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Pigment-Dispersing Factor Signaling and Circadian Rhythms in Insect Locomotor Activity

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Abstract

Though expressed in relatively few neurons in insect nervous systems, pigment-dispersing factor (PDF) plays many roles in the control of behavior and physiology. PDF's role in circadian timekeeping is its best-understood function and the focus of this review. Here we recount the isolation and characterization of insect PDFs, review the evidence that PDF acts as a circadian clock output factor, and discuss emerging models of how PDF functions within circadian clock neuron network of *Drosophila*, the species in which this peptide's circadian roles are best understood.

Keywords

Neuropeptide; Circadian; Neuromodulation; Pigment Dispersing Factor; Pigment Dispersing Hormone

1.1: Introduction

Pigment-dispersing factors (PDFs) are highly conserved 18-amino acid, α- amidated neuropeptides [1–3]. Though expressed in relatively few neurons in insect nervous systems [4–12], PDF plays many roles in the control of behavior and physiology, including circadian rhythmicity [13], geotaxis [14], sleep and arousal [15–18], copulation [19], flight [20], the modulation of visceral muscle contraction [21] and tracheal growth [22]. PDF's role in circadian timekeeping is its bestunderstood function and the focus of this review.

1.2: Isolation and Identification of PDFs As Clock Output Components

PDFs were first identified based on their similarity to crustacean pigment dispersing hormones (PDHs), which regulate the dispersion and migration of pigment in chromophores and photoreceptors, including daily rhythms of the latter [1,23]. Insect PDFs were first

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isolated based on their ability to induce pigment dispersion in crustaceans when applied exogenously and have very high sequence similarity to crustacean β-PDHs (Figure 1A) [23]. Insect brains contain small numbers of neurons, approximately six to 20, that are immunoreactive to antisera raised against crustacean PDH (PDHir neurons) [4–12]. Though the numbers and positions of PDHir neurons vary among the insect orders, most species studied contain anterior-ventrally located somata near the accessory medulla, an accessory visual neuropil [4–12]. These neurons typically extend neural processes within the accessory medulla, the optic lobes and the dorsal protocerebrum, as schematized for *Drosophila melanogaster* in Figure 1B [4–12]. Some species display additional PDHir somata and projections in other regions of the central nervous system, most notably in the tritocerebrum, suboesophageal ganglia, and the corpora cardiaca (e.g., [9,24]).

PDHir neurons with somata situated near the accessory medulla were implicated as circadian pacemakers in orthopteroid insects based on anatomical criteria, physiological observations, and ablation and transplant experiments [25]. The discovery that the PDHir neurons of the *Drosophila melanogaster* brain express the circadian clock gene *period* (*per*) [26] and that these neurons are missing in visual system mutants with weak or absent circadian rhythms further supported the hypothesis that PDHir neurons function as circadian pacemakers in insects [4,27].

The cloning of *Pdf* from *Drosophila melanogaster* [2] and subsequently from other insects (e.g., [3,28,29]), made it possible to address the role that this peptide plays in the control of circadian locomotor rhythms using molecular/genetic approaches. Flies bearing the loss-of-function Pdf^{01} mutation display a syndrome of circadian phenotypes. These mutants are characterized by the loss of the anticipatory morning peak of activity and an advanced evening peak of activity under light/dark (LD) conditions [13]. Pdf^{01} mutants also display significantly higher levels of arrhythmicity under constant darkness and temperature (DD), indicating that the ability to produce endogenous circadian rhythms is compromised in the absence of PDF [13]. Pdf^{01} mutants that do display rhythmic locomotion under DD have relatively weak rhythms with significantly shorter periods [13]. The loss of PDF in the fly is also accompanied by an inability to delay the evening peak of activity during long days [30] and the absence of increased nighttime activity in response to nocturnal light [31].

RNA-interference mediated knockdown of *Pdf* in the cockroach *Blattella germanica* resulted in significant increases in arrhythmicity in both LD and DD conditions, but had no obvious effects on the period of locomotor rhythms [29]. In contrast, *Pdf* knockdown in the cricket *Gryllus bimaculatus* resulted in a shortening of the free-running period but did not result in increases in arrhythmicity under DD conditions [32]. *Pdf* knockdown also reduced levels of nighttime activity in the cricket, and caused a more rapid resynchronization to shifted LD cycles [32]. Injection of PDH or PDF into the brains of free-running cockroaches and crickets produces dose-dependent phase changes in both insects [33,34]. Thus, PDF is required for normal circadian locomotor rhythms in several insects and likely acts to adjust the period or phase of circadian rhythms. However its specific roles may differ among species.

