SUPPLEMENTAL INFORMATION

This section includes additional text description accompanying:

7 Supplemental Figures

Two Supplemental Tables

Experimental Procedures

References

Diurnal changes in PDF signaling reflect day-length. We asked if differences in the photoentrainment schedule might affect the normal diurnal cycling we found in PDF sensitivity (S Figure 1A). Under 12:12 conditions and under long day conditions (16:8), values at ZT4 are nearly twofold larger in amplitude than values at ZT22. However, under short day conditions, (8:16), values were nearly three times larger at ZT4 than at ZT22. These results derive from at least 10 cells from at least 5 flies. Results generating full concentration-effect curves under SD and 12:12 are reported in Figure 1 and in Table 1. These results are congruent with reports that M neurons dominate the period of the circadian network during longer nights (Stoleru *et al.,* 2007).

PDF signaling complexes in brains in vitro are dynamic – evidence from Neuraminidase treatment. Adenylate cyclase 3 (AC3) functions in mammals are normally modified by glycosylation (Henion *et al.*, 2011). We reasoned that, because AC3 is a normal component of PDF receptor signaling complexes in M cells (Duvall and Taghert, 2012), some component(s) of these complexes could likewise be

normally modified by glycosylation. We therefore treated isolated brains dissected between ZT2-6 for 15 min with bath-applied neuraminidase (NM, 0.01 units/mL), a glycoside hydrolase that cleaves terminal glycosidic linkages of oligosaccharide chains attached to mucins, glycoproteins, and glycolipids. Following a 30 min washout, PDF responses were significantly reduced in both s-LNv and LNd in NA-treated versus vehicle treated brains (S Figure 1B and C): LNd are a distinct pacemaker cells type and representative of the so-called E neurons. These effects were enzyme concentration-dependent and not observed when the enzyme was first heat-inactivated (S Figure 1B). Likewise, pre-mixing NA and PDF, prior to adding the peptide to tissues, had no effect on the FRET response. These observations argue against direct NA degradation of the synthetic neuropeptide. With longer recovery times, PDF sensitivity in NA-treated brains, for both s-LNvs and LNds, regained full measure within two hours (S Figure 1D & E). The recovery process for s-LNv produced a t_{50} value of 106.7 min, with a slope of 19.3 (S Figure 1F); the t_{50} value for LNd was 80.04 min with a slope of 7.4) (S Figure 1H). Furthermore, the apparent degree of recovery for PDF sensitivity declined systematically for both pacemaker cell types over the course of the day (s-LNv: S Figure 1G; LNd: S Figure I). Together, these observations provide further evidence that critical aspects of PDF signaling complexes (their composition, number and/or location) are dynamic within pacemaker cell groups, as a function of time of day. It further suggests that the rates of dynamic change may be greatest in the morning hours. However, they do not specify which components of PDF signaling complexes are subject to this regulation.

D1-like Dopamine receptors in M cells are coupled to an AC different from AC3. We used a series of RNAi constructs to chronically knock down levels of different adenylate cyclases in the PDF neurons, including both s- and l-LNv, and tested maximal responses to 10^{-05} M Dopamine. The plot in S Figure 2 (top) reveals a distribution of values from measuring at least 10 cells, studied in at least three brains. RNAi for *Gsalpha60A* produces a strong diminution of responses. Likewise, RNAi for *AC13E* and *ACX-A* each produce strong decreases in DA responses ($p < 0.001$); RNAi for *AC76E* and for *ACX-C* produced a moderate though significant reduction ($p < 0.01$) and RNAi for *ACX-D* produced a mild reduction ($p < 0.05$). Importantly, RNAi for AC3 produced no reduction in DA responses nor did AC3 over-expression – both of which manipulations cause loss of PDF responses in these same neurons (Duvall and Taghert, 2012). We conclude D1-like dopamine responses in s-LNv are coupled to an AC different from AC3. Potential reasons for multiple responses in the present assay include the use of chronic knockdown as opposed to conditional (i.e., adult-specific) knockdown – Duvall and Taghert, 2012). Also, there are two potential D1-like GPCRs and they each could be coupled to different ACs.

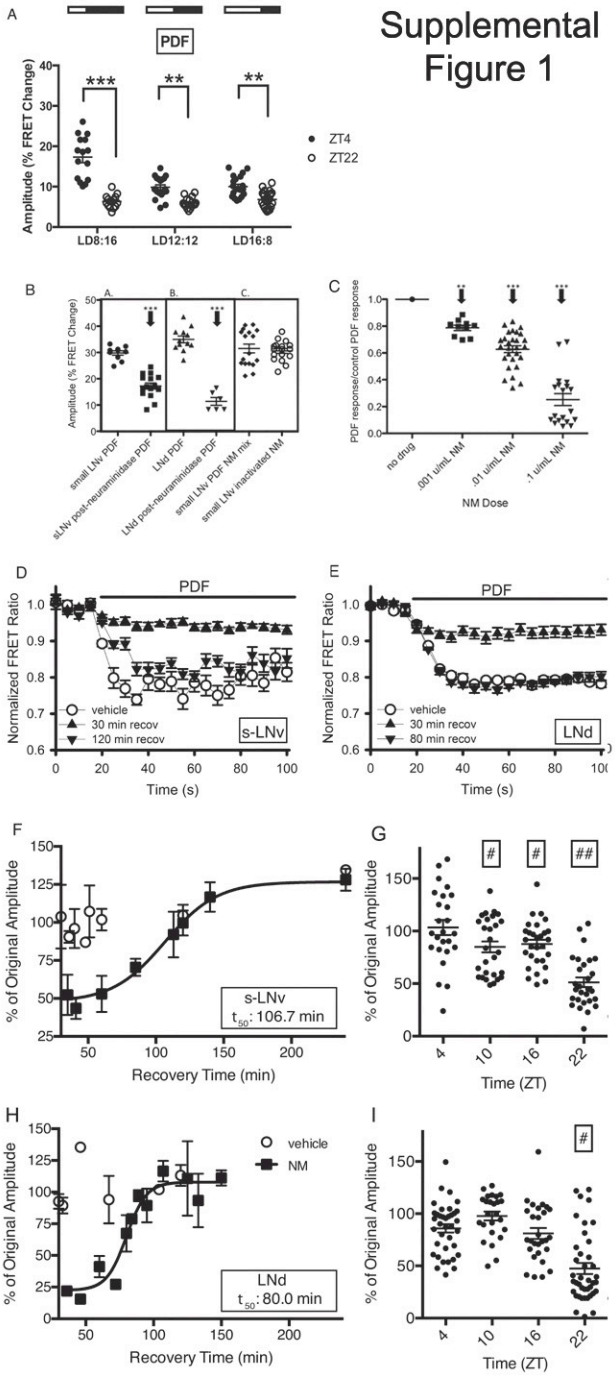
PDFR activation does not recruit β-arrestin-GFP translocation. We previously showed that in *hEK-293* cells, activation of 11 diverse, functionally-expressed fly neuropeptide receptors by their cognate ligands triggers *β-arrestin2-GFP (β-arr2-GFP)* translocation (Johnson *et al.*, 2003; 2004). Here we tested responses by the

