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# Cell Signaling Components of the Drosophila Circadian Pacemaker

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# Cell Signaling Components of the *Drosophila* Circadian Pacemaker

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ABBREVIATIONS:

| | |
|---|---|
| AC | adenylate cyclase |
| AKAP | A-kinase anchoring protein |
| cAMP | cyclic adenosine monophosphate |
| CFP | cyan fluorescent protein |
| CGRP | calcitonin gene-related peptide |
| cGMP | cyclic guanosine monophosphate |
| CT | circadian time |
| DA | dopamine |
| DH31/81 | Diuretic Hormone 31/81 |
| EPAC | Exchange protein activated by cAMP |
| FRET | Förster Resonance Energy Transfer |
| GPCR | G-protein coupled receptor |
| LNv | ventro-lateral neuron |
| LNd | dorso-lateral neuron |
| PDF | Pigment Dispersing Factor |
| PKA | Protein kinase A |
| NO | nitric oxide |
| ODQ | 1H-[1,2,4]Oxadiazolo [4,3-a]quinoxalin-1-one |
| Rut | rutabaga |
| SNAP | S-Nitroso-N-acetyl-DL-penicillamine |
| VIP | Vasoactive Intestinal Polypeptide |

| | |
|---|---|
| YFP | yellow fluorescent protein |
| ZT | Zeitgeber time |

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ABSTRACT OF THE DISSERTATION

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Daily rhythms in physiology are conserved throughout evolution. The molecular oscillations that underlie rhythms have been well characterized and are required in a specific set of pacemaker cells in the *Drosophila* brain for locomotor rhythms. These cells are divided into distinct subgroups and evidence suggests that subgroups of pacemaker cells may control distinct components of circadian behavior although these assignments are not rigid, and depend critically upon environmental conditions. Plasticity in the neural circuits controlling circadian behavior allow the animal flexibility in responding to fluctuations in environmental inputs, for example changing day length over seasons.

Intercellular communication between diverse clock cells is key for normal circadian behavior. This communication is mediated through the actions of the neuropeptide PDF (Pigment Dispersing Factor). Although increasing evidence suggests that downstream effectors differ between subgroups the molecular mechanisms of these differential responses remain unknown. Defining these mechanisms are of fundamental

importance to understanding the widespread and robust synchronizing actions of PDF across the circadian neuronal circuit. This thesis explores cell signaling components associated with the PDF receptor in two clock cell subgroups; small ventro-lateral neurons (small LNv) and dorsolateral neurons (LNd).

Using a genetically encoded sensor to measure changes in cAMP in the living *Drosophila* brain, I report that PDF receptor preferentially couples to specific signaling components. These groupings vary in composition depending upon receptor identity; small LNv cells express Gsα coupled receptors for the peptide DH31, PDF as well as dopamine. However, these three receptors show different coupling at the level of adenylate cyclase. PDF receptor is preferentially coupled to AC3 in the small LNv cells, however DH31 and dopamine responses are unaffected by manipulations of AC3. Surprisingly, another subgroup of PDF receptor expressing clock cells, LNd cells, do not rely upon AC3 to mediate PDF responsiveness. Instead, the PDF response in these cells is reduced after knockdown of AC78C. A class of scaffolding proteins called AKAPs (A-kinase anchoring proteins) are implicated in PDF responses in both subgroups presumably by allowing signaling complexes to form efficiently. My work suggests that downstream components form "signalosomes" in specific pacemaker cells in the *Drosophila* brain.

I report that small LNvs show diurnal variation in sensitivity to PDF peptide. My studies support a model in which modifications that affect localization of signaling components may play important roles in changes in receptor signaling. Previous work performed in the mammalian olfactory epithelium suggests that specific glycan modifications are required for AC3 enzymatic activity and that these modifications rely

upon a specific glycosyltransferase (β3GnT2). I report that knockdown of CG30036, the *Drosophila* homolog of β3GnT2, reduces PDF responses in small LNv cells and causes circadian disruption partially consistent with AC3 defects. Glycosylation sites are conserved among transmembrane ACs and I identified a separate glycosylstransferase (CG33145) that, when knocked down, reduces LNd PDF responses. These findings are consistent with a model of PDF receptor complexes that differ in their composition in different subgroups of pacemaker cells. This model provides a possible mechanism to explain the differences in downstream PDF responses between clock cell subgroups.

Additionally, I provide evidence that two forms of the Diuretic Hormone Peptide (DH31 and DH81) show differential preferences for two GPCRs (CG4395 and CG17415) in vivo. These two receptors are members of the CGRP family of peptide receptors, the third member of which is PDF receptor. DH81 is an 81 amino acid peptide and this greatly extends the length of known bioactive peptides, for which in vivo evidence is critical. Additionally, this finding suggests that, although they are encoded by the same gene, that peptide processing may functionally encode multiple neurotransmitter pathways. This finding may provide a mechanism for an additional level of signaling complexity within the *Drosophila* brain.

**CHAPTER 1**

INTRODUCTION:

Circadian Clocks

Organisms are equipped with endogenous circadian clocks to help anticipate and respond to daily changes in their environment. These pacemakers are robust and allow rhythms to persist with a period of ~24 hours even in the absence of entraining environmental cues from the earth's rotation. It is likely that the integrity of these rhythms is important for the maintenance of human health and circadian stress is associated with a host of medical problems including metabolic syndrome and cancer (Karlsson et al., 2001; DiLorenzo et al., 2003). Neuropeptides are key synchronizing agents of both *Drosophila* and mammalian clocks (Renn et al., 1999; Peng et al., 2003; Lin et al., 2004; Aton et al., 2005; Maywood et al., 2006). Circadian signaling is important for the integration of environmental timing and physiology; the observation that important rate-limiting metabolic enzymes are under circadian control highlights the pervasive influence of circadian pacemakers in normal biological function (Panda et al., 2002).

Circadian rhythms are generated on a cellular level by a set of rhythmic transcription and translation feedback loops generated by a set of conserved core clock proteins which are required a known groups of pacemaker cells (Dunlap 1999; Park et al., 2000). The first clock mutations were identified in *Drosophila* (Konopka and Benzer, 1971) and subsequent studies have extended these findings into the more complex set of genes required to support rhythmicity. One time-delayed negative feedback loop

involves the transcriptional activators clock (CLK) and cycle (CYC), which bind to and promote the transcription of the genes period (PER) and timeless (TIM). Eventually PER and TIM protein levels accumulate in the cytoplasm where they eventually dimerize and then are able to translocate back into the nucleus where they bind to and inhibit CLK/CYC and thus their own transcription (Sehgal et al., 1994; Hardin et al., 1990). The *Drosophila* circadian clock has served as a robust model for the influence of specific genes in the function of known neuronal circuits and behavior.

Peptide Signaling and the Clock

In *Drosophila*, these molecular rhythms are required in a specific group of about 150 cells in the brain that receive input from the external environment and that produce rhythmic behavioral output (Zerr et al., 1990; Ewer et al., 1992; Kaneko et al., 1997). When the critical pacemaker cells in the brain are disrupted, this leads to widespread arrythmicity in tissues throughout the organism (Yoshii et al., 2009). These 150 cells can be further subdivided into six bilateral anatomically distinguishable groups that are named based on their location in the brain (Figure 1) (reviewed in Nitabach and Taghert, 2008).

Both mammalian and *Drosophila* clocks consist of diverse groups of cells and intercellular communication between members of these clock networks is required for synchronization of both cellular and behavioral rhythms. Neuropeptides are critical mediators of intercellular communication between circadian pacemakers (Aton et al., 2005; Lin et al., 2004; Peng et al., 2003). Flies lacking the neuropeptide Pigment Dispersing Factor (PDF) or the PDF receptor show disruption of normal activity profiles

during a normal 12:12 light/dark cycle as well as a high degree of arrhythmicity under constant dark conditions (Figure 2) (Mertens et al., 2005; Renn et al., 1999; Stoleru et al., 2004; Hyun et al., 2005). PDF peptide is expressed by 2 specific pacemaker subgroups (large and small LNv) (see Figure 1) and the PDF receptor is expressed widely throughout the clock group (Im and Taghert, 2010; Lear et al., 2005l Mertens et al., 2005). The PDF receptor has been identified as a member of the family B (secretin-like) receptor, is most closely related to receptors for calcitonin and CGRP, and signals through cAMP and $Ca^{2+}$ (Hyun et al., 2009; Hewes and Taghert, 2001).

In mammals, the neuropeptide VIP (Vasoactive Intestinal Polypeptide) is a functional ortholog of PDF and also performs synchronizing functions in the circadian network in the brain (Aton et al., 2005). The VIP receptor VPAC2 also belongs to the secretin-like receptor family (Aton et al., 2005). The mammalian clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Klein et al., 1991). Striking similarities exist between the *Drosophila* and mammalian systems in the percentage of cells that express VIP and VPAC2 within the clock network as well as the behavioral effects of loss of this signaling pathway (Aton et al., 2005; Maywood et al., 2006; Colwell et al., 2002; Harmar et al., 2003). Parallel to the fly system VIP expressing cells receive inputs from the visual system (Tanaka et al., 1993) and about 30% of the VIP expressing cells also express VPAC2 suggesting that autoreceptor activity may also play a role in mammalian rhythms (Kallo et al., 2004). The signaling pathways activated downstream of these synchronizing peptides are important to define in vivo because circadian function in both mammals and *Drosophila* likely depends upon multiple

interacting rhythmic centers which are determined by the cellular physiology and the different clock subgroups.

Appropriate spatiotemporal patterns of PDF release and activation are key for proper coordination of the circuit. Levels of PDF peptide show daily variation in the dorsal projections of small LNvs suggesting that PDF release is temporally restricted and peaks in the morning (Park et al., 2000). No such variation has been observed in PDF receptor expression or downstream signaling although these components remain to be fully characterized and may confer temporal specificity to these pathways. In Chapter 4 I present evidence that two groups of pacemakers, small LNvs and LNds show diurnal variation in their sensitivity to PDF peptide.

Much previous work has focused on characterizing the importance of PDF signaling in the circadian system. One important question in the field has been: which cells respond to PDF? Due to the lack a reliable antibody for PDF receptor (Shafer et al., 2008), and the finding that GAL4/UASPDF-R strategies provide only incomplete rescue of behavioral phenotypes (Im and Taghert, 2010), previous work in the Taghert lab utilized a transgenic approach to address this question. Using an approximately 70kB transgene encoding myc-tagged PDF receptor under the control of its native promotor to map the expression pattern of the receptor they report that PDF receptor is expressed broadly, but not uniformly, throughout the clock network (Im and Taghert, 2010). These results support the hypothesis that PDF peptide produced by the LNv cells directly targets other members of the circadian network. Further studies in the Taghert lab have demonstrated that circadian neurons in the living brain respond to PDF by increasing cyclic nucleotide levels after application of peptide (Shafer et al., 2008). My work

extends these studies by using live-imaging techniques to elucidate downstream cell signaling components associated with the PDF response in specific clock cell subgroups. In Chapter 2 I identify a specific AC isoform that mediates PDF responses in small LNv cells, AC3. In Chapter 3 I analyze the roles of different ACs in the LNd cells and show that the PDF-R in these pacemakers is not uniquely coupled to AC3 as it is in the small LNv cells.

Pacemaker Subgroup Diversity

The importance of mechanisms that promote pacemaker synchronization can be appreciated in observation that *Drosophila* lacking the PDF neuropeptide or its receptor show core clock rhythms which are out of phase between pacemakers and disrupted locomotor rhythms (Renn et al., 1999; Hyun et al et al., 2009; Lin et al., 2004). PDF peptide null flies show abnormal cellular timekeeping: daily rhythms of nuclear translocation of the core clock protein PERIOD (Per) as well as daily rhythms in the intensity of Per straining are altered in multiple pacemaker subgroups. Interestingly, the precise nature of these alterations varies between subgroups (Lin et al., 2004). In the absence of PDF, small LNv cells remain rhythmic in their translocation of the PER proteins but become phase dispersed over time. In contrast, LNd cells remained phase coherent but became phase advanced over time (Lin et al., 2004). Rhythms in the intensity of PER immunostaining were maintained in small LNv but became phase-delayed and then diminished in LNds (Lin et al., 2004). These data indicate that PDF functions to synchronize pacemakers through intercellular communication, however, the molecular mechanisms responsible for this synchronization remain unknown.

The differential effects of the loss of PDF signaling between clock cells suggest that PDF signaling pathways differ between clock cell subgroups. Other studies have manipulated PDF release by examining mutant flies with abnormal PDF cell projections which cause higher levels of peptide to be released in the accessory medulla compared to those which cause higher levels to be released in the dorsal brain. Depending upon the brain region and pacemaker subgroups that received higher PDF levels the period was either lengthened or shortened; these results indicate that PDF functions to speed up the molecular oscillations of some subgroups of cells while slowing down other subgroups (Helfrich-Forster et al., 2000; Wulbeck et al., 2008). Likewise, genetically altering PDF cell physiology has pronounced effects on molecular oscillations in other pacemakers. For example, increasing PDF cell activity (by misexpressing chronically open sodium channels and theoretically increasing the level of released peptide and neurotransmitter) affects molecular oscillations in non-PDF producing cells (including LNds), without affecting core clock oscillations in PDF producing cells (including small LNvs) (Nitabach et al., 2006; Wu et al., 2008). Additional experiments which increase PDF signaling through expression of a tethered form of PDF to PDF receptor-expressing cells also induce complex behavioral rhythms with both long and short components, consistent with the hypothesis that PDF functions to speed up some clock cells and slows down others (Choi et al., 2009).

The role of the deep brain circadian photoreceptor crytochrome (CRY) in clock cell signaling also differs between clock cell subgroups (Im et al., 2011; Cusumano et al., 2009; Zhang et al., 2009). Several groups report that these pathways genetically interact to influence both locomotor activity as well as molecular oscillations in important

pacemaker cells (Zhang et al., 2009; Cusumano et al., 2009; Im et al., 2011). PDF receptor and CRY are co-expressed in a diverse subset of clock cells and in animals mutant for both *pdf* and *cry,* molecular rhythms are severely disrupted a group of clock cells, which includes the LNd cells while the small LNv cells show normal molecular oscillations (Im et al., 2011). This finding adds to the body of evidence that PDF signaling pathway components vary between pacemakers which may be predictably divided into subgroups, in which LNds behave differently than small LNvs.

Increasing evidence suggests that PDF signaling may have different downstream effectors between pacemaker subgroups although the molecular mechanisms of their differential responses remain unknown. Defining these mechanisms are of fundamental importance to understanding the widespread and robust synchronizing actions of PDF across the circadian neuronal circuit.

Signalosomes and Receptor Signaling

How do cells couple receptors to their downstream signaling components? A growing body of evidence indicates that signaling molecules form multi-protein complexes with preferred partners termed "signalosomes" (Dessauer, 2009). These signalosomes ensure that discrete lines of communications are associated with specific receptors, even those that share common components (for example cAMP or PKA). There are multiple ways that this clustering can be achieved but one group of proteins that has recently been highlighted for their association in forming signaling complexes with adenylate cyclases are A-kinase anchoring proteins (AKAPs). These proteins are so named due to their ability to bind protein kinase A but have also been demonstrated to

bind proteins both up and downstream of cAMP including phosphodiesterases, G protein coupled receptors and G proteins themselves (Rebois et al., 2006; Lavine et al., 2002). Evidence suggests that each AKAP binds a unique subgroup of AC isoforms although no general binding motif has been identified and different AKAPs may bind to different parts of the same AC isoform (Kapiloff et al., 2009). In Chapters 2 and 3 I present evidence that AKAPs play a role in PDF signaling in the *Drosophila* pacemakers, presumably by allowing efficient localization of signaling components.

Protein Modifications and PDF Signaling

One possible mechanism of differentiation between signaling pathways is the composition of signalosomes associated with the PDF receptor. Various protein modifications can play important roles in regulating the associations between members of signaling complexes as well as their appropriate subcellular localization (Rasmussen, 1992). Although the regulation of glycosylation status and its effects on protein function are not well understood there is increasing evidence suggests that these modifications play important roles in protein-protein interactions (Lis and Sharon, 1993). Nearly all proteins that travel through the ER (endoplasmic reticulum)–Golgi complex, including many receptors and transmembrane ACs, are likely to undergo N-linked glycosylation (Opdenakker et al., 1993). This modification can determine protein stability, folding, trafficking, and localization with important implications for cell–cell interactions, ligand–receptor binding affinity and intracellular signaling (Rasmussen, 1992). Indeed recent work shows that the TRPV1 receptor, which plays an important role in pain sensation, normally shows variable levels of glycosylation and that the loss of these modifications

leads to rapid desensitization of the receptor after application of capsaicin, although the trafficking of the receptor to the membrane remains intact (Veldhuis et al., 2012). Additionally, another recent report indicates that sensitivity of the type II TGF-β receptor is determined by N-linked glycosylation in a number of cultured human cell lines where it successfully hinders binding of TGF-β1 to TβRII and consequently renders cells resistant to TGF-β signaling (Kim et al., 2012). Notably in *Drosophila*, Notch receptor signaling is regulated by glycosylation events. The glycosyltransferase Fringe was first identified for its role in spatial restriction of Notch activation (Irvine and Wieschaus, 1999). Fringe modifies the extracellular domain of the receptor and these modifications alter the sensitivity of notch for one ligand versus another (Delta versus Serrate) (Irvine and Wieschaus, 1994; Panin et al., 1997; Bruckner et al., 2000; Moloney et al., 2000; Munro and Freeman, 2000).

In addition to receptors, other components of PDF signaling, including adenylate cyclases can be regulated by glycosylation. AC8 is a calcium-stimulated isoform that is selectively activated by capacitative calcium entry and that is thought to be regulated by localization of AC8 to lipid raft microdomains (Fagan et al., 1996; Smith et al., 2002). AC8 is modified by N-linked glycosylation and this modification is required to appropriately target AC8 to lipid rafts, although the un-glycosylated form of the enzyme remains capable of cAMP generation and can still be regulated by calcium (Pagano et al., 2009). N-glycosylation of newly synthesized protein may influence trafficking to the plasma membrane and can alter functional characteristics and likely to affect physiological regulation of multiple signaling components associated with G protein signaling.

Studies in the mammalian olfactory epithelium indicate that mammalian AC3 is heavily glycosylated and requires the function of a specific glycosyltransferase for these modifications, β3GnT2. Surprisingly, when the transferase itself is mutated AC3 loses not only the glycan modifications but also its enzymatic activity, even in response to direct stimulation (Henion et al., 2011). Sequences of predicted N-linked glycosylated are conserved on the extracellular loops of transmembrane ACs and mammalian AC2 is also known to be heavily glycosylated suggesting that these modifications may play a more general role in AC functions (Wong et al., 2000). In Chapter 4 I show evidence that knocking down specific glycosyltransferases also reduces PDF responses in pacemaker cells, consistent with a role in AC enzymatic function. Interestingly the transferase that plays a role in small LNvs, where AC3 mediated PDF responses is most closely related to the mammalian β3GnT2. However knockdown of a different transferase alters PDF responses in the LNd cells. Although the mechanisms by which glycosylation may interact with PDF signaling remain unclear, these findings are consistent with the hypothesis that signaling components differ between clock cell subgroups.

## Behavioral Outputs of the Circadian System

Locomotor activity is a robust and commonly used assay of circadian behavior in *Drosophila*. Wildtype flies show bimodal activity under a light regime of 12 hours of light followed by 12 hours of darkness (LD conditions) in which animals increase their activity before lights-on and again before lights-off in morning and evening anticipation peaks, respectively (Figure 2). When wild-type flies are released into constant darkness

(DD conditions) they maintain free-running behavioral rhythms with periods close to 24 hours in the absence of environmental cues. The ability of animals to free-run with a period close to 24 hours is due to the endogenous rhythms of the clock cells in the brain.

Evidence suggests that subgroups of pacemaker cells may control distinct components of circadian behavior (Grima et al., 2004; Stoleru et al., 2004; Stoleru et al., 2005). However these assignments are not rigid, and depend critically upon environmental conditions tested (Yoshii et al., 2005; Rieger et al., 2006; Rieger et al., 2009). Animals lacking PDF peptide producing cells or with arrhythmic PDF cells show a disruption of their normal morning activity under 12:12 light/dark conditions and disruption of other subgroups caused disruption of evening activity (Renn et al., 1999; Grima et al., 2004; Stoleru et al., 2004). These results suggested that "morning" (M) and "evening" (E) cells within the circadian network were responsible for components of daily behavior. However, under dim "moonlight" conditions, rhythmic clock gene expression in only four "evening" cells resulted in normal morning and evening peaks of activity (Rieger et al., 2009). Previous work also suggests that PDF from small LNv cells to a group of E cells (DN cells) are sufficient for morning activity (Lear et al., 2009; Zhang et al., 2010). Recent evidence has also suggested that PDF autoreceptors on M cells play a role in the allocation of activity and that activation of PDF pathways in these cells lead to increased "morningness" (Choi et al., 2012). Accumulating evidence indicates, while the roles that specific clock cells play in controlling circadian behavior are not rigid and can change under different environmental conditions, the circadian pacemaker is made of diverse cells that perform specialized functions.

I use a cell signaling assay to analyze cAMP responses to PDF, we might suppose that alterations that eliminate PDF cAMP responses and disrupt the signaling pathway throughout the clock network would lead to behavioral defects which phenocopy the pdf peptide and receptor mutants. My findings suggest that only very severe disruptions to PDF signaling are sufficient to produce behavioral effects and that our cell signaling assay shows some predictive value for circadian behavior.

CGRP Receptor Family

PDF receptor belongs to the Family B (secretin-like) group of GPCRs encoded in the *Drosophila* genome. PDF receptor belongs to the CGRP family of neuropeptide receptors, which also include the receptors encoded by CG17415 and CG4395 (Hewes and Taghert, 2001). There are 5 Secretin-like (family B) receptors predicted in the *Drosophila* genome and only three calcitonin gene-related peptide (CGRP) receptors (Hewes and Taghert, 2001). Previous work indicated that the CG17415 receptor is activated by the DH31 peptide (Johnson et al., 2005). Although CG17415 responds strongly to DH31 there was indication of a longer peptide that more potently activated CG4395 (J. Trigg, unpublished data). Manipulations of the gene encoding DH31 disrupt signaling for both receptors suggesting that a longer form of the DH31 peptide is the ligand for CG4395 (D. Jensen, unpublished data). Incrementally longer forms of the DH peptide increase the CG4395 responses in vitro coincident with the length of the peptide – with a maximal response in vitro from DH81 (J. Trigg, unpublished data).

A recent study reports that CG4395 plays a key role in courtship behavior in a subset of fruitless positive neurons (Li et al., 2011). Courtship phenotypes have not

previously been associated with DH31 mutants and this report adds support to the hypothesis that these DH31 and DH81 form two separate signaling pathways. DH81 represents the longest known signaling peptide and testing the function of this peptide in vivo is key to establishing its role in normal physiology. Although this work is ongoing, I was able to confirm increased sensitivity of CG4395 to DH81 in vivo using live imaging and these results are discussed in Chapter 5. This suggests that a single gene may essentially encode two different signaling pathways in the *Drosophila* brain and that DH81 is a potent activator the CGFP receptor encoded by CG4395.

Summary

This thesis focuses on elucidating downstream signaling components associated with the PDF receptor using live-imaging techniques in two specific subgroups of clock cells in the *Drosophila* brain. I present data that indicates that small LNvs show diurnal variation in their sensitivity to PDF peptide as well as in their ability to recover PDF responsiveness after glycosidase treatment. Additionally, I present evidence for in vivo signaling from a previously unrecognized neuropeptide, DH81 to the CGRP receptor encoded by CG4395. In the following chapters I present my results and discuss their broader applications for our understanding of the cell signaling events are key regulators of the circadian clock in the fruit fly.

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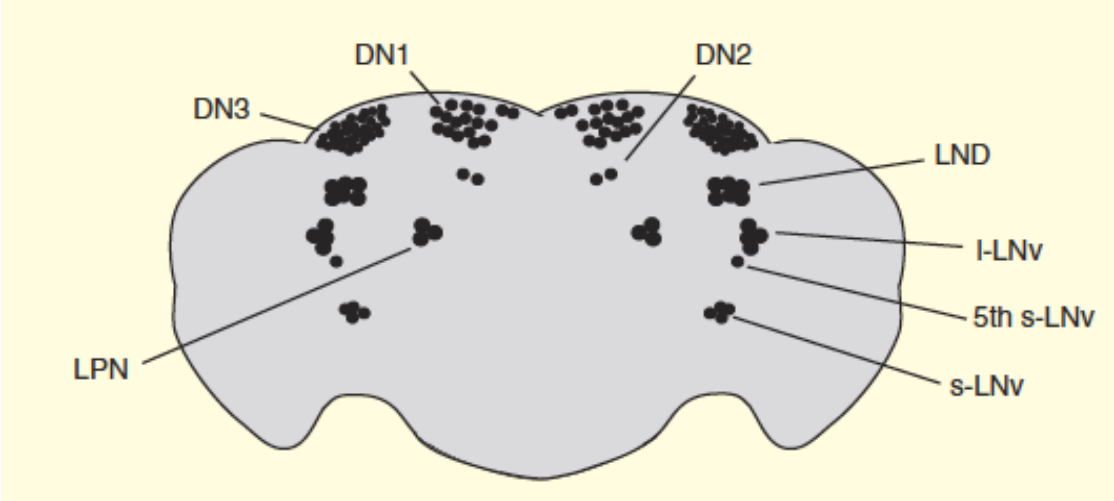


**Figure 1:** Pacemaker cells in the *Drosophila* brain. Subgroups are named based on their location in the brain. Two subgroups of LNvs (l-LNv and s-LNv) produce the peptide PDF. The two PDF receptor-expressing subgroups that are the focus of this thesis are the small LNvs (s-LNV) and the LNds (LND). (Image from Taghert and Nitabach, 2008).

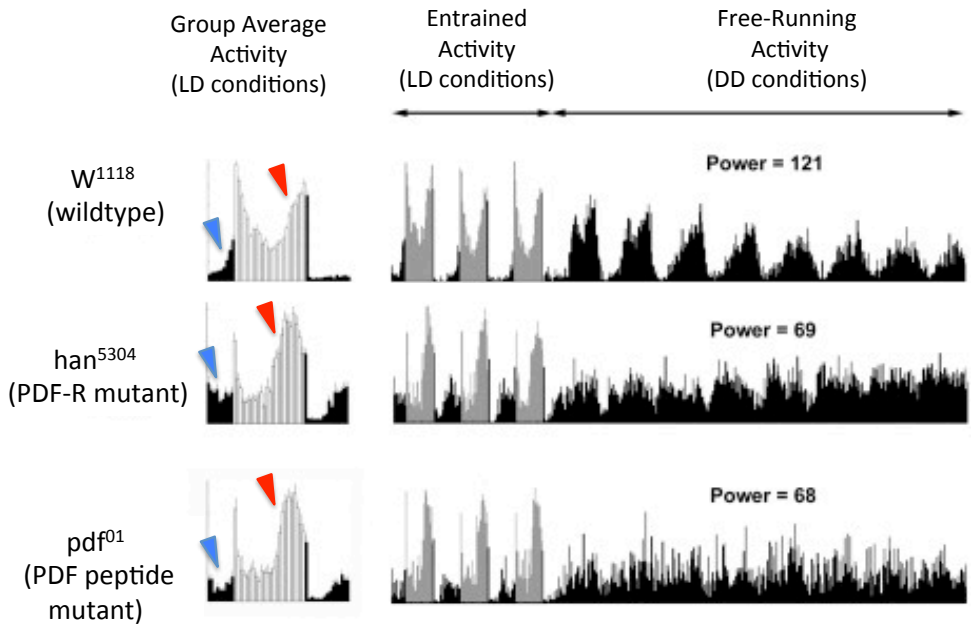


**Figure 2:** Circadian Locomotor Activities of wildtype, pdf-receptor and pdf-peptide mutants.

The locomotor activity records shown are population averages. The y-axis indicates relative levels of locomotor activity. White and black bars designate day and night phases, respectively. Activities in four LD cycles were pooled to show the average activity in LD phase (left). The 3 days of entrained activities in LD and the 8 days of free running activities in DD are shown at the right. The Han (pdf-receptor) and pdf peptide mutants display the common stereotypic feature that the evening peak is strikingly advanced (marked by red arrowhead) and the morning peaks are markedly reduced in the LD entraining period (marked by blue arrowhead). The activities of the Han (pdf-receptor) and pdf peptide mutants are also similarly arrhythmic in the free running condition (DD). (Image adapted from Hyun et al., 2005).

