronal functions required *gop*. Since *gop* mutants are poorly rhythmic, we assayed behavioral phase on the first day of DD, when circadian function is still evident in *gop* mutant flies (Figures 1A and 1B). To impact core clock function, we utilized reagents that impair CK2 activity and drove them specifically in PDF neurons using *pdf-Gal4*. We expressed the dominant negative *Timekeeper* (*Tik*) allele of the catalytic subunit of CK2 (CK2- α) or a double-stranded hairpin RNA targeting the regulatory subunit of CK2 (CK2- β ; β RNAi) under control of the GAL4 UAS (Lin et al., 2002). It has been shown previously that reductions of CK2 function through mutation of α or β subunits results in lengthened periods (Akten et al., 2003; Lin et al., 2002). We show here that targeted expression of UAS-*Tik* or UAS- β RNAi to PDF neurons results in a phase delay in circadian behavior on the first day of DD, consistent with period lengthening effects (Figures 3A, 3C, and 3E, data not shown). In contrast, *gop* flies exhibit an evening activity peak phase comparable to or perhaps advanced in comparison with that of wild-type (Figure 3B). When we assayed behavior in the double mutant flies (e.g., *gop*; *pdf-Gal4/+*; UAS-*Tik/+*), we found that these flies display a behavioral phase comparable to that of *gop*, i.e., early phase, but distinct from that of *GAL4/UAS-Tik* or *pdf-Gal4/UAS- β RNAi* (Figures 3D and 3F). These studies indicate that the effects of *gop* are epistatic to manipulation of PDF neurons by CK2. *gop* is thus required for these effects, suggesting that GOP acts downstream of PDF neurons to control circadian behavior.

gop Is Expressed in Putative PDF Target Areas

Given the similarity of *gop* to *pdf⁰¹* mutants, we wanted to determine if PDF expression and cycling are still intact in *gop* mutants. Using *gop/Df(1)ED411* flies, we assessed PDF levels in the small LN_v terminals and found high-amplitude cycling in these terminals (Figures 4A and 4B). Taken together with our genetic epistasis experiments (Figure 3), these data suggest that *gop* does not affect PDF expression or metabolism, but rather acts downstream of PDF to influence behavior.

To determine the anatomic locus of *gop* action, we also assessed *gop* transcript using real-time quantitative RT-PCR. We did not observe any significant diurnal rhythm using head RNA (data not shown). Of note, we found that the *gop* transcript is approximately 10-fold more concentrated in the head than in the body (head: body ratio = 11.8 ± 1.6 [mean \pm SEM], $n = 2$). However, given that the body produces about 3-fold more total RNA per fly, these data suggest that approximately one-fifth of the *gop* transcript may be present in the body.

Given the strong head expression of *gop* RNA, we assessed its distribution in whole-mount adult brains, using fluorescent in situ hybridization (Zhao et al., 2003). We observed the most prominent, reproducible, and anti-sense probe-specific signals from two areas of the dorsal brain: in a region near to or including the DN1 pacemaker neurons and in the region of the pars intercerebralis (PI), a cluster of neurosecretory cells in the dorsal midline that project into the median bundle (Figure 4C). Both of these cell groups have been implicated as key targets of PDF neurons (Jaramillo et al., 2004;