PDF receptor (CG13758), and by the two *Drosophila* D1-like receptors DopR1 (CG9652) and DopR2 (CG18741). As a control we tested the response of proctolin receptor (CG6986) to proctolin peptide (cf. Johnson *et al.*, 2003). In each of three independent transfections, dopamine routinely caused GFP translocation with either Dopamine receptor tested, and proctolin did likewise with cells expressing CG6986 in multiple independent transfections (S Figure 2 - bottom). Positive responses were especially evident in circumference measures, but also in many cross-section measures. However, in no experiments from any of three independent transfections did PDF application elicit such wholesale translocation in the case of PDFR-expressing cells, (S Figure 2). Likewise co-transfection with additional *GRK2* (two transfections; n = 10 cells) or with a mutant dynamin K44A (three transfections; n = 12 cells), to increase the sensitivity of the assays (Johnson *et al.*, 2003), did not produce PDFR-triggered GFP translocation. In addition, we tried *β-arrestin1*-GFP for the PDF-PDFR signaling system (two transfections, n= 10 cells), but saw no translocation. Once again, co-transfection with *GRK2* (two transfections; n = 10 cells) or with mutant *dynamin K44A* (three transfections; n = 12 cells) did not change that negative result.

PDFR and Ral-A co-localize in hEK-293 cells. To support the contention that if these two proteins can interact *in vivo*, we examined their sub-cellular distributions in living mammalian *hEK* cells following co-transfection. Both proteins were studied as fluorescent fusions: PDFR-mCherry and hRal-A-GFP (the kind gift of S. Ferguson, Western University, CN). In most cells, there were numerous incidents of co-

localization (S Figure 4): we observed adjacent or overlapping puncta, as well as Ral-A enriched vesicles surrounding receptor. The transient nature of many such co-localizing puncta became evident when the same field was repeatedly imaged over many seconds. Typically signals from both channels were lost when such puncta disappeared. We did not try to determine whether the loss of puncta was due to their dissolution or to their movement out of the focal plane. The trafficking and signaling properties of PDFR-mCherry fusion protein were very similar to wild type receptor. As measured in a single experiment, EC50 value of PDFR-mCherry for activation of co-transfected CRE-Luciferase was similar to wild type receptor (~1 micromolar), and the maximum amplitude value for the fusion receptor was approximately 80% of the wild type value.

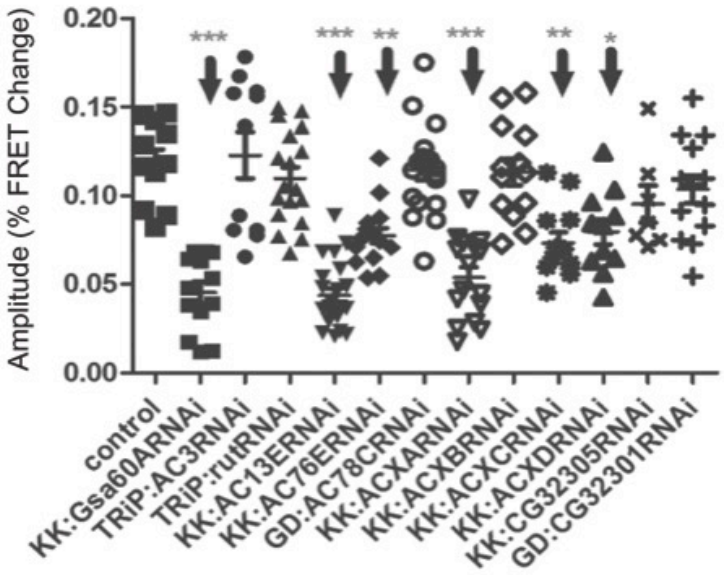
SUPPLEMENTAL FIGURES

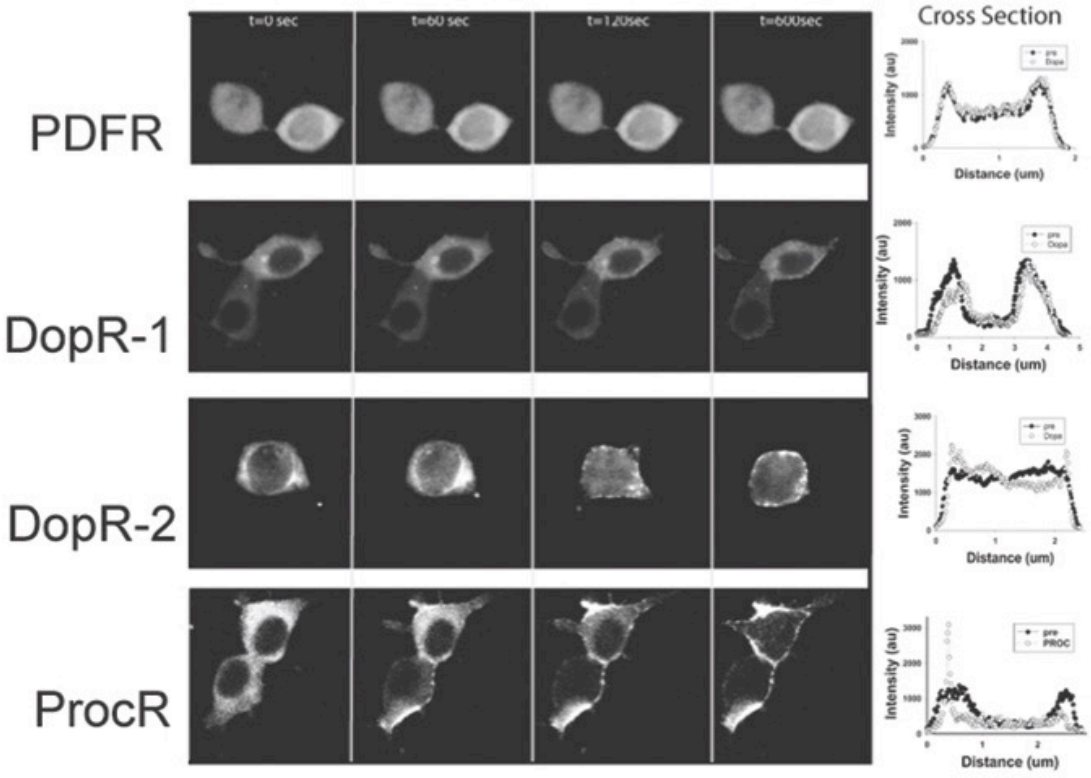


Supplemental Figure 1 – supporting Figure 1.