**CHAPTER 2:**

**PDF receptor preferentially couples to AC3 in small LNvs in *Drosophila***

This chapter includes portions of the manuscript:

Duvall LB, Taghert PH. (2012) The Circadian Neuropeptide PDF Signals Preferentially Through a Specific Adenylate Cyclase Isoform AC3 in M Pacemakers of *Drosophila*. PLoS Biol 10(6): e1001337.

**Principal Findings:**

Using live imaging of intact fly brains and transgenic manipulations, I show that adenylate cyclase AC3 underlies PDF signaling in small LNv cells. Genetic disruptions of AC3 specifically disrupt PDF responses: they do not affect other Gsα -coupled GPCR signaling in these cells, they can be rescued and they do not represent developmental alterations. Knockdown of the *Drosophila* AKAP-like scaffolding protein Nervy also reduces PDF responses. Flies with AC3 alterations show behavioral syndromes consistent with known roles of these pacemakers as mediated by PDF. Additionally, I present preliminary evidence that a phospho-AC3 antibody detectable signal in small LNv cells after putative PDF stimulation.

**Introduction:**

The importance of biological rhythms in the anticipation and response to daily environmental changes is underscored by their conservation throughout nature. In eukaryotes, these rhythms are generated by a set of core clock genes that contrive to produce interlocked feedback loops. Both mammalian and *Drosophila* circadian rhythms

are controlled by diverse groups of pacemaker neurons that express these core clock genes and proteins. In *Drosophila,* these rhythms are required in ~150 neurons, which can be subdivided into 6 bilateral anatomically distinct groups (Nitabach and Taghert, 2008). There appear to be two classes of pacemaker neuron in the fly brain which differ in many fundamental ways – these are termed M and E cells for historical reasons (Grima et al., 2004; Stoleru et al., 2004; Yoshii et al., 2004). Previous work indicates that these subgroups are functionally as well as anatomically distinct and that certain cells are associated with specific components of daily locomotor behavior. Importantly, these associations are subject to specific environmental conditions and they display considerable plasticity under different light and temperature conditions (Grima et al., 2004; Stoleru et al, 2004; Helfrich-Forster et al., 2000; Murad et al., 2007; Rieger et al., 2007; Zhang et al., 2010). This chapter focuses on small LNv cells, which comprise the M cell subgroup. These pacemaker subgroups communicate to synchronize with each other to produce coherent circadian rhythms (Lin et al., 2004; Lear et al., 2009). Neuropeptides are critical mediators of intercellular communication between pacemaker cells in both mammals and *Drosophila* and a number are expressed in the *Drosophila* clock cell system including the Pigment Dispersing Factor (PDF) (Aton et al., 2005; Helfrich-Forster et al., 1998; Park and Hall, 1998; Renn et al., 1999).

Loss of the PDF peptide or its receptor leads to abnormalities in circadian locomotor behavior including a reduction in morning anticipatory peak and a phase advance of the evening anticipatory peak under 12:12 LD Renn et al., 1999; Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005). Under constant conditions these flies show high levels of arrhythmicity or short, weak rhythms. PDF controls the amplitude and

phase of molecular rhythms of pacemaker cells (Lin et al., 2004; Yoshii et al., 2009). PDF's role in synchronization of clock cells indicates that its mechanism of action is largely within the cells of the clock network. The PDF neuropeptide is expressed by two specific pacemaker subgroups (large and small LNvs) and the PDF receptor is expressed widely, although not uniformly, throughout the circadian network in both M and E cell groups (Im and Taghert, 2010). The PDF receptor signals through calcium and cAMP although specific signaling components remain unknown (Hyun et al., 2005, Mertens et al., 2005). Signaling can be demonstrated in nearly all pacemaker cell groups in vivo (Shafer et al., 2008). Previous work indicates that small LNv cells increase cAMP levels in response to at least two neuropeptides, PDF and DH31 (Shafer et al., 2008). The PDF and DH31 receptors belong to the same class II (secretin) G-protein coupled receptor (GPCR) family (Hewes and Taghert, 2001). Both PDF (Hyun et al., 2005; Mertens et al., 2005) and DH31 receptors (Johnson et al., 2005) stimulate adenylate cyclases (AC) to produce cAMP in vitro, and in small LNv cells in vivo (Shafer et al., 2008), but the specific downstream components that differentiate the two peptide receptors remain unknown. Likewise, the basis for PDF's differential actions on the molecular oscillator in different pacemakers (Lin et al., 2004; Yoshii et al., 2009) has not yet been explained.

The present study seeks to find the identity of downstream components that are associated with PDF-R signaling pathways in different circadian pacemaker neurons? Specifically, using live imaging of intact fly brains, I identify the particular adenylate cyclase (AC) isoform that is associated with PDF signaling in small LNvs, commonly called M cells. Although some signaling components are common to both DH31 and PDF neuropeptide signaling, we report that DH31 signaling does not require the same AC in

the small LNv. This finding suggests that PDF signals preferentially through its favored AC, while other GPCRs, in the same identified pacemaker neurons, couple to other ACs. In Chapter 3 I present evidence that LNds, part of the E cell network, utilize different signaling components to form highly specific second messenger pathways.

**Results**

Epac1-camps is a genetically-encoded cyclic nucleotide sensor which can be visualized with subcellular resolution and which responds with great sensitivity to cAMP in *Drosophila* neurons (Shafer et al., 2008; Shakiryanova and Levitan, 2008; Tomchik and Davis, 2010; Crocker et al., 2010). Live brains expressing the reporter (using the gal4/UAS system) were imaged while saline was perfused through the line and responses were measured to a bolus presentation of peptide (Figure 1A). Small LNv neurons were easily identifiable by their position and morphology using a *Pdf*-gal4 driver and it was possible to obtain discrete readings from multiple cells within the same brain hemisphere.

In vitro assays indicate that Epac1camps has much higher sensitivity to cAMP than to other cyclic nucleotides (Nikolaev et al., 2003); however it was also shown that the sensor responds to changes in cGMP levels at the *Drosophila* neuromuscular junction (Shakiryanova and Levitan, 2008). Based upon in vitro studies of PDF signaling, we hypothesized that PDF receptor activation leads to increases in cAMP, not cGMP, levels in these pacemakers (Hyun et al., 2005; Mertens et al., 2005). To test this idea, I used SNAP (S-Nitroso-N-acetyl-DL-penicillamine (1)) as a Nitric Oxide (NO) donor, which is known to stimulate cGMP production (Schrammel et al., 1996). Addition of SNAP led to a measurable loss of the CFP/YFP FRET in small LNv, consistent with the interpretation

that the EPAC sensor detects increases in cGMP levels in addition to those of cAMP levels. SNAP responses were reduced in amplitude after pretreatment with a guanylate cyclase inhibitor 1H-[1,2,4]Oxadiazolo [4,3-a]quinoxalin-1-one (ODQ) (Supplemental Figure 1B). Importantly, ODQ pretreatment had no effect on PDF responses (Supplemental Figure 1A). Genetic over-expression of a cAMP-specific phosphodiesterase *dunce* reduced the amplitude of PDF responses (Supplemental Figure 1C), but had no effect on SNAP responses in small LNv cells (Supplemental Figure 1D). Together, these results are consistent with the supposition that in vivo, PDF signals through cAMP, not cGMP, in small LNv cells.

**Two adenylate cyclases (AC3 and AC76E) score positive in an in vivo RNAi screen targeting responses to PDF.**

The *Drosophila* genome encodes at least twelve ACs, five of which are expressed broadly, or at least broadly in the central nervous system (Flybase). The remaining cyclases (ACXA-E and CG32301 and CG32305) are thought to be expressed exclusively in the male germline (Cann et al., 2000; Flybase). *Rutabaga* (Rut) is the best characterized *Drosophila* ACs based on a mutagenesis screen for learning and memory phenotypes (Duerr and Quinn, 1982). Rut is expressed in the *Drosophila* brain and is stimulated by calcium and calmodulin (Livingstone et al., 1984). However, *rut* mutants showed normal PDF responses (data not shown), suggesting that a different AC(s) must mediate PDF-dependent signaling. This conclusion is also consistent with the lack of a circadian phenotype in *rutabaga* mutants (Levine et al., 1994). To test the role of other AC isoforms in PDF responses in small LNv cells, I performed a transgenic RNAi screen using constructs directed against 11 of the 12 ACs. Initial controls were performed with

and without UAS-*dicer2*, however expression of *dicer2* alone showed nonspecific effects on PDF responses and therefore all experiments presented were performed without dicer expression (data not shown). In small LNvs, two AC RNAi lines significantly reduced the amplitudes of PDF responses; *AC3* and *AC76E* (Figure 1C), although in neither case were PDF responses completely abrogated (Figure 1C, compare to 2nd column). In agreement with the initial *rut* mutant results, RNAi knockdown of *rut* mRNA had no effect on PDF responses. These results were consistent across different GAL4 lines (*Mai179*-gal4 and *tim*(UAS)-gal4) and therefore cannot be ascribed to differences in expression pattern or strength of the specific GAL4 driver used (data not shown).

**AC3 mediates PDF signaling in small LNv cells in adult stages.**

The results using *AC* RNAi could potentially be explained by deleterious effects on small LNv exerted by continuous RNAi expression throughout the neurons' period of development. To evaluate this possibility, I employed a temperature-sensitive genetic system that allows for normal development, followed by conditional induction of RNAi only in the adult fly. Animals raised at a permissive temperature (18°C), had gal4 activity blocked by a temperature sensitive gal80 transgene (tubulin-gal80$_{ts}$) (McGuire et al., 2003). After normal development, the flies were then moved to a higher temperature (29°C) at which the gal80 transgene is no longer active and the gal4 transgene can drive expression of the RNAi construct, as well as the Epac1camps sensor. When tested in this manner, adult-specific knockdown of *AC3*, but not of *AC76E*, resulted in a reduction of the PDF response in adult small LNvs (Figure 2A). This indicates that developmental effects likely cause the reduction observed in the initial RNAi screen for *AC76C*, while the reduction observed for the case of *AC3* RNAi indicates its mediation of PDF

responsiveness in adult small LNv pacemakers.

To further confirm AC3 as the candidate PDF-dependent AC and to exclude false positives (due to nonspecific RNAi knockdown), I performed further genetic tests using an independently generated *AC3* RNAi line from the Harvard TRiP project (TRiPAC3RNAi) in addition to the line used in the initial screen from the VDRC (now referred to as GD:AC3RNAi) (Perkins et al., update to TRiP collection) that targets a non-overlapping portion of the *AC3* RNA. The TRiP *AC3* RNAi line also produced a significant decrease in the amplitude of PDF responses. In addition, both the VDRC and the TRiP *AC3* RNAi lines were also tested in combination with flies that are deficient for the *AC3* gene region (*Df(2)LDS6*), to further reduce *AC3* levels. These *RNAi*/*Df* flies (hemizygous *AC3* mutants) showed a marked further reduction of the response to PDF neuropeptide in small LNv cells compared to responses in either single mutant genotype: *Df* /+ or *AC3* RNAi/ + (Figure 2B). Together these genetic experiments provide strong confirmation of my initial RNAi screening results, and support the hypothesis that AC3 is the principal mediator of PDF-dependent signaling in small LNv cells. Importantly, the consequences of knocking down AC3 were highly specific to PDF: even when combined with the deficiency, AC3 RNAi had no effect on small LNv cell responses to a closely related cAMP-generating neuropeptide, DH31 (Figure 2C) (Shafer et al., 2008). This indicates that, in these same neurons, DH31-R likely signals through a different AC.

**Over-expression of AC3, but not other ACs in M cells abrogates their PDF responses.**

I tested the effects of UAS-*rut*, -*ACXD*, -*AC76E*, -*AC3* and -*AC78C* to ask whether AC over-expression could affect PDF signaling *in vivo*. Novel constructs were first tested

for functionality by measuring *cre*-LUC responses to 10μM forskolin in *hEK* cells. All constructs tested showed an increased average response to forskolin compared to empty vector-transfected cells, although these did not reach significance (Supplemental Figure 2). I was surprised to find that, *in vivo,* over-expression of *AC3* completely abrogated PDF responses in M cells, while over-expression of all other constructs had no such effect (Figure 3A). This disruption was not due to developmental effects: delaying UAS-*AC3* induction until the adult stage after completion of normal development (using the gal80ts system), produced the same disruption of PDF responses (data not shown). In UAS-*AC3* flies, both DH31- and dopamine-elicited cAMP increases remained intact, indicating that the cells were demonstrably healthy and could respond normally to stimulation of other Gsα -coupled GPCRs (Figure 3B and C). These observations suggest that abnormally high levels of AC3 specifically disrupt the PDF signaling pathway, and add further proof that AC3 is a unique component of PDF signaling in M cells.

**UAS-*AC3* rescues the *AC3* RNAi phenotype.**

Knocking down *AC3* levels produced a diminution of PDF signaling in small LNv cells: to evaluate further the specificity of this effect we wished to employ an *AC3* rescue strategy. However, over-expressing the AC3 enzyme in small LNv cells above normal levels disrupted responsiveness to PDF (Figure 3A), suggesting that supra-normal levels of the AC3 enzyme can also lead to dysfunction. Therefore, I reasoned that a successful design to rescue the *AC3* knockdown would require a more moderate level of level of *AC3* over-expression. Because the gal4 system is temperature-sensitive, intermediate levels of *AC3* over-expression were achieved by raising the flies at 25°C and then moving them to 18°C overnight before imaging. This temperature shift could reduce

the activity of the gal4 driver, which could result in lower levels of UAS-*AC3* expression. Indeed this schedule of temperature changes reduced the disruptive effect of AC3 over-expression on responses to PDF in small LNvs (Figure 4A, second column). I wondered whether it could also maintain effective RNAi knockdown of the endogenous gene.

I confirmed that firstly the RNAi transgene is still active under this temperature regimen (Figure 4A, third column). This UAS-*AC3* RNAi line is directed against the 3' untranslated region (UTR) of *AC3* and can therefore be rescued potentially by expression of UAS-*AC3*, which includes only the *AC3* coding region. In fact, over-expression of UAS-*AC3*, with a temperature shift from 25°C to 18°C at adulthood, rescued the reduction in PDF responses otherwise observed in a UAS-*AC3* RNAi line (Figure 4A). Comparable over-expression of *AC78C* did not rescue this deficit and that result also confirms that the rescue was not due to simple dilution of the gal4 driver. Importantly, temperature down-shifted (25°C to 18°C) over-expression of *AC3* alone, which should result in a small overshoot of normal enzyme levels, shows a slight reduction in PDF responses compared to control (Figure 4A). This again suggested that normal levels of receptor and enzyme are key for normal function. Together these results provide strong evidence to support the hypothesis that AC3 is a specific AC isoform in small LNv cells whose levels are tightly controlled and that normally mediates responsiveness to PDF signaling.

**Over-expressing PDF receptor rescues loss of responsiveness to PDF due to overexpressed AC3.**

I pursued the AC3 over-expression condition to further evaluate the nature of the components of the PDF receptor signalosome in small LNv pacemakers. I reasoned that I

could perhaps counteract an imbalance between signaling components produced by AC3 over-expression if we also over-expressed the PDF receptor. In fact, over-expressing PDF-R using a UAS-*pdfr* transgene in combination with UAS-*AC3* fully rescued the PDF response back to control levels (Figure 4B). The combination of *AC3* over-expression with an additional copy of *pdfr* (under control of its own promoter within a ~70 kB transgene, termed *pdfr-myc*: (Im and Taghert, 2010) produced a partial rescue of the PDF response. The latter effect was smaller than that seen with UAS-*pdfr*, presumably because the induced level of *Pdfr* over-expression was greater with the UAS construct. Co-mis-expression of the closely-related neuropeptide receptor *dh31-R1* (CG17415 – (Johnson et al., 2005) along with *AC3* also gave a partial rescue of diminished PDF signaling due to AC3 over- expression, although these responses were still significantly lower than control and less than what I observed with co-misexpression of *Pdfr* and *AC3* (Figure 4B, third column). Together, these results suggest that (i) the diminution of PDF signaling that follows AC3 over-expression can be rescued by providing more PDF receptor, thus reducing the receptor/effector imbalance. It also suggests that (ii) the absolute ratio of PDF receptor to AC3 enzyme is important for normal neuropeptide signaling in small LNv cells.

***AC3* knockdown does not affect all Gs-coupled GPCR signaling in M cells.**

Both RNAi and over-expression screens suggested that PDF receptor associates preferentially to the AC3 adenylate cyclase in M cells, although expression profiling studies indicate that multiple AC isoforms are expressed in these identified pacemakers (Nagoshi et al., 2010). To determine the specificity of AC3 contributions to other peptide signaling pathways in M cells, I evaluated cAMP responses produced by other ligands for

Gsα coupled GPCRs. *Drosophila* DH31 (Diuretic Hormone 31) is a neuropeptide closely related to mammalian Calcitonin and its receptor (CG17415) is closely related to the Calcitonin receptor (Johnson et al., 2005). Activation of PDF receptor and DH31-R both lead to increases in cAMP and hence both are presumed coupled to Gsα (Mertens et al., 2005; Johnson et al., 2005); both increase cAMP in small LNv cells in vivo (Shafer et al., 2010). RNAi knockdown of the Gsα60A subunit disrupted both signaling pathways, as expected (Figure 5B and data not shown). Interestingly, over-expression of the *Drosophila* G protein Gsα60A also disrupted both PDF and DH31 signaling in small LNv cells and responses could be restored by over-expression of the cognate receptor along with Gsα60A (Figure 5 A and B). As mentioned above, neither knockdown nor over-expression of *AC3* affected DH31 responses (Figure 2C). I interpret these results to suggest that both PDF and DH31 receptors are coupled to Gsα60A, but that PDF-R subsequently signals through AC3 and DH31-R through a different AC.

**Knockdown of AKAP *nervy* reduces PDF responses.**

Scaffolding proteins play important roles in supporting assembly of specific signalosomes, which feature tight association between specific receptors and specific second messenger molecules (Dessauer, 2009). I hypothesized that scaffolding proteins may help explain the preference of PDF-R for coupling to AC3. In *Drosophila* there are four known AKAP (A-kinase anchoring proteins): molecules that bind to and help co-localize many components of cAMP signaling pathways (Dessauer, 2009). I tested the possible involvement of AKAPs as scaffolding proteins for PDF-R in small LNv cells using gene-specific RNAi constructs. Knockdown of the AKAP *nervy*, but not of the other three AKAPs, reduced PDF responses to an extent similar to that produced with the

AC3 RNAi (Figure 6A). As with *AC3*, *nervy* knockdown showed no effect on DH31 responses in small LNv cells (Figure 6B). When both *AC3* and *nervy* are knocked down together in the same small LNv cell PDF responses were disrupted to an even greater extent than with either RNAi alone, (Figure 6C). The results from single versus double RNAi constructs were generally consistent, although the comparison between TRiPAC3RNAi and TRiPAC3RNAi/nervyRNAi does not reach significance (Figure 6C). This finding suggests that nervy also plays a role in PDF signaling in small LNv cells, presumably by allowing PDF signaling components to effectively localize and thus promote efficient signaling.

**AC3 alterations affect circadian behavior.**

The foregoing data argue that AC3 mediates the cAMP generation produced by PDF in M cells. To what extent is circadian locomotor behavior affected by this disruption of this AC3 activity? RNAi knockdown of any single AC did not affect locomotor rhythms. However, combining *AC3* RNAi knockdown with a deficiency for the *AC3* region produced a very strong reduction in the morning anticipation peak, as well as higher levels of arrhythmicity under constant conditions (Figure 7B and Tables 1 and 2). The same features are also observed in UAS-*AC3* over-expression in PDF cells (Figure 7C and Table 1). Over-expression of UAS-*pdfr and UAS-AC3* together slightly reduced arrythmicity in DD compared to UAS-*AC3* alone (Table 1). However, the loss of morning anticipation seen in the UAS-*AC3* condition is not rescued by over-expression of the PDF receptor (Figure 7D). This suggests that, although the PDF FRET response is rescued (Figure 4B), additional (temporal) aspects of PDF signaling may contribute to normal circadian behavior in LD.

**AC3 phosphorylation is detectable in small LNv cells after putative PDF stimulation.**

Previous work in the mammalian olfactory epithelium indicates that, through the actions CaMKII AC3 is rapidly phosphorylated after stimulations and thus inactivated (Wei et al., 1998). This phosphorylation is rapid and occurs 10 – 20 seconds after activation. The mammalian and *Drosophila* sequences are highly conserved in this region and I reasoned that a *Drosophila* phospho-specific antibody for AC3 (AC3-P) would be likely to detect AC3 in small LNv cells. Due to the transitory nature of the signal I utilized a genetic approach to tether PDF to the target cells to use *Pdf*-gal4 to drive UAS-tethered-PDF so that the peptide is constitutively available to activate the PDF receptor (Choi et al., 2009). In three out of fourteen brains tested I detected AC3-P signal in 2 -3 small LNv cells (Figure 8). AC-P signal was never detected in large LNv cells; although these cells expressed the tethered PDF they have previously been shown to be unresponsive to PDF in vivo (Shafer et al., 2008). Although the relatively small number (3/14) of brains showing detectable levels of AC3-P signal suggests the transitory nature of the signal I show preliminary evidence that AC3-P is detectable in small LNv cells following putative PDF stimulation.

**Discussion**

Networks of pacemakers cells are synchronized by intercellular interactions (Stoleru et al., 2005). There is strong and diverse evidence that control of cAMP levels is a critical factor underlying pacemaker rhythmicity and synchronization. Daily changes in cAMP levels in SCN neurons contribute to setting the phase, period and amplitude of

PER2 cycles and thus represent an integral component of the clock mechanism itself (Cusumani et al.,2009). Furthermore, the RGS16 regulator sets the level of cAMP generation and its levels are likewise clock-controlled (O'Neill et al., 2008). Regarding synchronizing agents that couple diverse pacemakers, both PDF in the fly and VIP in the mouse produce cAMP increases in response to receptor activation, and these signals ultimately have access to the pacemaker mechanism in target cells (Yoshii et al., 2004l Lin et al., 2004; Lear et al., 2009 Doi et al, 2011; An et al., 2011; Park et al., 2000). Thus understanding the molecular components that control cAMP metabolism in pacemaker neurons, especially those downstream of receptors for the PDF and VIP modulators are significant goals for the field.

There are at least 12 different genes encoding adenylate cyclases in the fly genome, of which the best known is Rutabaga, a calcium- and calmodulin-sensitive AC.
Rut was first identified in a screen for mutations that affected learning and memory exhibited in an associative conditioning paradigm (Duerr and Quinn, 1982). The Rut cyclase displays the properties of a coincidence detector with its activity triggered by inputs from simultaneous activation of more than one GPCR (Tomchik and Davis, 2010). However, my studies indicate that, in M pacemakers, the PDF receptor is preferentially coupled not to Rut but to the adenylate cyclase encoded by *AC3*. In vitro studies suggest the AC3 cyclase may be inhibited by calcium (Iourgenko and Levin, 2000). The functional consequences of this specific signaling association, the physical basis that supports it, and the degree to which it may hold true in other PDF-responsive neurons in the *Drosophila* brain are important questions raised by this work.

The experiments that manipulated AC and PDF-R expression together indicate that

relative levels of AC enzyme and receptor are important determinants of normal PDF cAMP responses in small LNv pacemakers. Counter-intuitively, *AC3* over-expression was as effective in diminishing PDF responsiveness as was *AC3* knockdown. One possible explanation is that the abnormally high levels of AC3 result in incorrect subcellular localization of signaling components, which may preclude the ability of AC3 to contribute to cAMP generation. Within small LNv cells, only moderate expression of a UAS-*AC3* transgene could restore normal PDF responses after knockdown of endogenous *AC3*. Likewise, over-expressing AC3 together with PDF-R could restore the balance between receptor and effector, as indicated by the return of PDF responsiveness. Although these results may not generalize to all cell types or receptor pathways, it is notable that, for this circadian signaling pathway, appropriate levels of signaling components were as important as their simple presence or absence. The reliance on proper stoichiometry between receptor and AC is further evidence to support the hypothesis that PDF-R and AC3 exhibit a specific functional association within the M class of pacemaker cells.

One possible explanation for preferential coupling of PDF-R to AC3 is simply that it is the only one of the 12 adenylate cyclases to be expressed in these cells. However this explanation is inconsistent with at least two notable observations – first, small LNv cells in flies with a severe *AC3* knockdown *(Df2L;GDAC3RNAi)* still elevate cAMP levels normally in response to neuropeptide DH31. Second, according to recent profiling studies, multiple other ACs are normally expressed at appreciable levels in larval LNs and in adult LNv (Dahdal et al., 2010; Nagoshi et al., 2010). Interestingly, these studies indicate that *AC3* is not even the most abundant adenylate cyclase in adult LNv cells

(Nagoshi et al., 2010). Therefore, I favor an alternative explanation - that molecular specificity dictates the composition of different receptor pathways, with PDF-R residing in privileged association with AC3. A recent report by Choi and colleagues indicates that a tethered form of PDF increases morning allocation of circadian behavior while a tethered DH31 peptide does not (Choi et al., 2012). These results provide additional evidence that the signaling pathways are segregated. The connection between PDF receptor and AC3 is bolstered by my preliminary report that phosphor-AC3 signal is detectable in small LNv cells expressing tethered PDF. Although these are preliminary results and we have not yet excluded all possible artifacts (for example by treating the brain with a phosphatase to demonstrate that specificity of the antibody) this is an additional piece of evidence that PDF receptor activation is connected to AC3 in small LNv cells.