1.3: Mechanisms of PDF function in the clock neuron network of *Drosophila*

The circadian functions of PDF are best understood for *D. melanogaster*, whose clock neuron network can be manipulated with a precision unavailable in other species. This network consists of approximately 150 neurons, which support daily rhythms in clock gene expression and can be divided into nine distinct anatomical classes (Figure 1C) [35–37]. Two classes of clock neurons express PDF, which are readily divisible by anatomical criteria. The four to five large ventral lateral neurons (l-LN_vs) residing in each hemisphere project across the medulla of the ipsilateral optic lobe and across the posterior optic tract to the contralateral medulla and accessory medulla (Figure 1B) [4,13,24,38]. The four small ventral lateral neurons (s-LN_vs) in each hemisphere ipsilaterally innervate both the accessory medulla and dorsal protocerebrum (Figure 1B) where they reside in proximity to the projections of the other classes of clock neuron [4,13,24,38]. These dorsal projections display diurnal and circadian rhythms in PDF immunoreactivity, consistent with rhythmic PDF release in the dorsal brain [39].

Visualization of clock gene rhythms in various classes of neurons of Pdf^{01} mutants indicates that some clock neuron classes rely on PDF for the maintenance of coherent and synchronized molecular oscillations, including the PDF-expressing s-LN_vs themselves [30,40]. The fact that molecular rhythms within some clock neurons become progressively desynchronized under DD conditions fits well with the observation that locomotor rhythms of Pdf^{01} mutants are relatively strong during the first few days of DD but weaken progressively over the subsequent circadian cycles [13,40]. Flies lacking functional molecular clocks within the PDF neurons display high levels of arrhythmicity under DD conditions [41,42], indicating that circadian timekeeping within these neurons is critical for the maintenance of endogenous circadian rhythms. Furthermore, speeding up the molecular clock specifically within the PDF neurons shortens the free-running period of locomotor rhythms and speeds-up the molecular clocks within most PDF-negative clock neuron classes [43], an effect that requires PDF signaling [44]. Based on these results, PDF is hypothesized to function as a synchronizing factor within the network of clock neurons, a function remarkably similar to that of vasoactive intestinal polypeptide (VIP) in the clock center of the mammalian brain [45,46].

The identification of PDF's receptor PdfR (also known as Han) revealed that, like the receptor for VIP, it is a secretin-like G protein-coupled receptor that signals through increases in intracellular cyclic AMP (cAMP) [47–49]. The genetic loss of *PdfR* results in the same circadian behavioral phenotypes as those described for Pdf^{01} mutants [47–49]. The observation that the majority of clock neuron classes, including the PDF-expressing s-LN_vs but not the l-LN_vs, respond to bath-applied PDF peptide with cAMP increases provided further support of the hypothesis that PDF released from the LN_vs acts to coordinate the clock neuron network of *Drosophila* [50]. The rescue of *PdfR* expression within the clock neuron network of a loss-of-function *PdfR* mutant was sufficient for the rescue of the mutant's circadian behavioral phenotypes, further supporting the hypothesis that PDF acts to coordinate and synchronize circadian timekeeping throughout the clock neuron network

[47,51]. Furthermore the expression of membrane-tethered PDF peptide in the clock neurons of Pdf^{01} mutants rescued some aspects of the mutant's circadian behavior [52], further supporting the hypothesis that PDF's circadian function is based on its modulation of clock neurons.

Despite the evidence supporting a synchronizing function for PDF within the clock neuron network, other observations suggest a more complex role for PDF in circadian timekeeping. The loss of PDF has differential effects on the molecular clocks within the various clock neuron classes. The posterior dorsal neuron one ($DN1_p$) class appears to require PDF for the maintenance of molecular rhythms [30], whereas the dorsal lateral neurons (LN_ds) do not [30,40]). Strikingly, PDF has opposing effects on the synchrony of molecular rhythms within different clock neuron classes. For example, under free-running conditions the s-LN_vs become phase-dispersed with respect to their molecular rhythms in the absence of PDF [40], whereas the molecular clocks of the LN_ds display increased synchrony in the absence of PDF [30].

