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Rhythmic control of activity and sleep by class B1 GPCRs

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Abstract

Members of the class B1 family of G-protein coupled receptors (GPCRs) whose ligands are neuropeptides have been implicated in regulation of circadian rhythms and sleep in diverse metazoan clades. This review discusses the cellular and molecular mechanisms by which class B1 GPCRs, especially the mammalian VPAC2 receptor and its functional homologue PDFR in *Drosophila* and *C. elegans*, regulate arousal and daily rhythms of sleep and wake. There are remarkable parallels in the cellular and molecular roles played by class B1 intercellular signaling pathways in coordinating arousal and circadian timekeeping across multiple cells and tissues in these very different genetic model organisms.

The Class B1 GPCR Family

Members of the class B (also known as class 2 or secretin-receptor) family of G-protein coupled receptors (GPCRs) have been implicated in regulation of circadian rhythms and sleep. Of the six classes of GPCRs, class A and B receptors share similar seven-pass trans-membrane domains, but class B members have a large extracellular N-terminus that interacts with their endogenous ligands. Subfamily B1 consists of classical peptide hormone receptors which activate Gs G protein, cyclic adenosine monophosphate (cAMP), and, in some cases, phospholipase C (PLC) signaling (Figure 1). This review discusses the role of class B1 GPCRs, especially the mammalian VPAC2 receptor and its functional homologue PDFR in *Drosophila* and *C. elegans*, in regulating arousal and daily rhythms of sleep and wake.

Class B1 Control of Mammalian Circadian Rhythms and Sleep

Introduction to mammalian circadian neurobiology

Circadian rhythms control many aspects of mammalian physiology and behavior (Hastings et al., 2007) such as sleep-wake (Saper et al., 2005, Aston-Jones et al., 2001, Stermann et al., 1965), body temperature (Mendel and Raghavan, 1964), hormone secretion (Czeisler and Klerman, 1999), cardiovascular activation (Spoor and Jackson, 1966), renal function (Bonny

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Declaration of Interest

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and Firsov, 2013), cell division (Simmons, 1964, Matsuo et al., 2003), and metabolism (Bass, 2012). Wheel-running activity, for example, is frequently used as an *in vivo* measurement of clock output in rodents. These rhythms are easy to measure and highly precise (0.02 hour standard error in period observed in C57BL/6J mice) (Schwartz and Zimmerman, 1990) so that screens for genes, molecules, and cells that alter daily rhythms have succeeded to elucidate the underlying intracellular and intercellular mechanisms (Buhr and Takahashi, 2013, Mohawk et al., 2012).

The suprachiasmatic nucleus (SCN) is a master circadian pacemaker in mammals. Lesions of the SCN abolish circadian rhythms in wheel running and hormone levels (Stephan and Zucker, 1972, Moore and Eichler, 1972). The SCN shows glucose uptake rhythms *in vivo* that are higher during the day (Schwartz and Gainer, 1977). Electrical stimulation in the SCN shifts daily behavioral rhythms, much like light (Rusak and Groos, 1982). Remarkably, SCN transplants restore rhythms in SCN-lesioned animals with the period of the donor (Ralph et al., 1990). SCN cells (Webb et al., 2009) can generate cell-autonomous rhythms (Welsh et al., 1995). The intracellular mechanisms for rhythm generation are common to many cell types in mammals and rely on a transcription-translation feedback loop (Partch et al., 2014). Cells in the SCN appear to differ from most cells, however, in two critical ways that allow them to coordinate daily rhythms in the brain and body. Firstly, SCN cells receive monosynaptic inputs from the retina that are essential for synchrony to the local light/dark cycle (Hattar et al., 2002, Hattar et al., 2003). Secondly, SCN neurons can synchronize to each other so they can coordinately regulate rhythms in other cells and tissues (Dibner et al., 2010, Yamaguchi et al., 2003, Li et al., 2012). These properties allow the cells of the SCN to function as a master circadian pacemaker.

VIP and VPAC2 receptor

Vasoactive intestinal polypeptide (VIP) and its receptors provide a history of signaling rediscoveries. Identified in 1970 from small intestine extracts, VIP gained fame as a polypeptide with “an unusually wide range of biological activity” but with actions unlikely “to extend beyond the splanchnic circulation” (Said and Mutt, 1970). Originally thought to derive from “endocrine-like cells” in the gut (Polak et al., 1974), VIP modulated respiration, glucose homeostasis, and blood flow. In 1976, it was rediscovered as a neuropeptide synthesized by subsets of neurons in the central and peripheral nervous systems (Larsson et al., 1976). Over twenty years later, a major role for VIP and its receptors in the central nervous system was serendipitously discovered by biologists studying circadian rhythms (Colwell et al., 2003, Harmar et al., 2002, Shen et al., 2000).

The *vip* gene encodes a preprohormone which is processed into multiple peptide products including VIP and PHI (peptide histidine isoleucine). (Mikkelsen and Fahrenkrug, 1994). More needs to be learned about the processing of VIP. For example, little is known about the biological activity of PHI. Furthermore, VIP has been characterized as a 28 amino-acid peptide although recent efforts using mass spectrometry have found only a 13 amino-acid VIP catabolite in brain (Lee et al., 2010, Falth et al., 2006).

VIP binds the VPAC1 and VPAC2 (encoded by the *vipr1* and *vipr2* genes, respectively), and, to a lesser extent, PAC1 (encoded by the *pac1r* or *adycap1r1* gene). VPAC1 and

VPAC2 are both members of the class B1 GPCR family which can also be activated by PACAP (pituitary adenylate cyclase-activating peptide) (Dickson and Finlayson, 2009). Therefore it is necessary to distinguish which ligand (VIP, PHI or PACAP) and which receptors (PAC1, VPAC1 or VPAC2) contribute to particular circadian behaviors. This review summarizes the evidence that VIP/VPAC2 signaling is essential for synchronization of circadian cells to each other and for their entrainment to the light cycle. Less is known about the roles of PAC1 and VPAC1 receptors.