There is clear support for the concept of preferential coupling between GPCRs and specific ACs in multiple cell types, in addition to my own findings in *Drosophila* clock cells. Previous work in *Drosophila* (Ueno and Kiddokoro, 2008) suggests that individual cyclases play specific roles in G-protein signaling associated with gustation. Furthermore, studies of the GABAergic system in the mouse pituitary indicate that Type 7 adenylate cyclase is associated with ethanol and CRF sensitivity, although mRNA for four of the nine mammalian ACs are detected by microarray in pituitary tissue (Antoni et al., 2003). It has also been proposed that receptor/AC preference may depend upon environmental conditions: for example that the Type 7 preference of the CRF receptor in the mouse amygdala occurs only after phosphorylation of signaling components (Cruz et al., 2011). Without phosphorylation, CRF receptor couples preferentially to Type 9 adenylate

cyclase (Pronko et al., 2010). Thus, my results add to the body of evidence that highly specific associations between receptors and their downstream partners are key regulators of signaling.

There is evidence that signaling components within specific pathways do cluster, which may explain how generalized signaling molecules like cAMP and PKA are capable of targeting distinct downstream effectors. Much current work focuses on possible mechanisms for such localization. (Dessauer, 2009) and the concept of signalosomes has been proposed to describe the spatial sequestering of signaling pathway components to promote exactly this sort of specific association. Thus preferential AC3/PDF-R coupling may be achieved by localizing AC3 near to PDF receptors. Mechanisms for grouping signaling components may include their co-localization in lipid rafts; many of the components of cAMP signaling including G proteins, PDE, PKA and cyclic nucleotide gated channels are found in lipid rafts (Insel and Patel, 2009) and studies in human bronchial smooth muscle cells detected three different AC isoforms, which are present in distinct membrane microdomains and which respond to different neurotransmitters and hormones (Bogard et al, 2011).

In addition, it is likely that another clustering mechanism includes the formation of macromolecular structures through the use of scaffolding proteins that bind to signaling molecules, as first proposed by Stadel and Crooke (Stadel and Crooke, 1988). Later studies showed that ACs form large complexes with β-arrestins, G proteins and calcium channels (Davarre et al., 2001). The scaffolding protein InaD is required for normal localization of signaling components in the fly visual system including TRP and PLC (Tsunoda et al., 1997; Shieh and Zhu 1998; Scott and Zuker, 1998). Specialized signaling

components such as AKAPs (A-kinase anchoring proteins) can bind to receptors as well as kinases and adenylate cyclases (Dessauer, 2009). In *Drosophila*, AKAPs organize functionally discrete pools of PKA and disruption of these signaling complexes alters normal spatio-temporal signal integration and causes a loss of anesthesia-sensitive as well as long-term olfactory memory formation in flies (Lu et al., 2007). In our study, knockdown of AKAP *nervy* reduced PDF responses: These results lead to a hypothesis whereby, in small LNv pacemakers, PDF receptor preferentially couples to AC3 via a nervy-based scaffold system to produce normal circadian behavior (Figure 9). I emphasize that, while my results demonstrate a functional connection between AC3 and PDF-R, the basis for any physical connections has not yet been established.

Although my results provide an example of a specific receptor/enzyme paring in a subset of circadian clock cells, my evidence also suggests the exact details of PDF signaling in other *Drosophila* pacemakers may differ and we characterize a different subgroup of pacemakers, the LNds, in Chapter 3.

How well do the observations obtained with neuronal imaging predict or correlate with circadian locomotor behavior? Manipulations of *AC3* that severely disrupt PDF signaling in small LNv "M" cells were correlated with a loss of morning anticipation and increased arrythmicity in DD. Manipulations that only partially-reduce the FRET response (for example, single *AC3* or single *nervy* knockdown) resulted in normal circadian locomotor behavior or disruptions to some aspects but not all. The latter observations suggest that the animal is capable of compensating for reduced cAMP responses to PDF by M cells but not to a complete loss. This data argues for a contribution to behavior by PDF signaling via AC3 in M cells and stands in contrast to a

recent report by Lear et al. (2009). That group reported that PDF-R expression in "E" cells alone is sufficient for morning anticipation and that exclusive expression of PDF-R in M cells does not recover morning anticipation. I cannot reconcile these differences without further experimental efforts, but note that GAL80 techniques are not always sufficient to extinguish gene expression in vivo (L. Duvall, data not shown).

Depending on ambient conditions, (Helfrich Forster, 1998; Zhang et al., 2009), the small LNv cells contribute to normal morning anticipatory behavior and to maintenance of rhythmicity under constant dark conditions (Grima et al., 2004; Renn et al., 1999; Shafer et al., 2008; Blanchardon et al., 2001, Stoleru et alk 2005, Nitabach et al., 2006). However, in my study small LNv cells expressing *AC* RNAi remain normally responsive to at least two other neurotransmitters (DH31 and dopamine). Hence I suspect that much of the behavioral effect of knocking down *AC3* in M pacemakers is mainly due to loss of PDF signaling in them despite retention of additional inputs from PDF-independent source. Levels of PDF receptor and responsiveness to PDF are both high in small LNv cells, and absent (or barely detectable) in large LNvs (Shafer et al., 2008, Nagoshi et al., 2010; Park et al., 2000). Therefore I expect that *AC3* alterations in LNv cells (directed by *pdf*-GAL4) primarily affect PDF signaling in small LNvs, although we cannot exclude other *AC3*-dependent processes in both small and large LNvs.

Knockdown of Gsα60A levels of the M pacemakers lengthened the period in DD a behavioral effect opposite to those seen following loss of PDF, or LNv cell ablation – namely. Previous studies of Gsα60A in LNv cells also reported a long period phenotype (Iourgenko and Levin, 2000). Likewise selective expression in small LNv of *shibiri* (a dominant negative allele of the fly homolog to dynamin (Van der Blick and Meyerowitz,

1991) or of a chronically-open sodium channel (Nitabach et al., 2006) both produce long period phenotypes (Kilman et al., 2009; Wulbeck et al., 2009). Although I cannot rule out a PDF-dependent role in period lengthening in our Gsα60A experiments, my imaging data suggest the lengthened period phenotype may be explained by the fact that alterations of Gsα60A impact multiple signaling pathways.

My results demonstrate in *Drosophila* that, in small LNv (M) circadian pacemakers, a highly specific signaling cascade is activated in response to PDF. They suggest there exists a dedicated PDFR::AC3-dependent signaling pathway that functions to synchronize these particular clock cells. A different PDF signaling cascade is likely to operate in E pacemakers and is addressed in Chapter 3. The complete molecular details of these signaling complexes, their convergence with CRY signaling (Park et al., 2000) and their ultimate connections to the cycling mechanism are significant issues for future studies.

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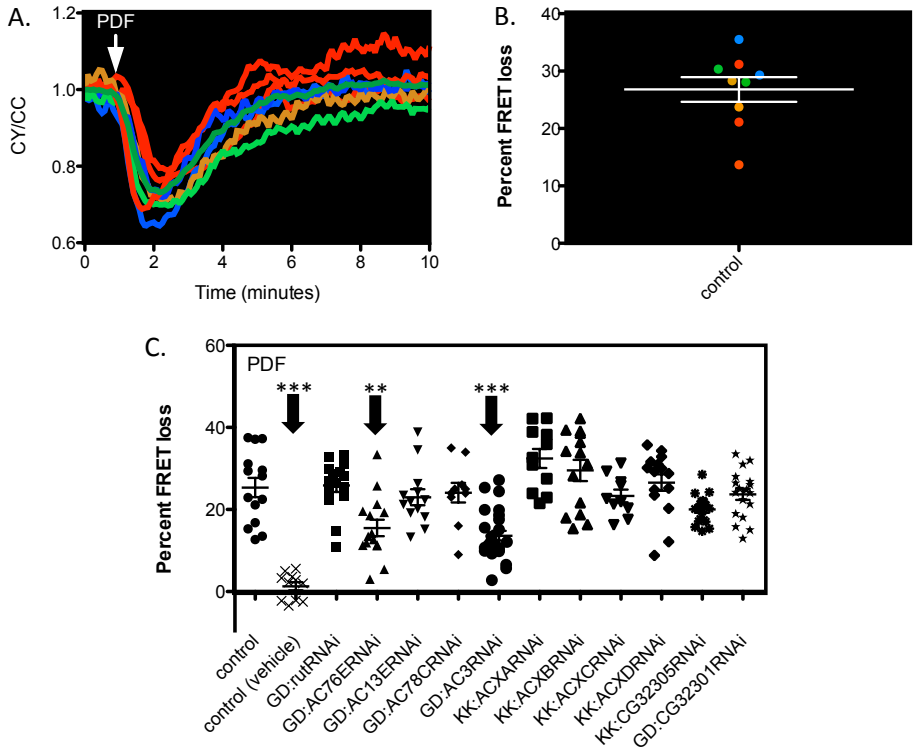



**Figure 1: Data collection of FRET responses and transgenic RNAi screen of ACs potentially coupled to PDF receptor in M cell pacemakers.**

A. Raw FRET imaging data (CY/CC) collected for 10 minutes (each trace represents an individual cell recorded as an ROI) to show FRET loss in response to bolus of PDF (marked by arrow) and recovery to baseline.

B. The scatter plot represents the maximal deflection from the initial imaging timepoint of the timecourse data shown in 1A. Error bars represent SEM.

C. Double stranded RNAi directed against 11/12 genes known to encode known adenylate cyclases in the *Drosophila* genome.

All genotypes include *Pdf*-gal4;Epac1camps and 1 copy of UASRNAi (except for control). Error bars denote SEM. ***, P<0.001 **, P<0.01 (compared with control).

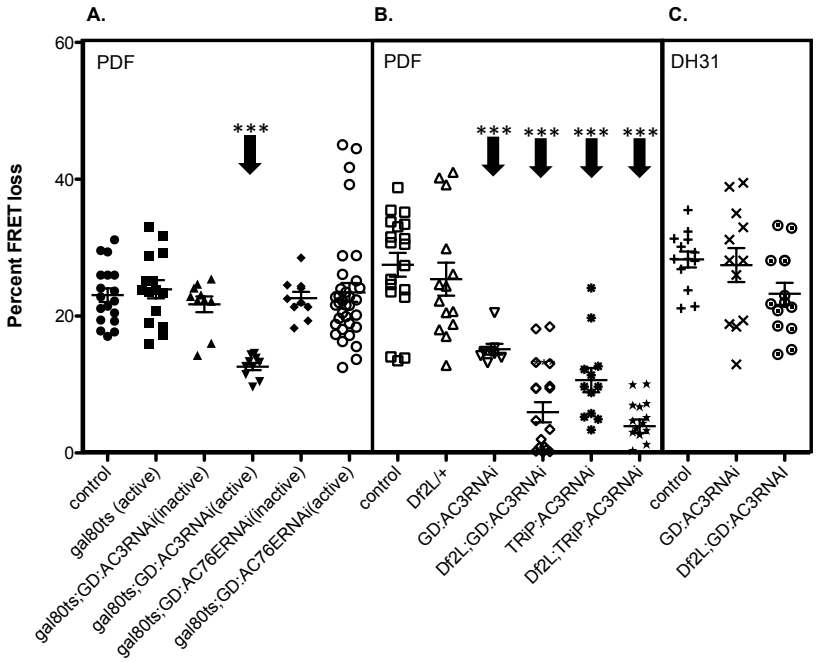



**Figure 2: A conditional transgenic RNAi test of AC involvement in PDF signaling in M cell pacemakers.**

A. Temperature sensitive gal80 was used to induce knockdown in adult cells only. Flies were raised at 18°C and moved to 29°C for 6 hours (inactive) – to allow for readable levels of Epac1camps sensor, or >36 hours (active). Adult induction of *AC3*RNAi (gal80ts;*AC3*RNAi (active)) significantly reduces the PDF response. Adult induction of *AC76E*RNAi shows no significant difference from control.
B. Genetic confirmation of *AC3* involvement was performed using two independently generated RNAi lines against *AC3* (GDAC3 and TRiPAC3) as well as flies that are deficient for the *AC3* gene region (*Df*(2L)*DS6*).
C. DH31 responses in M cells from flies with a knockdown of *AC3* in combination with *Df*(2L)*DS6*.

All genotypes include *Pdf*-gal4;Epac1camps. Error bars denote SEM. ***, P<0.001 (compared with control).

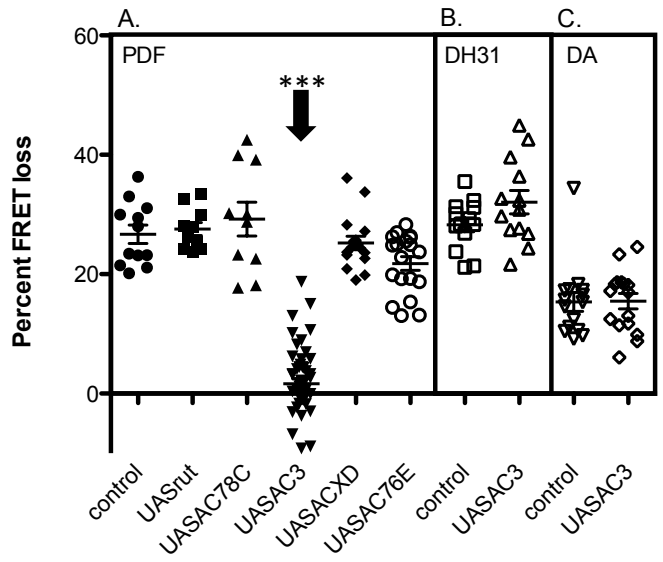



**Figure 3: Effects of over-expressing *AC* isoforms on different receptor signaling systems in M pacemakers**

A. *AC* over-expression effects on small LNv cell responses to neuropeptide PDF.
B. *AC3* over-expression effect on small LNv cell responses to neuropeptide DH31.
C. *AC3* over-expression effect on small LNv cell responses to dopamine.

All genotypes include *Pdf*-gal4;Epac1camps. Error bars denote SEM. ***, P<0.001 (compared with control).

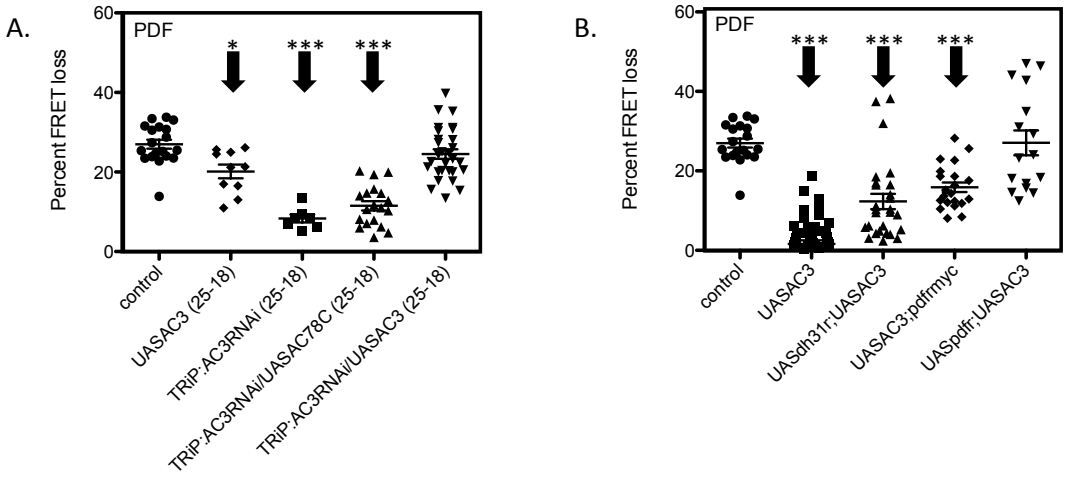



**Figure 4: Genetic rescues of *AC3* knockdown and over-expression effects in M cells**

A. Rescuing the loss of function state. Flies were raised at 25°C and moved to 18°C as adults for 12 – 15 hours before imaging to reduce levels of *AC3* over-expression. The effect of this schedule on the effects of AC3 knockdown (TRiP*AC3*RNAi) and *AC3* over-expression (UAS-*AC3*) is shown. The ability of over-expressing *AC78C* (TRiP*AC3*/UAS*AC78C*) and *AC3* (TRiP*AC3*RNAi/UAS*AC3*) to reverse the knockdown effect of AC3 RNAi are also shown.

B. Rescuing the gain of function state. Two *pdfr* over-expression genotypes were tested for their ability to affect AC3 over-expression: a UAS construct (UAS*pdfr*;UAS*AC3*) and a construct in which pdfr is driven by its endogenous promotor (UAS*AC3*;*pdfr*myc). For comparison the effects of co-mis-expressing a heterologous neuropeptide receptor is also shown (UAS*DH31R*/UAS*AC3*).

All genotypes include *Pdf*-gal4;Epac1camps. Error bars denote SEM. ***, P<0.001 *, P<.05 (compared with control).

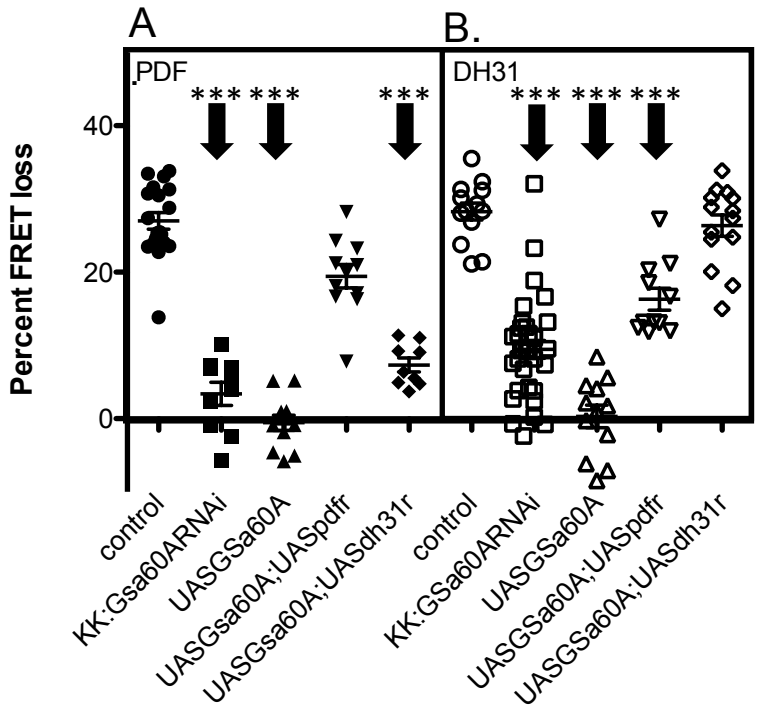



**Figure 5: Both PDF and DH31 responses are affected by altering $G_s$α60A levels.**

A. PDF responses following knockdown or over-expression of $G_s$α60A. $G_s$α60A over-expression effects were also measured in the context of over-expression of PDFR (UAS*$G_s$α60A*;UAS*pdfr*) or over-expression of DH31R (UAS*$G_s$α60A*;UAS*DH31R*).

B. DH31 responses following knockdown or over-expression of *$G_s$α60A*. *$G_s$α60A* over-expression effects were also measured in the context of over-expression of PDFR (UAS*$G_s$α60A*;UAS*pdfr*) or over-expression of DH31R (UAS*$G_s$α60A*;UAS*DH31R*).

All genotypes include *Pdf*-gal4;Epac1camps. Error bars denote SEM. ***, P<0.001 *, P<.05 (compared with control).

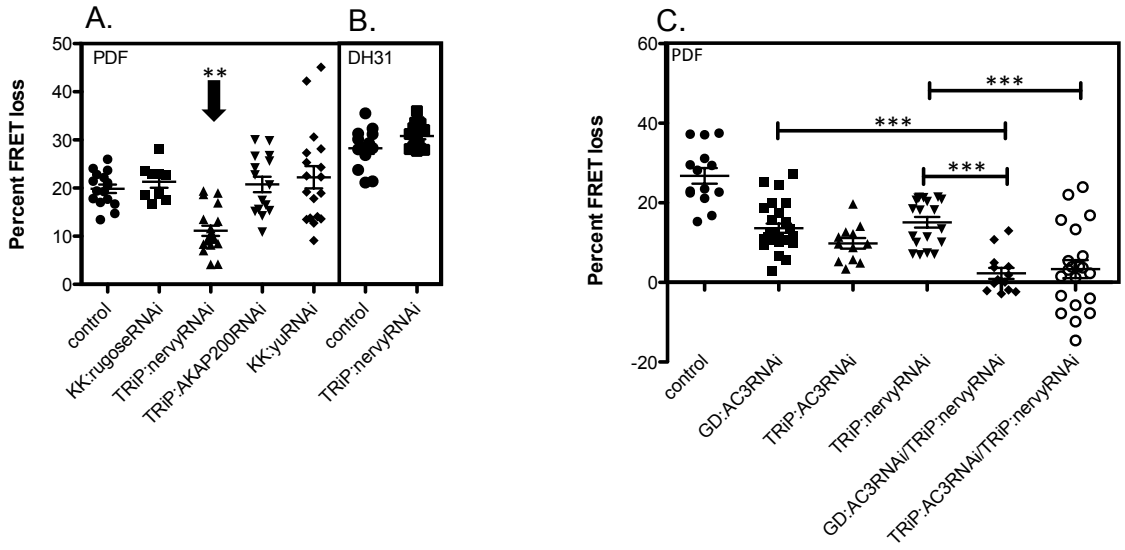



**Figure 6: Effects on PDF responses following RNAi knockdown of scaffolding protein RNAs in M cells.**

A. PDF responses of M pacemakers in flies expressing *nervy* RNAi.

B. DH31 responses of M pacemakers expressing *nervy* RNAi.

C. PDF responses of M pacemakers expressing AC3 and *nervy* RNAi. All transgenic lines are significantly different (<.001) from control and internal comparisons are highlighted by bracketed lines.

All genotypes include *Pdf*-gal4;Epac1camps. Error bars denote SEM. ***, P<0.001 (compared with control).

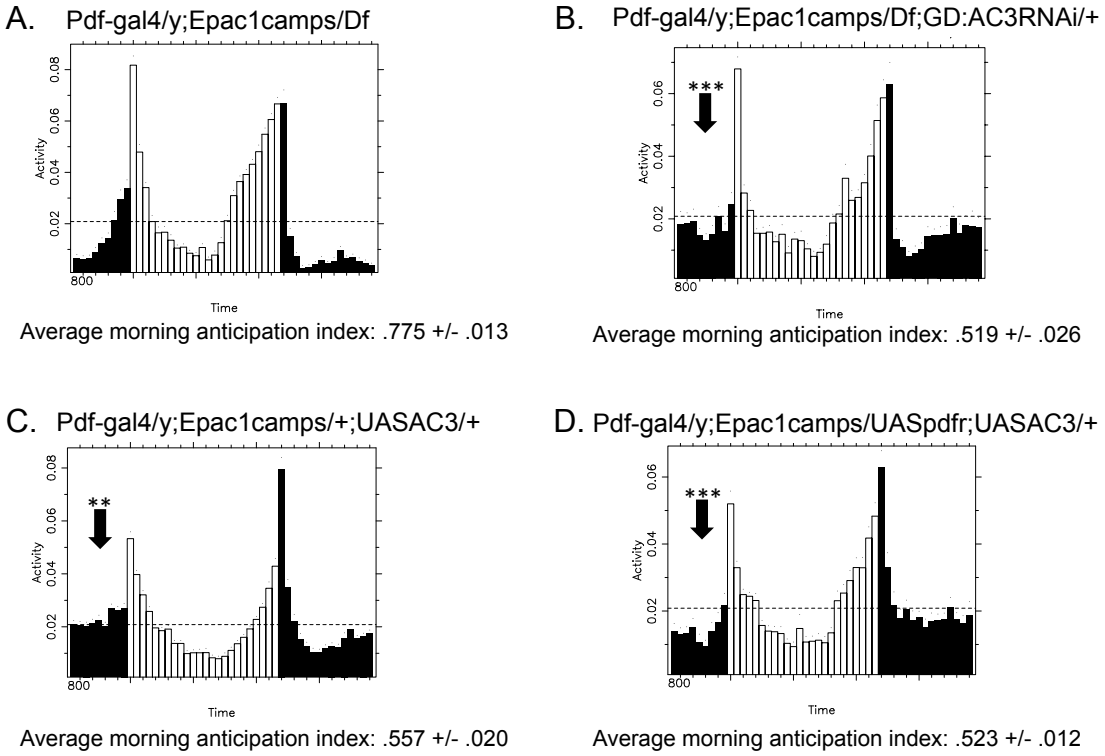



**Figure 7: Effects on circadian locomotor activity of altering AC3 in M pacemakers.**

A. Representative locomotor behavior of flies that are heterozygous for the *AC3* locus (Df(2L)*DS6*).
B. Representative locomotor behavior of flies that combine a knockdown of *AC3* by RNAi together with a deficiency for the *AC3* locus.
C. Representative locomotor behavior of flies over-expressing *AC3*.
D. Representative locomotor behavior of flies over-expressing *pdfr* and over-expressing *AC3*.

Morning anticipation index was calculated as (sum of activity 3 hours before lights-on)/(sum of activity 6 hours before lights-on). The average morning anticipation index was calculated from 3 replicates for each genotype. Error bars denote SEM. ***, P<0.001 (compared with control). Statisical analysis of morning anticipation is shown in Table 1 and behavioral outcomes for DD are shown in Table 2.

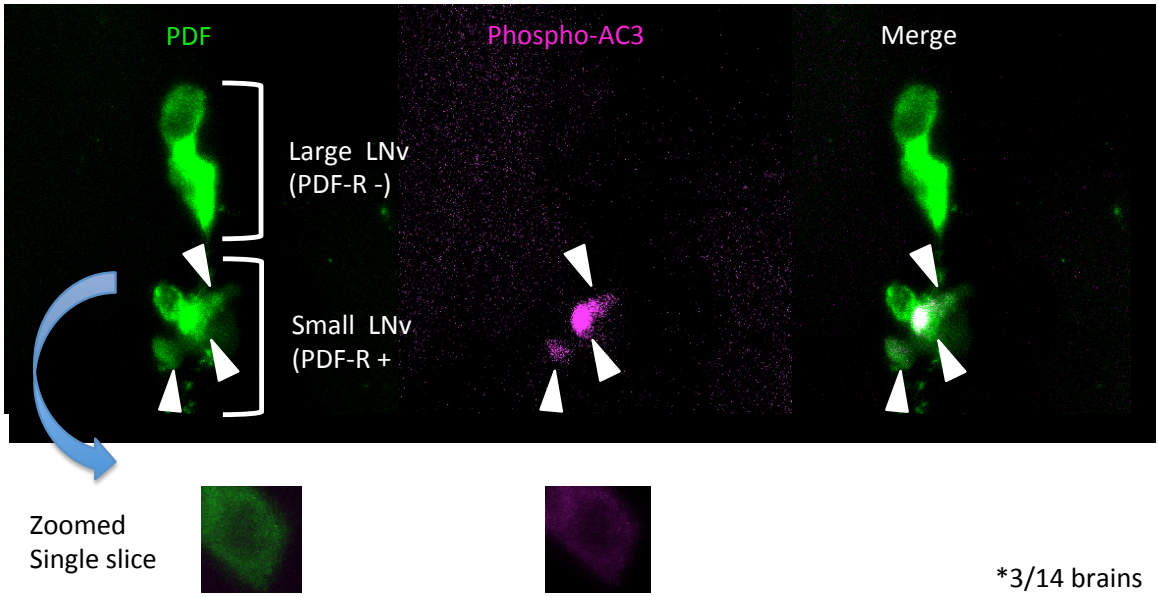



**Figure 8: Phospho-specific AC3 antibody detects signal in small LNv cells after putative PDF stimulation.**
Representative image from single brain with anti-PDF peptide staining and AC3-P signal in small LNvs.