(A). The diurnal rhythms of PDF sensitivity reflect photoperiodic entrainment conditions. Responses to a single concentration of 10^{-07} PDF shown for s-LNv (M pacemakers) in isolated brains dissected at either ZT4 (filled) or ZT22 (open) in 3-5 day old male adult flies raised in different photoperiods (LD8:16, LD12:12, and LD16:8). By application of Bonferroni's post-hoc test, ZT 4 values are significantly different from ZT22 values as indicated (** : $p<0.001$, *** : $p<0.0001$). **(B). Neuraminidase Treatment reduces the amplitude of PDF responses in both M (s-LNv) and E (LNd) pacemakers.** Left: Whole brains were treated with 0.1u/mL neuraminidase for 15 minutes before bath-applied PDF responses were measured from (A) s-LNv or (B) LNd pacemakers. (C) To test the direct effect of neuraminidase on PDF peptide, a mixture of PDF and neuraminidase (NM mix) was bath-applied. To test the requirement for neuraminidase enzymatic activity, whole brains were incubated with heat-inactivated neuraminidase (inactivated NM). All genotypes include *Pdf*-gal4;*Epac1camps* (for s-LNv measurements) or *Mai179*-gal4;*Epac1camps* (for LNd measurements). Right: Neuraminidase treatment *in vitro* reduces PDF responses in a dose-dependent manner. 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). **(C-I). FRET responses in both s-LNv and LNd recover from neuraminidase treatment *in vitro*, to a degree dependent on time-of-day.** Whole brains were incubated with various doses of neuraminidase for 15 minutes, allowed to recover for various amounts of time, and then assayed by Epac1camps FRET for responses to 10^{-06} PDF. (D). s-LNv responses at ZT2-6 recorded over 100 sec during peptide exposure, following indicated recovery time in Schneider's *Drosophila* culture media. (E). LNd responses at ZT2-6 recorded over 100 sec during peptide exposure, following indicated recovery time in Schneider's *Drosophila* culture media. (F). Timecourse of s-LNv recovery from neuraminidase treatment (squares) at ZT2-6 compared to vehicle control (circles) with t_{50} of 106.7 minutes and a slope of 19.3. (G). Whole brains were incubated with 0.01unit/mL neuraminidase for 15 minutes and allowed to recover in Schneider's *Drosophila* culture media for 60 minutes; responses to 10^{-06} PDF were then recorded in small LNvs. Genotype of all animals tested in (D, F and G) = *Pdf*-gal4;*Epac1camps*. (H.) Timecourse of LNd recovery from neuraminidase treatment (squares) at ZT2-6 compared to vehicle treated controls (circles) with t_{50} of 80.04 minutes and a slope of 7.4. (I). Whole brains were incubated with 0.01unit/mL neuraminidase for 15 minutes and allowed to recover in Schneider's *Drosophila* culture media for 60 minutes; responses to 10^{-06} PDF were then recorded in LNds. Genotype of all animals tested in (E, H and I) = *tim(UAS)*-gal4>*Epac1camps*. Error bars represent standard error. t_{50} for recovery curves were calculated using a Boltzmann sigmoidal fit. # = $p < 0.05$; ## = $p < 0.01$ (by Student's t test).

Supplemental Figure 2

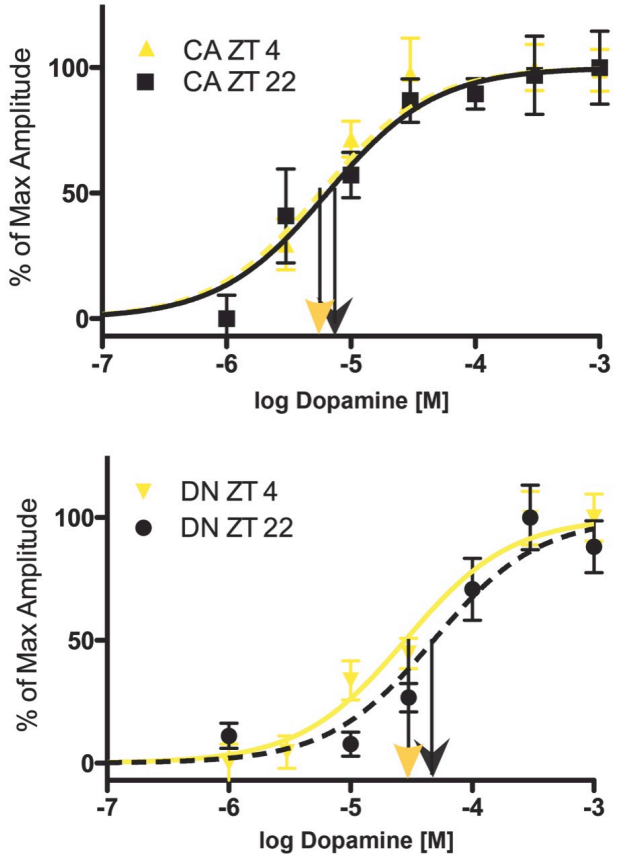




Supplemental Figure 2 – supporting Figure 3.

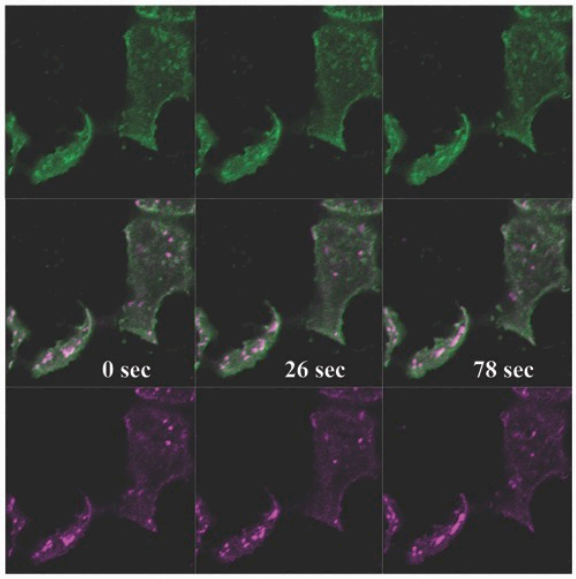
Top - transgenic RNAi screen of adenylate cyclases (ACs) potentially coupled to DA receptor(s) in M cell pacemakers. (A) Scatter plots of FRET responses in M cells chronically expressing double-stranded RNAi directed against *Gsa60A* (encoding the single Gs alpha) or any of 11 (of the 12 total) genes that encode known ACs in the *Drosophila* genome. All genotypes include *Pdf*-gal4;*Epac1camps* and one copy of UAS-*RNAi* (except for control). Error bars denote SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (compared with control). **β-arr2-GFP translocation by *Drosophila* peptide and dopamine GPCRs functionally expressed *in hEK-293* cells.** (Left) Single optical confocal images of cells transfected with *β-arrestin2-GFP* and with one of the four indicated GPCRs - PDFR: *CG13758*; Dop R1: *CG9652*; Dop R2: *CG18741*; ProcR: *CG6986*. At time zero, agonist was added GFP patterns were monitored over the ensuing 600 sec. (Right). Fluorescence Intensity at 600 sec plotted along the longest Cross Section. Cells expressing PDFR displayed no translocation of β-ARR2-GFP to the membrane or into puncta within the cytoplasm in any of the 22 cells imaged from 9 separate transfections. Dop1R1 displayed translocation in 4 of 6 cells (from 3 different transfections), Dop1R2 displayed positive translocation in 6 of 10 cells (from 3 different transfections), and proctolin displayed positive translocation in 9 of 10 cells (from 8 different transfections). We tested the peptides at doses of 10^{-06} and 10^{-05} M; we tested dopamine at 10^{-04} M.

