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E AND M CIRCADIAN PACEMAKER NEURONS USE DIFFERENT PDF RECEPTOR SIGNALOSOME COMPONENTS IN *DROSOPHILA*

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Abstract

We used real-time imaging to detect cAMP levels in neurons of intact fly brains to study mechanisms of circadian pacemaker synchronization by the neuropeptide PDF in *Drosophila*. PDF receptor (PDF-R) is expressed by both M (sLNv) and E (LNd) pacemaker sub-classes and is coupled to $G_{s\alpha}$ in both cases. We previously reported that PDF-R in M pacemakers elevates cAMP levels by activating the ortholog of mammalian Adenylate Cyclase 3 (AC3), but that *AC3* disruptions had no effect on E pacemaker sensitivity to PDF. Here we show that PDF-R in E pacemakers activates a different AC isoform, AC78C, an ortholog of mammalian AC8. Knockdown of *AC78C* by transgenic RNAi substantially reduces, but does not completely abrogate, PDF responses in these E pacemakers. The knockdown effect is intact when restricted to mature stages, suggesting a physiological and not a development role for AC78C in E pacemakers. The *AC78C* phenotype is rescued by over-expression of *AC78C*, but not by over-expression of the *rutabaga* AC. *AC78C* over-expression does not disrupt PDF responses in these E pacemakers, and neither *AC78C* knockdown nor its over-expression disrupted locomotor rhythms. Finally, knockdown of two AKAPs, nervy and AKAP 200 partially reduces LNd PDF responses. These findings begin to identify the components of E pacemaker PDF-R signalosomes and indicate they are distinct from PDF-R signalosomes in M pacemakers: we propose they contain AC78C and at least one other AC.

Keywords

Pigment Dispersing Factor; Neuropeptide; Signalosomes; *Drosophila melanogaster*; Circadian Circuit; Pacemaker Synchronization

INTRODUCTION

Neural circuits that coordinate daily locomotor rhythms utilize cell intrinsic molecular oscillators that produce robust 24 rhythms. Studies of such circadian neural circuits in both mice and in *Drosophila* suggest they are composed of multiple oscillators that are interconnected by synaptic and modulatory interactions that promote their sensitivity to environmental conditions and their synchronization (Welsh et al., 2010; Allada and Chung, 2010). Specific neuropeptides are critical synchronizing agents: in the mouse approximately 10% of the ~20,000 neurons in the suprachiasmatic nucleus express Vasoactive Intestinal

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Polypeptide (VIP); in the fly brain, a similar percentage of the ~150 circadian pacemaker neurons express its functional ortholog, Pigment Dispersing Factor (PDF). Genetic analyses suggest both VIP and PDF are instrumental for proper display of cellular and behavioral 24-hour rhythms. Thus definition of the neuropeptide signaling pathways that mediate pacemaker synchronization represents an important experimental goal for the field.

In *Drosophila*, pacemakers are categorized into M or E subgroups based on the preponderant actions they show with respect to the two prominent peaks of daily locomotor activity (Grima et al., 2004; Stoleru et al., 2004; Yoshii et al., 2003). Small LNvs principally are associated with morning activity, while LNds (along with several other subgroups) are associated with evening activity. These behavioral assignments are not rigid and can change under different environmental conditions (Rieger et al., 2009; Zhang et al., 2010; Peschel and Helfrich-Förster 2011). Understanding how M and E cells interact and how their hierarchical relationships can change due to network properties are key issues to help understand how a complex neural circuit controls locomotor behaviors. M cells release the 18 amino acid peptide PDF which targets its receptor, PDF-R in subsets of both M and E pacemaker subgroups (Shafer et al., 2008; Im and Taghert, 2010) but between M and E pacemakers, PDF: PDF-R signaling has different consequences.

For example, loss of PDF peptide alters molecular oscillations in multiple clock cells but M and E cell subgroups respond differently. Loss of PDF signaling desynchronizes daily rhythms of intensity and sub-cellular localization of PER staining in M cells, but produces an advanced phase and reduced amplitude of PER staining in E cells (Lin et al., 2004, Lear et al., 2005). In addition, mutations that increase overall PDF levels in the brain and expression of a membrane-anchored PDF (to effect constitutive autoactivation) both result in complex (i.e., multiple) behavioral rhythms (Choi et al., 2009; Wulbeck et al., 2008). This suggests PDF can accelerate some clock cells and slow others down (Yoshii et al., 2009). The interaction between PDF signaling and cryptochrome signaling pathways differs between M and E cells: double mutants of *pdf* and *cry* show normal molecular oscillations in M cells, while E cells show severely disrupted clock oscillations (Cusumano et al., 2009; Zhang et al., 2009; Im et al., 2011). Finally, we recently reported that PDFR in M cells is coupled to the AC3 isoform, but that disruptions of AC3 have no such effect on PDF responsiveness in E cells (Duvall and Taghert, 2011). This result suggested PDFR signalosomes in E cells must employ a different AC isoform(s); here we explore the identification of the E cell AC isoform(s).

Previous work suggests that the E cell subgroup is the primary driver of circadian locomotor behavior. Broad expression of tethered PDF in the clock network produces complex rhythms in pdf^{01}, even when it is not expressed in M cells (Choi et al., 2009). Lear and colleagues reported that expression of the PDF receptor exclusively in E cells provides rescue of locomotor behavior in *pdf-r* mutants, although it is incomplete (Lear et al., 2010; Im et al., 2011). These studies suggest that disruption of E cell PDF signaling results in predictable disruptions of circadian behavior. Based on the importance of PDF-R activation in E cells, the nature of PDF-R signalosomes is therefore a significant issue to understand mechanisms of circadian synchronization in the *Drosophila* brain.