VIP is synthesized by neurons within the ventral lateral suprachiasmatic nucleus (SCN) of the hypothalamus, a master circadian pacemaker (Abrahamson and Moore, 2001). The VPAC2 receptor is highly, widely and rhythmically expressed in the SCN (An et al., 2012). An important role for VIP in circadian biology was first demonstrated by its ability shift daily rhythms in arginine vasopressin (AVP) (Watanabe et al., 2000), spontaneous firing (Reed et al., 2001) and locomotor behavior (Piggins et al., 1995). These shifts require parallel changes in both cAMP/protein kinase A and PLC signaling (An et al., 2013). Activation of these signaling pathways induces expression of the *Per1* and *Per2* genes, canonical players in the timing of circadian cycling (Nielsen et al., 2002). Evidence for which of the class B1 receptors mediate the effects of VIP in the SCN comes from pharmacology and genetic manipulations.

The VPAC2R agonist Ro-25-1553 (Gourlet et al., 1997b) and antagonist PG99-465 (Moreno et al., 2000), but not the VPAC1R agonist [K15,R16,L27]VIP(1–7) (Gourlet et al., 1997a), change firing patterns in the SCN (Reed et al., 2002). Furthermore, daily application of the VPAC2R agonist can entrain daily rhythms in firing of wild-type, but not *Vipr2*^{-/-}, SCN neurons (Aton et al., 2005). These results implicate VPAC2R, not PAC1R or VPAC1R, in SCN function.

Transgenic mice overexpressing VPAC2R show a 0.5-hour shorter period of locomotor activity in constant darkness (Shen et al., 2000). These mice also adjust faster than wild-type mice to an advance in their light cycle (simulating a trip east across 6 time zones). Remarkably, mice that do not express VPAC2R also entrain faster to an advanced light cycle and have the additional phenotype of starting their activity up to 8 hours earlier than normal in a regular light schedule (Harmar et al., 2002). Surprisingly, most VPAC2R or VIP knockout mice show weak to no circadian locomotor rhythms in constant conditions and aberrant gene induction by light (Aton et al., 2005, Colwell et al., 2003, Harmar et al., 2002, Hughes et al., 2004, Dragich et al., 2010). Data from these studies suggest VIP and VPAC2R act on convergent Ca²⁺ and cAMP pathways important for generating circadian rhythms and synchronizing them to the local light schedule.

Does the loss of circadian rhythms in behavior and in tissues of mice deficient for VIP-VPAC2R signaling result from a loss of rhythms in individual cells or a loss of synchrony among rhythmic cells? Recordings of firing rate, intracellular calcium levels, and gene expression rhythms from many SCN neurons deficient for either VIP or VPAC2R revealed fewer rhythmic neurons and a wider range of periods (Aton et al., 2005, Brancaccio et al., 2013, Maywood et al., 2006, Brown et al., 2005). Because daily application of a VPAC2R agonist restores synchrony and the fraction of rhythmic cells in VIP-knockout SCN (but not

in the *vip2r* knockout), researchers have concluded that VIP-VPAC2R signaling is necessary for both circadian rhythms and synchrony (Aton et al., 2005).

The circadian system provides a unique opportunity to examine the dynamics of ligand-receptor interactions. For example, what is the relationship between VPAC2 receptor abundance and VIP release and how might their relative timing in the SCN affect circadian synchrony? A recent model incorporated the evidence that VIP release and VPAC2R expression are circadian and proposed that VIP can only synchronize cells in the SCN when released during the day (Ananthasubramaniam et al., 2014). Their model predicts that VPAC2R rhythms have little effect on synchrony, but can enhance rhythm amplitude if VIP release and VPAC2R levels peak together during the day (Figure 2a). The ubiquity and consequences of rhythms in both ligand secretion and receptor abundance are woefully understudied in any context.

What regulates VIP production and release? VIP expression is regulated at both transcriptional and translational levels in the SCN (Figure 2b). Two recent studies provide evidence for transcriptional regulation of the *Vip* gene by the transcription factor Lhx1 (Bedont et al., 2014, Hatori et al., 2014). They found that mice with Lhx1 conditionally deleted in the SCN entrain more rapidly to phase shifts in the light:dark cycle and tend to lose circadian rhythmicity in constant darkness. Both groups found that *Vip* mRNA is low in SCNs lacking Lhx1. Lhx1 protein dose-dependently activates a luciferase reporter fused to the *Vip* promoter, providing further evidence that Lhx1 activates *Vip* transcription (Hatori et al., 2014). Neurons within SCNs lacking Lhx1 show desynchronized circadian rhythms in firing rate (Hatori et al., 2014) and *Per2* expression (Bedont et al., 2014), phenocopying *Vip* and *Vipr2* knockout animals.

Translational inhibition of VIP is induced by the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) (Cao et al., 2013b). 4E-BP1 knockout mice show increased prepro-VIP expression and faster entrainment of locomotor and SCN rhythms to both advanced and delayed light cycles. Blocking VPAC2R signaling with PG99-465 reverses the quick entrainment. The researchers concluded that nighttime phosphorylation of 4E-BP1 by mammalian target of rapamycin (mTOR) allows 4E-BP1 to bind eukaryotic translation initiation factor 4E (eIF4E) and inhibit translation initiation of prepro-VIP. It is not known how the maximal disinhibition of VIP translation by mTOR phosphorylation of 4E-BP1 in the early night relates to the timing of VIP release. It is also unknown if and how the firing patterns of VIP neurons affect VIP release. Notably, although extracellular VIP likely degrades within a few minutes to hours (An et al., 2013), it can increase firing in SCN neurons for more than 2 hours depending on cAMP and PLC signaling, induction of *Per1* and CREB, and the fast delayed rectifier K⁺ current (Kudo et al., 2013). These results lead to a model where enhanced daytime firing increases daytime release of VIP within the SCN to induce long-term changes in membrane excitability and gene expression that support synchronization of SCN neurons to each other and to the environment. Together, 4E-BP1 and Lhx1 may drive daily rhythms in VIP availability.