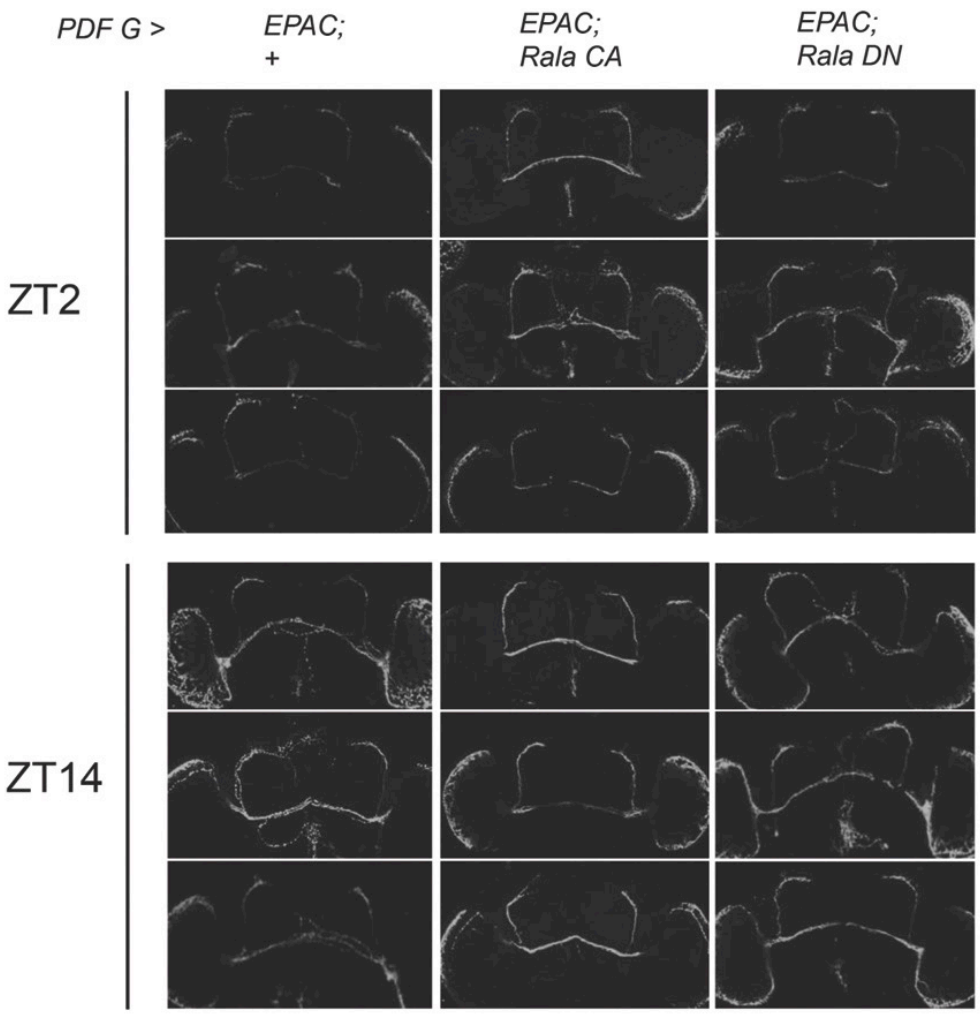
Supplemental Figure 3



Supplemental Figure 3 – supporting Figure 3.
Manipulation of RalA activity in M pacemaker neurons affects their daily cycle of DA sensitivity. **(top).** Concentration-effect curves for DA sensitivity following expression of *rala*^{CA} in PDF neurons at ZT4 (gold) and ZT22 (black). **(bottom)** Concentration-effect curves for DA sensitivity following expression of *rala*^{DN} in PDF neurons at ZT4 (gold) and ZT22 (black). All genotypes contain *Pdf*-GAL4 and UAS-*Epac-camps*. Downward arrows indicate values of EC50's. The calculated EC50 values are compiled in Table 1.