MATERIALS AND METHODS

Fly Rearing and Stocks

Drosophila were reared on cornmeal/agar supplemented with yeast at 25°C, unless otherwise indicated by experimental design. Male flies (age 2 to 5 days old) were moved to 29°C for 24 – 48 hours before imaging to increase UAS transgene expression. For temperature shift (tubulin gal80*ts*) experiments, crosses were maintained at 18°C to maintain

gal80*ts* suppression of gal4 and males were collected and moved to 29°C for 24 – 48 hours before imaging to allow UAS transgene expression. All gal4 and UAS lines used in this study have been described previously: UAS- *Epac1camps50A* (Shafer et al., 2008), *Mai179*gal4 (Cusumano et al., 2009), tim(UAS)gal4 (Blau and Young, 1999), UAS-*ACXD*, -*AC3*, -*AC78C*, -*AC76E* (Duvall and Taghert, 2012). The TRiP RNAi (UAS-TRiP*AC3*RNAi, UAS-TRiP-*nervy*RNAi, UAS-TRiP*AKAP200*RNAi, UAS-TRiP:*CG33958*, UAS-TRiP:*CG31183*), UAS-*rutabaga*, UAS-*dicer2*, tubulin gal80*ts* and *Df*(3)*LBSC553* lines were obtained through the Bloomington Stock Center (thanks to the Harvard TRiP RNAi project) and the UAS-GD*AC3*RNAi, UAS-*AC13*ERNAi, UAS-*AC78C*, UAS-*rut*RNAi, UAS-*ACXA*RNAi, UAS*ACXB*RNAi, UAS-*ACXC*RNAi, UAS*ACXD*RNAi, UAS-*yu*RNAi, UAS-*rugose*RNAi, UAS-KK:*CG32305*, UAS-*GD:CG32301* lines were obtained through the Vienna RNAi Stock Center.

Live Imaging

For epifluorescent FRET imaging, living brains expressing gal4-driven uas-*Epac1-camps* were dissected under ice-cold calcium-free fly saline (46mM NaCl, 5mM KCl, and 10mM Tris (pH 7.2). All genotypes include one copy of each transgene unless otherwise indicated. Dissected brains were placed on poly-l-lysine coated coverslips in an imaging chamber (Warner Instruments) and HL3 saline was perfused over the preparation (0.5 mL/minute) as described in Duvall and Taghert, (2012). Exposure times were 20 ms for YFP- FRET and 500 ms for CFP donor emission. Live FRET imaging was performed on individual cell bodies, YFP-FRET and CFP donor images were captured every 5 seconds with YFP and CFP images captured sequentially at each timepoint. Following 45 seconds of baseline YFP/CFP measurement the PDF peptide was injected into the perfusion line to result in a final concentration of 10^{-6} M. FRET readings were then continued to result in a total imaging timecourse of 10 minutes. PDF was synthesized by Neo-MPS (San Diego, CA).

FRET Imaging Data Analysis

For all experiments reported, we collected responses from at least 10 cells that were found in at least 5 brains for all genotypes. A region of interest (ROI) defined each individual neuron and for each, we recorded background-subtracted CFP and YFP intensities. The ratio of YFP/CFP emission was determined after subtracting CFP spillover into the YFP channel from the YFP intensity as in Shafer et al., 2008. The CFP spillover (SO) into the YFP channel was measured as .397. For each timepoint, FRET was calculated as (YFP-(CFP*SO CFP))/CFP. To compare FRET timecourses across different experiments FRET levels were normalized to initial baseline levels and smoothed using a 7-point boxcar moving average over the 10 min imaging timecourse, as in Duvall and Taghert, (2012). Statistical analysis was performed at maximal deflection from the initial timepoint by performing ANOVA analysis, followed by post-hoc Tukey tests using Prism 5.0 (Graphpad Software Inc).

RESULTS

AC78C scores positive in an *in vivo* RNAi screen targeting responses to PDF

We previously reported that PDF responses in LNd are completely abrogated by mutation of the PDF receptor and that PDF receptors are coupled to the $G_{s\alpha}$ subunit (Duvall and Taghert, 2012). However, while manipulations of adenylate cyclase 3 (AC3) alter PDF responsiveness in M pacemakers, they have no such effect on E cell PDF responses. That suggests that the two pacemaker subgroups use different signaling components (Duvall and Taghert, 2012). To pursue E cell PDF-R signaling components, we therefore performed an RNAi screen directed against 13 of the 14 predicted ACs in the *Drosophila* genome. At the time of our experiments no available RNAi line targeted ACXE, a cyclase that is predicted to be expressed in the male germline exclusively (Cann et al., 2000). We used the

*Mai179*gal4 driver to express UAS-RNAi transgenes in the three (of six) LNds that are known to express the PDF receptor and cryptochrome (Im and Taghert, 2010; Yoshii et al., 2009). By focusing on neurons that express the PDF receptor, we reasoned that a negative result would be more likely explained by an effect on PDF signaling. When individual AC isoforms were knocked down in LNds, *AC78C* RNAi significantly reduced PDF responses, although not completely (Figure 1A). Five ACs are broadly expressed in *Drosophila* tissues (Flybase, 2012; DroID.org, 2012: *AC78C, AC76E, AC3*, *rutabaga* and *AC13E*) and they arguably represent the strongest initial candidates for an LNd AC coupled to PDF-R. We therefore screened RNAi constructs for these five ACs (Fig 1B) with the addition of a *UASdicer2* transgene to increase efficiency of the RNAi knockdown (Dietzl et al., 2007). However this screen did not implicate any additional candidate ACs (Figure 1B): Expression of the *UASdicer2* transgene itself partially reduced PDF responses and only *AC78C* RNAi produced a further significant reduction in PDF response. However, that reduction was once again not complete, suggesting the involvement of an additional and, as yet, unidentified AC.