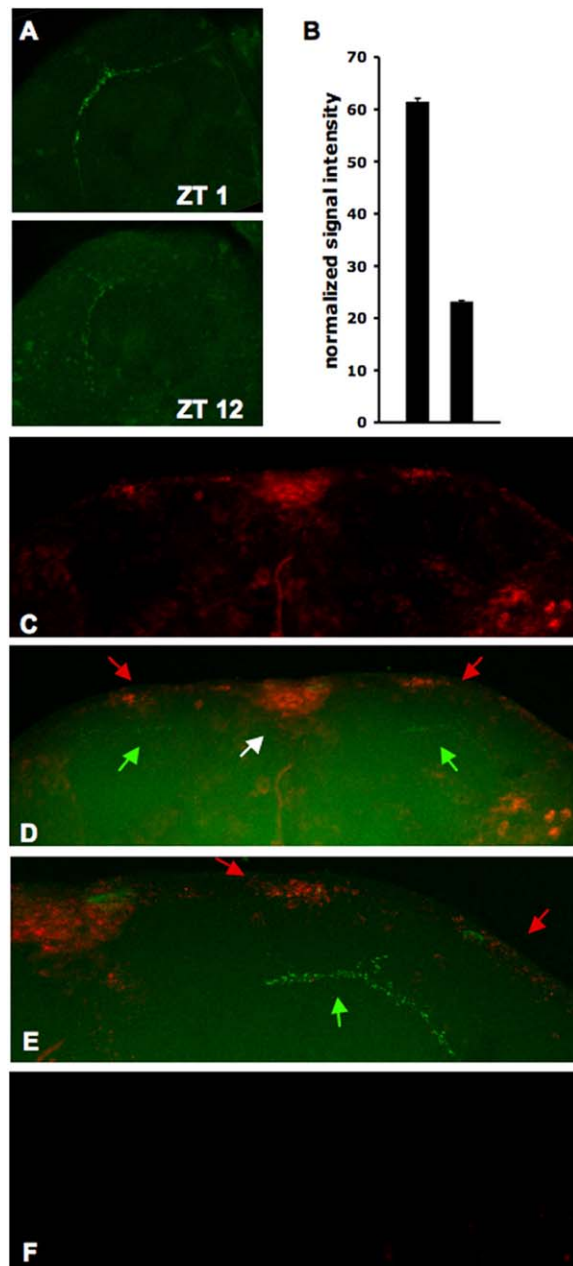


Figure 4. PDF and *gop* Expression

(A) PDF cycles in *gop* mutants. Maximum projections are shown for confocal sections encompassing the dorsal projections of the LNvs, as labeled by antisera to PDF. The panels show representative samples of *gop/ED411* adult brains at two time points (ZT 1 and ZT 12 [ZT, zeitgeber time]).

(B) Quantitation of average intensity of the LNV dorsal projections from a single experiment ($n = 16$ hemispheres). Error bars represent \pm SEM. Similar profiles were observed in three additional experiments (data not shown).

(C) Fluorescent in situ hybridization of *gop* antisense probe (red).

(D) Double labeling with PDF immunofluorescence (green). The white arrow indicates the pars intercerebralis region; red arrows indicate dorsal neuron regions; and green arrows indicate LNV dorsal projections.

(E) Higher magnification image of (D) to more easily visualize PDF projections.

(F) Sense probe.

Kaneko and Hall, 2000; Klarsfeld et al., 2004). We performed double labeling with anti-PDF antibodies and found that these *gop*-expressing cells (like DN1s) lie in proximity to the dorsally projecting terminals of the small LNvs (Figures 4D and 4E). While PDF and GOP cells do not appear to be synaptically connected, prevailing evidence suggests that PDF is able to act as a local hormone diffusing to more distant targets in the brain (Helfrich-Forster et al., 2000; Jaramillo et al., 2004). Thus, these expression data suggest potential explanations for the effects of *gop* on the circadian clock through its putative DN expression as well as its role in circadian output through expression in the PI.

Discussion

Here, we identify a mutant in a G protein-coupled receptor, *groom-of-PDF*, so named due to its striking phenotypic similarity to mutants in the neuropeptide gene, *pdf*. In light:dark cycles, both *pdf* and *gop* mutants display reduced or absent anticipation of the morning “lights on” transition and a phase advance of the evening activity peak. In addition, both mutants display free-running rhythms in constant darkness that decay over time. Both mutants also exhibit phase-advanced molecular rhythms after several days in constant darkness. The striking similarity between *gop* and *pdf* mutants strongly suggests that these two genes operate in a discrete pathway. Interestingly, both *pdf* and *gop* mutants were discovered as spontaneous mutations (Renn et al., 1999).