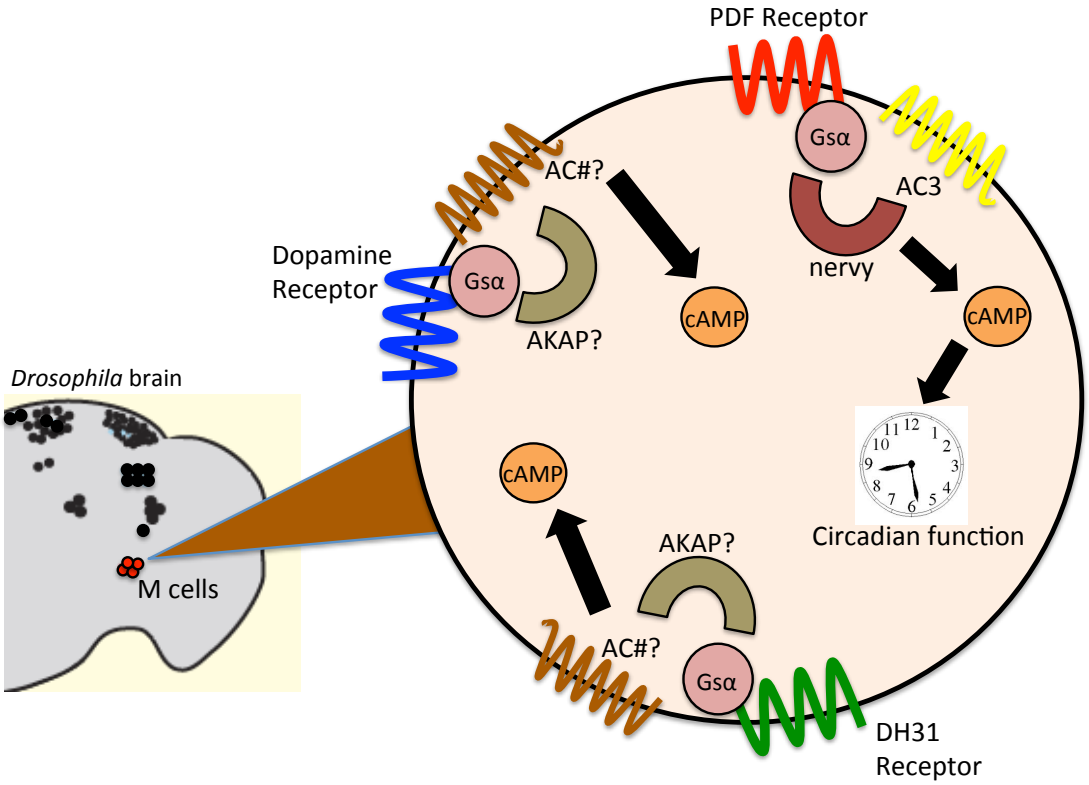
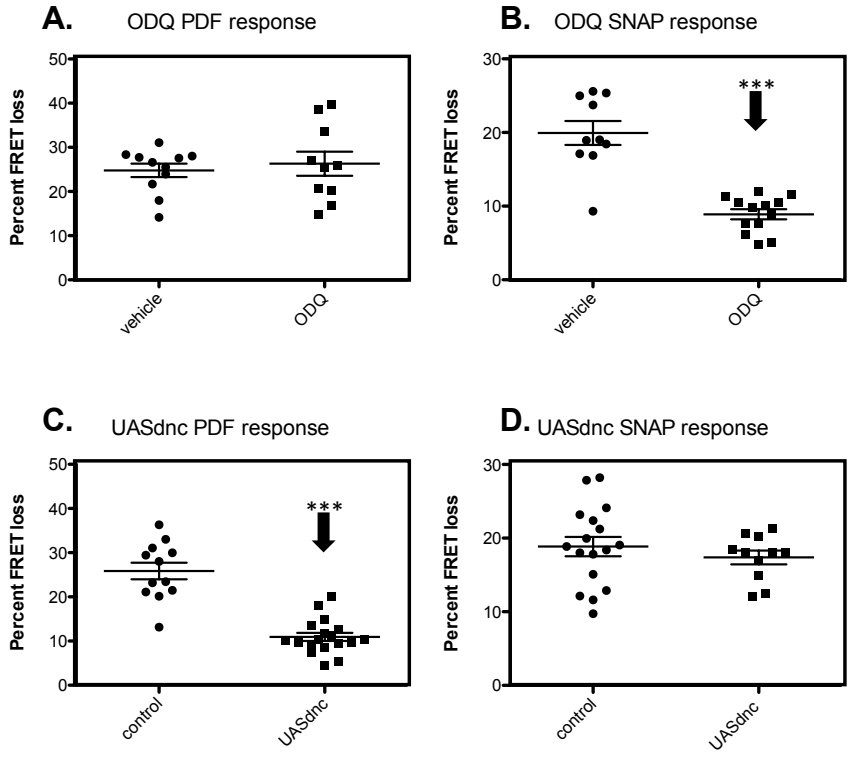


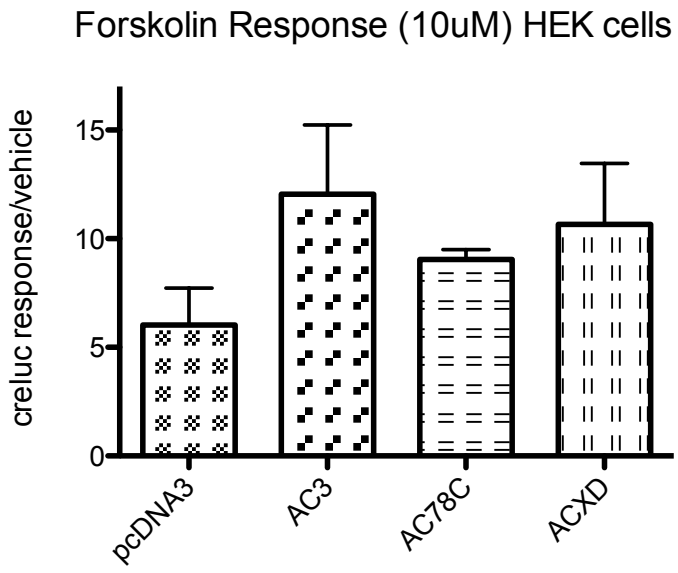

**Figure 9: A model for a circadian signalosome comprised of preferential PDFR:AC3:nervy coupling.**
M pacemakers respond to dopamine, DH31 and PDFR through $G_s\alpha$- coupled receptors: Activation of each receptor lead to increases in cAMP levels. Both DH31 and PDF receptors signal through $G_s\alpha$60A, however AC3 alterations affect PDF signaling without affecting DH31 responses. We propose that PDF signals through AC3 to affect circadian function but that dopamine and DH31 couple to other AC isoforms. Likewise, the AKAP nervy preferentially associates with the PDFR:AC3 signaling complex; other AKAPs support the $G_s\alpha$- coupled receptors that mediate responsiveness to DA and DH31.




**Supplemental Figure 1: PDF signals through cAMP, not cGMP in small LNv cells.**

A. Pretreatment of brains with guanylate cyclase inhibitor (ODQ) has no effect on PDF responses.
B. Pretreatment of brains with guanylate cyclase inhibitor (ODQ) significantly reduces SNAP responses.
C. Over-expression of cAMP specific phosphodiesterase dunce significantly reduces PDF response.
D. Over-expression of cAMP specific phosphodiesterase dunce has no effect on SNAP responses.

All genotypes include *Pdf*-gal4;Epac1camps. Error bars denote SEM. ***, P<0.001 (compared with control).




**Supplemental Figure 2: AC Over-expression in hEK cells.**
*Cre*-luc responses to forskolin in hEK-293 cells after transfection with AC overexpression constructs normalized to vehicle treated cells.

| Tukey's Multiple Comparison Test | Mean Diff. | q | $p<0.05$? | Summary | 95% CI of Diff |
|---|---|---|---|---|---|
| control versus *Pdf*$^{01}$ | 0.312 | 9.171 | Yes | *** | 0.1416 to 0.4824 |
| control versus *Df*(2L)/+ | 0.014 | 0.4115 | No | *ns* | −0.1564 to 0.1844 |
| control versus *Df*(2L)/GD:*AC3*RNAi | 0.2703 | 7.947 | Yes | *** | 0.09997 to 0.4407 |
| control versus UAS*AC3* | 0.2317 | 6.81 | Yes | ** | 0.06130 to 0.4020 |
| control versus UAS*PDF-R*;UAS*AC3* | 0.2663 | 7.829 | Yes | *** | 0.09597 to 0.4367 |
| control versus GD:*AC3*RNAi | 0.079 | 2.322 | No | *ns* | −0.09137 to 0.2494 |
| control versus TRiP:*AC3*RNAi | 0.1893 | 5.566 | Yes | * | 0.01897 to 0.3597 |
| control versus GD:*AC76E*RNAi | 0.041 | 1.205 | No | *ns* | −0.1294 to 0.2114 |
| control versus TriP:*nervy*RNAi | 0.2237 | 6.575 | Yes | ** | 0.05330 to 0.3940 |
| *Pdf*$^{01}$ versus *Df*2L/+ | −0.298 | 8.76 | Yes | *** | −0.4684 to −0.1276 |
| *Pdf*$^{01}$ versus *Df*2L/GD:*AC3*RNAi | −0.04167 | 1.225 | No | *ns* | −0.2120 to 0.1287 |
| *Pdf*$^{01}$ versus UAS*AC3* | −0.08033 | 2.361 | No | *ns* | −0.2507 to 0.09003 |
| *Pdf*$^{01}$ versus UAS*PDF-R*;UAS*AC3* | −0.04567 | 1.342 | No | *ns* | −0.2160 to 0.1247 |
| *Pdf*$^{01}$ versus GD:*AC3*RNAi | −0.233 | 6.849 | Yes | ** | −0.4034 to −0.06263 |
| *Pdf*$^{01}$ versus TRiP:*AC3*RNAi | −0.1227 | 3.606 | No | *ns* | −0.2930 to 0.04770 |
| *Pdf*$^{01}$ versus GD:*AC76E*RNAi | −0.271 | 7.966 | Yes | *** | −0.4414 to −0.1006 |
| *Pdf*$^{01}$ versus TRiP:*nervy*RNAi | −0.08833 | 2.597 | No | *ns* | −0.2587 to 0.08203 |

**Table 1: Quantification of morning anticipation index for LD behavior.**
Statistical analysis of morning anticipation behavior calculated as (total activity 3 h before lights-on)/(total activity 6 h before lights-on). Average morning anticipation was calculated from three replicates. Pdf01 genotype represents *Pdf*-null mutants, which have been widely studied and serve as an example of a total lack of morning anticipation. *p<0.05, **P<.0.01, ***p<.001. ns, not significant.

| Genotype | *n* | % Arrhythmic | Period (h) ± SEM | Power ± SEM |
|---|---|---|---|---|
| *Pdf*-gal4/y;*Epac1camps* | 59 | 2 | 24.3±0.09 | 81.7±3.6 |
| *Pdf*-gal4/y;*Epac1camps*/*Df*(2L) | 28 | 11 | 23.7±0.08 | 64.7±8.7 |
| *Pdf*-gal/y;*Epac1camps*/*Df*(2L);GD:*AC3*RNAi/+ | 33 | 82 | 23.9±0.20 | 39.7±8.6 |
| *Pdf*-gal4/y;*Epac1camps*/+;UAS*AC3*/+ | 46 | 83 | 24.0±0.45 | 26.9±5.2 |
| *Pdf* -gal/y;*Epac1camps*/UAS*PDF-R*;UAS*AC3*/+ | 39 | 64 | 24.3±0.40 | 23.2±4.0 |
| GD:*AC3*RNAi/+ | 29 | 7 | 23.6±0.12 | 70.2±5.5 |
| TRiP:*AC3*RNAi/+ | 39 | 20 | 23.3±0.05 | 43.9±4.6 |
| *Pdf*-gal4/y;*Epac1camps*/+;GD:*AC3*RNAi/+ | 31 | 0 | 24.4±0.07 | 84.0±4.1 |
| *Pdf*-gal4/y;*Epac1camps*/+;TRiP:*AC3*RNAi/+ | 30 | 3 | 24.1±0.07 | 81.6±6.7 |
| *Pdf*-gal4/y;*Epac1camps*/GD:*AC78C*RNAi | 54 | 6 | 24.1±0.06 | 88.7±4.1 |
| *Pdf*-gal4/y;*Epac1camps*/+;GD:*rut*RNAi/+ | 40 | 10 | 24.3±0.07 | 87.8±4.5 |
| *Pdf*-gal4/y;*Epac1camps*/GD:*AC13E*RNAi | 25 | 4 | 24.2±0.14 | 85.4±6.6 |
| *Pdf*-gal4/y;*Epac1camps*/+;GD:*AC76E*RNAi/+ | 52 | 2 | 24.2±0.06 | 99.0±3.9 |
| *Pdf*-gal4/y;*Epac1camps*/KK:*ACXA*RNAi | 17 | 0 | 24.5±0.16 | 46.0±4.1 |
| *Pdf*-gal4/y;*Epac1camps*/KK:*ACXB*RNAi | 29 | 14 | 24.7±0.23 | 101.6±5.8 |
| *Pdf*-gal4/y;*Epac1camps*/KK:*ACXC*RNAi | 22 | 17 | 23.7±0.09 | 75.8±14.7 |
| *Pdf*-gal4/y;*Epac1camps*/KK:*ACXD*RNAi | 20 | 0 | 24.3±0.13 | 111.5±8.7 |
| UAS*AC3*/+ | 24 | 8 | 23.8±0.18 | 56.2±9.1 |
| *Pdf*-gal4/y;*Epac1camps*/UAS*AC76E* | 30 | 20 | 24.4±0.11 | 59.4±5.6 |
| *Pdf*-gal4/y;*Epac1camps*/+;UAS*AC78C*/+ | 69 | 16 | 24.6±0.07 | 57.0±3.9 |
| *Pdf*-gal4/y;*Epac1camps*/+;UAS*ACXD*/+ | 69 | 28 | 24.7±0.08 | 41.3±3.4 |
| *Pdf*-gal4/y;*Epac1camps*/+;UAS*rut*/+ | 22 | 27 | 23.8±0.04 | 47.5±6.4 |
| *KK: Gsα60ARNAi*/+ | 23 | 4 | 24.1±0.20 | 45.2±4.7 |
| *UAS-Gsα60A*/+ | 34 | 0 | 24.3±0.5 | 110.5±5.5 |
| *Pdf*-gal4/y;*Epac1camps*/KK:*Gsα60A*RNAi | 24 | 0 | 25.0±0.06 | 60.2±6.2 |
| *Pdf*-gal4/y;*Epac1camps*/UAS*Gsα60A* | 56 | 64 | 24.9±0.27 | 22.3±3.1 |
| TRiP:*nervy*RNAi/+ | 26 | 15 | 23.4±0.17 | 61.1±7.7 |
| *Pdf*-gal4/y;*Epac1camps*/+;TRiP:*nervy*RNAi/+ | 34 | 29 | 23.8±0.09 | 48.2±4.9 |
| *Pdf*-gal4/y;*Epac1camps*/KK:*rugose*RNAi;+/+ | 22 | 32 | 25.2±0.16 | 60.0±5.8 |
| *Pdf*-gal4/y;*Epac1camps*/+;TRiP:*AKAP200*RNAi/+ | 20 | 20 | 24.5+0.03 | 60.0±0.9 |

**Table 2: DD behavioral outcomes grouped by genotype.**
Periods are calculated using chi-squared periodigram. Flies with a power <10 were scored as arrrythmic.

## CHAPTER 3

### PDF receptor signalosome components vary between pacemaker subgroups

This chapter includes portions of the manuscript:

Duvall LB, Taghert PH. (2012) The Circadian Neuropeptide PDF Signals Preferentially Through a Specific Adenylate Cyclase Isoform AC3 in M Pacemakers of *Drosophila*. PLoS Biol 10(6): e1001337.

**Principal Findings:**

Using real-time cAMP imaging of intact fly brains I report that PDF receptor is a Gs $\alpha$ coupled receptor which increases cAMP levels in dorsolateral neurons (LNds), a representative E cell subgroup in the *Drosophila* brain. These findings parallel my results in small LNv (M) cells reported in the previous chapter. However, unlike small LNv cells, disruptions of AC3 levels have no effect on PDF receptor-expressing LNds. Knockdown of a different adenylate cyclase (AC78C) reduces PDF responses in these cells although this candidate remains unconfirmed because it does not interact with the relevant AC78C deficiency, because overexpression of AC78C does not disrupt PDF responses and because AC78C disruption does not affect locomotor rhythms. However, this partial reduction in PDF responsiveness due to AC78CRNAi is rescued by overexpression of AC78C. I report that knockdown of two AKAPs, nervy and AKAP 200 partially reduces LNd PDF responses.

**Introduction**:

Multiple lines of evidence suggest that downstream signaling pathways differ between clock cell subgroups. Previous studies have suggested a dichotomy between the so-called M and E cell subgroups (Grima et al., 2004; Stoleru et al., 2004). Although the

M and E classifications were derived from their roles in control of morning and evening bouts of locomotor behavior respectively, these behavioral assignments are not rigid and can change under different environmental conditions (Rieger et al., 2007; Zhang et al., 2010). Despite the plasticity in the circadian circuit, many studies have also highlighted the differences in clock signaling and function between the M and E subgroups. Small LNvs belong to the M pacemaker subgroup and the E pacemaker subgroup consists of a diverse group of cells that include the LNds (Grima et al., 2004; Stoleru et al., 2004). Loss of PDF peptide alters molecular oscillations in multiple clock cell subgroups but M and E cell subgroups respond differently. Loss of PDF signaling desynchronizes per staining rhythms in small LNvs but causes a phase advance and reduced amplitude in per staining rhythms LNds (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 behavioral rhythms (Choi et al., 2009; Wulbeck et al., 2008). This suggests PDF can accelerate some clock cells and slow others down. The interaction between PDF signaling and cryptochrome signaling pathways also differs between M and E cells; double mutants of *pdf* and *cry* show normal molecular oscillations in small LNv M cells, while E cells, including LNds, show severely disrupted clock oscillations (Im et al., 2011).

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 report that expression of the PDF receptor exclusively in E cells provides rescue of locomotor behavior in *pdf-r* mutants, although incomplete (Lear et al.,

2010; Im et al., 2011). These studies suggest that disruption of E cell PDF signaling will result in predictable disruptions of circadian behavior.

One possible mechanism that might account for the differences in PDF effects between M and E cells is that signaling components might differ between clock cell subgroups. My previous work described in Chapter 2 suggests that signaling components are sequestered into signaling complexes in small LNvs. Based on the importance of PDF-R activation in E cells, the nature of PDF-R signalosomes is a significant issue in mechanisms of circadian synchronization. Therefore, in this study I investigate the signaling components that mediate PDF responses in PDF receptor expressing LNds, as a representative E cell subgroup.

**Results**:

I used fly rearing and imaging methods as described in the previous chapter (see Materials and Methods section).

***Gs $\alpha$* alterations affect multiple LNd cell responses**

To evaluate PDF signaling in an identified and representative E cell subgroup I investigated PDF-R expressing LNd (the CRY+/PDFR+ subset of LNd (hereafter I refer to these as LNd), using the *Mai179*-gal4 driver - Cusamano et al., 2009; Yoshii et al., 2009; Im and Taghert, 2010)). I first confirmed that PDF induces cAMP, not cGMP, responses in these neurons (Supplemental Figure 1). I also confirmed that LNd PDF responses are dependent upon PDF-R; flies with the strong *pdfr* mutation ($han^{5304}$) display no LNd responsiveness (as was been previously reported for M cells by Shafer et al., 2008) (Figure 1A). In M cells, the PDF receptor is coupled to Gs $\alpha$ and signaling can

be disrupted by either reducing Gsα levels using RNAi, or by overexpressing high levels of Gs $\alpha$ using a UAS*Gsα* transgene (Chapter 2). I found that both Gsα manipulations reduced PDF responses in LNds as well (Figure 1B). LNds are not responsive to the neuropeptide DH31 but they do respond to dopamine with cAMP increases: dopamine responses were also reduced by Gsα manipulations (data not shown). As in the small LNvs, this observation suggests Gsα manipulations disrupt multiple signaling pathways in LNd subgroup.

**AC3 manipulations do not affect LNd cell PDF responses**

Based on the historical importance of the *rut* AC in the literature, I first tested a mutation for the *rutabaga* AC and found normal LNd PDF responses (data not shown); likewise, RNAi directed against *rutabaga* also had no effect on LNd PDF responses (Figure 1C). I quickly turned therefore to the simplest hypothesis; which predicts that LNd PDF-R responses, as in small LNv, involve AC3 specifically. I was surprised to find that in the case of *AC3*, neither RNAi knockdown (combined with a *AC3 Df*) nor *AC3* overexpression altered PDF responses in this E-type clock cell subgroup (Figure 1C). Because this result proved contrary to my initial prediction I thought it was important to test alternative explanations. To eliminate the possibility that cryptic genetic or technical factors could have affected this outcome, I tested small LNv responses in the same brains (using the *Mai179*-gal4 driver) in which LNd cells proved responsive (data not shown). Importantly, small LNv responses were still reduced as expected. Thus, AC3 manipulations only affected PDF responses in small LNvs and not in LNds.

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

My results suggest that LNd PDF responses are not mediated by AC3 - at least not

exclusively. I therefore repeated an RNAi screen directed against 11 of the 12 known ACs encoded in the *Drosophila* genome. When individual AC isoforms were knocked down in LNd AC78C RNAi significantly reduced PDF responses, although it did not completely abrogate PDF responses (Figure 2). I also repeated this screen against the five ACs that are broadly expressed in *Drosophila* tissues (Flybase, 2012; DroID.org, 2012): AC78C, AC76E, AC3, rutabaga and AC13E (as these are arguably are strongest candidates) with the addition of a *UASdicer2* transgene to increase efficiency of the RNAi knockdown (Dietzl et al., 2007). Although expression of the *UASdicer2* transgene itself reduced PDF responses (compare control column in Figure 3 to control column in Figures 1 and 2), AC78C again reduced (although not completely abrogated) PDF responses. However this screen did not implicate any additional candidate ACs (Figure 3).

**Knockdown of AC78C in adult cells reduces LNd PDF cell responses**

As in small LNv, deleterious effects due to continuous expression of AC78C RNAi may explain the reduction in PDF responses. To test this explanation, I employed a conditional genetic system (tubgal80ts), which allows normal development followed by induction of RNAi only in the adult fly (McGuire et al., 2003). When flies were allowed to develop normally, induction of AC78C RNAi in adult flies produced a reduction in PDF responses consistent with my initial screening results (Figure 4A). Expression of the tubgal80ts transgene alone had no effect on PDF responses (Figure 4A). This suggests that the reduction of LNd responsiveness is not due to developmental defects and that in adults, AC78C plays a role in mediating PDF responses in these pacemakers.

**Additional AC manipulations do not have any effect on LNd PDF responses**

To perform genetic confirmation of the AC78C phenotype in LNds, I crossed RNAi expressing flies to animals with a deficiency for the AC78C region (there are no other available RNAi lines available that target AC78C). Addition of the AC78C relevant deficiency did not further reduce the PDF response in LNd cells (Figure 4B). Thus, my proposal that AC78C mediates the LNd PDF response remains unconfirmed at present. It is possible that the RNAi effect is nearly complete and that the addition of a Deficiency for the region will therefore not enhance the phenotype. A related hypothesis is that multiple ACs may contribute to LNd PDF responses. I therefore tested several combinations of AC RNAi lines and, although the AC78C reduction was maintained, no combination of RNAi lines significantly reduced PDF responses beyond AC78C knockdown alone (Figure 5). I also tested the combination of AC3 overexpression (which totally disrupts small LNv PDF responses) with AC78C RNAi and this combination did not further reduce LNd cAMP responses (Figure 5, last column). Finally, adding a *UASdicer* transgene to increase the RNAi knockdown efficiency (Dietzl et al., 2007) did not reveal any additional candidate ACs (see Figure 3).

**Overexpression of AC isforms has no effect on LNd PDF responses**

I tested the effects of overexpression of UAS-*ACXD*, -*AC78C*, -*AC76E*, -*rut* and -*AC3* on LNd PDF responses. Based upon my findings reported in Chapter 2, I anticipated finding that overexpression of the relevant AC (possibly AC78C) would abrogate PDF responses in these cells. However, none of the overexpression constructs tested had any effect on LNd PDF responses (Figure 6). This lack of effect suggests that, either AC78C is not the mediator of PDF responses in this cell subgroup, or that LNd signaling pathways are less sensitive to overexpression of AC78C and may use some

compensatory mechanism(s) to maintain PDF responsiveness.

**Combination of AC78C overexpression construct with AC78CRNAi**

To further test the hypothesis that AC78C mediates PDF signaling in LNd, I combined the RNAi transgene with the AC78C overexpression construct. Because the RNAi transgene targets a portion of the AC78C coding region it targets both the endogenous AC78C as well as the overexpression construct. However, overexpression of UASAC78C with AC78C restores PDF responses in LNds, which is not simply due to gal4 dilution because overexpression of UASrut does not rescue PDF responses in these cells (Figure 7).

**AKAPs nervy and AKAP200 knockdown reduce LNd PDF response**

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 (Chapter 2). In similar fashion, I tested the possible involvement of AKAPs as scaffolding proteins for PDF-R in LNd cells using gene-specific RNAi constructs. I report that knockdown of either *AKAP200* or *nervy* reduces LNd PDF responses (Figure 8). Knockdown of *AKAP200* reduces PDF responses to about 40% of their original levels whereas knockdown of *nervy* only reduces PDF responses to about 65% of their original levels. These findings suggest that there may be multiple signalosomes that can mediate PDF responses in LNds.

**AC78C manipulations do not result in 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). Therefore, I expected that disruptions to PDF responses in these cells would phenocopy

animals with PDF-R expressed only in the M cell subgroup (small LNv) and show an advance in the evening peak in LD conditions and weak short rhythms in DD (Hyun et al., 2005). However, AC78C manipulations that reduced PDF responses did not result in behavioral deficits. This behavioral result is consistent with the partial reduction in PDF responsiveness (my previous results suggest that only severe reductions in PDF responsiveness affect locomotor behavior). Additionally, to date, no combination of genetic elements that partially reduce LNd PDF responses (AC/AKAP/Df etc.) shows circadian disruptions consistent with a total loss of PDF signaling.

**Discussion:**

Simply put, the set of AC3 manipulations that caused a disruption of PDF responsiveness in M pacemakers had no such effect in E pacemakers. Importantly, RNAi-mediated disruption of Gs $\alpha$ affected both subgroups indicating that the gal4 driver strength is sufficient. Multiple lines of evidence have suggested that 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). 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 (Lin et al., 2004). (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 molecular rhythm, while M cells continue to cycle normally (Zhang et al., 2009; Park et al,. 2009; Im et al., 2011). Different subsets of E cells have previously been implicated in control of evening anticipation and, even when AC3 is

altered in all clock cells, the evening peak retains its proper phase (Grima et al., 2004; Stoleru et al., 2004; Mutad et al., 2007; Lear et al., 2009), again suggesting that AC3 is not a required enzyme in E type pacemaker cells (data not shown). These findings are consistent with the hypothesis that there are two functionally different PDF signaling pathways operating in different pacemaker cell types.