Genetic confirmation of a role for *AC78C* in E cell PDF responses

We evaluated the *AC78C* RNAi phenotype in two ways. First we asked whether the loss-of-function phenotype could be explained by developmental, as opposed to physiological, effects of AC78C in E cells. Thus we employed a conditional genetic design (via *tub*-gal80^{ts}), which permits normal development followed by induction of RNAi only in the adult fly (McGuire et al., 2003). When induction of *AC78C* RNAi was restricted to adult stages it nevertheless produced a reduction in PDF responses that was quantitatively similar to results following continuous knockdown (Figure 2A). Expression of the *tub*-gal80^{ts} transgene alone had no effect on PDF responses (Figure 2A). This suggests that the reduction of E cell PDF responsiveness is not due to developmental defects and that, in mature LNd, AC78C plays a substantial role in mediating PDF responses in these pacemakers.

As a second test, we combined *AC78C* RNAi with different UAS-*AC* over-expression constructs to determine if the loss-of-function phenotype could be reversed. Because the VDRC *AC78C* RNAi transgene targets a portion of the AC coding region (Vienna *Drosophila* RNAi Center), both the endogenous *AC78C* as well as the over-expression RNA are targeted in this design. Nevertheless, over-expression of *AC78C* cDNA fully restored PDF responsiveness in E cells expressing *AC78C* RNAi. In contrast, over-expression of the *rut* AC did not fully rescue PDF responses in such E cells (Figure 2D). The latter observation provides a measure of rescue specificity, and also rules out the possibility that the reversal was due to simple dilution of GAL4's efficacy, due to the presence of an additional UAS transgene. Overexpression of *rutabaga* provides a small increase in PDF responsiveness in the *AC78C*RNAi background suggesting that *rutabaga* partially substitutes for *AC78C*. This is in contrast to the situation we found earlier in small LNv, in which *rutabaga* showed no level of substitution for the relevant cyclase (AC3). This observation highlights another difference between M and E cell signaling.

Compound *AC* RNAi knockdowns do not further reduce E cell PDF responses

To further evaluate the candidacy of *AC78C* in E cell PDF responses, we combined *AC78C* RNAi with a deficiency for the *AC78C* region. We did not observe any further decrement in the E cell PDF response, suggesting the *AC78C* RNAi knockdown is effectively complete (Figure 2B). That finding again raises the possibility that one or more additional ACs normally contribute to E cell PDF responses. Using this logic, we therefore tested several pairwise combinations of *AC* RNAi lines. However, no combination of RNAi lines significantly reduced PDF responses beyond the level found with *AC78C* knockdown alone

although the combination of AC78C and AC3 knockdown produces a subpopulation of cells that fail to respond to PDF (Figure 2C). This suggests that there may be a subset of LNds that depend specifically on AC78C/AC3. We also combined a UAS-*AC3* with *AC78C* RNAi – *AC3* overexpression totally and specifically disrupts PDF responses in M cells (Duvall and Taghert, 2012). However, this combination did not further reduce the E cell PDF-generated cAMP responses either (Figure 2C).

Overexpression of AC isoforms had no effect on LNd PDF responses

We previously reported that in small LNvs, PDF-R is associated with AC3 and that AC3 over-expression in that M pacemaker type specifically disrupted PDF signaling (Duvall and Taghert, 2012). On that basis, we anticipated a finding wherein overexpression of the relevant AC in LNds (possibly AC78C) would abrogate PDF responses in that E pacemaker cell type. We therefore tested the effects of UAS-mediated overexpression of -*ACXD*, -*AC78C*, -*AC76E*, -*rut* and -*AC3* on PDF responses in E pacemakers, but we saw no effects (data not shown). This suggests that LNd signaling pathways are less sensitive to overexpression of cognate ACs and may use some compensatory mechanism(s) to maintain PDF responsiveness. Regardless of the exact mechanism, this observation highlights another difference between PDF responses in small LNvs versus LNds.

AKAPs nervy and AKAP200 knockdown reduced LNd PDF responses

Scaffolding proteins such as AKAPs can bind to signaling components to organize efficient signaling (Dessauer 2009). Knockdown of the AKAP nervy reduced PDF responses in small LNv (Duvall and Taghert, 2012). In similar fashion, we tested the possible involvement of four candidate AKAPs as scaffolding proteins for PDF-R in LNd cells using gene-specific RNAi constructs. Knockdown of either *AKAP200* or *nervy* reduced LNd PDF responses (Figure 3). Knockdown of *AKAP200* reduces PDF responses to about 40% of their original levels whereas knockdown of *nervy* only reduced PDF responses to about 65% of their original levels. These findings are consistent with the possibility that multiple signalosomes normally mediate PDF responses in LNds.

AC78C manipulations did not result in a circadian locomotor phenotype

Previous work suggests that PDF signaling within the E cell subgroup is largely responsible for normal circadian locomotor behavior (Choi et al., 2009; Lear et al., 2009; Im et al., 2011). Because LNds make up only a subset of E cells in the *Drosophila* brain, and because the *Mai179*gal4 driver is relatively weak and restricted to a small subset of cells, we primarily used a stronger driver, expressed broadly throughout the clock network (*tim(UAS)*gal4) to drive UAS transgenes in locomotor assays. We expected that disruptions to PDF responses by AC knockdown in these cells might phenocopy animals with PDF-R expressed only in the M cell subgroup (small LNv). Specifically, we looked for an advance in the evening peak in LD conditions and weak short rhythms in DD (cf. Hyun et al., 2005). However, *AC78C* manipulations that partially reduced PDF responses in E pacemakers did not result in behavioral deficits, even with the addition of a UAS*dicer2* transgene to increase RNAi knockdown efficiency (Figure 4). A single experiment using the *Mai179*gal4 driver to drive *AC78C* RNAi gave similar behavioral results (Table 1; Supplementary Figure 1). This result is consistent with the view that partial reductions in PDF responsiveness are not sufficient to produce behavioral changes. Indeed, our previous results suggested that only AC knockdowns that produce severe reductions in PDF responsiveness also affect locomotor behavior (Duvall and Taghert, 2012). Additionally, to date, no combination of genetic elements that partially reduce LNd PDF responses (AC RNAi/AKAP RNAi /AC78C *Df* etc.) shows circadian disruptions consistent with a total loss of PDF signaling (data not shown).