But how can either loss of or enhanced VIP-VPAC2R signaling in the SCN each lead to quicker entrainment? One hypothesis is phase tumbling: VIP speeds entrainment by

transiently shifting some cells more than others, reducing the synchrony among SCN cells so that they can adjust to the new light schedule more rapidly (An et al., 2013). When added to the SCN slice, 20 or 50 nM VIP can entrain SCN rhythms (An et al., 2011) but, above 150 nM, VIP can decrease circadian amplitude for several days (An et al., 2013). At concentrations above 150nM, VIP disperses the phase relationships among SCN cells and accelerates entrainment of the SCN to temperature cycles in vitro. Similarly, injection of VIP in vivo accelerates entrainment of daily locomotor rhythms to a subsequent shift in the light schedule. However, chronic high levels of VIP (as in 4E-BP1 knockout mice) or low levels (as in the case of mice lacking VIP or Lhx1 in the SCN) reveal there are other mechanisms that can speed entrainment. There is evidence from SCN co-cultures that, in the absence of VIP-mediated synchrony, other neuropeptides can mediate synchrony (Maywood et al., 2011) and that, for example, vasopressin may normally slow entrainment (Yamaguchi et al., 2013). Thus, VIP-VPAC2R signaling between approximately 20 and 150 nM appears to promote circadian synchrony among SCN cells and slow their entrainment to the ambient light cycle while abolishing or increasing VIP-VPAC2R signaling outside of this range leads to reduced synchrony among the circadian cells and faster entrainment to the environment.

It is worth noting in the context of sleep and circadian biology that small populations of VIP-positive neurons also reside in the retina, olfactory bulb (OB), hippocampus, amygdala and neocortex. VPAC2 is expressed at low levels in grey matter almost everywhere in the brain. A recent report found that VIP-VPAC2R signaling is also required for circadian rhythms in the OB and olfactory performance (Miller et al., 2014). Future studies should focus on the potential that daily rhythms in the brain and body depend on local or diffusible VIP-to-VPAC2R signaling.

PACAP and PAC1 receptor

PACAP was identified in 1989 in a screen for neuropeptides that activate adenylate cyclase in the pituitary.(Miyata et al., 1989) Both the originally discovered 38-amino acid isoform (PACAP-38) and a 27-amino acid isoform act as endogenous ligands (Miyata et al., 1990) derived from the same prepro-PACAP mRNA (Vaudry et al., 2000). Subsequently, PACAP has been implicated in photic entrainment of circadian rhythms.

PACAP is found in the terminals of intrinsically photosensitive retinal ganglion cells (ipRGCs).(Hannibal, 2002) Glutamate and PACAP are co-stored in ipRGCs that innervate the SCN (Engelund et al., 2010). These cells also express melanopsin, a photopigment critical for responses to light level including pupil constriction, synchronization of daily rhythms to the daily light cycle and melatonin suppression (Panda et al., 2002, Ruby et al., 2002). Intriguingly, little is known about the role of PACAP in these non-imaging light responses (Engelund et al., 2012, Kawaguchi et al., 2010).

PACAP activates two class B1 receptors in the SCN, PAC1R and VPAC2R. Within the brain, PAC1R is thought to be the primary mediator of PACAP responses in the OB, thalamus, hypothalamus, hippocampus and cerebellum (Hashimoto et al., 1996, Shioda et al., 1997). PAC1R protein expression has yet to be reported, but PAC1R mRNA level is much higher in the ventral SCN (Hannibal, 2002), consistent with its role in photoentrainment.

PACAP can shift SCN rhythms although published results appear to conflict. In one study, 1 μ M PACAP-38 applied during the subjective day produced a 3.5 h advance in the daily rhythm in SCN firing and had little effect at other times of day (Hannibal et al., 1997). In another study, 1nM PACAP-38 applied *in vitro* or *in vivo* during the day had little effect, but produced large shifts similar to light when applied during the night (Harrington et al., 1999). It may be that PACAP typically acts as a co-transmitter with glutamate at some times to mediate photoentrainment (Michel et al., 2006, Shibata and Moore, 1993, Chen et al., 1999). For example, the PACAP receptor antagonist, PACAP6–38, blocks or potentiates glutamate-induced phase shifts when applied during in early or late subjective night, respectively (Chen et al., 1999). Three independent strains of PACAP knockout mice shift less to light at night, but differ in the magnitude of the effect and in their circadian periods (Colwell et al., 2004, Hashimoto et al., 2001, Hamelink et al., 2002, Kawaguchi et al., 2010, Kawaguchi et al., 2003, Beaulieu et al., 2009). Furthermore, mice deficient for PAC1R have a slightly shorter circadian period in running-wheel activity and aberrant responses to light (Hannibal et al., 2001, Hannibal et al., 2008). Future studies should test cell-type specific roles for PACAP and PAC1R in gating of photic information to the SCN and take advantage of new, more specific, PAC1R agonists and antagonists (Dickinson et al., 1997, Moro and Lerner, 1997, Tatsuno et al., 2001, Uchida et al., 1998, Beebe et al., 2008).

Growth Hormone Releasing Hormone (GHRH) receptor

In contrast to PAC1 and VPAC2 receptors, there is limited evidence that other class B1 receptors play a role in circadian rhythmicity. GHRH and its receptor, GHRHR, for example, have been shown to promote non-rapid eye movement (NREM) sleep (Parker et al., 1969, Takahashi et al., 1968), but have not been tested in circadian biology.