The short period phenotypes of the rhythmic subsets of *Pdf* and *PdfR* mutants suggest that PDF normally acts to lengthen the period of the circadian clock [13,47–49]. However, when PDF levels are increased in the dorsal protocerebrum through the ectopic expression of *Pdf*, the free-running period of locomotor rhythms is not simply lengthened [53,54]. Though *Pdf* overexpression is associated with increases in the period of locomotor rhythms in some individuals, it also causes an increase in the incidence of arrhythmicity and internal desynchronization, a condition marked by the presence of multiple periodicities in individual flies [53,54]. Such PDF-induced behavioral desynchrony was accompanied by a loss of synchrony among the clock neuron classes, with the s-LN_vs along with a subset of dorsal neurons (DNs) displaying short-period molecular rhythms, with other DNs and a subset of the LN_ds displaying longer-period rhythms [30]. Similar effects are observed when PDF neurons are constitutively hyperexcited through the directed expression of a modified Na^+ channel [55]. Furthermore, when PdfR is constitutively activated specifically in the s-LN_vs using a membrane-tethered PDF peptide, the behavioral output of these neurons (the anticipatory morning peak of activity) was phase-advanced, consistent with an accelerated molecular clock in these neurons [56]. These results indicate that PDF has differential effects on neuronal oscillators, increasing the period of some while decreasing the period of others, at least when PDF is overexpressed or PdfR is constitutively activated.

1.4: Network Properties of PDF function in *Drosophila*

The results outlined in the preceding two paragraphs indicate that PDF has several and in some cases opposing effects within the clock neuron network. The nature of *PdfR* expression within the network provides a partial explanation for these findings. Because specific antisera for the immunocytochemical visualization of PdfR expression do not exist and the *PdfR* gene is too large for standard transgenic methods of expression mapping, Im and Taghert (2010) created flies in which the entire *PdfR* gene was introduced with a C-terminal MYC tag into loss-offunction *PdfR* mutants [57]. This large (~70kB) construct (*PdfR-MYC*) fully rescues the behavioral phenotypes of *PdfR* mutants and allows PdfR-MYC expression to be assayed using anti-MYC immunocytochemistry [57]. This expression

mapping revealed that the clock neuron network is mosaic for *PdfR* expression, with only approximately half of the neurons expressing the receptor [57] (Figure 1C). PdfR expression is not uniform within most clock neurons classes, for example, only half the LN_ds and less than half the $DN1_p$ and DN3 neurons expressed detectable levels of PdfR-MYC [57]. Live imaging experiments using cAMP sensor specifically expressed within the PdfR-positive and -negative LN_ds revealed that PdfR-expressing LN_ds but not PdfR-negative LN_ds, respond to bath-applied PDF [44]. Thus, only subsets of clock neurons are receptive to PDF. *PdfR* expression is closely associated with the expression of *cryptochrome* (*cry*), a blue light circadian photoreceptor [58,59], and flies lacking both *PdfR* and *cry* function display severe circadian phenotypes [59–61].

The mosaic nature of *PdfR* expression in the clock neuron network may partially explain some of the findings outlined above. For example, the internal desynchronization observed when PDF levels are increased in the dorsal protocerebum [30,53,54] likely reflects desynchronization between the PdfR-positive and PdfR-negative clock neurons. Furthermore, the fact that only half of the LN_ds express PdfR [57] may explain why their molecular oscillations are more synchronous in the absence of PDF [30], as PDF could normally act to desynchronize these two types of LN_ds. Thus, PDF could act as a synchronizing factor among PdfR-expressing neurons while acting to desynchronize classes composed of PdfR-positive and -negative clock neurons. In support of this hypothesis, synchronization of PDF-negative neurons to experimentally slowed PDF neurons requires PdfR and neurons lacking PdfR expression do not synchronize to slowed PDF neurons [44]. Furthermore, the strength of molecular clock coupling to PDF neurons varies among PdfR-expressing neurons, with some clock neurons being strongly coupled and others showing relatively weak coupling to PDF neuron clocks [44]. In fact, when the speed of the molecular clock within the PDF neurons is specifically altered, the presence of a physiological connection between PDF neurons and their PdfRexpressing targets does not always insure the coupling of their molecular clocks [44]. Thus, the efficacy of PDF mediated synchronization likely varies even among clock neurons expressing *PdfR*.