DISCUSSION

Multiple lines of evidence suggest PDF signaling differs between clock cell subgroups. (i) Loss of PDF has distinct effects on PERIOD protein cycling in LNv (M cells) versus non-LNv cells (E cells) (Lin et al., 2004). Both cell groups continued to show cycling in PER immunostaining levels and localization but, while M cells become phase-dispersed in PER cycles, E cells remain synchronized with altered phase and amplitude of PER accumulation. (ii) In *pdf/cry* and *pdfr/cry* double mutants, a subset of E cells show a phase advance and/or severe attenuation (Im et al., 2011) of the PER protein molecular rhythm, while M cells continue to cycle normally (Zhang et al., 2009; Park et al,. 2009; Im et al., 2011). (iii) Knockdown of *AC3* disrupts small LNv PDF responses but has no such effect in LNds. These earlier findings are consistent with the hypothesis that there are at least two functionally different PDF signaling pathways that normally operate in different pacemaker cell types.

The present results provide strong evidence that AC78C is a candidate AC for mediating a portion of PDF responses in LNd. Importantly, the partial reduction in PDF responsiveness in LNds is fully rescued by overexpression of *AC78C*, but only partially rescued by overexpression of *rutabaga*. However, we emphasize that there likely remain one or more additional ACs that contribute to PDF responses in these pacemakers, and that they remain as yet uncharacterized. The partial reduction in LNd PDF responses is still observed when *AC78C* is knocked down only in adult stages, and this reduction remains incomplete when *AC78C* RNAi is combined to a number of other UAS-*AC* RNAi lines. Hence we have been unable to completely abrogate the PDF responses in E pacemakers. The addition of the deficiency for the *AC78C* genetic region does not further reduce the PDF response, which is yet more evidence suggesting the contribution of another AC(s). The additional LNd PDFR-associated AC(s) may have been missed among the isoforms that we tested because it produced a false negative(s): it is technically possible that one or more *AC* RNAi lines does not appropriately target its cognate cyclase for degradation.

A number of studies suggest that the basic unit of AC function is a dimer (Rodbell, 1980) and that AC homodimerization may be required for normal trafficking to the plasma membrane (Gu et al., 2001; Seebacher et al., 2001; Gu et al., 2002). Previous work indicated that ACs may also form heterodimers: these may exhibit characteristics different from either of the single isoforms, or of homodimers. For example, AC2 and AC5 form heterodimers that show higher sensitivity to $G_{s\alpha}$ signaling than either of the components individually (Baragli et al., 2007). Although ACs can form specific and preferred interactions with other signaling molecules, it is likely that more passive mechanisms of coupling are also used – that signaling components are not directly interacting, but localized nearby each other in the plasma membrane (Dessauer, 2009). It is possible that LNds use less stringent coupling of PDF-R to specific ACs than do small LNvs. Thus, although there is a preference for AC78C coupling in LNds, it is possible that PDF signaling can in fact access two or more ACs isoforms to generate some sufficient level of cAMP. Alternatively, the neurons may assemble and segregate signalosomes bearing different ACs whose activation instigates different functional outcomes. In sum, we favor the hypothesis that AC78C and at least one additional AC together mediate PDF responses in E pacemakers like the LNds (Figure 5). Whether they do so jointly for instance as heterodimers, or in distinct fashion, remains to be determined.

To what extent do different signaling components actually result in differences in downstream PDF signaling between clock cell subgroups? If the considered endpoint is simply the production of cAMP, then it is possible that signalosomes with different components may nevertheless perform the same function. We observe that both M and E

cell subgroups respond to PDF with an increase in cAMP and that LNds respond with slightly higher amplitude (see: Duvall and Taghert, 2012). Our analysis has not revealed any temporal differences in PDF responses or recovery between these two subgroups, although more subtle differences - sub-second differences in kinetics or microdomain differences – are not easily detected by the imaging techniques we have used (Nikolaev et al., 2003). Alternatively, PDF-R signalosome differences between pacemaker cell types, as highlighted by AC isoform differences, may indicate fundamental differences in signaling networks, within which cAMP elevations may play important local roles. The extent to which differences in the networks that generate cAMP account for the differences in downstream effects of PDF in different clock cell subgroups helps define a testable hypothesis.

PKA is important for maintaining normal locomotor rhythms, although PKA alterations do not affect core clock proteins themselves (Majercak et al., 1997; Park et al., 2000). This has been taken to mean that a likely role for cAMP and PKA in the *Drosophila* circadian timing system might be in the flow of information between pacemaker cells and output pathways. However, these previous studies were performed with mutant flies that make it difficult to disambiguate the roles of individual pacemaker subgroups.

Why would a single neuropeptide receptor utilize multiple signaling pathways in different target cells? Recent studies have implicated feed-forward mechanisms in neuropeptide circuit modulations, often as a method of regulating competing circuit elements (Jing et al., 2007, Wu et al., 2010; reviewed by Taghert and Nitabach, 2012). Differential signalosome compositions may provide a mechanism for a single neuropeptide like PDF to differentially modulate components of the circadian circuit. In fact, the connections between *Drosophila* clock cells suggest that PDF may be working through such a mechanism. There are two sources of PDF in the *Drosophila* brain; the large and small LNvs (Helfrich-Förster, 1998). Previous work suggests that there are direct anatomical connections between some nodes of the circadian circuits; small LNv and large LNvs are directly connected and the small LNvs send projections to the dorsal brain where the E cells, including LNds are located (Helfrich-Förster et al., 2007; reviewed in Sheeba, 2008). Additionally, previous studies have also suggested that PDF released from the large LNvs can act on dorsal cells over a longer distance (Cusumano et al., 2009; Shafer and Taghert, 2009). This suggests that PDF from large LNvs can act directly on dorsal (E) clock cells and indirectly through the small LNv (M) cells. A feedforward model provides a mechanism for PDF to act differentially in its actions to promote synchrony of the circadian neural circuit in *Drosophila* and to allow flexibility in the regulation of multiple, competing oscillators.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Allada R, Chung BY. Circadian organization of behavior and physiology in *Drosophila*. Annu Rev Physiol. 2010; 72:605–24. [PubMed: 20148690]

Baragli A, Grieco ML, Trieu P, Villeneuve LR, Hebert TE. Heterodimers of adenylyl cyclases 2 and 5 show enhanced functional responses in the presence of G αs. Cellular Signaling. 2007; 20:480–492.