GHRH activates the release of growth hormone (GH) from somatotroph cells in the pituitary, opposing the inhibitory action of somatostatin. GH, subsequently, acts on peripheral organs either directly or via insulin-like growth factor 1 (IGF-1) to reduce the release of GHRH (Obal and Krueger, 2004). Slow wave sleep coincides with large bursts of GH secretion. Sleep deprivation reduces GH release and slow wave activity during the recovery after sleep deprivation enhances GH release (Parker et al., 1969, Takahashi et al., 1968). Injection of GHRH into the cerebral spinal fluid enhances sleep (Obal et al., 1988, Zhang et al., 1999), although systemic administration can impair sleep (Steiger et al., 1992). Thus, GHRH is described as a NREM-promoting peptide.

Genetic and pharmacological experiments indicate that the GHRHR regulates NREM duration and intensity. *Lit/lit* mice (Zhang et al., 1996) carry a point mutation that prevents GHRH from binding with its receptor. *Dw/dw* rats, like *lit/lit* mice, have reduced GHRH receptor binding and mRNA levels (Obal et al., 2001). Both models have reduced NREM sleep (Zhang et al., 1996). Blocking GHRH signaling with a GHRH receptor antagonist (N-Ac-Tyr¹,D-Arg²)-GHRH(1–29)-NH₂ (Obal et al., 1991) or an antibody against GHRH (Obal et al., 1992) reduces NREM sleep duration and intensity and the response to sleep deprivation. Inhibition of GHRH signaling can also be achieved through GH activation of endogenous negative feedback loop, resulting in attenuated NREM (reviewed in (Obal and Krueger, 2004)). Finally, reducing GHRHR levels with siRNA or activity with an antagonist

in somatosensory cortex decreases slow wave power during NREM sleep (Obal et al., 1992). Thus, GHRHR signaling, perhaps within neocortex, is necessary for normal NREM sleep duration and intensity.

Class B1 Control of *Drosophila* Circadian Rhythms and Sleep

Circadian rhythms in *Drosophila*

Circadian rhythms in *Drosophila* are traditionally studied by examining different behaviors--eclosion rhythms and locomotor activity--either entrained to an external time cue (light or temperature cycles) or in constant environmental conditions. When entrained to an external cue (normally a 12h:12h light:dark (LD) cycle), flies predominantly hatch in the morning (Konopka and Benzer, 1971). In this review we focus our attention on light-dependent entrainment of daily locomotor activity, since loss of a class B1 neuropeptide or its receptor alters photic entrainment without disrupting synchronization to temperature cycles (Busza et al., 2007, Yoshii et al., 2005).

When entrained to a 12h:12h LD cycle flies display two prominent bouts of activity, one around lights-on (dawn) and the other around lights-off (dusk) (Figure 3A). These activity bouts are not mere responses to the light transitions because (i) flies increase their activity prior to the light transitions (termed morning and evening anticipation) and (ii) these activity bouts persist when released into constant darkness (DD, termed morning and evening peak, Figure 3B). When released into constant darkness and temperature, flies maintain free-running activity rhythms with a period close to ~24 hours (Figure 3C). All these behaviors depend on a functional molecular clock harbored by a network of ~150 neurons localized in eight anatomically distinct neuron groups (Figure 3D), four of which are ventrolateral—large lateral ventral (ILN_v), small lateral ventral (sLN_v), lateral posterior (LPN), lateral dorsal (LND)—and four of which are dorsal (DN1a, DN1p, DN2, DN3) (Shafer et al., 2006, for review, see Allada and Chung, 2010, Nitabach and Taghert, 2008).

PDF and circadian rhythms

The earliest indication that Pigment Dispersing Factor (PDF, also known as Pigment Dispersing Hormone) is involved in circadian rhythms came from the finding in the mid 90s that PDF is expressed in the ventral subset of circadian pacemakers (Helfrich-Forster, 1995). PDF is expressed in both the sLN_vs and the ILN_vs. However, it wasn't until the late 90s that the role of PDF in circadian rhythms was demonstrated (Renn et al., 1999). Null mutations of the *pdf* gene (*pdf*⁰¹) demonstrated that PDF is necessary for proper eclosion rhythms (Myers et al., 2003), rhythmic anticipatory activity in LD, as well as maintaining free-running rhythms in constant darkness (Renn et al., 1999). The PDF receptor (PDFR) was discovered independently by three different groups in 2005 (Hyun et al., 2005, Lear et al., 2005, Mertens et al., 2005) and, as is typical for class B1 receptors, signals through cAMP. Just like the *pdf*⁰¹ flies, *PDFR* loss-of-function mutants have no morning anticipation, phase advanced evening anticipation, and become arrhythmic in DD. Thus, PDF/PDFR signaling is required to coordinate daily rhythms in fly larvae, pupae, and in adults.

PDF and eclosion rhythms

PDF released from LNvs regulates eclosion rhythm by acting on cells in the prothoracic gland (PG) (Figure 4A) (Myers et al., 2003). These cells possess their own molecular clock and eclosion rhythms persist when an entrainment cue is present but become arrhythmic under constant conditions in the absence of PDF. This indicates that PDFR functions to transduce circadian timekeeping signals not only in the brain, but also in peripheral tissues. Indeed, it has recently been demonstrated that circadian rhythms of secretion of waxy pheromones are controlled *via* PDF-PDFR signaling to oenocytes (the pheromone-producing cells in the epidermis) (Krupp et al., 2013).