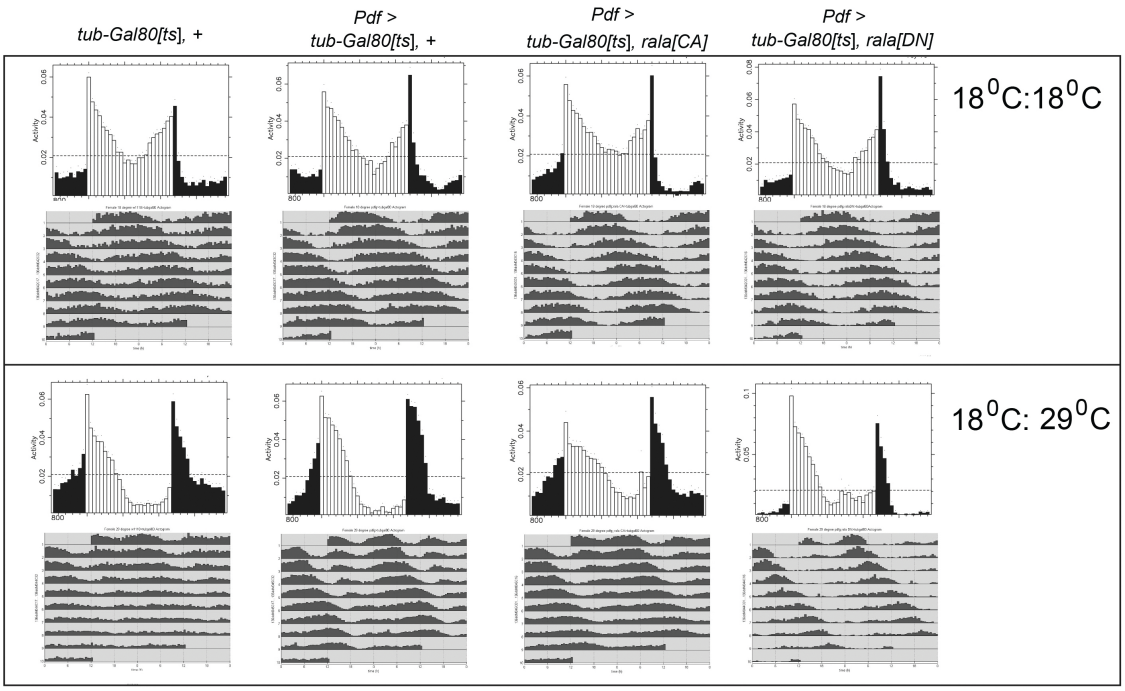
Supplemental Figure 4





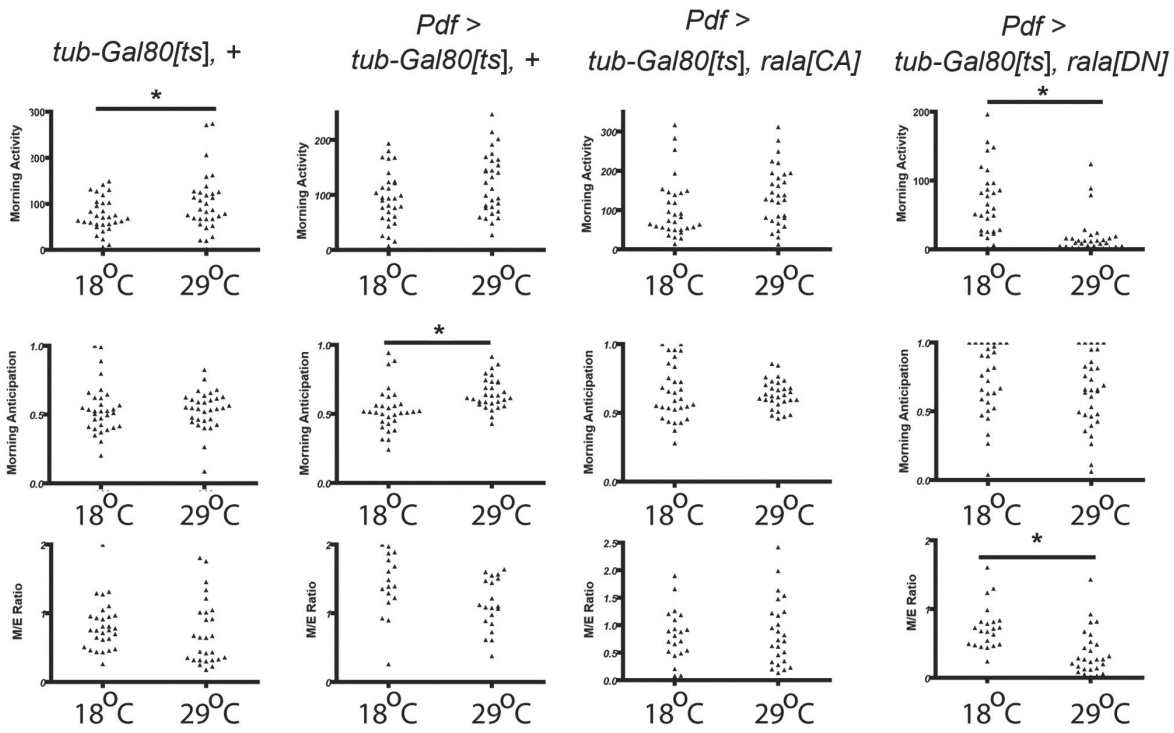
Supplemental Figure 4 – supporting Figure 4.
(top): Co-localization of Ral-A and PDFR in *hEK-293* cells. Single optical confocal images of representative of cells transfected with human *rala-GFP* (green) and *Pdfr-mCherry* (magenta). In the merged images, the numbers refer to seconds elapsed from the first image of the series. **(bottom): PDF neurons are morphologically normal despite chronic expression of either *rala*^{CA} or *rala*^{DN} genetic variants**. The figure shows adult (day 2-5) male *Drosophila* brains that were immunocytochemically stained in wholemount for an epitope on the PRO sequence of the PDF neuropeptide precursor (anti-PAP - Renn et al., 1999). Brains were dissected and stained from either ZT2 or ZT14, as indicated. Three representatives (of five brains examined) are shown for each genotype and condition.

Supplemental Figure 5



Supplemental Figure 5 – supporting Figure 5.

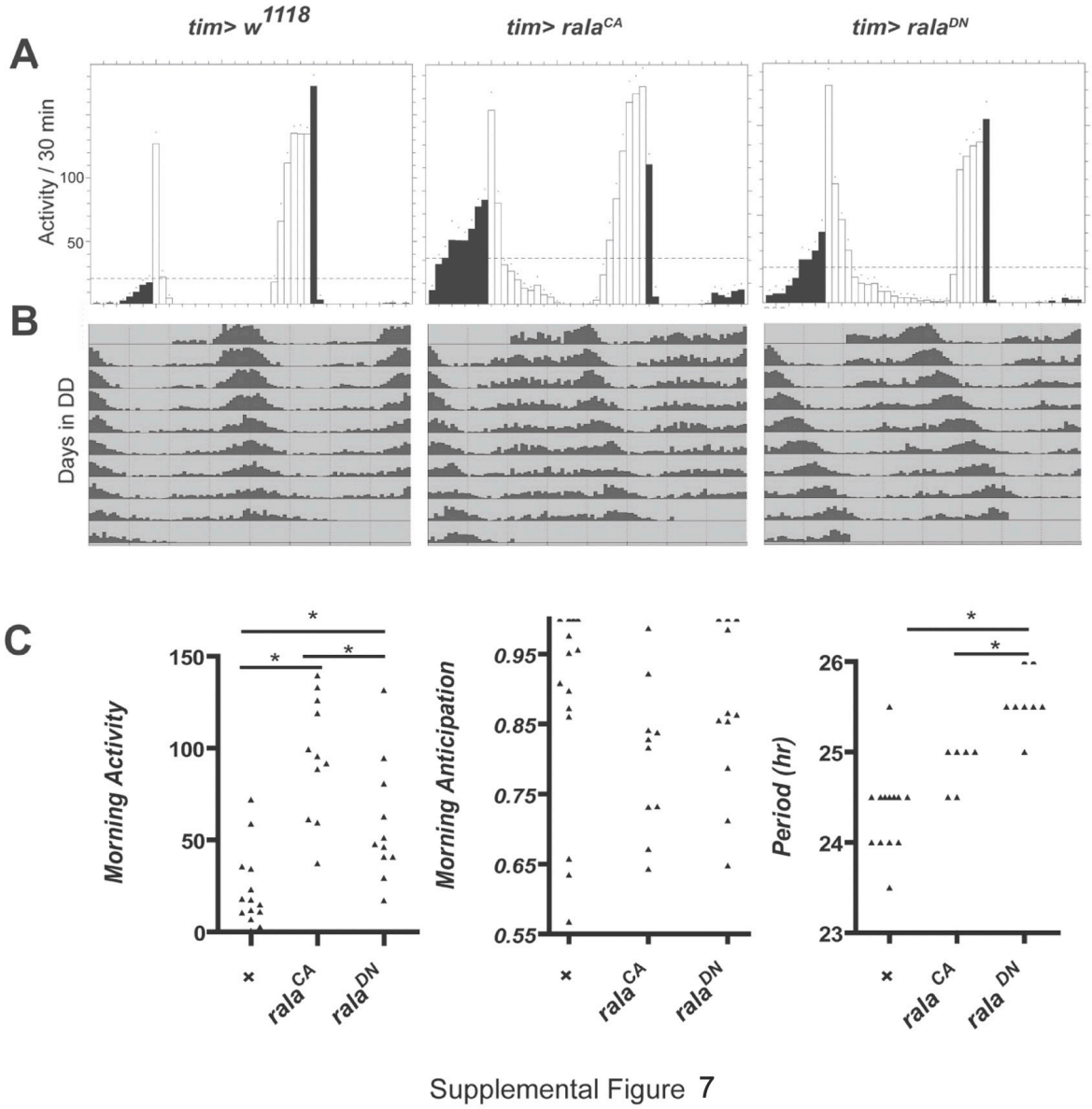
Conditional down-regulation of RalA activity in adult M neurons affects diurnal and circadian locomotor patterns. LD group eductions (for days LD1-6) and average group actograms (for DD days 1-9) are shown for four different genotypes raised at 18ºC throughout their lifetime (18ºC:18ºC) or following a shift to 29oC) at adult eclosion (18ºC:29ºC). The four genotypes are listed at the top (n=16 for all in this single experiment). Results shown are from a single experiment and representative of two independent experiments. Analysis of the Morning Peak of behavior on DD1, compiled for the two experiments jointly, is presented in Supplemental Figure 11. Analysis of periodic behavior under constant conditions (DD3-9) for the two experiments is presented in Supplemental Table 2: the circadian period of the four genotypes is not different when tested at 18ºC; when tested at 29ºC, flies containing the *rala*^{DN} transgene exhibited a significantly longer period (Table 2*)*. The shortening of τ in flies containing the *gal80*^{ts} transgene at higher temperatures may suggest this background inadvertently harbors the *SLIH* allele of *per* (cf. Hamblen *et al*., 1998).