AC78C is a candidate AC for mediating PDF responses in LNd, however this result remains unconfirmed. Although the reduction in PDF responses is maintained when AC78C is knocked down only in adult stages and this reduction is maintained when AC78C is crossed to a number of other UASAC RNAi lines I have been unable to completely abrogate the PDF responses in LNd. However, the partial reduction in PDF responsiveness in LNds is rescued by overexpression of AC78C but not by overexpression of rutabaga. The addition of the deficiency for the AC78C genetic region does not further reduce the PDF response, suggesting the contribution of another AC(s). It is possible that one of my other RNAi lines does not appropriately target its cyclase for degradation and leads to a false negative in our RNAi screen. I favor the possibility that an additional (currently unidentified) AC mediates that PDF response in the LNd. Hence it remains to be determined how uniform are the components of PDF signalosomes in the M versus E pacemaker cell types.

To what extent do different signaling components actually result in differences in downstream PDF signaling between clock cell subgroups? It is possible that signalosomes with different components may be performing the same function. I observe that both M and E cell subgroups respond to PDF with an increase in cAMP and that LNds respond with slightly higher amplitude (data not shown). My analysis has not

revealed any temporal differences in PDF responses or recovery between these two subgroups, although sub-second differences in kinetics are not likely to be reported by my sensor (Nikolaev et al., 2003). The extent to which differences in the pathways that generate cAMP in response to PDF account for the differences in downstream effects in clock cell subgroups remains an important area for future study. An important cAMP target, PKA, has been shown to be important for maintaining normal locomotor rhythms although PKA alteration do not seems to affect core clock proteins themselves (Majercak et al., 1997; Park et al., 2000). This suggests 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 although these studies were performed with mutant flies that make it difficult to disambiguate the roles of individual pacemaker subgroups. Knockdown of a catalytic subunit of PKA (PKA-C1) using a broad clock driver (*tim*gal4) phenocopies *pdf* null mutants in LD and results in even more severe DD arrythmicity, and knockdown only in PDF cells (*pdf*gal4) does not affect locomotor rhythms (W. Li, unpublished data). These findings suggest that PKA signaling downstream of PDF receptor in PDF-negative clock cells (which include E cells) plays a key role in mediating circadian behavior. However, reductions in PKA signaling in M cells do not.

Why would a single neuropeptide receptor utilize multiple signaling pathways? Recent studies have implicated feed-forward mechanism in neuropeptide circuit modulations (Jing et al., 2007, Wu et al., 2010; Taghert and Nitabach, 2012). Differential composition of signalosomes may provide a mechanism for the single neuropeptide, PDF to differentially modulate components of the circadian circuit. Although the precise composition of the signaling complexes that mediate PDF responses in LNd remain

incompletely defined, this possibility provides a mechanism for PDF to act broadly to reconfigure the circadian neural circuit in *Drosophila*.

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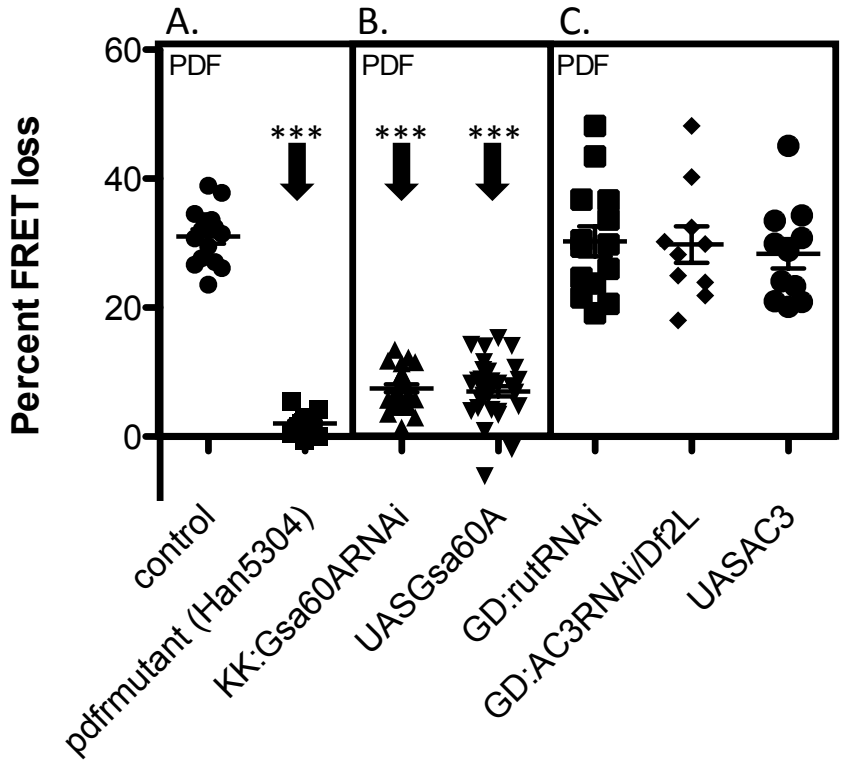



**Figure 1: Effects of manipulating Gs and AC3 levels in E cell subgroup.**

A. PDF responses in PDF-R expressing LNd cells (E cells). Flies with the severe PDF-R mutation Han5304 show no response to PDF.
B. PDF responses in PDF-R expressing LNd cells (E cells). Both knockdown and overexpression of Gs60A significantly reduce PDF responses in E cells.
C. PDF responses in PDF-R expressing LNd cells (E cells) in genotypes that most severely disrupt M cell PDF responses. Knockdown (Df2L/AC3RNAi) and overexpression of AC3 do not affect E cell PDF responses.

All genotypes include *Mai179*-gal4;Epac1camps. Error bars denote SEM. ***, $P<0.001$ (compared with control).

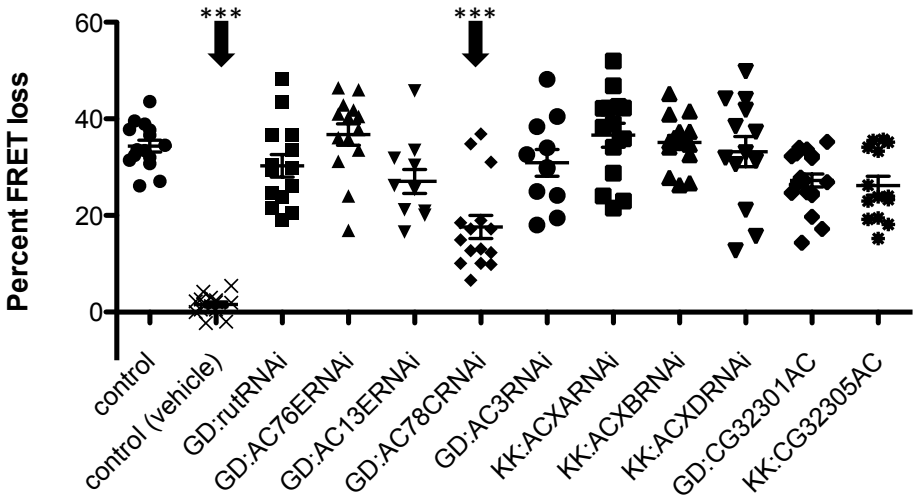



**Figure 2: AC78C scores positive in an RNAi screen directed against LNd cell PDF responses.**

Double stranded RNAi directed against 11/12 genes known to encode known adenylate cyclases in the *Drosophila* genome.

All genotypes include *Mai179*-gal4;Epac1camps and 1 copy of UASRNAi (except for control). Error bars denote SEM. ***, P<0.001 (compared with control).

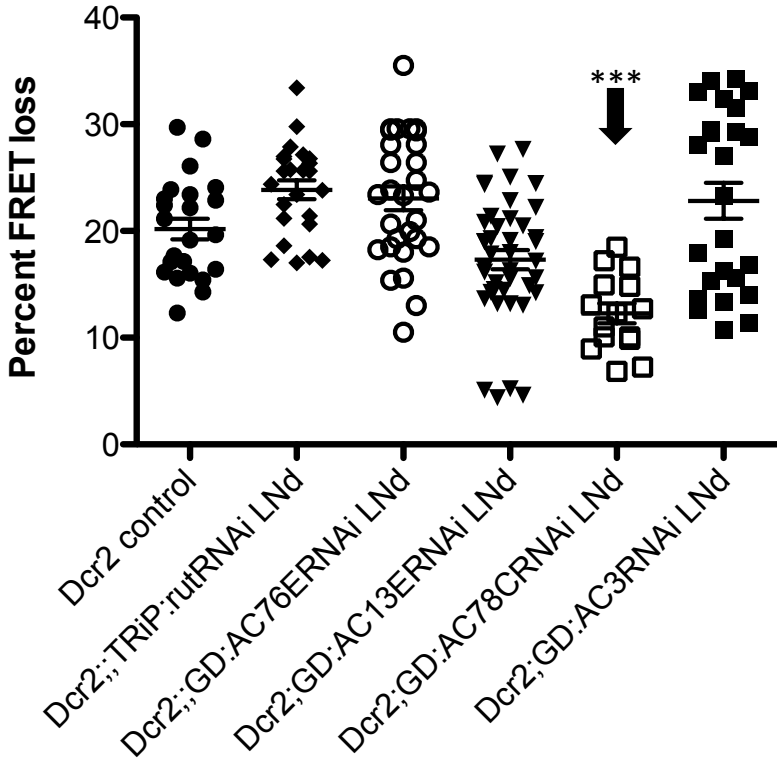



**Figure 3: AC78C remains the only RNAi line which reduces PDF responses in LNd cells but does not abrogate PDF responses.** Addition of the UASdicer reduces PDF responses but does not reveal any additional hits. All genotypes include *Mai179*-gal4;Epac1camps and 1 copy of UASRNAi (except for control). Error bars denote SEM. ***, P<0.001 (compared with control).

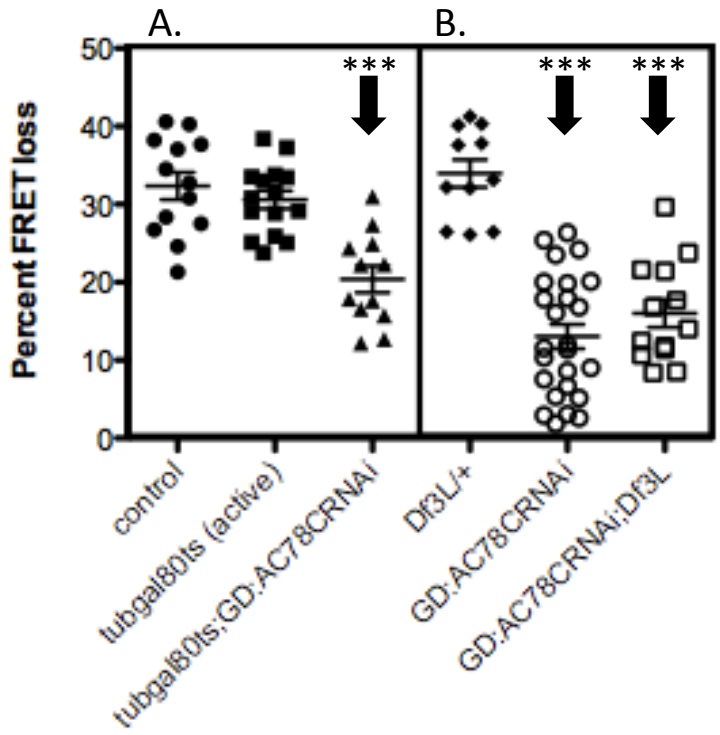



**Figure 4: Adult specifc knockdown of AC78C reduces PDF responses but addition of relevent Deficiency does not further reduce PDF response in LNds.**

A. Adult specific expression of AC78C RNAi reduces PDF responses although tubgal80 transgene expression alone has no effect on PDF response.
B. Addition of AC78C relevent deficiency does not further reduce PDF response when combined with RNAi knockdown.

All genotypes include *Mai179*-gal4;Epac1camps and 1 copy of UASRNAi (except for control). Error bars denote SEM. ***, P<0.001 (compared with control).

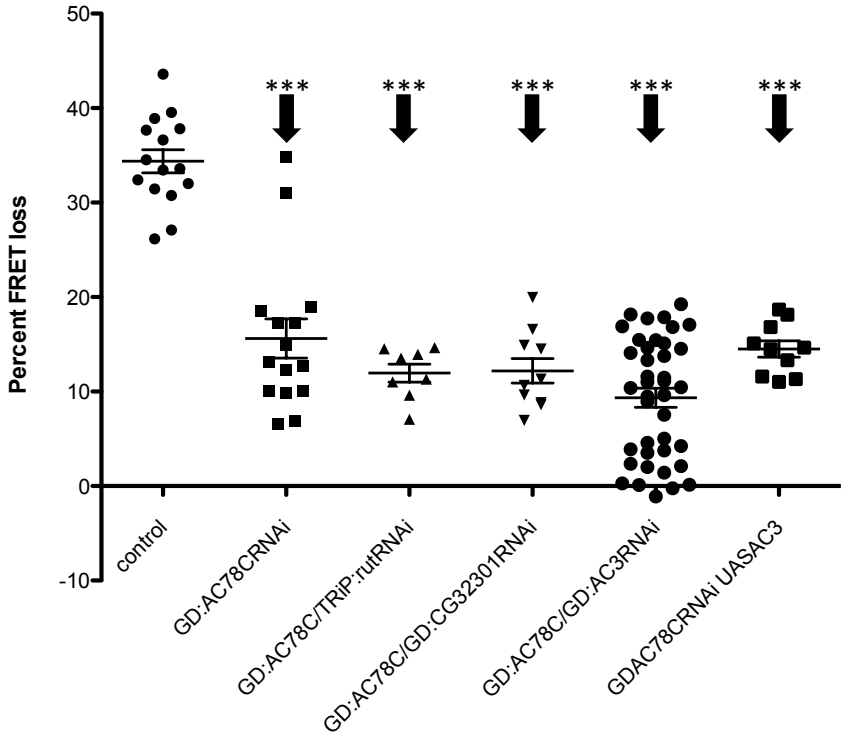



**Figure 5: Knockdown of multiple ACs does not further reduce LNd PDF repsonses.**
Combinations of AC knockdowns do not reduce PDF responses beyond single AC78C RNAi alone.
All genotypes include *Mai179*-gal4;Epac1camps and 1 copy of UASRNAi (except for control). Error bars denote SEM. ***, P<0.001 (compared with control).

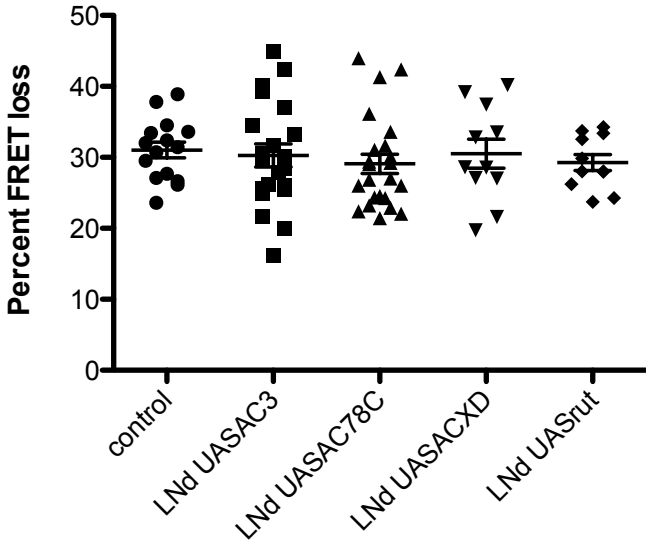



**Figure 6: Overexpression of AC isforms does not affect LNd PDF responses.**
All genotypes include *Mai179*-gal4;Epac1camps and 1 copy of UAS transgene (except for control). Error bars denote SEM. None are significantly different from control.

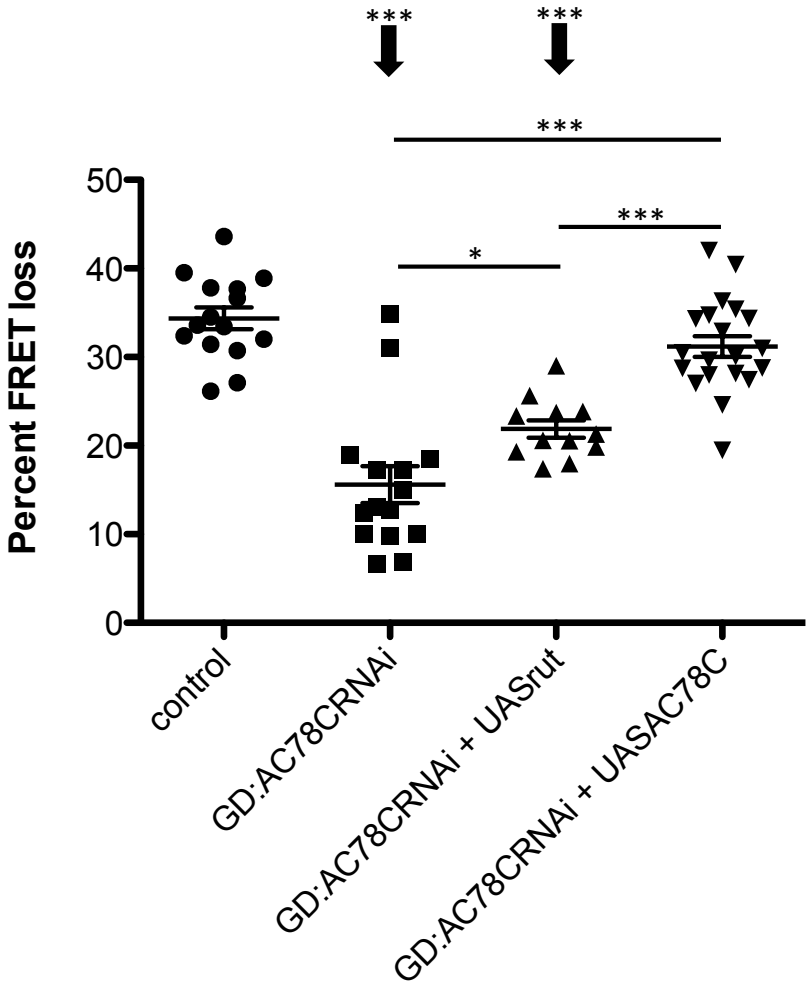



**Figure 7: Overexpression of AC78C, but not rutabaga rescues PDF responsiveness of LNds.**
All genotypes include *Mai179*-gal4;Epac1camps and 1 copy of UAS transgene (except for control). Error bars denote SEM. . ***, P<0.001 *,P<0.05 (Arrows denote comparisons with control, internal comparisons are shown as horizontal lines between genotypes).

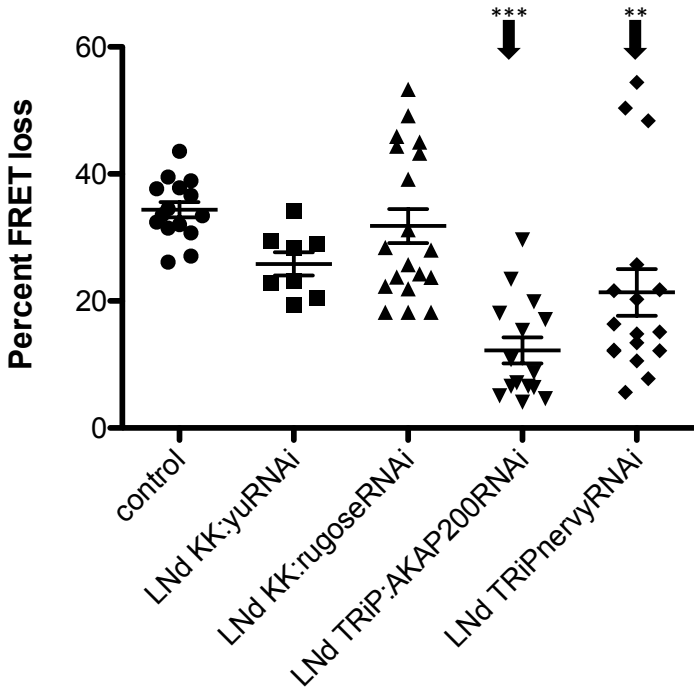
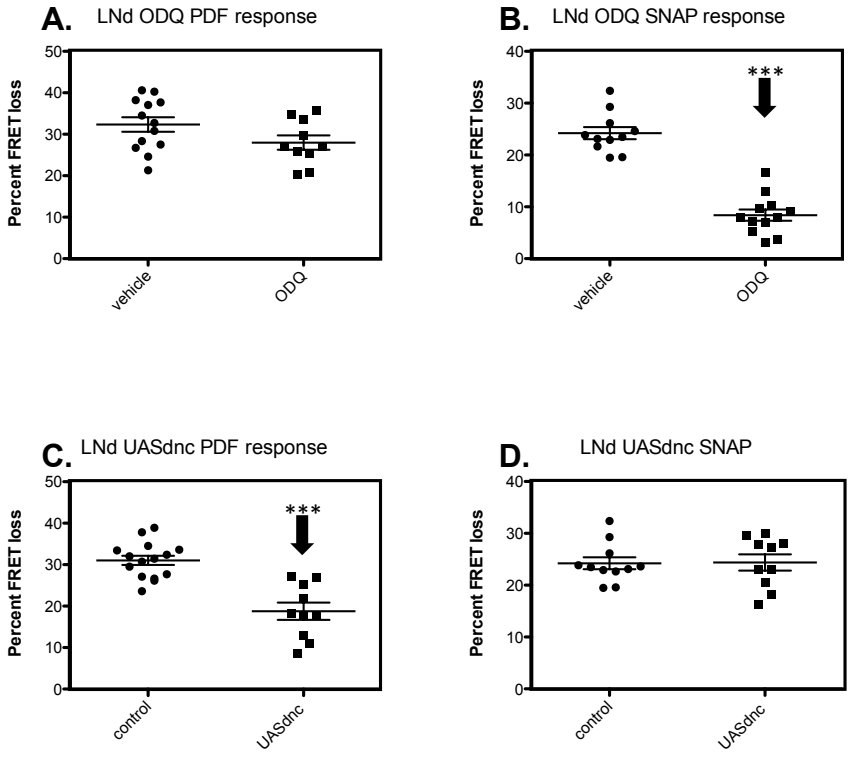



**Figure 8: Effects on PDF responses following RNAi knockdown of scaffolding protein RNAs in LNds.**
Knockdown of either AKAP200 or nervy reduces LNd PDF responses.
All genotypes include *Mai179*-gal4;Epac1camps and 1 copy of UAS transgene (except for control). Error bars denote SEM. ***, P<0.001 **, P<0.01 (compared with control).




**Supplemental Figure 1: PDF signals through cAMP, not cGMP, in LNd cells.**

A. Pretreatment of brains with guanylate cyclase inhibitor (ODQ) has no effect on PDF responses.
B. Pretreatment of brains with guanylate cyclase inhibitor (ODQ) significantly reduces SNAP responses.
C. Over-expression of cAMP specific phosphodiesterase dunce significantly reduces PDF response.
D. Over-expression of cAMP specific phosphodiesterase dunce has no effect on SNAP responses.

All genotypes include *Mai179*-gal4;Epac1camps. Error bars denote SEM. ***, P<0.001 (compared with control).

**CHAPTER 4:**

**Daily Changes in PDF Receptor Signaling**

**Principal Findings:**

I report a diurnal rhythm in sensitivity to the PDF neuropeptide in two pacemaker subgroups, small LNvs and LNds. In both groups the sensitivity observed at ZT4 is higher than that observed at ZT22 and, at least in small LNvs, this rhythms persists under constant conditions. I report that genetic disruption of glycosylation impairs PDF-generated cAMP responses in two subgroups of pacemaker cells in *Drosophila*: small LNv and LNd cells. I find evidence that RNAi induced knockdown of specific glycosyltransferases (CG30036 in small LNvs and CG33145 in LNds) reduce PDF cAMP responses in these pacemaker subgroups and result in circadian locomotor defects. Additionally, both subgroups of cells recover PDF responsiveness after exposure to a drug that disrupts glycan modifications. The speed of recovery from this drug treatment also shows diurnal rhythmicity, with a peak phase that resembles that of the rhythm in PDF sensitivity. These observations and their correlation suggest that PDF receptor signaling complexes can occupy different sub-cellular distributions and/or functional states. Furthermore, it suggests there is normally a cyclic ~24-hr rhythm in the flux between different states.

**Introduction**:

PDF receptor signaling plays important roles in synchronizing clock cells and promoting normal circadian locomotor behavior (Renn et al., 1999; Lin et al., 2004;

Hyun et al., 2005; Lear et al., 2005). Previous studies have suggested that PDF is released from small LNv dorsal projections in the morning based upon cycles of levels of PDF peptide detected in these projections (Park et al., 2000). However, subsequent reports have called into question the requirement for oscillations in PDF peptide levels for normal circadian behavior (Kula et al., 2006). No such rhythms in the levels of the PDF receptor have been reported although the possibility that receptor signaling may show circadian variation has never been addressed in the literature. Data from SCN cultures suggest that VIP, the mammalian homolog of PDF, is rhythmically released (Shinohara et al., 1994).

Daily changes in PDF signaling may provide important timing cues for the clock network. Previous studies suggest that PDF communicates phase information from small LNvs to other pacemakers that in turn, control circadian locomotor activity (Stoleru et al., 2005). It is unknown whether PDF's synchronizing functions are accomplished by signaling constantly or in a gated fashion. Gating could occur by restricted release of peptide, or changes in receptor availability or modification. Data collected from rat SCN suggests daily rhythms in the release of VIP, the mammalian homolog of PDF (Albers et al., 1990; Shinohara et al., 1994; Aton et al., 2005). Although it is not clear theoretically that the signal must be gated, a number of groups have suggested that PDF signaling is gated: small LNvs are most electrically excitable in the morning, when PDF release is thought to be maximal (Cao et al., 2008; Sheeba et al., 2008). Inducing constitutive PDF availability through the expression of a tethered form of the peptide results in abnormal complex locomotor rhythms (Choi et al., 2009). Post-translational modifications can play

diverse roles in protein localization and trafficking; PDF receptor signaling may show diurnal variation under the control of these mechanisms (Rasmussen, 1992).

Glycosylation plays important roles normal protein function, for example in folding, stability, trafficking, localization or protein-protein interactions (Rasmussen, 1992; Lis et al.,1993; Opdenakker et al., 1993). Most proteins that travel through the ER-Golgi complex are modified to varying degrees (Opdenakker et al., 1993). Transmembrane proteins, including GPCRs and ACs, are often modified by the addition of carbohydrate moieties (Rasmussen, 1992; Wong et al., 2000). However, the regulation of glycosylation status and its complex effects on protein function remain difficult to predict in many cases. Glycosylation is an enzyme dependent site-specific process (Rasmussen, 1992). For example, specific enzymes, located in the Golgi network add these sugars to proteins as they are translated. PDF signaling components are likely to be affected by these carbohydrate modifications that may represent post-translational modifications that affect daily changes in PDF receptor activation.