PDF and circadian locomotor activity

PDF signaling is important for the generation of coherent locomotor rhythms and the actions of PDFR activation are varied and cell specific (Figure 4B). In LD conditions, loss of PDF or PDFR causes an attenuation of the morning anticipation as well as a phase advance in evening anticipation, and when released into DD a majority of these flies are arrhythmic, with the remaining rhythmic flies having a shortened period of ~21h (Renn et al., 1999). The effects of PDF on circadian rhythm are mediated via the sLNvs, since lLNvs do not show oscillation of clock proteins when released into DD (Kaneko et al., 2000, Shafer et al., 2002, Veleri et al., 2003, Yang and Sehgal, 2001), and knockdown of PDF using RNA interference (RNAi) limited to sLNvs reduces morning anticipation in LD and causes arrhythmicity in DD (Shafer and Taghert, 2009). PDF released from sLNvs acts in an autocrine fashion on sLNvs as well as on a specific subset of DN1s and LNds (Im and Taghert, 2010, Shafer et al., 2008, Yao and Shafer, 2014). Rescue of PDFR in DN1s and LNds restores morning anticipation and free-running period in DD (Zhang et al., 2010, Lear et al., 2009), rescue of PDFR in sLNvs is important for sustaining rhythms in DD (Lear et al., 2009, Zhang et al., 2009), and rescue of PDFR in LNds restores evening anticipation (Guo et al., 2014). These studies indicate that PDF signaling from sLNvs to PDFR-expressing circadian clock neurons is essential for proper network synchronization and expression of circadian rhythms.

The lLNvs are part of the light input pathway to the clock, since they receive indirect connections from the compound eye and colocalize with axon terminals of the extraretinal photoreceptors, the Hofbauer-Buchner eyelets, in the accessory medulla (Helfrich-Forster et al., 2002, Helfrich-Forster et al., 2007). Ablation of lLNvs reduces phase shifts induced by brief light pulses late at night (Shang et al., 2008). In addition, the lLNvs increase their firing rate upon light exposure, an effect mediated cell-autonomously by direct light sensing through the blue-light deep-brain photoreceptor CRYPTOCHROME (CRY) in these cells (Fogle et al., 2011, Sheeba et al., 2008). However, although PDF secretion from lLNvs is not necessary for circadian rhythms, it plays a modulatory role, since increased PDF release from lLNvs causes a period shortening in DD and an increase in morning activity in LD by acting on PDFR in sLNvs (Choi et al., 2012). Taken together, these distinct roles for sLNv- and lLNv-derived PDF signals lead to a model in which lLNvs provide light information to the circadian network through PDF signaling to the sLNvs, which then relay those signals to the rest of the network by their own secretion of PDF (Choi et al., 2012).

The two activity bouts present in daily rhythms of *Drosophila* are regulated by a dual-oscillator network that separates the individual cells of the circadian circuit into two main groups, the morning (M) and the evening (E) cells, a distinction based on their ability to drive the respective activity bouts. Cell ablation studies and cell specific rescue of clock mutations identified the PDF-positive sLN_vs as M cells and the 5th PDF negative sLN_v as well as the LN_ds as E cells (Grima et al., 2004, Stoleru et al., 2004). However, the effect of PDF is not limited to the morning activity as evidenced by the fact that the evening anticipation in flies without PDF or PDFR is phase advanced (Hyun et al., 2005, Lear et al., 2005, Mertens et al., 2005, Renn et al., 1999).

PDF induces a coherent behavioral rhythm by synchronizing the different oscillators within the circadian clock. PDF does so by slowing down the clock in the E-cells and speeding it up in M-cells. This is supported by two independent observations (i) loss of *PDF* or *PDFR* causes the different circadian neurons to oscillate without a coherent rhythm (Lin et al., 2004, Peng et al., 2003, Yoshii et al., 2009) and (ii) increasing PDF signaling causes split molecular and behavioral rhythms with a long and a short period (Choi et al., 2009, Helfrich-Forster et al., 2000, Nitabach et al., 2006, Wu et al., 2008, Yoshii et al., 2009). Furthermore, altering the speed of molecular oscillations in PDF cells also causes changes in speed of molecular oscillations in non-PDF clock cells (Stoleru et al., 2005, Yao and Shafer, 2014), and this effect is dependent on PDF signaling (Yao and Shafer, 2014). In addition, changing the speed of molecular oscillations in non-PDF clock cells alters behavioral rhythms even in the absence of PDF signaling (Yao and Shafer, 2014).

PDF's role in synchronizing M and E cells is mediated by its cyclical expression in the dorsal terminals of sLN_v neurons, which is highest around dawn (Park et al., 2000), coincident with greatest electrical activity and, hence, secretion (Cao and Nitabach, 2008, Cao et al., 2013a). Consistent with the role of PDF in synchronizing the different oscillators is the observation that PDFR is expressed in most of the circadian neuron classes (including sLN_vs but not ILN_vs), while there is only limited expression outside the circadian clock (Im et al., 2011, Im and Taghert, 2010, Shafer et al., 2008). Based on these data one might conclude that phasic release of PDF is necessary to induce behavioral rhythms. However, constitutive activation of PDFR in non-PDF clock cells using a membrane-tethered PDF isoform (t-PDF) restores robust behavioral rhythms in *pdf⁰¹* flies (Choi et al., 2009, Choi et al., 2012). These flies exhibit complex free-running rhythms simultaneously expressed long- and short-period components, similar to those induced by ectopic constitutive expression of normal secreted PDF (Helfrich-Forster et al., 2000). In this scenario, the absence of phasic PDFR activation causes dispersal of molecular oscillations among various classes of clock cells, while constitutive activation of PDFR permits those various classes to simultaneously drive locomotor rhythms. Thus in addition to changing the pace of the molecular clock, PDF also acts as a gating signal that enables non-PDF clock neurons to drive locomotor rhythms at their respective intrinsic molecular oscillator pace.