1.5: PDF as a circulating peptide hormone

In addition to the clock-associated PDF neurons of the central brain, four to eight PDF neurons are present in the posterior abdominal segments of *Drosophila*'s ventral nerve chord (AbPdf neurons) (Figure 1C) [4,5,24], a pattern of expression that is also found in the locust *Locusta migratoria* [7], the blowfly *Phormia terraenovae* [5], and the cabbage fly *Delia radicum* [62]. In adult *Drosophila*, these neurons project out of ventral nerve cord and terminate superficially along the surface of the rectum and at the junction of the posterior midgut, anterior hindgut and the ureters [5,21]. PDF likely serves as a circulating peptide hormone in insects. Some insects appear to release PDH from the corpora cardiaca, the brain's neurohemal organ [9] and the AbPdf neurons are strong candidates as a source of circulating PDF [7], The AbPdf neurons do not express the molecular clock and PDF from these neurons is not required for normal locomotor rhythms in *Drosophila* [63]. However circulating PDF adjusts the phase of the molecular clock in oenocytes, the peripheral sites of pheromone production in the fly [64]. Interestingly, the normal phasing of the oenocyte molecular clock requires PDF in both the LN_vs and the AbPDF neurons [64]. Thus,

circulating PDF appears to influence at least some non-neuronal peripheral clocks in *Drosophila*.

1.6: The Cellular Basis of PDF Signaling

Moasic expression of PdfR among clock neurons may not fully explain the varied effects of PDF within the circadian system. How can the same peptide functioning through the same G protein-coupled receptor have opposing effects on different clock neuron targets? One possible explanation comes from the discovery that different clock neuron classes utilize different adenylyl cyclases (ACs) for PdfR signaling. In the s-LN_vs PdfR signaling produces cAMP increases through AC3, while AC78C (an ortholog of mammalian AC8) and at least one additional unidentified adenylyl cyclase are used in the *PdfR*-expressing LN_ds [65,66]. These findings indicate that clock neurons differ in PdfR signalosome composition, leading to the hypothesis that distinct signalosomes transduce cAMP increases into distinct or perhaps even opposing effects on the molecular clocks of PDF-receptive neurons [66].

The PdfR signaling pathway in clock neurons has been partially revealed and is summarized in Figure 2. Upon binding to PDF peptide, PdfR activates Gαs (also known as Gsα60A) [56,67], resulting in activation of ACs and the production of cAMP [49,50]. This action is opposed by the phosphodiesterase Dunce, whose abundance is regulated by GW182, a protein essential for microRNA-mediated gene silencing [67].

How do PdfR-induced cAMP increases eventually lead to changes in the phase or amplitudes of molecular clocks and/or changes in the membrane properties of target neurons? Recent work has established that PdfR-induced cAMP increases activate protein kinase A (PKA) in clock neurons [68,69]. PKA signaling enhances the stability of the Period (PER) and Timeless (TIM) protein, resulting in changes in the phase and/or period of the molecular clocks within target clock neurons [68,69]. It is interesting to note that the *Drosophila* cyclic-AMP response element binding protein B (dCREB2), a well-established mediator of cAMP/PKA pathway, interacts with the core molecular clock feedback loop [70]. Furthermore, a mutation of *dCREB2* produces circadian locomotor phenotypes remarkably similar to those of *Pdf* or *PdfR* mutants [70]. Thus, dCREB2 is a clear candidate component of PdfR signalosomes.

Though PdfR is thought to signal predominantly through increases of cAMP, it can produce changes in Ca^{2+} [49,69]. Though the acute excitation of PDF neurons does not result in measureable Ca^{2+} changes in PdfR-expressing LN_ds [71], focal application of PDF does cause small Ca^{2+} increases in a subset of the $DN1_p$ class of clock neurons [69]. Furthermore, PdfR has recently been identified as an IP_3/Ca^{2+} coupled receptor required for the maintenance of flight [20]. Thus, it appears that PdfR signaling causes Ca^{2+} increases at least in some PdfR-expressing neurons, including some clock neurons [69,72]. Finally, PDF signaling causes depolarization in some neurons, including the $DN1_p$s, which display increased firing rates in response to the focal application of the peptide [69].