In *Drosophila*, Notch is an example of a specific membrane receptor whose function depends upon glycosylation. Notch signaling is required for numerous aspects of normal development and its ligand specificity is regulated by its glycosylation status (Mumm and Kopan, 2000). In the Golgi network, the Notch receptor is modified on the extracellular domain by a specific glycosyltransferase, FRINGE (Panin et al., 1997). Although the biochemical mechanism remains unclear, glycosylated Notch receptor shows a preference for the ligand Delta versus the ligand Serrate, while unmodified receptor shows the opposite preference (Irvine and Wieschaus, 1994; Panin et al., 1997; Bruckner et al., 2000; Moloney et al., 2000; Munro and Freeman, 2000).

Other components of GPCR signaling are likely to be regulated by glycosylation as well. In mammals, AC8 is a calcium-stimulated adenylate cycalse isoform that is specifically activated by capacitative calcium entry (CCE) and is localized with other CCE components in microdomains of lipid rafts (Fagan et al., 1996; Smith et al., 2002). AC8 is modified by N-linked glycosylation and mutant forms of the AC that are un-glycosylated are not appropriately trafficked to the CCE component-containing lipid rafts, although the unmodified AC8 is still responsive to calcium (Pagano et al., 2009). These findings support the idea that signaling components are sequestered into complexes and that carbohydrate modifications can be important determinants for signaling complex formation and trafficking.

Based upon the results described in Chapter 2, I have proposed that AC3 is the mediator of PDF responses in small LNvs in the *Drosophila* brain. Previous work in the mammalian olfactory epithelium indicates that AC3 is heavily glycosylated and depends upon a specific glycosyltransferase to add the appropriate sugars (Henion et al., 2011). When these modifications are lost, AC3 loses both basal and forskolin induced enzymatic activity, although the protein is trafficked normally to the primary cilia in olfactory epithelium (Henion et al., 2011). Although no specific mechanism is proposed for the role of these modifications in mediating enzymatic activity the predicted extracellular site for glycosylation is conserved across all transmembrane adenylate cyclases in mammals (Henion et al., 2011). Mammalian AC2 is heavily glycosylated normally and the conservation of the target glycosylation site suggests that these modifications may play a previously unappreciated role in cAMP generation (Wong et al., 2000).

In this chapter, I pursued these two general questions. First I investigated the potential for diurnal changes on sensitivity of small LNvs to PDF over the circadian day. Second, I tested the effect of disrupting specific components of glycosylation on PDF-generated cAMP responses in two groups of pacemaker cells in the *Drosophila* brain.

**Results**:

I tested PDF cAMP responses in small LNvs and LNds using Epac1camps FRET sensor with methods described in previous chapters. (See Materials and Methods section).

**PDF sensitivity shows circadian variation in small LNvs and LNds**

Changes in immunohistochemical staining of PDF in small LNv nerve terminals is generally assumed to reflect a daily change in the rate of PDF release (Park et al., 2000). More PDF is thought to be released in the photophase than in the scotophase, although this interpretation is clearly not authoritative. However, changes in PDF receptor sensitivity have not yet been assessed. I therefore tested PDF responses to low doses of the peptide in the small LNv subgroup of clock cells. Previous observations have suggested that there is no variation in PDF cAMP responses: however these were performed with supra-maximal doses of PDF (O. Shafer, personal communication; L. Duvall, unpublished data). I therefore re-examined this issue by testing doses of PDF that are close to the threshold of detection using the FRET sensor. I report that PDF sensitivity at very low doses ($10^{-9}$ M) does show clear time-of day sensitivity under 12:12 light/dark regimens while doses at $10^{-8}$ M show no significant differences over the day (Figure 1A and B). Small LNvs are most sensitive to PDF at ZT4 and show least

sensitivity to low doses of PDF at ZT22 (Figure 1B). I also tested sensitivity to PDF in another group of clock cells, LNds, at the timepoints identified in small LNvs as having minimum (ZT22) and maximum (ZT4) PDF sensitivity. As in small LNvs, responses to $10^{-8}$ M PDF are not significantly different between these two timepoints, but responses to $10^{-9}$ M are significantly greater at ZT4 compared to ZT22 in LNds (Figure 1 C and D).

To more fully characterize the changes in pacemaker PDF sensitivity I performed a full dose-response curve for PDF responses at the minimum and maximum timepoints in both pacemaker subgroups (ZT22 and ZT4). I tested PDF at doses ranging from $10^{-7}$ to $10^{-11}$ M in at least 6 brains collected from at least 2 replicate timepoints. Small LNvs are more sensitive to PDF at ZT4 (EC50: $3.367 \times 10^{-10}$) compared to ZT22 (EC50: $3.361 \times 10^{-9}$) (Figure 2A). LNds are more sensitive to PDF at ZT4 (EC50: $3.626 \times 10^{-10}$) compared to ZT22 (EC50: $2.767 \times 10^{-9}$) (Figure 2B). In short there is a full log unit difference in efficacy for this peptide ligand over the course of a 24-hour day.

To test whether these rhythms persist under constant conditions I tested PDF responses at two intermediate doses of PDF that had shown differential PDF responses at ZT22 compared to ZT4 ($5x10^{-9}$ M and $5x10^{-10}$ M). Although all responses measured in DD are reduced in amplitude compared to their LD counterparts (compare to Figure 2), small LNvs retain higher sensitivity to PDF at CT4 compared to CT22 (Figure 3). This is not explained by a slow degradation in PDF responsiveness in constant conditions because the increased sensitivity persists at CT28 (after 1 full day in DD) compared to CT22 (data not shown).

**Neuraminidase treatment reduces PDF responses in both small LNvs and LNds**

It is likely that at least some PDF receptor signaling components are modified by glycosylation and previous work suggests that carbohydrate modifications are key for normal AC3 cAMP generation in mammals (Henion et al., 2011). To test the effect of a loss of glycan modifications on PDF responses in clock cells in *Drosophila* I treated whole brains with bath-applied neuraminidase (0.01 units/mL), a glycoside hydrolase enzyme that cleaves the glycosidic linkages. After 15 minutes in a bath of HL3 saline and neuraminidase, PDF responses were significantly reduced in both small LNvs and LNds (Figure 4A and B). This reduction is dose dependent (Supplemental Figure 1) and when the enzyme is head-inactivated there is no effect on PDF responses indicating that the enzymatic activity of neuraminidase reduces PDF responses (as opposed to some other bath component or interaction) (Figure 4C). To exclude the trivial possibility that the neuraminidase preparation directly affects the PDF peptide used for stimulation, I added a neuraminidase/PDF mixture. Mixing neuraminidase and PDF has no effect on the PDF cAMP response measured in pacemaker cells, indicating that residual neuraminidase does not reduce PDF sensitivity by directly degrading the PDF peptide (Figure 4C).

**PDF responses recover after neuraminidase treatment**

Neuraminidase is an enzyme that disrupts glycan modifications and therefore affects many signaling molecules. To ensure that enzyme treatment does not cause irreparable damage to neurons I allowed whole brains to recover in Schneider's culture media after the 15 minute neuraminidase incubation period. Both small LNvs and LNds regained full PDF responsiveness within two hours (Figure 5 A and B). Bath treatment with vehicle (saline instead of neuraminidase) had no effect on PDF responses (Figure 5

A and B, red circles). Recovery curves were calculated using a Boltzmann sigmoidal fit and LNds show recovery at a shorter timepoint (V50: 80.04 min) than small LNvs (V50: 106.7 min) (Figure 5 A and B).

**Neuraminidase recovery shows diurnal variation**

Glycan modifications are generally thought to occur co-translationally within the Golgi apparatus and it is therefore unlikely that the original drug-disrupted signaling molecules are re-modified at the plasma membrane after neuraminidase treatment (Rasmussen, 1992). This suggests that the recovery of PDF cAMP responses reflects rebuilding of new signaling complexes. I reasoned that the recovery from neuraminidase treatment is likely to reflect endogenous rates of trafficking of new receptor complexes to the plasma membrane and that the rates of receptor trafficking may coordinate with the rhythms observed in PDF receptor sensitivity. I tested PDF responses after 60 minutes of culture-media recovery post-neuraminidase treatment in both small LNvs and LNds. The PDF responses were tested at four timepoints throughout the circadian day. Animals were housed under 12:12 light:dark conditions and dissected at ZT 4, 10, 16 and 22. In both subgroups of cells tested PDF responses were most reduced at ZT22 and were highest at ZT4 (Figure 6 A and B) reflecting less recovery after 60 minutes from neuraminidase treatment at ZT22 and more complete recovery at ZT4.

**Knockdown of glycosyltransferase encoded by CG30036RNAi reduces small LNv PDF responses**

The *Drosophila* genome encodes at least seven β1,3-galactosyltransferase (Gal/GalNAcβ1,3GlcNAc) glycosyltransferases (Altmann et al., 2003; Flybase, 2012). I tested the effect of RNAi knockdown of a subset these genes for an effect on PDF

responses in small LNv and LNd cells based upon the finding that a specific member of this family is required for AC3 in the mammalian olfactory epithelium (Henion et al., 2011). I used double-stranded RNAi to knock down specific glycosyltransferase isoforms.

In small LNv cells the knockdown of the glycosyltransferase encoded by CG30036 reduces PDF responses (Figure 7A). CG30036 is the most closely related gene to the glycosyltransferase identified for its role in modifying mammalian AC3 in the olfactory epithelium. In small LNv cells, RNAi directed against several other glycosyltransferases had no effect on the PDF response. These CG30036 RNAi animals also show subtle (but not significant) disruptions in their circadian behavior, a trend towards a reduction of the morning peak of anticipation as well as increased levels of arrythmicity under constant conditions (Figure 8 B and D). These changes are consistent with, although less severe than, the behavioral defects associated with disruption of AC3.

**Knockdown of glycosyltransferase encoded by CG33145RNAi reduces LNd PDF responses**

In LNd cells, knockdown of the glycosyltransferase encoded by CG33145 reduced PDF responses, although knockdown of other related glycosyltransferases had no such effect (Figure 7B). These CG33145 knockdown animals show normal behavior under light/dark conditions but are almost entirely arrhythmic under constant conditions (95% AR) (Figure 8 C and D). The few animals that remain rhythmic display a shortened period compared to controls (Figure 8D).

**Discussion**:

My previous results suggest that signaling pathway components are sequestered into specific signaling complexes (termed signalosomes) with the PDF receptor in a preferred associated with AC3 in the small LNv cells in *Drosophila*. These findings suggest that protein-protein interactions may be key regulators of signaling pathways and may help to differentiate pathways that use common components. In this section, I report daily changes in the sensitivity of the small LNvs and LNds to PDF and corresponding daily changes in the rates of recovery of PDF responsiveness after bath treatment with a glycosidase that reduces PDF responses. Here I discuss the significance and possible connections between these two sets of observations.

PDF acts as a synchronizing agent among the pacemaker cells in the *Drosophila* brain. Although a number of previous studies suggest that PDF signals in a gated fashion and that it is released in the morning from small LNvs (Park et al., 2000) it is possible that PDF signals continuously throughout the day. Gating of PDF signaling could occur through a number of mechanisms including: restricted release of the peptide, receptor availability through levels of protein or receptor modifications or through restrictions in downstream signaling components. There is a suggestion that PDF release is temporally restricted (Park et al., 2000) but other possibilities have not been fully investigated. In mammals, there is evidence that VIP, a synchronizing agent in the SCN, is released with circadian rhythmicity (Albers et al., 1990; Shinohara et al., 1994). Overexpression of the VPAC2 receptor shortens the circadian period however continuous application of a VPAC2 receptor agonist lengthens the period. These seemingly inconsistent results may be explained because, receptor overexpression alone may have different temporal effects

on signaling than constitutive levels of peptide alone (Shen et al., 2000; Pantazopoulos et al., 2010). Modeling of the VPAC2 signaling pathway suggests that some of the most sensitive parameters relate to AC activation and deactivation, suggesting the factors other than peptide release may play important roles daily signaling (Hao et al., 2006).

I found that small LNvs and LNds are most sensitive to the PDF peptide early in the photophase (ZT4) and that the cells are least sensitive late in the scotophase (ZT22). I also report that recovery rates of PDF responses show similar circadian changes; clock cells are able to recover from neuraminidase treatment more quickly in the morning (ZT4) than at night (ZT22). By measuring recovery rates at different circadian times of day I reason that endogenous rates of trafficking of signaling molecules are revealed. However, this assumes that neuraminidase treatment does not affect normal trafficking of signaling components to the plasma membrane.

Why would trafficking rates vary over the course of the day? Previous work suggests that PDF release is highest in the morning, based upon peptide staining levels in the dorsal projections of small LNv cells, although rhythmic release may not be a requirement for normal circadian behavior (Park et al., 2000; Kula et al., 2006). My findings suggest that the fraction of PDF receptors that represent the active signaling component itself may also undergo daily changes. If that is true, then in the morning, cells may normally be "primed" to respond maximally to the daily PDF release due to increased trafficking of the signaling components leading to increased sensitivity. The change in sensitivity is approximately 10-fold which is substantial and may represent a genuine point of physiological significance, i.e., the time of high sensitivity is the time of high signaling, and vice versa. Higher sensitivity to PDF in the morning is also

consistent with the suggestion that PDF signaling and cryptochrome signaling converge when CRY signaling is maximal – at dawn (Im et al., 2011). However, at present I have no experimental evidence linking the changes in PDF sensitivity to functionally important changes in PDF signaling. Future experiments could attempt to gain such knowledge by (for example) trying to create a state or static "high" or "low" PDF sensitivity (without mutating the *Pdf* of *PDFr* loci) and asking whether there are behavioral outcomes consistent with impaired PDF signaling.

One unanswered question that remains is: what is the effective dose of PDF that clock cells normally receive when PDF is released endogenously? At supramaixmal doses of PDF daily variation in sensitivity is not observed, therefore the endogenous daily dose must fall below that threshold for this phenomenon to have biological relevance. Thus one suggestive prediction from this line of reasoning is that normally PDF levels are released in limiting amounts, such that changes in PDF sensitivity translates into daily changes in PDF action.

Whole-brain treatment with neuraminidase, which broadly disrupts glycosyl modifications, reduces PDF responses in both small LNv and LNd cells. This finding indicates that glycosylation is required for some component of PDF responsiveness in both clock cell subgroups tested, although it does not directly implicate any specific molecule(s). Many transmembrane proteins, a group that includes both GPCRs and ACs in *Drosophila*, can be modified by glycosylation and this can affect a number of functions including folding, localization, as well as protein-protein interactions (Rasmussen, 1992). However, previous results from the mammalian olfactory epithelium that indicate that AC3 requires specific carbohydrate modifications as well as the finding

that the most closely related *Drosophila* glycosyltransferase (CG30036) reduces PDF responses in small LNvs suggest that ACs are likely targets of these modifications (Henion et al., 2011; Wong et al., 2000). This finding supports the possibility that both mammalian and *Drosophila* ACs utilize glycan modifications for their function.

Both of the clock cell subgroups that I tested recovered from neuraminidase treatment within an hour. This finding was important because it very simply indicates that the enzyme treatment has not caused irreversible damage to the cell. Glycosylation generally occurs co-translationally and glycosyltransferases generally reside in the Golgi apparatus, therefore it is unlikely that PDF signaling proteins are re-modified at the plasma membrane (Rasmussen, 1992). This suggests that the recovery observed is due to the rebuilding of new, functional signalosomes and not just the re-modification of existing signaling components. The diurnal variation in recovery suggests that there is diurnal variation in the rate at which active PDF receptor complexes appear in the plasma membrane. It is not yet clear how these oscillations are achieved and whether or not these are post-translational events. Additionally, it remains to be seen whether this variation in sensitivity is a PDF-specific effect or whether clock cells oscillate in their sensitivity to other neurotransmitters with the same or different phase.

In spite of these unanswered questions, my findings suggest that protein modifications are important for normal signaling underlying the critical synchronization function provided by the neuropeptide PDF. Additionally, PDF sensitivity shows substantial diurnal variation: this suggests that PDF receptor signaling, not just changes in release of PDF, may provide a critical timing role in the neural circuits that underlie normal circadian physiology.

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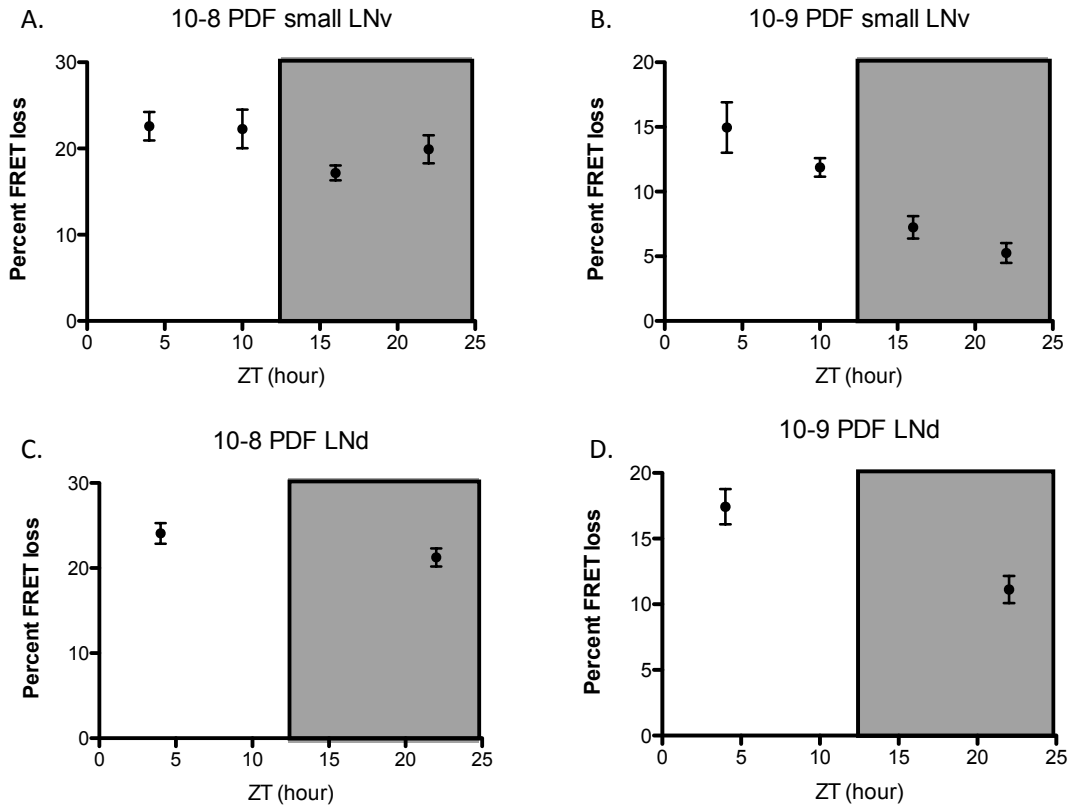
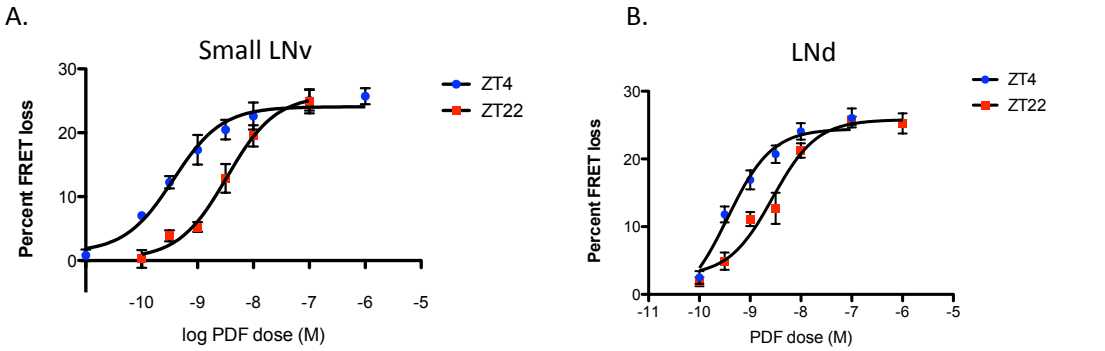



**Figure 1: Daily variation in sensitivity of small LNv and LNd cAMP response to low doses of PDF peptide.**

Whole brains were collected at different timepoints and then tested for maximal PDF responses.

A. Small LNvs show no daily rhythms in maximal response to $10^{-8}$ M PDF.
B. Small LNvs show daily rhythms in maximal responses to$10^{-9}$ M PDF with greatest response at ZT4 and least response at ZT22.
C. LNds show no difference in maximal response to $10^{-8}$ M PDF between ZT22 and ZT4.
D. LNds show greater response to to$10^{-9}$ M PDF at ZT4 compared to ZT22.

All data points include data collected from $\geq$10 cells from $\geq$5 brains at 2 independently collected replicates. Small LNv genotypes (A and B) include one copy of *pdf*-gal4;UAS-Epac1camps. Genotypes used for LNds (C and D) include one copy of *tim(UAS)*-gal4;UAS-Epac1camps. Error bars denote SEM




| | ZT4 | ZT22 |
|---|---|---|
| log(agonist) vs. response (three parameters) | | |
| Best-fit values | | |
| Bottom | 1.279 | 0.2484 |
| Top | 24.07 | 25.86 |
| LogEC50 | -9.433 | -8.474 |
| EC50 | 3.687e-010 | 3.361e-009 |
| Span | 22.79 | 25.61 |
| Std. Error | | |
| Bottom | 1.707 | 1.372 |
| Top | 0.9926 | 1.747 |
| LogEC50 | 0.1273 | 0.1310 |
| Span | 1.791 | 1.942 |
| 95% Confidence Intervals | | |
| Bottom | -2.105 to 4.663 | -2.475 to 2.972 |
| Top | 22.10 to 26.04 | 22.39 to 29.33 |
| LogEC50 | -9.686 to -9.181 | -8.733 to -8.214 |
| EC50 | 2.062e-010 to 6.593e-010 | 1.847e-009 to 6.115e-009 |
| Span | 19.24 to 26.34 | 21.76 to 29.47 |
| Goodness of Fit | | |
| Degrees of Freedom | 113 | 106 |
| R square | 0.6251 | 0.6269 |
| Absolute Sum of Squares | 4077 | 4770 |
| Sy.x | 6.007 | 6.708 |
| Number of points | | |
| Analyzed | 116 | 109 |

| | ZT22 | ZT4 |
|---|---|---|
| log(agonist) vs. response (three parameters) | | |
| Best-fit values | | |
| Bottom | 2.711 | -1.747 |
| Top | 25.80 | 24.45 |
| LogEC50 | -8.558 | -9.441 |
| EC50 | 2.767e-009 | 3.626e-010 |
| Span | 23.09 | 26.20 |
| Std. Error | | |
| Bottom | 1.468 | 4.561 |
| Top | 1.492 | 1.064 |
| LogEC50 | 0.1355 | 0.1932 |
| Span | 1.782 | 4.234 |
| 95% Confidence Intervals | | |
| Bottom | -0.2071 to 5.629 | -10.83 to 7.335 |
| Top | 22.84 to 28.77 | 22.33 to 26.57 |
| LogEC50 | -8.827 to -8.289 | -9.825 to -9.056 |
| EC50 | 1.488e-009 to 5.143e-009 | 1.495e-010 to 8.793e-010 |
| Span | 19.55 to 26.63 | 17.77 to 34.63 |
| Goodness of Fit | | |
| Degrees of Freedom | 96 | 86 |
| R square | 0.6363 | 0.5675 |
| Absolute Sum of Squares | 3221 | 2486 |
| Sy.x | 5.793 | 5.376 |
| Number of points | | |
| Analyzed | 99 | 89 |

**Figure 2: Dose response of PDF sensitivity of two pacemaker subgroups collected at two circadian timepoints (ZT4 and ZT22).**

Doses of PDF ranging from $10^{-11}$ M to $10^{-6}$ M were tested at two different circadian (ZT4 and ZT22) timepoints in small LNvs (A) and LNds (B).

Animals were entrained under 12:12 LD conditions for at least three days prior to imaging.

A. Small LNvs show higher sensitivity to PDF at ZT4 (EC50: $3.687 \times 10^{-10}$ M) compared to ZT22 (EC50: $3.361 \times 10^{-9}$ M)

B. LNds show higher sensitivity to PDF at ZT4 (EC50: $3.626 \times 10^{-10}$ M) compared to ZT22 (EC50: $2.767 \times 10^{-9}$ M).

Genotypes used for small LNvs include *Pdf*-gal4; UAS-Epac1camps. Genotypes used for LNds include one copy of *tim(UAS)*-gal4;UAS-Epac1camps. Error bars denote SEM.

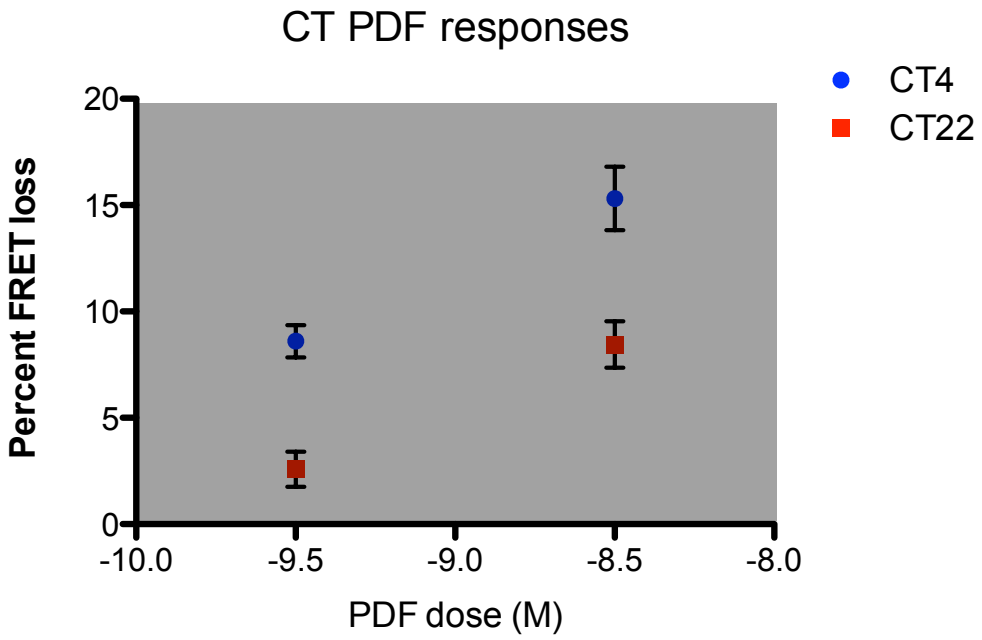



**Figure 3: Small LNv PDF sensitivity shows circadian variation under constant conditions.**
Two doses of PDF were tested at two different circadian timepoints in small LNvs. Animals were entrained under 12:12 LD conditions for at least three days and released into DD prior to imaging.
All genotypes include *Pdf*-gal4;UAS-Epac1camps. Error bars denote SEM.