Since there is only one receptor for PDF, how is it possible that activation of the same receptor is slowing down the clock in E cells and speeding up the clock in M cells? The answer to this could lie in the ability of PDFR to distinctly couple with different subtypes of adenylate-cyclase specific to M and E cells (Duvall and Taghert, 2012, Duvall and Taghert,

2013). Knockdown of adenylate cyclase 3 (AC3) using RNAi disrupts PDF signaling in M cells but not E cells (Duvall and Taghert, 2012), while RNAi against adenylate cyclase 87C (AC87C) disrupts PDF signaling in E cells but not M cells (Duvall and Taghert, 2013). Thus critical pathways of circadian synchronization are mediated by highly specific second messenger components. In support of this, two recent studies demonstrated that PDFR mediated increases in intracellular cAMP affects the molecular clock differently in separate sets of clock neurons. While cAMP, via PKA, stabilizes the clock protein TIMELESS (TIM) in DN1s (Seluzicki et al., 2014), it stabilizes the clock protein PERIOD (PER) in sLNvs (Li et al., 2014). Moreover, AC3 disruptions in small LNv did not alter GPCR signaling by other ligands that elevate cAMP levels in these neurons (dopamine and the neuropeptide DH31) (Duvall and Taghert, 2012). Hence, within sLNvs, PDFR signaling occurs via discrete molecular pathways that are distinct from those controlled by other cAMP-elevating ligands. In addition to targeting specific molecular components of the oscillator mechanism, PDFR in different cells varies in its ability to influence the molecular oscillator. While four of the six LNs express PDFR, only two of those PDFR-positive LNs synchronize their molecular oscillations to the pace of oscillation in sLNvs (Yao and Shafer, 2014). These studies support the hypothesis that PDF signaling components within target cells are sequestered into “circadian signalosomes” whose compositions differ between different pacemaker cell types. Future studies should address the molecular mechanisms that underlie the organization of these “circadian signalosomes”.

DH44 and circadian rhythms

A recent study identified neurons of the pars intercerebralis (PI) as a target region for output signals of the clock (Cavanaugh et al., 2014), consistent with earlier speculations that predicted the PI to be the major target of clock cells (Hall, 2003). Combining RNA sequencing transcriptome profiling, immunocytochemistry, and cell-specific genetic manipulations revealed that the neuropeptide DH44 is a critical output of a specific subset of PI neurons for regulating circadian rhythms (Cavanaugh et al., 2014), although the cellular targets of DH44 as a clock output remain unknown. Since PI neurons arborize extensively throughout the fly brain and also release their signaling molecules into the hemolymph, they can either act specifically on pre-motor circuits or more systemically prime the entire body. There are two class B1 receptors through which DH44 signals (Hector et al., 2009, Johnson et al., 2004). Both are expressed in the brain and signal through increases in cAMP. Future work should include identifying the cellular targets of DH44 released from PI cells, and through which DH44 receptor it acts.

Sleep in *Drosophila*

The fruit fly *Drosophila melanogaster* has proven to be a productive model organism for investigating the basic mechanisms of sleep regulation. Sleep in flies is defined operationally as a period of 5 minutes or more of locomotor inactivity. *Drosophila* are diurnal animals that mostly sleep at night, but also during a midday “siesta” (Hendricks et al., 2000, Shaw et al., 2000) (Figure 5). Fly sleep shares all the behavioral hallmarks that define sleep in higher animals, including adoption of a stereotyped body posture, increased sensory arousal threshold, changes in neural activity, circadian regulation, and homeostatic response to sleep deprivation (Hendricks et al., 2000, Shaw et al., 2000, van Alphen et al.,

2013). Moreover, genes regulating sleep as well as the basic neural architecture of sleep circuits are conserved in animal evolution (Parisky et al., 2008, Sehgal and Mignot, 2011).

PDF and sleep

In addition to coordinating rest:activity rhythms PDF is also a wake-promoting agent. Under natural conditions the role for PDF is most likely to increase light mediated arousal during the day since (i) ILNvs directly respond to light (Fogle et al., 2011, Sheeba et al., 2008), (ii) cell ablation of ILNvs increases daytime sleep (Shang et al., 2008), and (iii) increasing electrical activity of LNvs suppresses sleep during the night (Parisky et al., 2008, Shang et al., 2008, Sheeba et al., 2008). In addition to light, PDF-positive LNvs are also under control of sleep promoting GABAergic neurons which regulate sleep by acting on ionotropic RDL receptors (defined by their resistance to the insecticide and GABA_A antagonist dieldrin) (Agosto et al., 2008, Parisky et al., 2008, Chung et al., 2009) and metabotropic GABA_B receptors (Gmeiner et al., 2013). LNvs also receive wake-promoting signals from dopaminergic neurons which act through the DOPR receptor (Lebestky et al., 2009, Shang et al., 2011). The sources of both sleep-regulating signals are currently unknown and future work should address this question. The wake-promoting role of PDF largely emanates from the ILNvs, which signal to sLNvs but also other unknown targets, as demonstrated by the fact hyperexcitation of ILNvs is still wake-promoting in the absence of sLNvs (Sheeba et al., 2008).

DH31 and sleep

The neuropeptide DH31 acts as a wake-promoting signal that awakens the fly late at night in anticipation of dawn (Kunst et al., 2014). DH31 is expressed in a subset of DN1 clock neurons, which also express PDFR (Kunst et al., 2014). Activation of PDFR in these neurons suppresses sleep late at night, similar to what can be seen with increased DH31 signaling, and this suppression of sleep is dependent on DH31 expression (Kunst et al., 2014). As mentioned earlier, PDF signaling to DN1s is sufficient for circadian rhythms in DD (Zhang et al., 2010, Lear et al., 2009). However, DH31 has no effect on circadian rhythms, neither does activation of PDFR in DH31-expressing DN1s (Kunst et al., 2014). Thus, this PDF-to-DH31 peptide relay is a sleep specific output of the circadian clock, which functions independent of circadian rhythms. The targets of sleep-regulating DH31 signals remain unknown, and future work should address this important issue.