We have abundant evidence that the gene mutated in the *gop* mutant is the peptide GPCR CG13758. Complementation testing maps the *gop* phenotype away from other mutations on its X chromosome and to an area of 3A region containing just six candidate genes including CG13758. CG13758 transcripts are severely disrupted by a retrotransposon insertion in the third exon corresponding to a portion of the N-terminal extracellular domain. None of the mutant cDNAs analyzed would produce a wild-type full-length receptor. These data strongly suggest that the disruption of CG13758 is largely responsible for the *gop* phenotype.

Our genetic and phenotypic analysis suggests that GOP acts downstream of PDF. First, PDF remains rhythmically expressed in *gop* mutants. Second, alteration of circadian period through manipulation of clock genes in PDF⁺ neurons is blocked in *gop* mutants, indicating that *gop* is required for PDF neuronal effects on circadian phase. Although essential for PDF neuron action, the *gop* transcript is most strongly expressed in cells thought to be the targets of PDF neurons in the dorsal brain, likely including the pars intercerebralis and perhaps the DN1s (Figure 4; Jaramillo et al., 2004; Kaneko, 1998; Klarsfeld et al., 2004). Indeed, we observed changes in PER expression in a subset of DN1s in the dorsal brain (Figure S3). Finally, we have preliminary data that the extracellular domain of GOP can bind PDF using an in vitro assay (Supplemental Experimental Procedures; Figure S4). Moreover, this binding appears to be specific. It is competed by PDF but not by another neuropeptide, proctolin, and significant binding is not observed with an unrelated peptide (Figure S4). Further

studies using broader sets of ligands and quantitative affinity assessments in combination with cell-based signaling studies will be necessary to definitively identify GOP as the PDF receptor. Indeed, in an accompanying report in this issue of *Neuron*, such studies have been performed and the authors have identified CG13758 as a PDF receptor (Mertens et al., 2005). The behavioral phenotype described here suggests that GOP may be the sole PDF receptor in *Drosophila*.

The *gop* expression pattern is also consistent with a role as a PDF receptor. *gop* expression was noted most prominently in the dorsal brain. As previously noted, there is a wealth of evidence (see the Introduction) that PDF release into the dorsal brain mediates circadian behavior. Here, we find *gop* transcript expression in parts of the dorsal brain in the regions of the LNV terminals, including areas near the pacemaker DN1 neurons as well as the pars intercerebralis. We favor the idea that these sites of receptor expression mediate circadian behavior. While we cannot definitively identify these cells as the DN1 group, it is interesting to note that PER expression in a subset of DN1s is altered in *gop* mutants (Figures S3E and S3F), consistent with potential GOP function in these neurons. Nonetheless, cell-specific double-labeling experiments will be necessary to demonstrate clearly *gop* expression in DN1 pacemaker neurons.

Our studies also clearly reveal a role for GOP in feedback onto the core oscillator. Previous studies described binding of biotinylated PDF directly on subsets of LNs and DNs (Peng et al., 2003). We do not observe significant *gop* expression in the LNs yet we find that molecular oscillations in the LNs are altered (Figure S3). We favor the notion that the effects of *gop* on LN oscillations are not cell-autonomous, but instead reflect altered network function. Anatomic studies have defined a potentially reciprocal circuit between LNVs and DN1s (Kaneko and Hall, 2000). Such a circuit may be necessary to reinforce molecular cycling under constant conditions. We propose that altered signaling in the DN1s due to loss of *gop* may modulate feedback onto the LNVs and result in advanced PER cycling in these cells. Consistent with this hypothesis we observed reduced PER staining in a subset of DN1s in *gop* mutants.