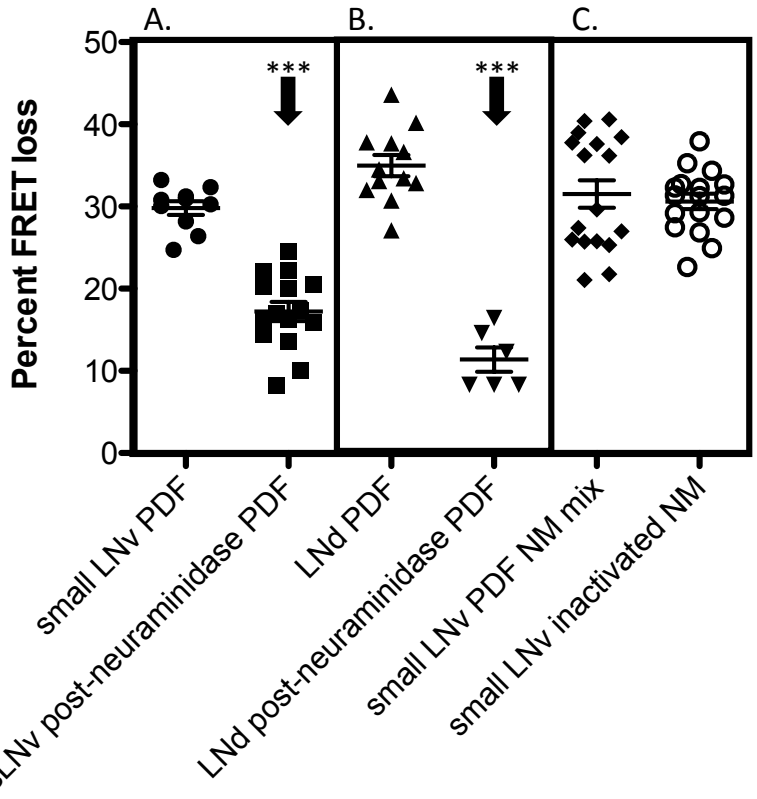



**Figure 4: Neuraminidase Treatment reduces PDF responses in both small LNvs and LNds.**

A. Whole brains were treated with .1unit/mL neuraminidase for 15 minutes before bath applied PDF responses were measured from small LNv cells.
B. Whole brains were treated with .1unit/mL neuraminidase for 15 minutes before bath applied PDF responses were measured from LNd cells.
C. To test the direct effect of neuraminidase on PDF peptide, a mixture of PDF and neuraminidase was bath applied. To test the requirement for neuraminidase enzymatic activity, whole brains were incubated with heat-inactivated neuraminidase.

All genotypes include *pdf*-gal4;Epac1camps (for small LNv cells) or *Mai179*-gal4;Epac1camps (for LNd cell measurements). Error bars denote SEM. ***, P<0.001 (compared with control).

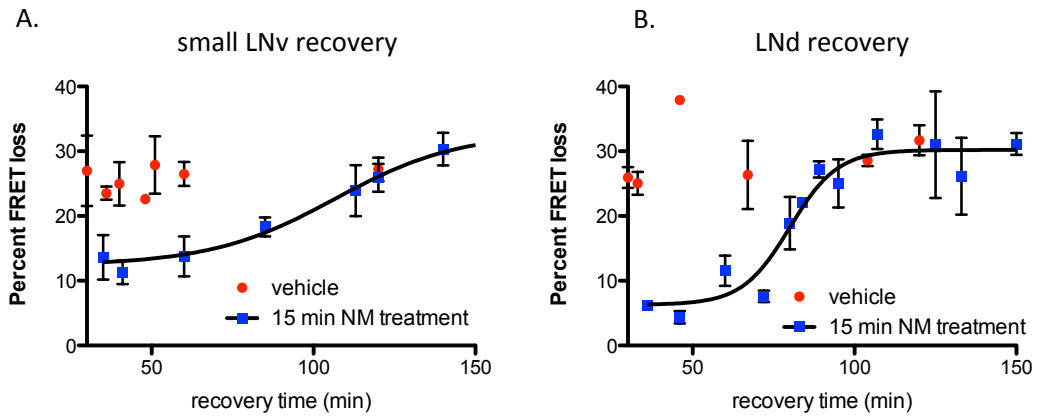



**Figure 5: Small LNvs and LNds recover PDF responsiveness after neuraminidase treatment.**

A. Small LNvs recover from neuraminidase treatment (blue squares) compared to vehicle control (red circles) with V50: 106.7 minutes. Genotype of all animals tested include *Pdf*-gal4;Epac1camps.
B. LNds recover from neuraminidase treatment (blue squares) compared to vehicle treated controls (red circles) with V50: 80.04 minutes. Genotype of all animals tested include *tim(UAS)*-gal4;Epac1camps.

Error bars represent SEM. V50 for recovery curves were calculated using Boltzmann sigmoidal fit.

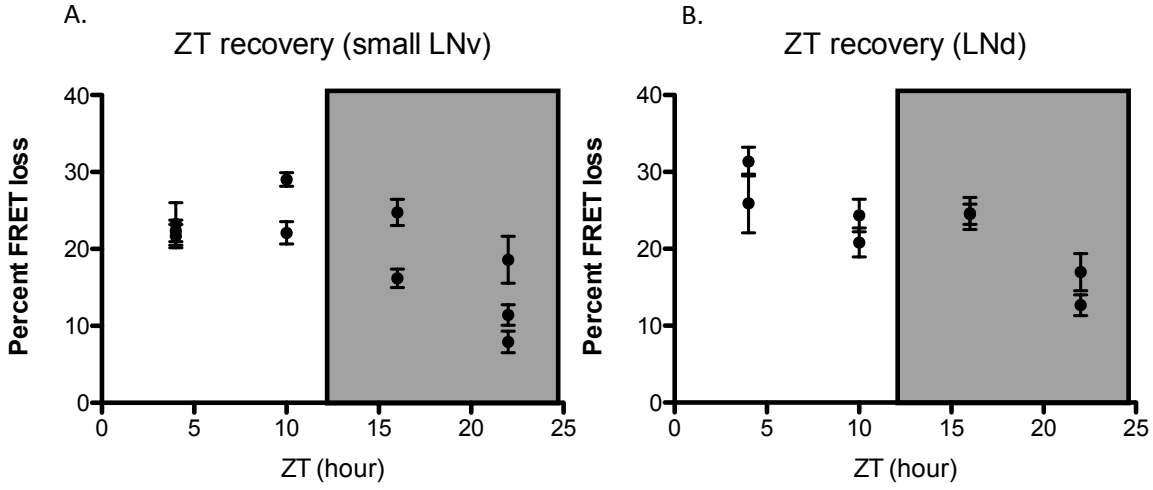



**Figure 6: Daily variation in recovery of PDF cAMP responsiveness after neuraminidase treatment.**
Whole brains were incubated with .01unit/mL neuraminidase for 15 minutes and allowed to recover in Schneider's *Drosophila* culture media for 60 minutes and PDF responses were recorded in small LNvs (A) and LNds (B).
Error bars represent SEM. Small LNvs were imaged using *Pdf*-gal4;Epac1camps and LNd cells were imaged using *tim(UAS)*-gal4;Epac1camps.

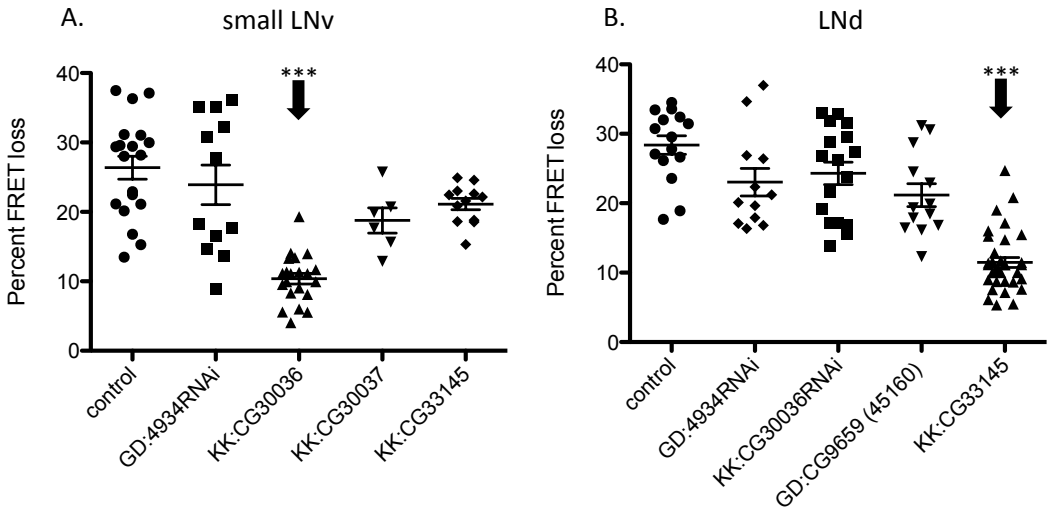
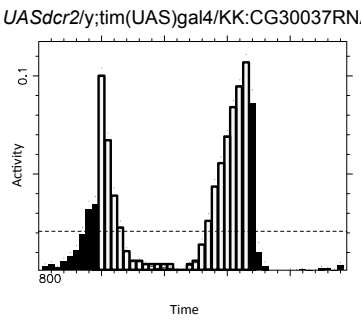



**Figure 7: Knockdown of specific glycosyltransferases reduces PDF responses in small LNv and LNds.**

A. Knockdown of the glycosyltranferase encoded by CG30036 reduces PDF responses in small LNvs.
B. Knockdown of the glycosyltransferase encoded by CG33145 reduces PDF responses in LNds.

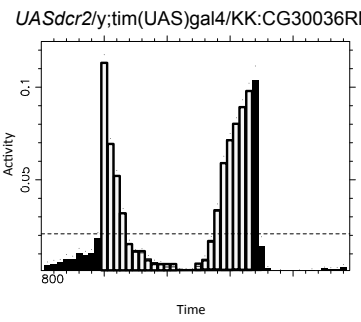
Small LNvs were imaged using *Pdf*-gal4;Epac1camps and LNds were imaged using *tim(UAS)*-gal4;Epac1camps. Error bars denote SEM. ***, P<0.001 (compared with control).

A.



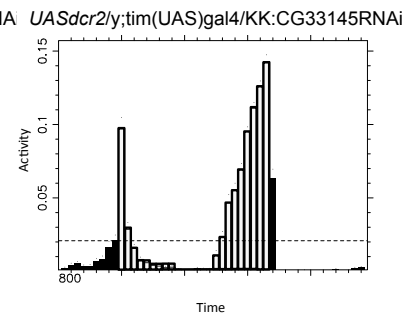

Average morning anticipation index: .898

B.




Average morning anticipation index: .746

C.




Average morning anticipation index: .803

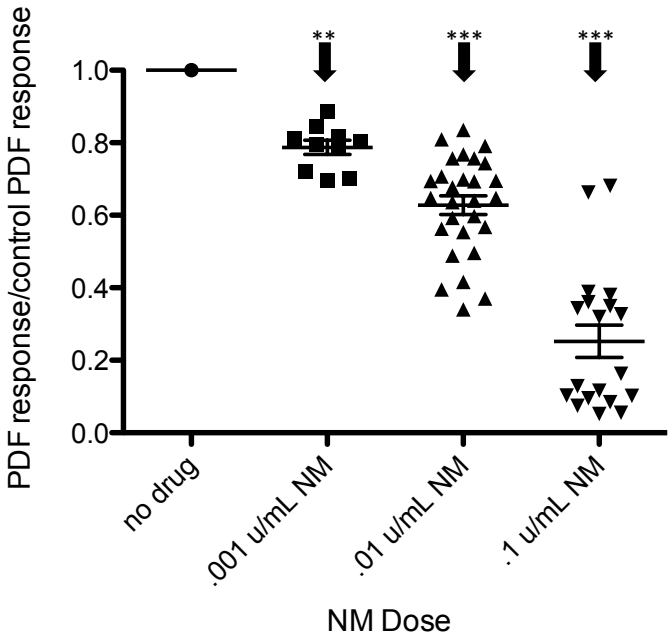
D.

| Genotype | n | % Arrythmic | Period (h) +/- SEM | Power +/- SEM |
|---|---|---|---|---|
| UAS-dcr2/y;tim(UAS)gal4/+ | 60 | 0 | 24.1 +/- .08 | 78.2 +/- 6.3 |
| *UASdcr2*/y;tim(UAS)gal4/KK:CG30037RNAi | 51 | 14 | 23.9 +/- .2 | 41.8 +/- 8.4 |
| *UASdcr2*/y;tim(UAS)gal4/KK:CG30036RNAi | 48 | 63 | 24.0 +/- .5 | 26.9 +/- 5.2 |
| *UASdcr2*/y;tim(UAS)gal4/KK:CG33145RNAi | 56 | 96 | 22.5 | 12 |

**Figure 8: Circadian locomotor behavior of glycosyltransferase knockdown.**

A. LD behavior of knockdown of the glycosyltranferase encoded by CG30037 has no effect on circadian locomotor behavior.
B. LD behavior of knockdown of the glycosyltransferase encoded by CG30036.
C. LD behavior of animals with knockdown of glycosyltransferase encoded by CG33145.
D. DD locomotor behavior of control animals as well as knockdown of CG30037, CG30036 and CG33145.

All genotypes include UAS-dicr2;*tim*(*UAS*)-gal4. Filled bars in A-C represent lights-off and open bars represent lights-on. Periods were calculated using chi-squared periodigram.




**Supplemental Figure 1: Neuraminidase treatment reduces PDF responses in a dose-dependent manner.**
Whole brains were incubated with various doses of neuraminidase for 15 minutes and then assay for cAMP response to 10-6 PDF. Y-axis represents ratio of PDF responses to vehicle treated (no drug) control responses.

All genotypes include *pdf*-gal4;Epac1camps. Error bars denote SEM. ***, P<0.001 **, P<0.01 (compared with vehicle-treated control).

**CHAPTER 5:**

**DH81 is a potent activator of the CGRP receptor encoded by CG4395**

This chapter includes data that is part of a planned submission:

> Jensen D, Trigg J, **Duvall LB**, Schooley DA, Taghert PH. De-orphaning paralogous neuropeptide receptors in *Drosophila*: the endogenous ligand for the second dh31 receptor (calcitonin receptor-like) is an extended, 81-amino acid form of dh31. [In preparation].

LBD performed in vivo cAMP imaging experiments.

**Principal Findings:**

I provide in vivo support for differential sensitivity of cAMP generation of two Calcitonin Gene-Related Peptide (CGRP) GPCRs to long and short forms of the activating peptide (DH81 and DH31 respectively). Functional expression in cell lines suggests CG17415 is preferentially sensitive to the shorter peptide DH31, and that the orphan receptor CG4395 is preferentially sensitive to increasingly longer forms of DH31. The most potent peptide for CG4395 is the longest potential form of DH31 retaining its PRO sequences, the 81 amino acid peptide DH81. I show that in the *Drosophila* brain the DH31 peptide activates CG17415 and CG4395 at comparable levels. Whereas DH81 is 100 times more potent in activating CG4395. These findings provide strong support for the hypothesis that the endogenous ligand for the CG4395 receptor is the 81 amino acid peptide DH81.

**Introduction:**

In *Drosophila*, there are five neuropeptide receptors that belong to the Family B (secretin-receptor like) group of G protein coupled receptors (Hewes and Taghert, 2001).

These Secretin R-like receptors signal predominantly via Gsα-coupled cAMP generation (Wimalawansa, 1996). Two of these five GPCRs are related to mammalian CRF-Receptor and represent receptors for the *Drosophila* peptide DH44 (Johnson et al., 2004; Johnson et al., 2005). The other three *Drosophila* Family B receptors, including the receptor for PDF, are more related to mammalian receptors for the peptides calcitonin and CGRP. The other two receptors in this group include CG17415 and CG4395. The first is a receptor for the peptide DH31 (Johnson et al., 2005) both in cell assays as well as in the living *Drosophila* brain (Johnson et al., 2005; Shafer et al., 2008) while CG4395 remains an orphan.

Our laboratory and that of David Schooley (University Nevada Reno) have engaged in a collaboration to identify the endogenous CG4395 ligand. To date these efforts have used in vitro assays (functional expression of the CG4395 receptor in a mammalian cell line - D. Jensen, JS Trigg et al., unpublished data). They have assayed purified peptides from *Drosophila* heads as well as the activity of synthetic peptides and together this work has supported the following hypothesis. The peptide DH31 is a potent ligand for CG4395 to nearly the same extent as for CG17415. Furthermore, manipulations of the gene that encodes the DH31 peptide greatly decrease endogenous ligand activity for both CG17415 and CG4395. These data suggest CG7415 and CG4395 may represent two separate receptors for the same DH31 peptide. There are several instances wherein two receptors are dedicated to a single *Drosophila* neuropeptide, including *Drosophila* tachykinin (Monnier et al., 1992; Birse et al., 2006; Poels et al., 2007), dromyosuppressin (Johnson et al., 2003), DH44 (Johnson et al., 2004), to name a few.

However, there is reason to think the endogenous ligand for CG4395 is not the 31 AA peptide DH31. When purified, active fractions from tissue extracts have mobilities different from synthetic DH31, suggesting there is an endogenous peptide ligand distinct from DH31. Also, incrementally longer forms of the DH31 peptide (increasing N-terminal extensions) increase the CG4395 responses in vitro – with a maximal response in vitro from the longest version, an 81 amino acid peptide called DH81 (D. Jensen, J. Trigg, et al., unpublished data).

Neuropeptides are processed into their mature forms from larger protein precursors. These precursors have conserved "pre" and "pro" regions that play dedicated roles in appropriately trafficking the peptide into the regulated secretory pathway. The "pre" signal peptide sequence at the N terminus largely consists of hydrophobic residues and specifies insertion into the membrane (Strauss et al., 1977). This portion is removed in the endoplasmic reticulum by a membrane bound signal peptidase (Turner, 1984). The subsequent pro-protein is then trafficked to the Golgi apparatus and is packaged into secretory granule (Palade, 1975). The pro region of the peptide is often important for normal folding and is usually removed by limited proteolysis in secretory granules (Loh et al., 1984). The longer pro-protein forms of the DH31 peptide are named based on the amino acid length of the peptide. Therefore, DH81 represents the longest pro-DH31 peptide with 81 amino acids (Figure 1). Peptide precursors are modified by specific sets of enzymes and are generally thought to be inactive until they are expressed in their mature forms (Van de Ven et al., 1993).

Although this work is ongoing, these results suggest that a single gene may essentially encode two different signaling pathways in the *Drosophila* brain and that

DH81 is a potent activator the CGRP receptor encoded by CG4395. The work I describe in this chapter tests this hypothesis by asking whether the in vitro results predict in vivo results: specifically, whether, DH81 activates CG4395 in vivo more potently than does DH31.

**Results:**

**DH81 peptide activates CG4395 in hEK cell assays**

Diuretic hormones play key roles in *Drosophila* physiology and signal through G protein coupled receptors that are closely related to the PDF receptor (Johnson et al., 2005). (D. Jensen, JS Trigg, et al., unpublished data). In vitro work measuring cAMP generation following transient receptor transfection in hEK cells suggests that the CG17415 and CG4395 receptors have differential preferences for long versus short forms of the DH31-related peptides, and that the CG4395 receptor shows higher sensitivity to DH81 with an EC50 of $3.72 \times 10^{-9}$ whereas DH31 has an EC50 of $3.29 \times 10^{-7}$. However, the receptor encoded by CG17415 shows roughly equal sensitivity to either form of the peptide: the EC50 for DH81 is $1.13 \times 10^{-8}$ M and EC50 for DH31 is $1.93 \times 10^{-8}$ M (J. Trigg, unpublished data).

**DH31 activates both CG17415 and CG4395 in the *Drosophila* brain**

I tested cAMP responses in the living brain using the genetically encoded cAMP sensor Epac1camps and methods described in previous chapters. I tested responses to both DH31 and DH81 peptide superfusion using cells known to express CG17415: large ventro-lateral neurons (large LNv) and in a separate group of neurons that express CG4395: a set of ellipsoid body cells (EB cells) of the Central Complex. The

identification of the EB neurons as preferentially expressing CG4395 and not CG17415 comes from unpublished anatomical data comparing GAL4 and anti-receptor antibody immunohistochemistry (B Leung, S Waddell, P Taghert, unpublished). Dissection and imaging methods were identical to those described in Chapter 2 with the exception that peptides were dissolved in 1% BSA instead of 1% DMSO to maximize stability of the DH81 peptide.

Whole brains were dissected and cAMP responses were quantified for concentrations of DH31 ranging from $10^{-6}$ M to $10^{-10}$ M in cells that express CG17415 (large LNv) and CG4395 (EB cells). Large LNvs were visualized using *pdf*-gal4 driver. EB cells were visualized using *CG4395*-gal4 driver. DH31 activates both receptors with similar sensitivity: EC50 for large LNv (CG17415): $4.498 \times 10^{-9}$, EC50 for EB cells (CG4395): $5.225 \times 10^{-9}$ (Figure 2).

**DH81 preferentially activated CG4395 compared to CG17415 in the *Drosophila* brain**

I report that DH81 addition activates cAMP responses in CG4395-expressing EB cells (EC50: $6.338 \times 10^{-11}$) with much higher sensitivity compared to the CG17415-expressing large LNv (EC50: $1.073 \times 10^{-8}$) (Figure 3).

**Discussion:**

These findings suggest that a single gene, which encodes the DH31 neuropeptide, may activate two distinct signaling pathways depending upon the peptide processing. Two related receptors show different sensitivity to each of the DH31-related peptides. Our findings suggest that peptide processing may play a previously unappreciated role in

generating signaling complexity, as DH81, which represents a form of pro-DH31, preferentially activates the receptor encoded by CG4395.

The initial findings, reported using hEK cell transfections, were confirmed using the live-brain cAMP FRET imaging. The congruence between these two methods confirms the reliability of our in vivo imaging method. DH81 represents the longest known neuropeptide to date; therefore, testing the functionality in vivo is a key step in demonstrating the biological relevance of this neuropeptide. Studies are ongoing in the Taghert and Schooley labs to confirm expression of the DH81 peptide in *Drosophila* head; this is important to establish to confirm that DH81 represents an endogenous signaling peptide.

Diuretic hormone and PDF are among the group of neuropeptides expressed in the circadian clock network. Although these receptors belong to the same subfamily of class B GPCRs, my previous findings in Chapters 2 and 3 suggest that even closely related receptors may show preferential coupling to different downstream components. This is confirmed by the finding that expressing tethered PDF peptide on cells that express both PDF-R and DH31-R (CG17415) alters circadian rhythms; however expressing tethered DH31 does not cause these effects (Choi et al., 2012). This result suggests that activation of these two closely related receptors results in distinct signaling cascades in the same cell. A recent study reports that the third member of this subfamily, CG4395, plays a key role in courtship behavior in a subset of fruitless positive neurons (Li et al., 2011). Courtship phenotypes have not previously been associated with DH31 alterations and this report adds support to the hypothesis that these DH31 and DH81 form two separate signaling pathways.

In mammals, CGRP receptors associate with Receptor Activity Modifying Proteins (RAMPs) which are important for ligand binding and surface expression of the receptor as well as playing roles in modifying signaling (Barwell et al., 2012). Co-expression of mammalian RAMP1 or the *Drosophila* RCP 1 with CG7415 (DH31-R) receptor permits cAMP generation in vitro although co-transfection with RAMP or RCP only slightly improves CG4395 response profile (Johnson et al., 2005; J. Trigg, unpublished data). These findings suggest that CG17415 and CG4395 require accessory proteins for their function. This finding is consistent with the finding that CG4395 shows higher sensitivity to both DH31 and to DH81 peptides in vivo compared to the in vitro response, as does the PDF receptor (J. Trigg, unpublished data). These differential sensitivities suggest that there may be accessory protein partners in the *Drosophila* brain that are not present in hEK cells that modify receptor signaling. These receptors may form larger signaling complexes as suggested in previous chapters and the association of each receptor with different downstream components may provide a mechanism to explain how such closely related receptors, that use common signaling components may effect such different behavioral outputs.

In humans, CGRP has been associated with migraine and these receptors as well as their accessory RAMPs are under study as promising therapeutic targets (Raddant and Russo, 2011). The elucidation of CGRP receptor function may eventually serve as model for migraine in *Drosophila* and may also aid in the development of novel targets for the treatment of migraine.

My previous findings discussed in chapter 4 suggest that receptor sensitivity may change over the course of the day. It is not clear whether or not other CGRP receptors

undergo similar changes however it is important to note that these dose-response curves were not collected under strict 12:12 LD entrainment (although all were collected during the light phase).

The extent to which the longer form of the DH81 peptide is expressed in the brain and the possibility that other peptide pathways may utilize similar pro-protein mechanisms to generate signaling diversity remain important areas that are open for future research and may allow insight into the complexity of neuropeptide signaling. These findings may also provide additional ligands which may de-orphan receptors that currently have no known activators or reveal additional ligands for those receptors that have already been characterized.

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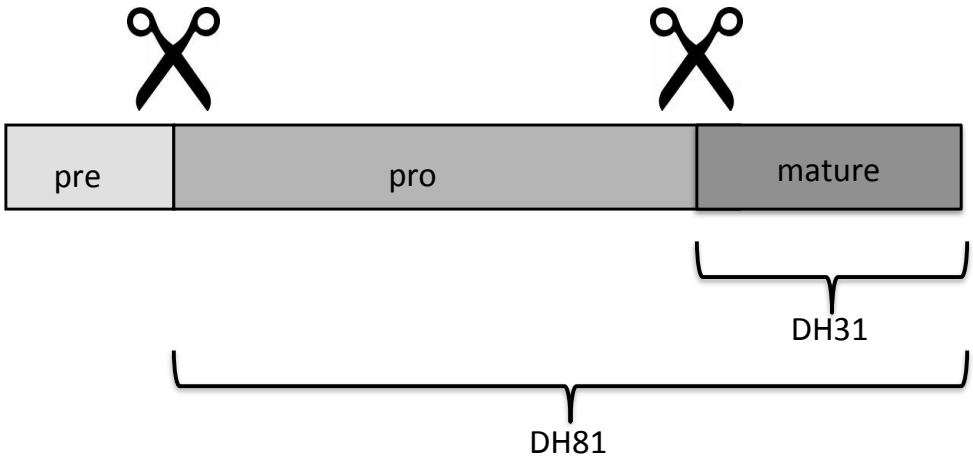
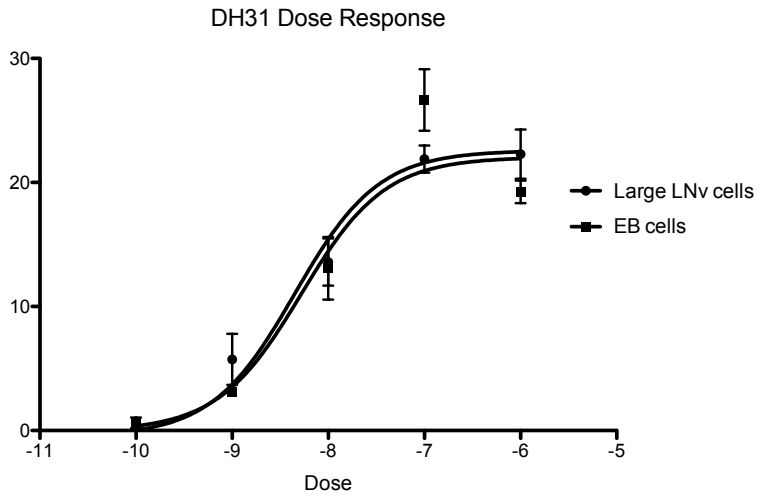



**Figure 1: Peptide processing of Diuretic Hormone 31.**
CG13094 encodes the peptide Diuretic Hormone 31. The immature peptide is processed but recent evidence suggests that the pro-peptide DH81 (81 amino acids in length) functions as an endogenous signaling molecule distinct from the mature peptide DH31.