Class B1 Control of *C. elegans* Locomotion and Lethargus

Recently, homologs of the PDF pathway were characterized in *Caenorhabditis elegans* (Janssen et al., 2009, Janssen et al., 2008). BLAST homology searches using *Drosophila* PDFR as template identified the *pdf-1* gene in *C. elegans*. A pharmacological screen for activation of PDFR then uncovered three worm peptides, PDF-1a, PDF-1b, and PDF-2, encoded by two genes, *pdf-1* and *pdf-2*. Each of these peptides display moderate to strong homology to *Drosophila* PDF and crustacean Pigment Dispersing Hormone. Cell culture studies indicate that splice isoforms of the *C. elegans* PDFR couple to G_{qs} like other class B1 GPCRs. At the molecular level, the PDF signaling system is conserved within the worm.

The PDF peptides, both *pdf-1* and *pdf-2*, are expressed in a variety of cells (Janssen et al., 2008, Janssen et al., 2009). Within the nervous system, both peptides are expressed, with limited overlap, in sensory neurons, interneurons, and motorneurons. Though fewer in number compared to the peptides, the PDFR is also expressed in sensory neurons, mostly mechanosensory. PDFR is predominantly found in interneurons, motorneurons, and muscle cells. Based on expression patterns alone, the worm PDF signaling system is poised for sensorimotor function.

Initial phenotypic characterization of *pdf-1*, *pdf-2*, or *pdf-1* null mutants affirm this expectation (Janssen et al., 2009, Janssen et al., 2008, Meelkop et al., 2012). All mutants exhibit subtly to strongly altered locomotion parameters used for navigation, including the frequency of backwards movements, called reversals, and turning that lead to directional changes. Within the PDF peptides, however, worms with null mutations in *pdf-1* and *pdf-2* alter different features of locomotion, consistent with their distinct expression patterns. The *pdf-1* null mutants exhibit lower speeds and higher reversal frequencies, whereas the *pdf-2* null mutants only exhibit slightly altered reversal behavior. The *pdf-1* null mutants copy the *pdf-1* null mutants' behavior.

Are these altered locomotion patterns consistent with a conserved function of the PDF signaling system? Of the two peptides, PDF-1 function has been studied in far greater detail. These studies reveal a consistent role in arousal behaviors, suggesting that PDF function is indeed conserved. Circadian behaviors are not fully characterized in *C. elegans*. However, developing worms enter a behaviorally quiescent, sleep-like state called lethargus during each larval molt with rhythmic precision (Raizen et al., 2008). Further, the timing of the developmental molt is regulated by LIN-42, the worm homolog of PER, suggesting a genetic link between circadian rhythms in flies and control of developmental timing in the worm (Jeon et al., 1999, Monsalve et al., 2011). Interestingly, exit from the lethargus state depends on PDF-1 secretion and PDFR function in mechanosensory neurons (Choi et al., 2013). Thus in developing worms, PDF-1 drives arousal via modulation of sensory thresholds. Adult worms also exhibit timed switches in foraging strategy. These behavioral switches manifest in transitions from "roaming" and "dwelling," described by active exploration of the environment and navigational quiescence, respectively (Flavell et al., 2013). Interestingly, here too PDF-1 is involved in the transition from dwelling to roaming. Similar mechanisms potentially underlie the findings of another study that demonstrates the dependence of male worms' mate searching on PDF-1 signaling (Barrios et al., 2012). Thus in adults as well, in different contexts, PDF-1 consistently modulates arousal states. These studies indicate that PDF function is conserved in the worm. Further molecular and cellular analysis of PDF signaling in the worm will continue to provide mechanistic insight into this conserved signaling system.

Conclusions

Class B1 neuropeptide signaling can be found throughout the circadian systems of both flies and mammals. In *Drosophila*, PDF/PDFR influences sleep and circadian rhythms at many stages of neural processing. In mammals, VIP/VPAC2R regulates circadian synchrony within the SCN and OB, and likely elsewhere in the brain. However, there are still many

questions that remain to be answered, especially in respect to the influence of the activation of class B1 receptors in target cells. The many parallels between class B1 signaling in the circadian control systems of *Drosophila* and mammals are remarkable, and the combined efforts exploiting these two model organisms to address the many fascinating remaining questions are highly synergistic.

Recent work in *Drosophila* has begun to investigate how PDF can affect electrical properties short-term as well lead to more long-term changes of protein expression and stability (Li et al., 2014, Seluzicki et al., 2014). Furthermore, while PDF has been extensively researched for the last decade, DH31 and DH44 research is still in its early stages and much more investigation needs to be done to understand their roles in regulating behavior and physiology.

The SCN depends on multiple neuropeptides to balance the degree of synchrony among circadian cells and tissues with environmental conditions. For example, during short days, the cells are more in phase than during the long days of summer (Farajnia et al., 2014, Meijer et al., 2012). The specifics of how this is accomplished are not fully understood. The seasonal changes in the strength of intercellular signaling could adjust the degree of cell-cell synchrony either through changes in neuropeptide release, receptor levels, or intracellular signaling pathways. Interestingly in this regard, it has been shown in *Drosophila* that light inhibits outputs from PDF neurons through indirect miRNA regulation of downstream PDFR signaling (Picot et al., 2007, Zhang and Emery, 2013). In addition, the relative importance of specific neuropeptides may change with environmental conditions, age, or sex. Multiple ligands and multiple receptors may allow for expanded functional plasticity. Since ligands acting through class B1 receptors all elevate intracellular cAMP, it remains unclear whether, and if so how, signals through multiple class B1 receptors expressed in the same cell are distinguished.