Blau J, Young MW. Cycling *vrille* expression is required for a functional *Drosophila* clock. Cell. 1999; 99(6):661–71. [PubMed: 10612401]

Cann MJ, Chung E, Levin LR. A new family of adenylyl cyclase genes in the male germline of *Drosophila melanogaster*. Dev Genes Evol. 2000; 210(4):200–206. [PubMed: 11180822]

Choi C, Fortin JP, McCarthy E, Oksman L, Kopin AS, Nitabach MN. Cellular dissection of circadian peptide signals with genetically encoded membrane-tethered ligands. Curr Biol. 2009; 19:1167–1175. [PubMed: 19592252]

Cusumano P, Klarsfeld A, Chelot E, Picot M, Richier B, Rouyer F. PDF-modulated visual inputs and cryptochrome define diurnal behavior in Drosophila. Nat Neurosci. 2009; 12:1431–1437. [PubMed: 19820704]

Dessaur CW. Adenylyl Cyclase-A-kianse Anchoring Protein Complexes: The Next Dimension in cAMP Signaling. Mol Pharmacol. 2009; 76:935–941. [PubMed: 19684092]

Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblauer S, Couto A, Marra V, Keleman K, Dickson BJ. A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. Nature. 2007; 448:151–156. [PubMed: 17625558]

Duvall LB, Taghert PH. The circadian neuropeptide PDF signals preferentially through a specific adenylate cyclase isoform AC3 in M pacemakers of *Drosophila*. PLoS Biol. 2012; 10(6):e1001337. [PubMed: 22679392]

Grima B, Chelot E, Xia R, Rouyer F. Morning and evening peaks of activity rely on different clock neurons of the *Drosophila* brain. Nature. 2004; 431:869–873. [PubMed: 15483616]

Gu C, Sorkin A, Cooper DMF. Persistent interactions between the two transmembrane clusters dictate the targeting and functional assembly of adenylyl cyclase. Curr Biol. 2001; 11:185–190. [PubMed: 11231154]

Gu C, Cali JJ, Cooper DMF. Dimerization of mammalian adenylate cyclases. Eur J Biochem. 2002; 269:413–421. [PubMed: 11856299]

Helfrich-Förster C. Development of pigment-dispersing hormone-immunoreactive neurons in the nervous system of *Drosophila melanogaster*. J Comparative Neuro. 1998; 380(3):335–354.

Helfrich-Förster C, Shafer OT, Wulbeck C, Grieshaber E, Rieger D, Taghert P. Development and morphology of the clock-gene-expressing lateral neurons of *Drosophila melanogaster*. J Comp Neurol. 2007; 500:47–70. [PubMed: 17099895]

Hyun S, Lee Y, Hong ST, Bang S, Paik D, Kang J, Shin J, Lee J, Jeon K, Hwang S, Bae E, Kim J. *Drosophila* GPCR Han is a receptor for the circadian clock neuropeptide PDF. Neuron. 2005; 48:267–278. [PubMed: 16242407]

Im SH, Taghert PH. PDF receptor expression reveals direct interactions between circadian oscillators in *Drosophila*. J Comp Neurol. 2010; 518:1925–1945. [PubMed: 20394051]

Im SH, Li W, Taghert PH. PDFR and CRY signaling converge in a subset of clock neurons to modulate the amplitude and phase of circadian behavior in *Drosophila*. PLoS One. 2011; 6:e18974. [PubMed: 21559487]

Jing J, Sweedler JV, Cropper EC, Alexeeva V, Park JHm, Romanova EV, Xie F, Demrbow NC, Ludwar BC, Weiss KR, Vilim FS. Feed-forward compensation mediated by the central and peripheral actions of a single neuropeptide discovered using representational difference analysis. J Neurosci. 2010; 30:16545–16558. [PubMed: 21147994]

Lear BC, Merrill CE, Lin JM, Schroeder A, Zhang L, Allada R. A G protein- coupled receptor, groom-of-PDF, is required for PDF neuron action in circadian behavior. Neuron. 2005; 48:221–227. [PubMed: 16242403]

Lear BC, Zhang L, Allada R. The neuropeptide PDF acts directly on evening pacemaker neurons to regulate multiple features of circadian behavior. PLoS Biol. 2009; 7:e1000154. [PubMed: 19621061]

Lin Y, Stormo GD, Taghert PH. The neuropeptide pigment-dispersing factor coordinates pacemaker interactions in the *Drosophila* circadian system. J Neurosci. 2004; 24:7951–7957. [PubMed: 15356209]

Majercak J, Kalderon A, Edery I. *Drosophila* melanogaster deficient in protein kinase A manifests behavior-specific arrhythmia but normal clock function. Mol Cell Bio. 1997; 17:5915–5922. [PubMed: 9315649]

McGuire SE, Le PT, Osborn AJ, Matsumoto K, Davis RL. Temporal and Regional Gene Expression Targeting with the Conventional GAL4/UAS System in *Drosophila*. A. *Dros*. Res Conf. 2003; 44:153.