1.7: A summary of PDF functions in the *Drosophila* nervous system

The limited expression of PDF within the *Drosophila* nervous system, along with highly specific molecular genetic tools for the manipulation of PDF- and PdfR-expressing neurons in the fly, have allowed researchers to identify diverse functions for this peptide. The many circadian and non-circadian roles played by PDF likely reflect the existence of diverse sets of PDF targets (Figure 3) and multiple modes of signaling downstream of PdfR. The targets of PDF are likely to reside in both the central nervous system and peripheral tissues and to include both clock and nonclock cells [21,57,64,73]. It seems clear that the study of PDF, its cellular targets, its modes of signaling, and its behavioral and physiological functions will continue to enrich our understanding of neuromodulatory control for many years to come.

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Highlights

- We recount the isolation and characterization of insect pigment-dispersing factors.
- We discuss the expression of pigment-dispersing factor within insect central nervous systems.
- We review the evidence that pigment-dispersing factor acts as a circadian clock output factor in insects.
- We present a model of pigment-dispersing factor function within the circadian clock neuron network of *Drosophila*.

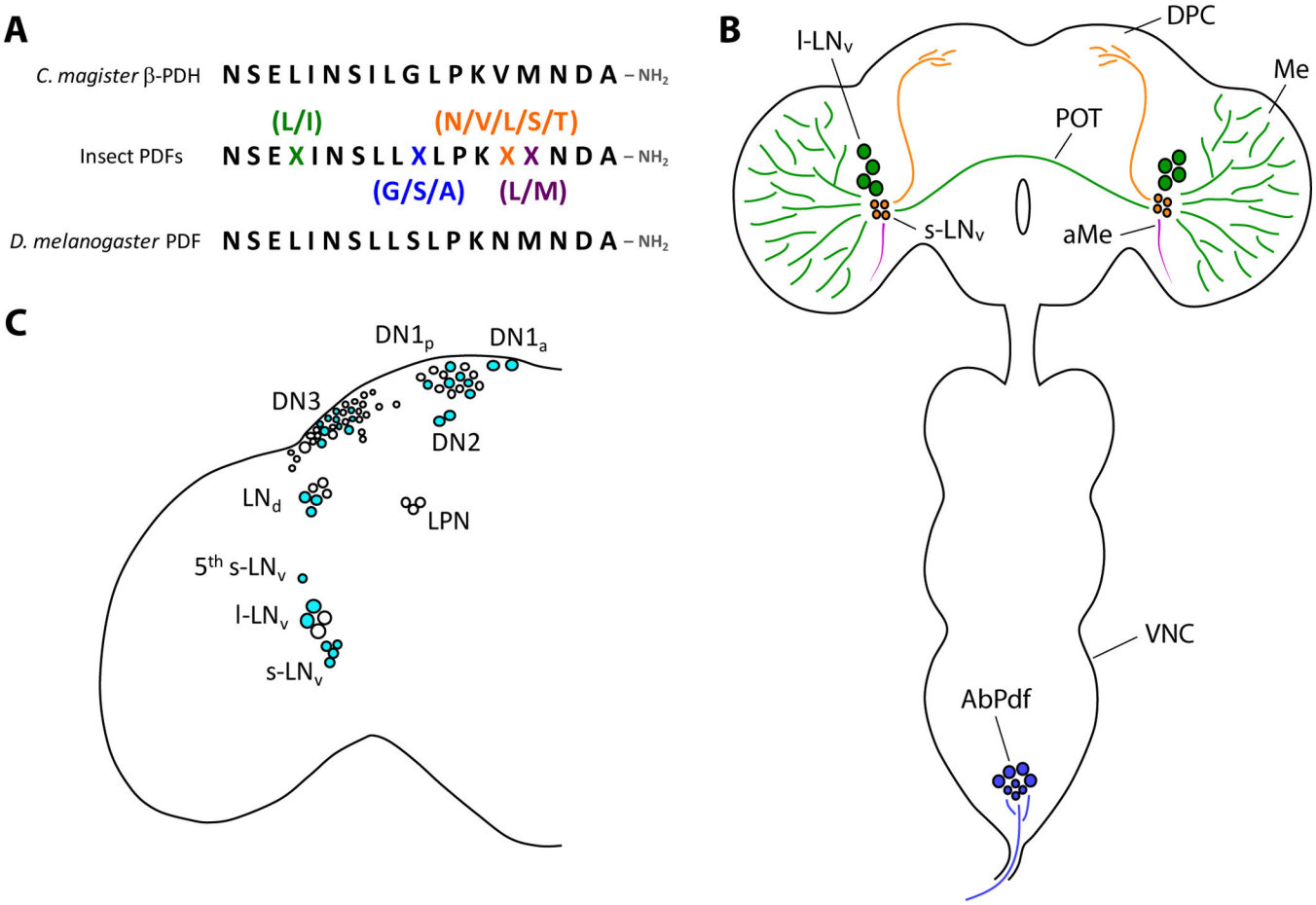

Figure 1.