List of Abbreviations

AC3	Adenylate cyclase 3
AC87	Adenylate cyclase 87C
AVP	Arginine vasopressin
cAMP	3'-5'-cyclic adenosine monophosphate
CALCR	Calcitonin receptor
CALCRL	Calcitonin receptor-like receptore
CHRH1	corticotrophin-releasing hormone receptor 1
CHRH2	corticotrophin-releasing hormone receptor 2
CRY	Cryptochrome
DD	Constant darkness
DH31	Diuretic hormone 31

DH44	Diuretic hormone 44
DH44 R1	Diuretic hormone 44 receptor 1
DH44 R2	Diuretic hormone 44 receptor 2
DN1a	anterior dorsal neurons 1
DN1p	posterior dorsal neurons 1
DN2	dorsal neurons 2
DN3	dorsal neurons 3
GCGR	Glucagon receptor
GH	growth hormone
GABA	γ -amino-butyric acid
GHRH	growth hormone releasing hormone
GHRHR	growth hormone releasing hormone receptor
GIPR	Gastrin inhibitory polypeptide receptor
GLP1R	Glucagon receptor-related receptor 1
GLP2R	Glucagon receptor-related receptor 2
GPCR	G-protein coupled receptor
IGF-1	insulin-like growth factor 1
LD	Light:dark
ILNv	large lateral ventral neuron
LNd	lateral dorsal neuron
LPN	lateral posterior neuron
mTOR	mammalian target of rapamycin
NREM sleep	non-rapid eye movement sleep
OB	olfactory bulb
PAC1	Pituitary adenylate cyclase-activating peptide type 1 receptor
PACAP	Pituitary adenylate cyclase-activating peptide
PDF	pigment dispersing factor
PDFR	pigment dispersing factor receptor
PER	Period
PI	Pars intercerebralis
PTHr1	parathyroid hormone receptor 1
PTHr2	parathyroid hormone receptor 2

PHI	Peptide Histidine Isoleucine Amide
PKA	Protein Kinase A
PLC	phospholipase C
RNA	Ribonucleic acid
RNAi	RNA interference
SCN	suprachiasmatic nucleus
SCTR	secretin receptor
sLN_v	small lateral ventral neuron
TIM	Timeless
VIP	vasoactive intestinal peptide
VPAC1	vasoactive intestinal peptide receptor 1
VPAC2	vasoactive intestinal peptide receptor 2

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Figure 1. Phylogenetic tree of class B1 GPCRs

Constructed using an all-against-all comparison of protein sequence, the tree illustrates sequence similarity among the 25 known members of the family. Only *Vipr2* in mammals and *Pdfr* in flies have been shown to play important roles in daily rhythms. Human genes are in red, *Drosophila* genes are in blue, and *C. elegans* gene are in green. Modified from (Harmar, 2001). Color version of this figure is available online.

Figure 2. Regulation of mammalian circadian rhythms by VIP and VPAC2R

(A) A computer model simulates the essential role for VIP and its receptor, VPAC2, in synchronizing circadian rhythms among SCN neurons (Ananthasubramaniam et al., 2014). The model reveals that the relative timing of VIP release and VPAC2R availability impacts synchrony and rhythms amplitude. For example, VIP released during subjective day enhances synchrony among rhythmic neurons (top). Release during the night fails to support circadian synchrony (middle). In contrast, time of peak daily VPAC2R expression can enhance rhythms amplitude, but has little impact on synchrony when VIP is released during the day (yellow circles) and fails to rescue the loss of synchrony when VIP is released at night (green squares; bottom).

(B) VIP/VPAC2 signaling cascade in the SCN. (upper right) Transcription of *Vip* depends on the transcription factor Lhx1. Translation initiation of *Vip* is blocked by a complex formed by 4E-BP1 and eIF-4E. This can be disinhibited by mTOR-dependent phosphorylation. The translated product, prepro-VIP, undergoes steps of cleavage and packaging before it is released, presumably when the neuron fires action potentials with the appropriate pattern and rate. (Lower left) When VPAC2R is activated by VIP, both Gs and Gq pathways mediate induction of the circadian clock genes, Period 1 and 2 (Per), through concurrent activation of cAMP and PLC and subsequent phosphorylation of CREB. Color version of this figure is available online.

Figure 3. *Drosophila* circadian control network

(A) Average locomotor activity plot of 4–7 day old wild-type flies in 12 h of light and 12 h of dark conditions per 15-minute bin. Light and dark bars indicate activity levels during light and dark, respectively. Increases in activity are present prior to lights-on (morning anticipation) and lights-off (evening anticipation) transitions. (B) Average locomotor activity on the first day of constant darkness dark bars indicate subjective night and light bars subjective day. Two distinct peaks of activity (morning and evening peak) are recognizable. (C) Actogram for a group of wild-type flies in constant darkness. The height of vertical bars indicates level of activity during a 30-min interval. Each horizontal line contains two days of activity data, with the second day of data on one line repeated on the first day of data on the following line to ease visualization of the circadian period. Under DD, activity bouts occur at the same time each day, indicating a free-running period of near 24 h. (D) Map of the different groups of circadian clock cells and their anatomical position within the fly brain. Color version of this figure is available online

Figure 4. Regulation of *Drosophila* circadian rhythms by PDF

(A) Schematic of PDF dependent control of eclosion rhythms. Rhythmic release of PDF from LNvs (red) acts on Prothoracic Ganglion neurons (PG, light blue) to control eclosion (see text for details). (B) Role of class B1 neuropeptides for the control of rhythmic locomotor behavior. PDF, rhythmically released from sLNvs acts on evening cells (light blue, 5th sLNv and LNd) and DN1 (green) to control various facets of circadian locomotor behavior. From DN1s, the circadian information is propagated via unknown signals to pars intercerebralis cells (PI, purple) which secrete DH44 to control locomotor rhythms in constant darkness. In addition, DN1 also release DH31 to control sleep late at night. sLNvs also receive light dependent PDF input from ILNvs which promotes morning anticipation (see text for details). Color version of this figure is available online.

Figure 5. *Drosophila* sleep

Average sleep plot of 4–7 day old wild-type flies in 12 h of light and 12 h of dark conditions per 30 minute bin. The corresponding light:dark cycle is represented by the white (light phase) and the grey (dark phase) background. Flies mostly sleep during the night and have a second prominent sleep bout during the day.