Supplemental Figure 6

Supplemental Figure 6 – supporting Figure 5.

Conditional down-regulation of RalA activity in adult M neurons affects the Morning Peak of activity on DD1. Scatter plots of Morning Activity (total activity 3 hr before lights-ON), Morning Anticipation (total activity 3 hr before/activity 6 hr before light-ON) and M/E ratio are shown for each of the four genotypes listed across the top. All flies were raised at 18°C. For each measure, 18°C and 29°C refer to the temperature at which adults were maintained and tested. * indicates $P < 0.01$ (by Student's t test). Effects on τ are complied in Supplemental Table 2.



Supplemental Figure 7 – supporting Figure 5.
Locomotor activity rhythms in flies expressing *rala* variants under the *tim*(UAS)-Gal4 driver. (A): LD group eductions for control (n=13) flies, UAS-*rala*^{CA} (n = 10 flies) and UAS-*rala*^{DN} (n= 8 flies). (B): Average group actograms in DD. (C). Activity measures in DD: Total morning activity (Left), Morning anticipation (Center), and circadian period (Right). * indicates $p < 0.01$; ** indicates $P < 0.001$. See also Supplemental Table 2.

SUPPLEMENTAL TABLES

Gene	Genotype	n	N	AR%	TAU	PWR	WID	SNR	Act-DAY	Act - NIGHT
	tim > Dcr2	76	6	5%	24.30	103.1	6.5	2.4	15.8	11.4
Sec5	*tim > Dcr2, GD28873*	16	1	13%	24.21	106.7	6.1	2.0	20.3	20.9
	tim > Dcr2, JF02676	30	4	37%	25.20**	69.0	6.0	1.7	7.9	10.1
Sec6	*tim > Dcr2, JF02623*	9	2	78%	24.04	66.8	6.0	1.5	8.0	6.0
Sec10	*tim > Dcr2, JF02633*	19	2	32%	24.03	57.8	6.5	1.6	10.6	9.7
Sec15	*tim > Dcr2, KK105126*	31	3	87%	27.38***	10.2	2.5	0.4	6.5	8.9
	tim > Dcr2, JF02649	14	1	36%	24.17	25.4	2.9	0.6	7.2	4.1
Exo70	*tim > Dcr2, JF02876*	19	2	32%	25.27***	43.6	3.7	1.0	15.	13.7
Exo84	*tim > Dcr2, JF03139*	11	2	82%	25.45	13.3	4.9	0.7	8.05	7.68

Supplemental Table 1. Supporting Figures 5 & 6.
Rhythmic locomotor activity in days DD3-9 for flies following knockdown of genes encoding various exocyst subunits.

For circadian period (TAU), all values were compared by a Student's T test to that in the *tim> Dcr2* control stock. ***: $p < 0.001$; **: $p < 0.01$).
n = # flies; N = # experiments; AR% = % of arrhythmic flies; PWR = power; WID = activity peak width; SNR = signal to noise; Act-Day = average activity per 30 min in subjective day; Act-Night = average activity per 30 min in subjective night.

Genotype	n	N	AR%	TAU	PWR	WID	SNR	Act-Day	Act-Night
Pdf > Epac50A	25	2	12	24.55	58.4	5.8	1.02	16.7	12.1
Pdf > rala^{DN} / Epac50A	27	2	30	25.48**	60.3	5.3	1.26	11.6	9.3
Pdf > rala^{CA} ; Epac50A	28	2	11	24.50	55.4	5.0	0.97	13.1	8.9
Pdf > Dcr2	25	2	10	25.18	68.8	6.6	0.99	6.1	18.1
Pdf > Dcr2;;rala RNAi	31	2	58	26.43**	25.8	4.1	0.79	6.0	11.4
w^{11118} > Dcr2;;rala RNAi	30	1	3	23.79	132.1	6.8	2.01	28.7	18
Pdf > w^{11118}	28	2	0	25.23	65.19	6.6	1.06	11.2	16.5
Pdf > 2xtpdf	28	1	38	24.33	44.3	5.3	0.76	13.3	8.3
Pdf > 4xtpdf	30	1	47	24.34	31.8	4.3	0.61	10.4	5.8
Pdf > rala^{DN}	42	3	17	25.81	68.6	7.1	1.18	13.1	20.1
Pdf > rala^{DN} / 2xtpdf	42	3	5	24.55*	80.5	6.1	1.65	25.8	21.8
Pdf > rala^{DN} / 4xtpdf	39	3	28	24.70*	53.5	4.4	1.11	17.1	13.2
Pdf > rala^{DN} / 2xScrpdf	41	2	46	25.52	46.1	5.2	0.87	13.2	14.6
Pdf > rala^{DN} / 4xScrpdf	37	2	46	26.18	38.7	5.8	0.93	11.9	12.0
Pdf > rala^{DN} / 4xdh31	27	1	56	25.96	26.7	4.4	0.71	9.1	11.8
Pdf > rala^{CA}	44	3	18	24.17	50.8	4.8	0.75	15.6	10.7
Pdf > rala^{CA} / 2xtpdf	42	3	19	23.83	51.7	4.8	1.06	24.9	13.6
Pdf > rala^{CA} / 4xtpdf	40	3	58	24.06	27.8	3.5	0.82	21.4	16.8
Pdf > rala^{CA} / 2xScrpdf	43	2	33	24.16	39.6	3.9	0.72	24.2	18.4
Pdf > rala^{CA} / 4xScrpdf	38	2	47	23.85	33.5	3.1	0.66	16.2	10.9
Pdf > rala^{CA} / 4xdh31	22	1	27	23.91	39.8	4.4	0.60	17.9	8.5
tim > w^{11118}	77	6	1	24.30	88.2	6.1	1.61	17.0	14.1
tim > rala^{DN}	78	6	0	25.00**	94.0	6.0	1.52	15.4	17.2
tim > rala^{CA}	36	3	11	24.27	21.0	1.3	0.90	24.2	5.0
w^{11118} > rala^{DN}	70	3	1	23.58	80.3	5.9	1.24	32.4	29.2
w^{11118} > rala^{CA}	38	3	33	23.94	51.3	5.1	1.00	21.5	12.3
tim > Dcr2	25	2	20	24.46	67.8	7.2	2.17	16.5	18.6
tim > Dcr2;; rala RNAi	20	2	100	-	-	-	-	6.6	7.5
w^{11118} > tub gal80^{ts} 18^{o}C-18^{o}C	29	2	45	26.75	28.2	5.0	0.8	17.8	23.2
Pdf > tub gal80^{ts} 18^{o}C-18^{o}C	29	2	28	26.67	40.2	6.4	1.0	22.5	29.6
Pdf > tub gal80^{ts}, rala^{DN} 18^{o}C-18^{o}C	28	2	11	27.19	4.72	8.7	1.5	16.4	26.8
Pdf > tub gal80^{ts}, rala^{CA} 18^{o}C-18^{o}C	27	2	26	26.90	61.2	9.4	1.6	17.0	27.0
w^{11118} > tub gal80^{ts} 18^{o}C-29^{o}C	27	2	15	25.40	72.4	5.8	1.6	12.7	11.4
Pdf > tub gal80^{ts} 18^{o}C-29^{o}C	28	2	11	25.82	76.5	6.8	1.6	24.7	20.6
Pdf > tub gal80^{ts}, rala^{DN} 18^{o}C-29^{o}C	26	2	8	26.33**	90.9	7.3	2.2	11.4	8.7
Pdf > tub gal80^{ts}, rala^{CA} 18^{o}C-29^{o}C	28	2	0	25.79	82.0	7.0	1.8	23.2	19.3