(**A**) The highly conserved sequence of insect PDFs. Insect PDFs have highly similar sequences to crustacean β-PDH hormones. Crustacean nervous systems also contain α-PDF, to which insect PDFs display no homology. Top: the sequence of β-PDH from *Cancer magister*. Middle: a generalized amino acid sequence for insect PDFs. Variable amino acids are indicated by a colored X with common variants shown above or below the variable amino acid. Bottom: the sequence of PDF from *Drosophila melanogaster*. (**B**) A schematic of PDF neuron anatomy in the *Drosophila* central nervous system. The three classes of PDF neurons, the large ventral lateral neurons (l-LN$_v$s), the small ventral lateral neurons (s-LN$_v$s) and the abdominal PDF neurons (AbPdf) are indicated. The l-LN$_v$s innervate the medullae (Me) of the optic lobes and project across the posterior optic tract (POT). The s-LN$_v$s project to the dorsal protocerebrum (DPC). Both the l-LN$_v$s and s-LN$_v$s project to the accessory medullae (aMe), shown in magenta. The AbPdf neurons reside in the abdominal ganglia of the ventral nerve cord (VNC), project to the viscera and are a likely source of circulating PDF. Note, PDF is also expressed within neurons of the tritocerebrum of adult *Drosophila* (not shown), though they undergo programed cell death in the days following adult eclosion [13]. (**C**) A cell body map of clock neurons in the *Drosophila* brain. The various neuron classes are indicated. PdfR expression is indicated in cyan. PdfR expression in the lateral posterior neurons (LPNs) was not determined.