CG13758/*gop* encodes a class B GPCR neuropeptide receptor most closely related to calcitonin receptors. Of note, rhythmic calcitonin receptor expression has been observed in the mammalian circadian pacemaker, the suprachiasmatic nucleus (Panda et al., 2002). More compelling is the apparent role of peptidergic class B GPCR signaling in both fly and mammalian circadian clocks. Like *gop* mutants, genetic knockout of the VPAC2 receptor, a class B GPCR that is activated by VIP and pituitary adenylate cyclase-activating peptide (PACAP), results in a loss or alteration of behavioral and molecular rhythms (Harmar et al., 2002). Thus, these studies reveal underlying similarities in clock mechanisms beyond the core transcriptional feedback loops. The identification of a G protein-coupled receptor essential for PDF neuronal action represents an important step toward elucidating the molecular and neural pathways that transform core molecular oscillations into daily behavioral rhythms.

Experimental Procedures

Behavioral Analyses

The activity levels of male progeny (0 to 8 days old) were assayed using Trikinetics Activity Monitors (Waltham, MA). For LD analyses, activity levels from each fly were normalized and averaged over 4 days (Zhao et al., 2003). For DD behavior, χ^2 periodogram analyses were performed using Clocklab (Actimetrics, Evanston, IL). Rhythmic flies were defined as those in which the χ^2 power was ≥ 10 above the significance line. Statistical significance was determined by Student's t test.

Transcript Analysis

Total RNA was obtained from adult *Drosophila* heads, using the Trizol method (Invitrogen), and standard RT-PCR was performed using ThermoScript RT-PCR reagents (Invitrogen). See the Supplemental Experimental Procedures for primer sequences and details of real-time PCR.

PDF Immunohistochemistry

Adult *Drosophila* (0 to 7 days old) were entrained to 3–5 days of LD prior to dissection. Staining was performed as described previously, using Rat anti-PDF (Lin et al., 2002; Park et al., 2000). Samples were imaged using confocal microscopy (Nikon C1). For quantitation, maximum projections of confocal series were subject to blind scoring using Image J (NIH). For each brain hemisphere, the average intensity was determined over the length of the dorsal LNV terminal, with background subtraction. The projection with the highest signal intensity was assigned a value of 100, and remaining samples were normalized to that value. Statistical significance was determined by Student's t test.

Fluorescent In Situ Hybridization

A 2.0 kb fragment of the *gop*/CG13758 cDNA was subcloned in both orientations into pCR2.1 (Invitrogen). Probe generation and in situ hybridization was carried out on *yw* brains essentially as previously described (Zhao et al., 2003), with some exceptions: brains were fixed in 4% paraformaldehyde for 45 min and brains were treated with 10 μ g/ml proteinase K for 30 s prior to hybridization. For double labeling, tissue was reblocked for 15 min in TNB (Molecular Probes TSA protocol) and incubated with rabbit anti-PDF diluted 1:10,000 in TNB for at least 1.5 hr at room temperature, then detected with a fluorescent anti-rabbit secondary antibody at 1:200 (Jackson). Probes were also tested against brains overexpressing *gop* to confirm the specificity and efficacy of the probes to penetrate throughout the whole brain (data not shown). These flies were generated by crossing a UAS-based EP line (EY11851; BDGP) upstream of *gop* to *tim-Gal4*. Hybridization was performed on at least two to five brains per experimental condition and was repeated seven times. Images were collected using confocal microscopy (Nikon C1).

Transgenic Flies

A BglII/XhoI fragment from pET-*Tik* (Lin et al., 2002) was cloned into pUAST to generate transgenic flies. For the pUAS- β RNAi construct, full-length CK2- β (clone RE31047; Invitrogen) was subcloned into pWiz as inverted repeats spaced with a white gene intron (Lee and Carthew, 2003). All constructs were sequenced and injected into *y^{1w}^{67c23}* *Drosophila* embryos (CBRC Transgenic Fly Core; Charlestown, MA).

Supplemental Data

Supplemental data include four figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this report online at <http://www.neuron.org/cgi/content/full/48/2/221/DC1/>.

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