Murad A, Emery-Le M, Emery P. A subset of dorsal neurons modulates circadian behavior and light responses in *Drosophila*. Neuron. 2007; 53:689–701. [PubMed: 17329209]

Nikolaev VO, Bunemann M, Hein L, Hannawacker A, Lohse MJ. Novel single chain cAMP sensors for receptor-induced signal propagation. J Biol Chem. 2003; 279:37215–37218. [PubMed: 15231839]

Park SK, Sedore SA, Cronmiller C, Hirsh J. Type II cAMP-dependent protein kinase-deficient *Drosophila* are viable but show developmental, circadian, and drug response phenotypes. J Biol Chem. 2000; 275:20588–20596. [PubMed: 10781603]

Peschel N, Helfrich-Förster C. Setting the clock – by nature: Circadian rhythm in the fruitfly *Drosophila melanogaster*. FEBS Letters. 2011; 585(10):1435–1442. [PubMed: 21354415]

Rieger D, Wülbeck C, Rouyer F, Helfrich-Förster C. Period gene expression in four neurons is sufficient for rhythmic activity of Drosophila melanogaster under dim light conditions. J Biol Rhythms. 2009; 24(4):271–82. [PubMed: 19625729]

Rodbell M. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. Nature. 1980; 284:17–22. [PubMed: 6101906]

Seebacher T, Linder JU, Schultz JE. An isoform-specific interaction of the membrane anchors affects mammalian adenylyl cyclase type V activity. Eur J Biochem. 2001; 268:105–110. [PubMed: 11121109]

Shafer OT, Kim DJ, Dunbar-Yaffe R, Nikolaev VO, Lohse MJ, Taghert PH. Widespread receptivity to neuropeptide PDF throughout the neuronal circadian clock network of *Drosophila* revealed by real-time cyclic AMP imaging. Neuron. 2008; 58:223–237. [PubMed: 18439407]

Shafer OT, Taghert PH. RNA-interference knockdown of *Drosophila* pigment dispersing factor in neuronal subsets: the anatomical basis of a neuropeptide's circadian function. PLoS One. 2009; 4(12):e8298. [PubMed: 20011537]

Sheeba V. The *Drosophila melanogaster* circadian pacemaker circuit. J Genetics. 2008; 87(5):485–493. [PubMed: 19147937]

Stoleru D, Peng Y, Nawathean P, Rosbash M. A resetting signal between *Drosophila* pacemakers synchronizes morning and evening activity. Nature. 2005; 438:238–242. [PubMed: 16281038]

Taghert PH, Nitabach MN. Peptide neuromodulation in invertebrate model systems. Neuron. 2012; 76:82–97. [PubMed: 23040808]

Ueno K, Kidokoro Y. Adenylyl cyclase encoded by AC78C participates in sugar perception in *Drosophila melanogaster*. Eur J Neurosci. 2008; 28:1956–1966. [PubMed: 19046378]

Welsh DK, Takahashi JS, Kay SA. Suprachiasmatic nucleus: cell autonomy and network properties. Annu Rev Physiol. 2010; 72:551–77. [PubMed: 20148688]

Wu Y, Cao G, Pavlicek B, Luo X, Nitabach MN. Phase Coupling of a circadian neuropeptide with rest/activity rhythms detected using a membrane-tethered spider toxin. PLoS Biol. 2008; 11:273.

Wu JS, Vilim FS, Hatcher NG, Due MR, Sweedler JV, Weiss KR, Jing J. Composite modulatory feedforward loop contributes to the establishment of a network state. J Neurophysiol. 2010; 103:2174–2184. [PubMed: 20181731]

Wulbeck C, Grieshaber E, Helfrich-Förster C. Pigment-dispersing factor (PDF) has different effects on *Drosophila's* circadian clocks in the accessory medulla and in the dorsal brain. J Biol Rythms. 2008; 23:409–424.

Yoshii T, Wulbeck C, Sehadova H, Veleri S, Bichler D, Stanewsky R, Helfrich-Förster C. The neuropeptide Pigment-dispersing factor adjusts period and phase of *Drosophila's* clock. J Neuroscience. 2009; 29:2597–610.

Zhang L, Lear BC, Seluzicki A, Allada R. The CRYPTOCHROME photoreceptor gates PDF neuropeptide signaling to set circadian network hierarchy in *Drosophila*. Curr Biol. 2009; 19:2050–2055. [PubMed: 19913424]

Zhang Y, Liu Y, Bilodeau-Wentworth D, Hardin PE, Emery P. Light and temperature control the contribution of specific DN1 neurons to *Drosophila* circadian behavior. Curr Biol. 2010; 20(7): 600–605. [PubMed: 20362449]

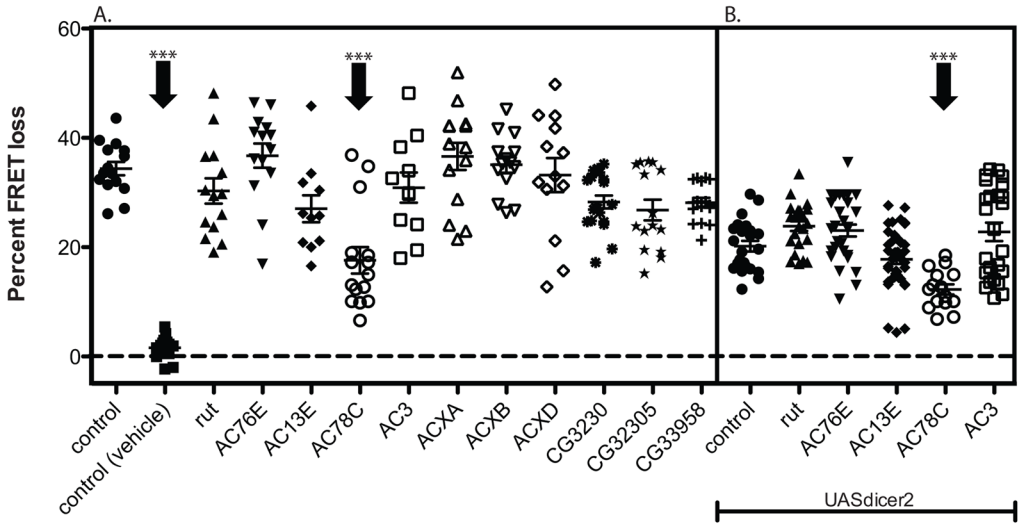

Figure 1. Knockdown of AC78C reduced PDF cAMP responses in LNds

A. RNAi screen directed against most of the predicted *ACs* in *Drosophila.* Only the *AC78C* RNAi significantly reduces PDF responses in LNd pacemakers.