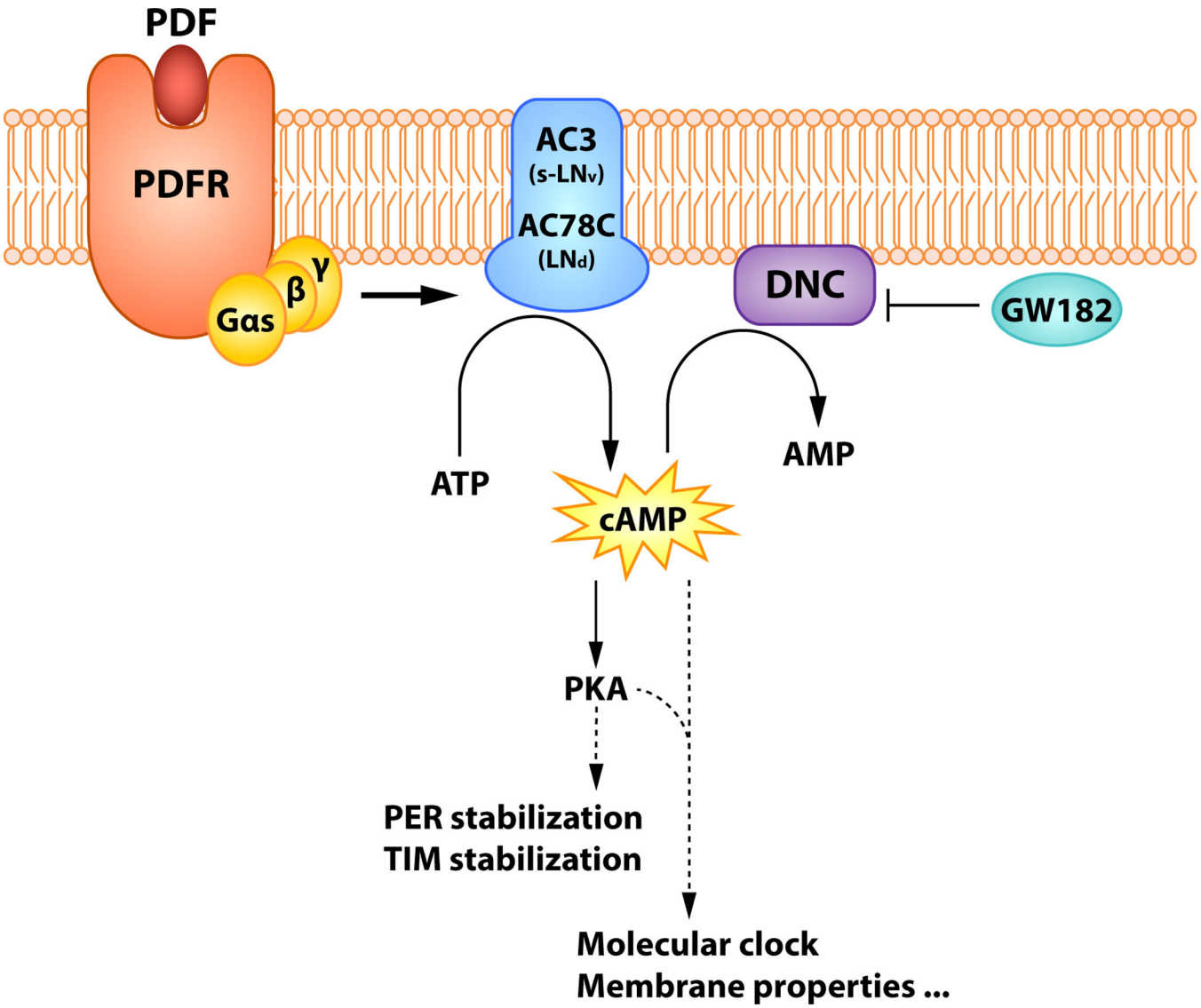

Figure 2.

A schematic representation of the PdfR signaling pathway. PdfR signals through Gαs and activates distinct adenylyl cyclases (ACs) in different classes of PdfR-expressing clock neurons. The action of PdfR is opposed by the phosphodiesterase Dunce (DNC), the abundance of which is regulated by GW182, a protein essential for microRNA-mediated gene silencing. Increases of cAMP levels stimulate protein kinase A (PKA), which leads to the stabilization of PERIOD (PER) protein, thereby adjusting the molecular clock. Please see section 1.6 for a full description.

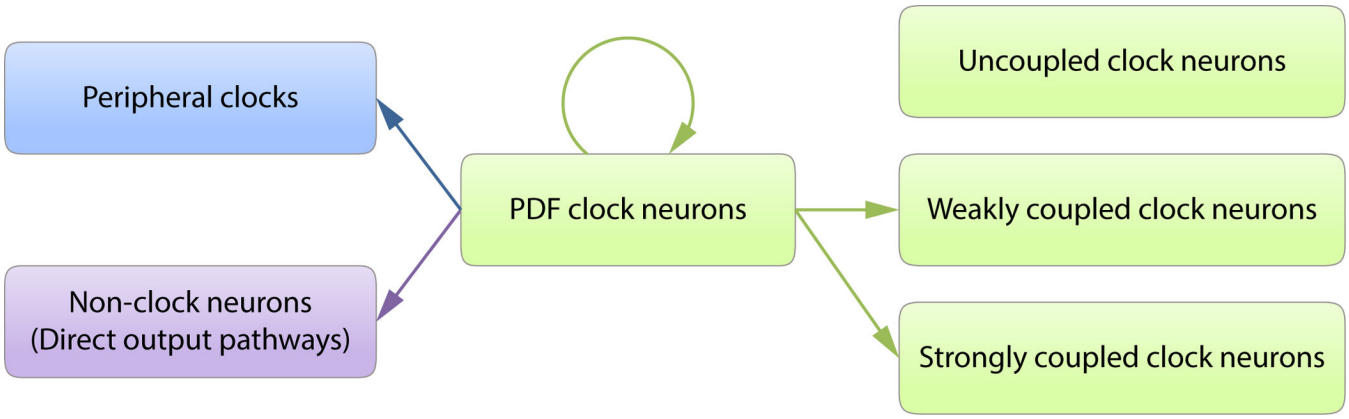

Figure 3.

A summary of PDF function in the circadian system of *Drosophila*. PDF-negative clock neurons are variably coupled to PDF-expressing clock neurons, with some PdfR-expressing neurons strongly coupled and others more weakly coupled to the molecular clocks of PDF neurons. Other clock neurons do not express PdfR and do not appear to be physiologically connected to PDF neurons. PDF also modulates neurons that do not express the molecular clock and these neurons may represent output targets of the circadian system. Finally, circulating PDF, coordinates molecular clocks in peripheral tissues and mediates non-circadian functions of PDF. Please see sections 1.5 and 1.7 for details.