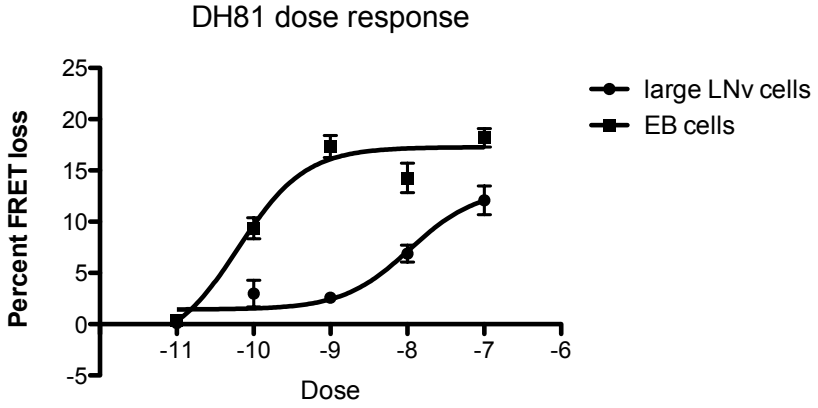



| | Large LNv cells | EB cells |
|---|---|---|
| log(agonist) vs. response (three parameters) | | |
| Best-fit values | | |
| Bottom | -0.4630 | -0.03874 |
| Top | 22.58 | 22.04 |
| LogEC50 | -8.347 | -8.282 |
| EC50 | 4.498e-009 | 5.225e-009 |
| Span | 23.04 | 22.08 |
| Std. Error | | |
| Bottom | 1.643 | 1.113 |
| Top | 1.122 | 0.9679 |
| LogEC50 | 0.1896 | 0.1670 |
| Span | 1.782 | 1.373 |
| 95% Confidence Intervals | | |
| Bottom | -3.743 to 2.817 | -2.252 to 2.175 |
| Top | 20.34 to 24.82 | 20.11 to 23.96 |
| LogEC50 | -8.725 to -7.969 | -8.614 to -7.950 |
| EC50 | 1.882e-009 to 1.075e-008 | 2.432e-009 to 1.123e-008 |
| Span | 19.48 to 26.59 | 19.34 to 24.81 |
| Goodness of Fit | | |
| Degrees of Freedom | 73 | 95 |
| R square | 0.7066 | 0.7369 |
| Absolute Sum of Squares | 2626 | 3222 |
| Sy.x | 5.998 | 5.824 |
| Number of points | | |
| Analyzed | 76 | 98 |

**Figure 2: Dose response curve receptors encoded by CG17415 and CG4395 to DH31 peptide.**

Large LNvs (expressing CG17415) and EB cells (expressing CG4395) were assayed with doses of DH31 that varied from $10^{-6}$ M to $10^{-10}$ M. Each dose was tested on at least 10 different cells collected from at least 5 different brains.

Large LNv data was collecting from male flies expressing *Pdf*-gal4;Epac1camps, EB cell data was collected from males expressing *4395*-gal4;Epac1camps.




| | large LNv cells | EB cells |
|---|---|---|
| log(agonist) vs. response (three parameters) | | |
| Best-fit values | | |
| Bottom | 1.436 | -2.372 |
| Top | 13.17 | 17.27 |
| LogEC50 | -7.969 | -10.20 |
| EC50 | 1.073e-008 | 6.338e-011 |
| Span | 11.74 | 19.65 |
| Std. Error | | |
| Bottom | 0.5800 | 2.183 |
| Top | 1.408 | 0.6748 |
| LogEC50 | 0.2265 | 0.1522 |
| Span | 1.400 | 2.177 |
| 95% Confidence Intervals | | |
| Bottom | 0.2783 to 2.593 | -6.651 to 1.908 |
| Top | 10.36 to 15.98 | 15.95 to 18.60 |
| LogEC50 | -8.421 to -7.517 | -10.50 to -9.900 |
| EC50 | 3.792e-009 to 3.038e-008 | 3.189e-011 to 1.260e-010 |
| Span | 8.944 to 14.53 | 15.38 to 23.91 |
| Goodness of Fit | | |
| Degrees of Freedom | 74 | 195 |
| R square | 0.5590 | 0.4100 |
| Absolute Sum of Squares | 917.7 | 9202 |
| Sy.x | 3.522 | 6.869 |
| Number of points | | |
| Analyzed | 77 | 198 |

**Figure 3: Dose response curve of receptors encoded by CG17415 and CG4305 to DH81 peptide.**

Large LNv cells (expressing CG17415) and EB cells (expressing CG4395). Large LNv cells (expressing CG17415) and EB cells (expressing CG4395) were assayed with doses of DH81 that varied from $10^{-7}$ M to $10^{-11}$ M. Each dose was tested on at least 10 different cells collected from at least 5 different brains.

Large LNv data was collecting from male flies expressing *Pdf*-gal4;Epac1camps, EB cell data was collected from males expressing *4395*-gal4;Epac1camps.

## CHAPTER 6:

## Conclusions and Future Directions

Each individual chapter contains a specific a discussion section, therefore I will focus on the larger questions raised by this work in this final Conclusions section.

In this dissertation study I present evidence for receptor-specific "signalosomes" in circadian cells in the *Drosophila* brain. More generally, this provides a possible mechanism for the differentiation of signaling pathways that utilize common signaling molecules, but which lead to different downstream effects.

Additionally, I present evidence that sensitivity to the PDF peptide shows daily variation in two subgroups of clock cells which suggests that PDF receptor signaling, not just changes in release of PDF, may provide a critical timing role in the *Drosophila* circadian clock.

### What are the components of signalosomes?

In Chapter 2, I presented evidence to argue for the participation of a specific adenylate cyclase isoform, AC3 and scaffolding protein nervy in PDF receptor signaling in small LNv pacemakers. It is likely that many other pathway components, such as phosphodiesterases, are also sequestered in signaling complexes. Therefore, a clear area of future study is to investigate the physical interactions of the proteins that compose the proposed circadian signalosomes. Although I identified certain adenylate cyclases and scaffolding proteins that mediate PDF signaling in specific clock cells, my studies did not address the possible physical interactions between these proteins. Additionally, it is likely that there are a number of other proteins that are involved in shaping PDF

responses in clock cells including kinases, arrestins and phosphodiesterases (Gervasi et al., 2010).

One method to test for physical interactions between proteins is to perform immunoprecipitation followed by western blot or mass spectrometry. Immunoprecipitation relies upon affinity-based co-purification of interacting partners and identification by antibody probes or identifiable mass spectrometry patterns. This effort is currently limited by the availability of antibodies that accurately report PDF receptor, AKAPs and ACs in *Drosophila*. Both the PDF receptor and adenylate cyclases are transmembrane proteins that are easily denatured and may therefore prove difficult as subject for analysis by co-immunoprecipitation procedures. However, co-immunoprecipitation has been successfully used to identify interactions between AKAPs and PKA (Herberg et al., 2000) AC1 and ERK (Gros et al., 2006) as well as AC with GPCRs, G proteins, PKA and phosphatases in mammalian neurons (Davare et al., 2001). A proteomic approach may also be complicated by the possibility that signaling components may couple in specific subgroups of cells or even only under specific environmental conditions. Also, large multi-protein complexes may not report indirect interactions between members in the same larger complex. Signaling complexes that are limited in their expression or that are transient in nature are likely to be overwhelmed by signal from other cells in a whole-brain preparation, although new techniques are being developed to better mimic cellular conditions (Jain et al., 2011). Although questions of protein-protein interactions may be addressed in a more tractable way using transient expression of signaling proteins in vitro, this may also be problematic if cell lines lack all of the components required for the normal physiological signalosome.

One approach is to tag certain components and then to perform an immunoprecipitation using this tag and then probe using antibodies for other signalosome components. Along this line, it may possible to use a MYC-tagged version of PDF receptor which is under the control of its native promoter (Im and Taghert, 2010). One possible drawback of this approach is that the modifications of the proteins for tagging may affect the interactions, however, the PDF receptor-MYC transgene rescue all known genetic deficiencies associated with loss of PDF receptor function (Im and Taghert, 2010). Although the PDF receptor is relatively sparsely expressed in the brain, other components such as ACs are likely to be broadly expressed in many neurons and probably interact with a large number of other proteins (Nagoshi et al., 2010).

Another possible approach to evaluate signaling complex interactions is to use methods available in vivo to report proximity of signalosome proteins using fluorescent reporters that can be monitored in real time (Padilla-Para and Tramier, 2012). These methods allow cell-specific recording from the living brain although my work also suggests that interpretation of studies that require overexpression of proteins must be interpreted with caution because high levels of overexpression of signaling proteins (AC3 and Gs) were capable of disrupting normal signaling. One option may be to express tagged proteins that will produce FRET when they are in close proximity; one possible drawback to this approach is that FRET measurements rely upon very close (<10nm) apposition of proteins and signal may not be observed if signaling proteins form larger complexes with intermediate partners (Schaufele et al., 2005). In addition, there are complications when using bi-molecular versus uni-molecular FRET approaches that involve interpreting the stoichiometry of the relevant over-expressed molecules. With a

FRET based assay it would be possible to assay the formation of signalosomes in the absence of scaffolding molecules (like AKAPs). Based upon my findings in Chapter 2, that the AKAP nervy reduces PDF responses in small LNvs, I would expect that close localization of AC3 and PDF receptor would be reduced in a background with compromised scaffolding proteins.

**What are the specific roles of glycosylation in signaling?**

Another avenue of investigation that is suggested by this work is further study of the role of glycosylation in modifying signaling proteins involved in PDF responses. These carbohydrate modifications may play important roles not only in protein function and accessibility but also in determining protein localization and may therefore also lead to changes in the formation and composition of signalosomes (Marshall, 1972).

Many transmembrane proteins are glycosylated co-translationally in the endoplasmic reticulum and it is likely that many of the components of PDF receptor signaling pathways are modified by glycosylation, but it remains unclear which specific proteins depend upon these changes (Marshall, 1972; Lis et al., 1993; Altmann et al., 2001; Wong et al., 2011). This possibility could be investigated by using antibodies directed against PDF receptor or AC3 and treating them with glycosidase, like PNGase-F that removes these modifications (e.g., Henion et al., 2011). Those proteins that are normally heavily glycosylated will be detected as a broad band in a western blot assay under baseline conditions and that after treatment with glycosidase these proteins will be detected as a more restricted band with a lower molecular weight. However, these experiments rely upon the development of reliable antibodies for the detection of PDF

receptor along with AKAP and ACs in *Drosophila* or the development of tagged versions of these proteins that maintain their biological function.

Another possible approach to answer this question may be to make targeted mutations of the consensus sequences regions for specific types of glycosylation in genes that encode components of the PDF receptor signaling pathway. The sequence for N-linked glycosylation is most commonly Asn-Xaa-Ser/Thr (Marshall, 1972). Mutations that prevent modifications that are required for normal PDF responses would be expected to show reduced FRET responses to PDF addition.

**How are signalosomes regulated?**

My findings in Chapter 4 suggest that PDF sensitivity varies by time of day under a 12:12 light/dark environment with maximum sensitivity in the morning, when PDF is thought to be released (Park et al., 2000). Although different cell groups use different signaling components we still don't know if these components might mediate some of the circadian rhythmicity to PDF responses. It is possible that these modifications might alter PDF responses and that these modifications underlie the observed daily changes in PDF sensitivity.

It is unknown whether or not this rhythmicity is under the control of the circadian clock or if it is downstream of changes in light levels, or perhaps following rhythmic activation by the ligand PDF. To inquire about the role of the clock, these experiments could be performed in a clock mutant background, for example $per^{01}$ or $per^{S}$ and/ or they could be performed under constant conditions to see if they persist. These changes may also be due to changes in light/dark conditions – it would therefore be interesting to test

daily changes in PDF sensitivity in *cryptochrome* mutants. Cryptochrome is a deep brain photoreceptor and has been shown to interact genetically with PDF signaling pathways, although the exact mechanisms are unknown (Cusumano et al., 2009; Im et al., 2011; Zhang et al., 2009). PDF ligand activation in the morning could lead to a profound reduction in PDF activation later in the day, possibly due to receptor desensitization of endocytosis. During some forms of LTD induction AMPA receptors in Purkinjie cells show desensitization that last for more than 10 hours, suggesting that changes in receptor sensitivity may occur over the course of hours (Ito and Karachot, 1990).

**What is downstream of cAMP in clock cell PDF responses?**

The Epac1-camps FRET sensor detects changes in cyclic nucleotide levels in the living brain (Shafer et al., 2008). PKA is the primary target of cAMP and is likely to be downstream of PDF receptor in behaviorally relevant clock cells, based upon the finding that reduction of PKA signaling in clock cells closely phenocopies the PDF peptide/receptor null mutation and its elevation produces an opposite phenotype (W. Li, unpublished data). I have confirmed that that changes to PKA do not feed back to directly affect the measured PDF cAMP response in clock cells (L. Duvall, unpublished data) although I cannot detect alterations of PKA signaling using the Epac1camps FRET sensor.

PKA sensors, including FRET-based sensors, have been used previously and recent advances have made them much more tractable for use in the living brain (Depry and Zhang, 2011). It likely that PDF addition would also lead to a PKA FRET response in clock cells and that PKA responses play a role in normal locomotor behavior.

Different clock cell subgroups may show differences in PKA responses. In addition to PKA, there are numerous other pathways that are downstream of cAMP including Epac as was as Rac/Ras signaling (Ridley, 1994, de Rooj et al., 1998; Kawasaki et al., 1998). If PKA signaling underlies PDF's effects on circadian rhythms then I expect that the genetic alterations that reduced cAMP responses will also reduce PKA FRET responses in a similar manner. Recent advances allow for concurrent imaging of cAMP and PKA in living cells (Aye-Han et al., 2012).

**How do signalosomes affect rhythms at the circuit level?**

How can these studies contribute to an understanding of the broad role PDF plays in the circadian network? There are a number of previous studies that indicate that neuropeptides function to re-configure hardwired neuronal networks to add plasticity to the anatomical connections (recently reviewed by Taghert and Nitabach, 2012). Specifically, neuropeptides utilize feed-forward mechanisms in which a specific node X acts directly on another node Z and also indirectly through an intermediate node Y. One example of neuropeptide modulation through feed-forward mechanisms is found in the circuits that control egestive and ingestive behavior in Aplysia (Wu et al., 2010). It is likely that PDF acts in a similar manner, that PDF from large LNv cells, which are directly sensitive to light, acts directly on E cells to control circadian behavior as well as through the intermediate small LNv cells (Figure 1). Feed-forward loops are commonly thought to help to reconfigure networks between plastic states (Marder and Bucher, 2007; Bargmann, 2012; Brezina, 2010). This may allow a neuromodulator like PDF to act

broadly in multiple sites of plasticity (possibly mediated by different signaling complexes) to promote network states beyond the anatomical connections.

A number of previous studies have indicated that PDF signaling in M cells (small LNv) differ from PDF signaling in E cells (including LNds). In E cells PDF signaling combines with cryptochrome (CRY) signaling to sustain molecular oscillations but in M cells this interaction does not take place (Im et al., 2011). It is possible that different signaling components, including ACs may help to determine which downstream pathways are activated.

One open question that remains is the sufficiency of specific signalosome components to convey signaling properties that control specific component of circadian behavior. One possible experiment to test this would be to transplant small LNv signaling components (AC3/nervy) into other pacemaker cells that are thought to control other aspects of circadian behavior (Grima et al., 2004; Stoleru et al., 2004). Recent work suggests that PDF receptor activation in LNvs shifts the balance of circadian activity from evening to morning (Choi et al., 2012). This would predict that replacing endogenous signaling components with LNv PDF-R signaling complexes in other clock cells would result in a shift of circadian behavior from evening to morning.

Another possible avenue of exploration if the trafficking of the PDF receptor and how its subcellular localization relates to its function as well as the identification of possible chaperone proteins. Studies of neuronal AMPA receptors suggest that patterns of receptor movements are not generated by a single interacting molecule such as a scaffolding molecule, but are instead the results of an ensemble of coordinated molecules (Hoze et al. 2012). Changes in receptor localization are activity dependent and, after

induction of LTD the amount of receptor that is recycled versus that which is targeted for degradation determines the extent of synaptic depression (Fernandez-Monreal et al., 2012). These findings highlight the importance of subcellular localization and membrane sorting to determine the outcome of synaptic plasticity.

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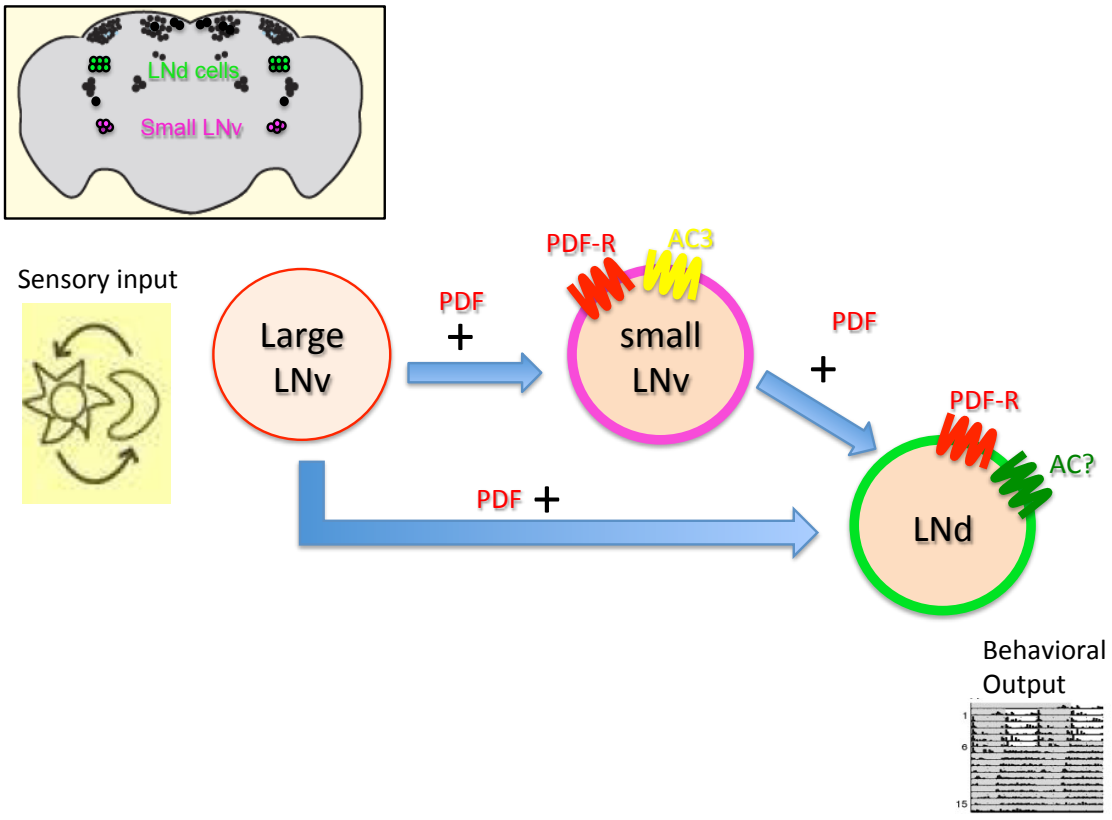


**Figure 1: Model for feedforward functions of PDF in the circadian system of *Drosophila*.**

## MATERIALS AND METHODS

### Fly Rearing and Stocks:

*Drosophila* were reared on cornmeal/agar supplemented with yeast and reared 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. For temperature shift UAS*AC3*/TRiP*AC3*RNAi rescue experiments males were reared at 25°C and moved to 18°C for 12 – 16 hours before imaging to reduce gal4 driven expression of AC3. All gal4 lines used in this study have been described previously: *Pdf*(m)gal4 [64], UAS- Epac1camps50A (Shafer et al., 2008), Mai179gal4 (Cusumano et al., 2009), and *4395*-gal4 (Johnson et al., 2005). The TRiPRNAi (UAS-TRiP*AC3*RNAi, UAS-TRiP-*nervy*RNAi, UAS-TRiP*AKAP200*RNAi), UAS *Gsα60A*, UAS-*rutabaga,*UAS-*dunce,* UAS-*dicer2* tubulin gal80*ts,* Df*(3L)BSC199* and Df(2)*LDS6* lines were obtained through the Bloomington Stock Center (thanks to the Harvard TRiP RNAi project) and the UAS-*Gsα60A*RNAi, 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:*CG30036*RNAi, UAS-KK:*CG3036*RNAi, UAS-KK:*CG33145*RNAi and UAS-CG:*CG9659*RNAi 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 10 mM Tris (pH 7.2). All lines tested included one copy each of gal4, (pdf-gal4 used for small LNv cells and Mai179gal4 for PDF-R(+)LNd cells) and Epac1camps. All genotypes include one copy of each transgene unless otherwise indicated. Full genotypes are available in Supplemental Table 1. For the RNAi AC screen and for pharmacological experiments, whole brains were placed at the bottom of a 35 x 10 mm plastic FALCON Petri dish (Becton Dickenson Labware) as in Shafer et al., (2008), incubated in HL3 saline, and substances tested by bath application. For all remaining experiments, dissected brains were placed on poly-l-lysine coated coverslips in an imaging chamber (Warner Instruments) and HL3 was perfused over the preparation (.5 mL/minute). Microscopy was performed through a LUMPL 60x/1.10 water objective with immersion cone and correction collar (Olympus) on a Zeiss Axioplan microscope. Excitation and emission filter wheels were driven by a Lambda 10-3 optical filter changer and shutter control system (Sutter Instrument Company) and controlled with SLIDEBOOK 4.1 software (Intelligent Imaging Innovations). Images were captured on a Hamamatsu Orca ER cooled CCD camera (Hamamatsu Photonics). Exposure times were 20 ms for YFP-FRET and 500ms for CFP donor. 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 bath added/injected into the perfusion line to result in a final concentration of $10^{-06}$ M. DH31 and DH81 tested in Chapter 5 were added at varying concentrations in 1% BSA. FRET readings were then continued to result

in a total imaging timecourse of 10 minutes. ODQ and dopamine were purchased from Sigma. Synthetic DH31 and DH81 were provided by David Schooley and PDF was produced by (Neo MPS, San Diego CA).

**Neuraminidase treatment:**

Whole brains were incubated in a 35 x 10 mm plastic FALCON Petri dish (Becton Dickenson Labware) with .01 unit/mL Neuraminiase (Sigma-Aldrich Co.) in Phosphate buffer with .3% BSA in HL3 saline for 15 minutes. After 15 minutes the Neuraminidase/HL3 mixture was washed out and replaced with Schneider Media with 10 % FBS and insulin (Schneider's Insect Media (Sigma-Aldrich Co.), 10% FBS (Serum Source International), 1:100 L-Glutamine 200mM (Gibco - Life Technologies Co.) 1:100 Penicillin/Streptimycin 10000U (Gibco - Life Technologies Co.), human Insulin 10ug/ml (Sigma-Aldrich Co.) and brains were allowed to recover. After recovery brains were moved into HL3 saline and imaged as described previously.

**Circadian Timepoint Imaging:**

To collect FRET responses at specific timepoints, flies were entrained to a 12:12 light/dark schedule for at least three days prior to imaging. Whole brains were dissected at ZT (X) placed in individual 35 mm dishes with HL3 saline. Peptide was bath applied and imaging was stopped after cells responded and FRET levels were stable for at least 9 timepoints. All data collection occurred +/- 20 minutes of ZT timepoint for dissection.

**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 minute imaging timecourse. 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).

**Over-expression Constructs:**

Over-expression constructs were built by PCR construction from cDNA derived from adult heads (*Canton S*) and subcloned into *P{cDNA3}* and *P*{UAS-*attb*} vectors. The original *AC3* clone was a kind gift from Lonny Levin (Weill Cornell Medical College).

The sequences of all primers used in this study are:

AC3(BamHI)5':GGATCCATGGAAGCAAATTTGGAGAACGGTC;

AC3(EcoRV)3':GATATCCTATTCTAGCAAAGACTGACATTCT; AC78C 3':

CTATAACGCATCGTTGTGGCTCTTCGATAT; AC78C nested 3':

ACTTAGACCCAGTGAGTGCGCGTACTCGG ; AC78C 5':

ATGGACGTGGAACTCGAAGAGGAGGAGGAG ; AC78C nested 5':

GCATAGCAATAGACAGAATCCTCCGCCACA;

AC76E 3': CTACAATTTCCCATCGAAAGGTGTCTTTAC; AC76E nested 3':

ATCAACAGCAACTGGGTGACGATCGGTGAT;

AC76E 5': ATGGTAAATCACAATGCGGAAACTGCGAAA; AC76E nested 5':

GCCACTAGCTACACGCCACCGCTTTTCGCC; ACXD5':

ATGGACTCCTACTTCGACTCGGCC; ACXD3':

CTAGTCTTCTTTGGTTGGCGCGGCC.

**In vitro Signaling Assays:**

HEK cells were tested using a *cre*- M forskolin 24 hours post-transfection with different UAS-*AC* constructs that had been subcloned into p{CDNA3}. All constructs were co-transfected with *cre-luc* and compared to empty- vector transfected cells. [0.5 µg creluc and 2.5µg PDF-R and 2.5µg AC]. 4 hours after forskolin addition, cells were lysed and luciferin added followed by bioluminescence measurement using a Victor-Wallac plate reader. Measurements were normalized to vehicle treated control, performed in triplicate and each genotype was tested in triplicate.

**Locomotor Activity:**

Male flies were loaded into Trikinetics Activity Monitors 4-6 days after eclosion (Trikinetics Inc.). Locomotor activity was monitored for 6 days under 12:12 light/dark and then for 9 days under constant darkness (DD) conditions. Anticipation index was calculated as in Im and Taghert, (2010) as [activity for 3 hours before lights-on]/[activity

for 6 hours before lights-on]. To analyze rhythmicity under constant conditions we normalized activity from DD days 3 -9 and used X2 periodigram with a 95% confidence cutoff as well as SNR analysis Levine et al., (2002). Arrhythmic flies were defined by having a power value <10.

**Immunohistochemistry:**

Whole brains were dissected and fixed (4% paraformaldehyde in NaHPO4) for 45 minutes. Brains were then washed with PBS to remove fix and blocked in .3% Normal Goat Serum in PBS for at least one hour. Brains were then incubated with primary antibody (GP anti-AC3P 1:250; mouse anti-PDF 1:500) overnight at 4°C. After washing brains were then incubated with secondary antibody for 2 hours at room temperature. After a second wash step in PBS with .1% Triton-X brains were then cleared with increasing concentrations of glycerol and then mounted under VectaShield (VectorLabs Inc.). All images were taken using confocal microscopy (Bakewell Neuroimaging Laboratory, Department of Anatomy and Neurobiology).