B. Addition of UAS*dicer2* to increase RNAi knockdown does not reveal additional candidates for ACs mediating PDF response and does not enhance the *AC78C* RNAi phenotype.

All genotypes include *Mai179*-gal4;Epac1camps and 1 copy of UASRNAi (except for control). Error bars denote SEM. ***, P<0.001 (compared with control). Statistical analysis was performed at maximal deflection from the initial timepoint by performing ANOVA analysis, followed by post-hoc Tukey tests. Detailed information about RNAi lines is included in the Materials and Methods section.

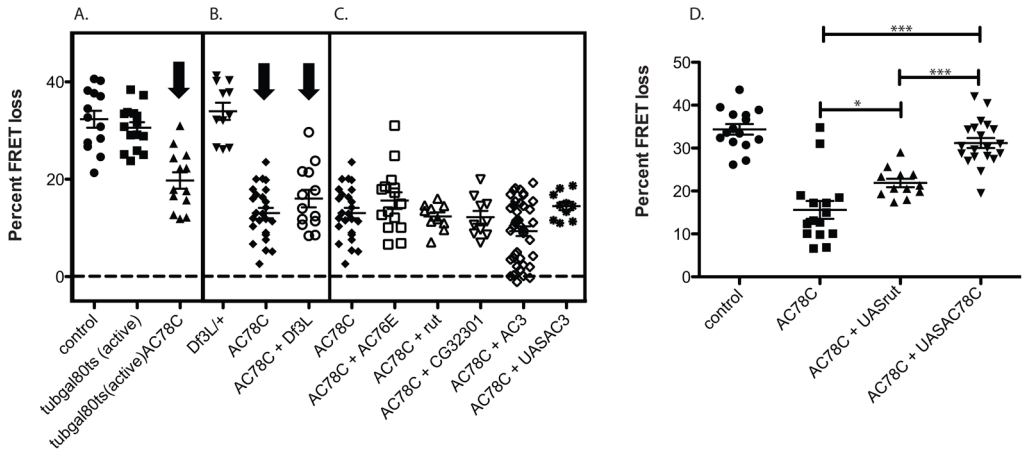

Figure 2. *AC78C* RNAi partially reduces PDF responses in adult LNds

A. AC78C RNAi expressed only in adult cells reduced PDF response in LNds (expression of *tubgal80^{ts}* alone has no effect). Animals with "active" GAL4 were transferred to 29°C as described in Materials and Methods to inactivate inhibition by the *tub-GAL80* transgene.

B. The *AC78C* RNAi phenotype is not enhanced by combination with a cognate deficiency.

C. Pairwise combinations of RNAi constructs for *AC78C* and other ACs does not enhance the *AC78C* RNAi phenotype (none are significantly different from AC78C RNAi alone by Tukey's Multiple Comparison Test).

D. The *AC78C* RNAi-mediated reduction in PDF cAMP responses in LNd is rescued by over-expression of *AC78C* but not of *rutabaga*.

All genotypes include *Mai179*-gal4;Epac1camps and 1 copy of UASRNAi (except for control). Error bars denote SEM. ***, P<0.001 *, P<.05 (compared with control, internal comparisons are shown and brackets between genotypes compared). Statistical analysis was performed at maximal deflection from the initial timepoint by performing ANOVA analysis, followed by post-hoc Tukey tests

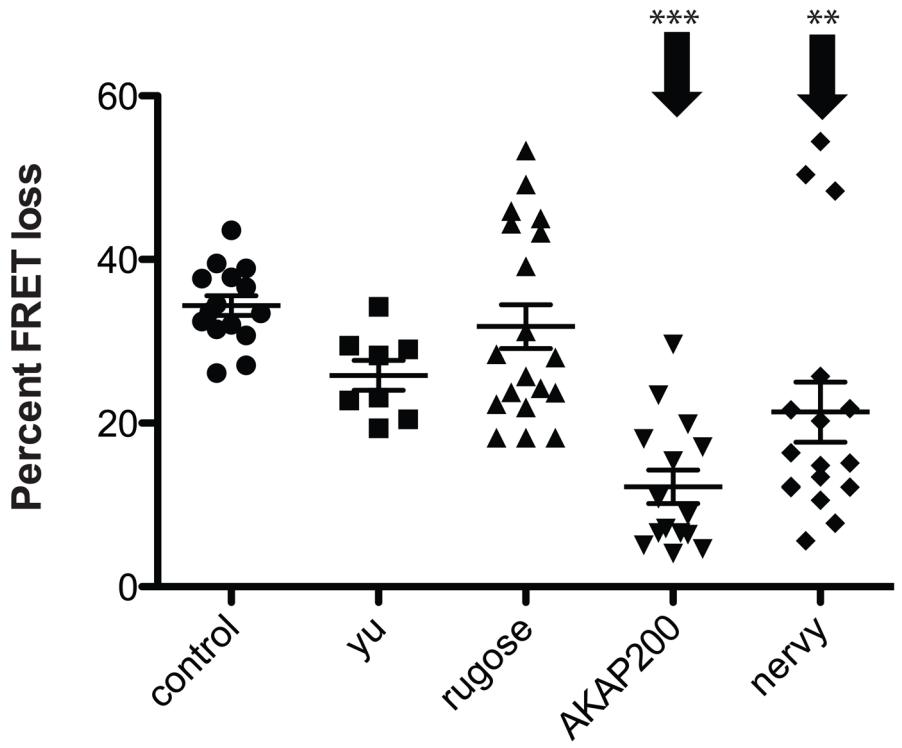

Figure 3. RNAi-mediated knockdown of two potential AKAPs reduces cAMP elevation by PDF in LNd pacemakers