Supplemental Table 2. Supporting Figures 5 & 6.

Rhythmic locomotor activity in days DD3-9 for flies with alterations of *rala* and of *pdf* signaling.

rala RNAi = TRiP HMS01365. By a Student's T test, the value for tau in *Pdf* > *Epac50A/rala^{DN}* was significantly different from that of *Pdf* > *Epac50A* ($p < 0.001$), but that of *Pdf* > *Epac50A/rala^{CA}* was not. The value in *Pdf* > *Dcr2;rala RNAi* was significantly different from that in *Pdf* > *Dcr2.* The value in *Pdf* > *rala^{DN} /2xtpdf and Pdf* > *rala^{DN} /4xtpdf* were significantly different from that in *Pdf* > *rala^{DN}*, but those in *Pdf* > *rala^{DN} /2xScrpdf, Pdf* > *rala^{DN} /4xScrpdf, Pdf* > *rala^{DN} /4xtDH31, Pdf* > *rala^{CA} /2xtpdf, Pdf* > *rala^{CA} /4xtpdf, Pdf* > *rala^{CA} /2xScrpdf, Pdf* > *rala^{CA} /4xScrpdf, Pdf* > *rala^{CA} /4xtDH31* were not. The value in *tim* > *rala^{DN}* was significantly different from that of *tim* > *w^{1118} ,* but that in *tim* > *rala^{CA}* was not. (**: $p < 0.001$; *: $p < 0.01$). For genotypes that included *tub-gal80^{ts}*, tau values were not significantly different (by Tukey's post hoc test) when flies were raised and tested at 18^{0}C (18^{o}C-18^{o}C), but when raised at 18^{0}C then tested at 29^{0}C (18^{o}C-29^{o}C), flies expressing *rala^{DN}* were significantly different from the others ($p < 0.01$).

n = # flies; N = # experiments; AR% = % of arrhythmic flies; PWR = power; WID = activity peak width; SNR = signal to noise ratio; Act-Day = average activity per 30 min in subjective day; Act-Night = average activity per 30 min in subjective night.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Drosophila stocks

We studied the following RNAi knockdown models of exocyst components: *sec5* (*CG8843*) – JF02676 and GD28873; *sec6* (*CG5341*) – JF02623; *sec10* (*CG6159*) – JF02633; *sec15* (CG7034) – JF02649, KK105126; *exo70* (*CG7172*) – JF02876; *exo84* (*CG6095*) - JF03139. All stocks tested contained one copy of UAS-*dcr2*.

We used *Pdf*-Gal4 and the $gal80^{ts}$ stock to conditionally express $rala^{CA}$ and $rala^{DN}$ variants by raising and testing flies at the permissive temperature, 18°C; alternatively, such flies were raised at 18°C until adult eclosion then moved to the restrictive temperature, 29°C, for at least 3 days before starting behavioral tests.

Locomotor Activity

We tested each genotype in two to three separate experiments (representing independent genetic crosses). We calculated 'Morning Activity' as the total activity recorded in the 3 hr before lights-on, and a 'Morning Anticipation' index as the "activity for 3 h before lights-on/activity for 6 h before lights-on" (Im and Taghert (2010). The M/E ratio (Morning-to-Evening Peak ratio) was computed following Choi *et al.*, 2012. Briefly: we smoothed DD1 activity profiles of individual flies by computing 5-point moving averages (each point represents a 30 min activity value). We determined morning peak and evening peak by the maximum activity around the subjective dawn and dusk. Then, using the smoothed data, we determined the duration of a peak from a starting point of continuous activity increase towards the

peak to the end of a continuous activity decrease from the peak, allowing one step in both increasing and decreasing durations. Finally, we calculated morning peak and evening peak as the sum of smoothed activity in this duration. We calculated the M/E ratio as morning peak over evening peak.

Neuraminidase treatments.

Whole brains were incubated in a 35 x 10 mm plastic FALCON Petri dish (Becton Dickenson Labware) with 0.01 unit/mL Neuraminidase (Sigma-Aldrich Co.) in Phosphate buffer with 0.3% BSA in HL3 saline for 15 minutes. After 15 minutes the Neuraminidase/HL3 mixture was washed out and replaced with Schneider Media with 10 % fetal bovine serum (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/Streptomycin 10000U (Gibco - Life Technologies Co.), human Insulin 10ug/ml (Sigma-Aldrich Co.) and brains were allowed to recover for various lengths of time at RT, then moved into HL3 saline and imaged as described previously.

Analysis of concentration-effect curves.

In an effort to examine the question further, a single data set (dopamine treatment at ZT4) was chosen, as this data set had the largest number of distributions that showed significant difference to a single Gaussian (of 7 concentrations tested, 4 showed a significant difference with at least one test and 2 with both). These distributions were fit by eye with the sum of 2 Gaussian

distributions, with means and standard deviations of the 2 components and the fraction in one component adjustable (the fraction in the second component was set to (1-fraction in the first component). (The "first component" was arbitrarily defined as the component with the smaller mean.) These fits indicated that the means of both components increased in a similar fashion with concentration, giving EC50 values of 3.8 ± 0.5. 4.8 ± 3.2 and 2.5 ± 1.8 μM for the means for a single Gaussian and the first and second components of the fits with the sum of 2 Gaussians (best fitting parameter value ± estimated standard error of the fit value). This result indicated that if two components were present it was likely that they had similar responses to dopamine treatment. Finally, the fraction of cells in the first component showed no dependence on dopamine concentration. The fraction in the first component had a mean of 0.51 ± 0.25 (mean ± SD; range 0.16 – 0.85) and did not show a significant change with dopamine concentration (regression coefficient did not differ from zero; P = 0.96).