Knockdown of *AKAP200* and of *nervy* significantly but partially reduced LNd PDF responses. All genotypes include *tim(UAS)*-gal4;Epac1camps and 1 copy of UASRNAi (except for control). Error bars denote SEM. ***, P<0.001 **, P<.01 (compared with control). Statistical analysis was performed at maximal deflection from the initial timepoint by performing ANOVA analysis, followed by post-hoc Tukey tests

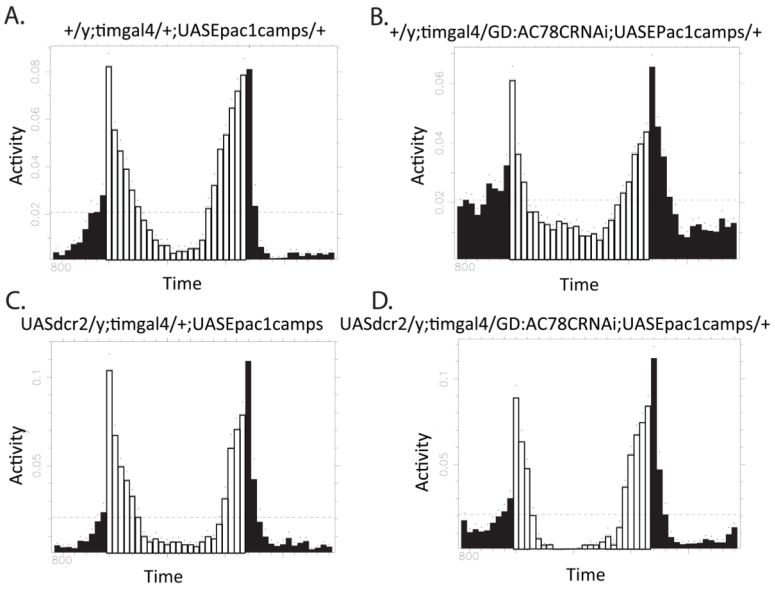

Figure 4. Knockdown of AC78C does not result in locomotor defects in circadian behavior

A. Representative locomotor behavior of control flies expressing the Epac1camps sensor under control of *tim(UAS)*-gal4 driver.

B. Representative locomotor behavior of flies with knockdown of AC78C in pacemaker cells.

C. Representative locomotor behavior of control flies expressing UAS-dicer2 transgene in addition to Epac1camps under *tim(UAS)*-gal4 driver.

D. Representative locomotor behavior of flies expressing RNAi directed against AC78C in addition to UASdicer2 transgene to increase RNAi efficiency.

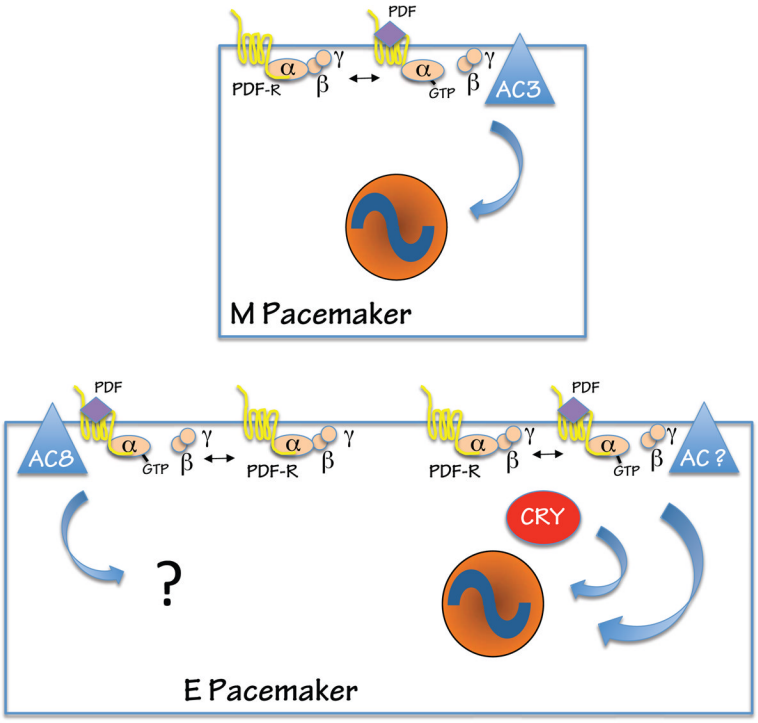

Figure 5. Model for PDF-R signaling in M versus E cells in *Drosophila*

PDF-R associates with AC3 in small LNv cells but signalosome components differ in LNds. In this E cell subgroup PDF signaling relies on AC78C (AC8) and at least one other AC.

Table 1

Behavioral outcomes in constant darkness, grouped by genotype.

Genotype	n	% Arrhythmic	Period (h) +/− SEM	Power +/− SEM
+/*y*;*tim*(UAS)-*gal4*/+; *Epac1camps*/+	60	2	24.3 +/− .07	73.9 +/− 3.2
+/*y*;*tim*(UAS)-*gal4*/GD:*AC78C*RNAi; *Epac1camps*/+	59	8.4	24.0 +/− .06	88.6 +/− 3.8
UAS*dcr2*/*y*;*tim*(UAS)-*gal4*/+; *Epac1camps*/+	60	11.2	24.4 +/− .15	80.6 +/− 4.2
UAS*dcr2*/*y*;*tim*(UAS)-*gal4*/GD:*AC78C*RNAi; *Epac1camps*/+	35	8.5	24.4 +/− .21	89.9 +/− 6.7
+/*y*;*Mai179*-*gal4*/GD: *AC78C*RNAi; *Epac1camps*/+	30	0	24.1 +/− .1	64.2 +/− 8.5