Overall, there is no compelling reason to think that there is more than one population of cells that differ in the response to treatment. If there were, it seems likely that the concentration-effect curves for the two populations are very similar. These considerations do not demonstrate that there is only a single population of cells, but strongly indicate that analyzing the data in terms of a single population is justified.

RNAi screen of ACs potentially coupled to dopamine receptor in M cell pacemakers.

We studied transgenic fly stocks that expressed double-stranded RNAi's directed against Gsα and 11 of the 12 genes annotated to encode adenylate cyclases. All genotypes included one copy of *Pdf*-Gal4; UAS-*Epac1camps* and one copy of UAS-*RNAi* (except for control); these stocks did not contain UAS-*dcr2*. *Drosophila* were reared at 25°C and moved to 29°C for 2 days before imaging to increase UAS transgenic expression. Live brains were dissected under ice-cold calcium-free fly saline (46 mM NaCl, 5 mM KCl, and 10 mM Tris (pH 7.2)). The dissected brains were placed on poly-l-lysine-coated coverslips in an imaging chamber (Warner Instruments), and HL3 was perfused over the preparation (0.5 mL/minute). During FRET imaging, dopamine was injected into the perfusion line to result in a final concentration of 10^{-06} M. Each point shows individual s-LNv cell's maximal amplitude response to dopamine. For each genotype, responses from at least 10 cells and from at least 5 flies were collected. Error bars denote SEM. *** $p<0.001$, ** $p<0.01$ (compared with control).

β-arrestin2-GFP translocation assays.

hEK-293 cells were transfected using 25 μg of LipofectAMINE and 12 μg of DNA total per 4×10^6 cells. Cells were transfected with a 5:1 ratio of *GPCR* DNA and *β-arrestin2-GFP* (*βarr2-GFP*) DNAs. The dominant negative dynamin mutant (K44A, Zhang *et al.*, 1997) was included in some transfections, at a ratio of 5:1:1 (*GPCR:β-arr2-GFP:K44A).* Some experiments included overexpression of a G protein receptor kinase (GRK2) by including it in transfections at a ratio of 5:1:1 (*GPCR:β-arr2-GFP:GRK2*). Cells were maintained and microscopically analyzed as described above

PDF or dopamine was dissolved into the serum-free media and added to the imaging dish after 3 minutes of baseline recording. The intensity of β-arr2-GFP was measured before and after addition of pharmacological agents using Image J software (NIH). A straight line was drawn to bisect the cell and the intensity profile was plotted and exported into Sigma Plot for further analysis. A segmented line was then drawn around the circumference of the cell and again the intensity profile was plotted and exported into Sigma Plot for further analysis. Positive *β-arr2-GFP* translocation was scored whenever GFP fluorescence intensity increased 2-fold or more at plasma membrane loci – using either the cross-section or circumference measurements.

RalA/PDFR co-localization assays

We transfected *hEK-293* cells with a 1:1 ratio of *Pdfr::mCherry* pcDNA3 and *hRalA::EGFP-C1* in T25 flasks that were seeded the day before with 2 x 10^6 cells. We used a total of 10 ug of DNA and 20ul Lipofectamine 2000 (Invitrogen) diluted in Opti-MEM I Reduced Serum Medium (Invitrogen) in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. The DNA:Lipofectamine was incubated in a humidified, 37^{o}C and 5% CO_2 incubator for 5 hours. The cells were then split into five poly-lysine coated 35mm MatTek glass bottom dishes (8 x 10^5 cells/dish). For further details see Johnson *et al.*, 2003. The cells were incubated overnight and imaged on a Nikon A1 confocal microscope at 37^{o}C and under 5% CO_2 in DMEM. Images were collected throughout cell depths, and closely studied at or near cell surfaces.

Immunocytochemistry.

We studied male flies (2-5 days old) with dissection, fixation, and immunocytochemical visualization of pro-PDF as described in Renn *et al.* (1999). We used guinea pig anti-proPDF (PAP-59-IV;) diluted 1:1000, in PBS with 0.3% Triton X-100 (PBS-TX). Alexa-Fluor (568) conjugated secondary antisera (goat) were from Molecular Probes (Invitrogen, Carlsbad, CA) and were diluted 1:1000 in PBS-TX. When comparing PDF Immuno-reactivity levels between genotypes, care was taken to insure that the immunocytochemistry was conducted in parallel using the same reagents, aliquots of antisera, and exposure and rinse times.. Immunocytochemically labeled brains were arranged on poly-lysine-coated coverslips, dehydrated in a graded glycerol series, and mounted in Hard-Set Vectashield (Vector Laboratories, Burlingame, CA).

CRE-luciferase signaling assays.

hEK-293 cells were transfected using 25 μg of LipofectAMINE and 12 μg of DNA total per 4×10^6 cells, with a 5:1 ratio of *PDFR-mCherry* and *Cre-luciferase* DNAs. Assays were performed as described previously (Mertens *et al.*, 2005).

SUPPLEMENTAL REFERENCES

Barak. L. S., Ferguson, S. S., Zhang, J., and Caron, M. G. A β-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. J. Biol. Chem. 1997; 272: 27497-27500.

Hamblen MJ, White NE, Emery PTJ, Kaiser K, Hall JC. Molecular and Behavioral analysis of four period mutants in Drosophila melanogaster encompassing extreme short, novel long and unorthodox arrhythmic types. Genetics 1998; 149(1): 165-178.

Henion TR, Faden AA, Knott TK, Schwarting GA. β3GnT2 maintains adenylyl cyclase-3 signaling and axon guidance molecule expression in the olfactory epithelium. J Neurosci. 2011; 31(17):6578-6586.

Johnson EC, Bohn LM, Barak LS, Birse RT, Nässel DR, Caron MG, Taghert PH. Identification of *Drosophila* neuropeptide receptors by G protein-coupled receptors-beta-arrestin2 interactions. J Biol Chem. 2003; 278(52):52172-52178.

Johnson EC, Bohn LM, Taghert PH. *Drosophila* CG8422 encodes a functional diuretic hormone receptor. J Exp Biol. 2004; 207(Pt 5):743-74.

Stoleru D, Nawathean P, Fernández MP, Menet JS, Ceriani MF, Rosbash M. The *Drosophila* circadian network is a seasonal timer. Cell. 2007; 129(1):207-219.

Zhang, J., Barak, L. S., Winkler, K. E., Caron, M. G., and Ferguson, S. S. A central role for β-arrestins and clathrin-coated vesicle-mediated endocytosis in β-2-adrenergic receptor resensitization. Differential regulation of receptor resensitization in two distinct cell types. J. Biol. Chem. 1997; 272, 27005-27014.