Surprising gene expression patterns within and between PDF-containing circadian neurons in *Drosophila*

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To compare circadian gene expression within highly discrete neuronal populations, we separately purified and characterized two adjacent but distinct groups of *Drosophila* adult circadian neurons: the 8 small and 10 large PDF-expressing ventral lateral neurons (s-LNvs and l-LNvs, respectively). The s-LNvs are the principal circadian pacemaker cells, whereas recent evidence indicates that the l-LNvs are involved in sleep and light-mediated arousal. Although half of the l-LNv–enriched mRNA population, including core clock mRNAs, is shared between the l-LNvs and s-LNvs, the other half is l-LNv– and s-LNv–specific. The distribution of four specific mRNAs is consistent with prior characterization of the four encoded proteins, and therefore indicates successful purification of the two neuronal types. Moreover, an octopamine receptor mRNA is selectively enriched in l-LNvs, and only these neurons respond to in vitro application of octopamine. Dissection and purification of l-LNvs from flies collected at different times indicate that these neurons contain cycling clock mRNAs with higher circadian amplitudes as well as at least a 10-fold higher fraction of oscillating mRNAs than all previous analyses of head RNA. Many of these cycling l-LNv mRNAs are well expressed but do not cycle or cycle much less well elsewhere in heads. The results suggest that RNA cycling is much more prominent in circadian neurons than elsewhere in heads and may be particularly important for the functioning of these neurons.

PDF neurons | microarrays | cycling mRNAs

Many biochemical, physiological, and behavioral processes are governed by a circadian clock, which results in daily oscillations with a period of approximately 24 h. Circadian phenomena have been studied in multiple eukaryotic and even prokaryotic systems, and a large body of evidence now indicates that the biochemical underpinnings of eukaryotic molecular clocks include negative feedback loops of transcription (1–3). These cell-autonomous feedback loops also regulate the expression of so-called "output genes," many of which regulate circadian functions other than core timekeeping (1). In addition to this transcriptional regulation, posttranscriptional modifications such as phosphorylation regulate the stability and activity of clock proteins, and therefore also contribute to accurate timing as well as to robust mRNA and protein oscillations (4–8).

In *Drosophila*, there are about 75 clock neurons on each side of the adult brain. They control adult *Drosophila* locomotor activity, which peaks twice a day in anticipation of the dawn and dusk transitions. The clock neurons are divided into seven classes based on their anatomical locations and characteristics (9, 10). There are three groups of dorsal neurons (DN1, DN2, and DN3), a lateral posterior neuron, and three groups of lateral neurons. These are the dorsal lateral neurons and the two groups of lateral neurons: the small ventral lateral neurons (s-LNvs) and the large ventral lateral neurons (l-LNvs). Although many genes are expressed similarly in all clock cells, there are interesting exceptions. We have manually purified different sets of circadian neurons and characterized two genes, one of which is specific for LNvs and the other for DNs (11). Moreover, only LNvs express the neuropeptide PDF: all l-LNvs and four of the five s-LNvs on each side of the brain are PDF-positive. This neuropeptide has a widespread influence on other clock cells (12). Moreover, the two classes of LNvs have different projection patterns: the s-LNvs project to the dorsal protocerebrum, whereas the l-LNvs project to the second optic neuropil and to the contralateral LNv region (13, 14).

As suggested by these different projections, the s-LNvs and l-LNvs make distinct contributions to behavior. The s-LNvs control the timing of the morning peak of locomotor activity and are also the key pacemaker neurons under constant darkness conditions (15, 16). The l-LNvs, in contrast, have recently been implicated in light-mediated arousal and sleep as well as in phase-shifting in the dawn-advance zone (17–19). Consistent with light sensitivity, the l-LNvs manifest spontaneous tonic and bursting action potentials regulated by acute changes in light intensity (20).

As a first step to identify genes that might contribute to l-LNv– and s-LNv–specific functions, we characterized these two mRNA populations by separately purifying the neurons based on size as well as on PDF expression and by comparing their mRNA profiles with that from generic head neurons labeled by ELAV expression. Although core clock mRNAs are enriched in both sets of mRNAs as expected, a surprisingly large fraction of the l-LNv–enriched mRNA population, about 50%, is not shared with s-LNv–enriched mRNAs. A roughly similar number of enriched s-LNv RNAs are not enriched in the l-LNv RNA population. Proteins previously noted with s-LNv or l-LNv expression patterns have corresponding mRNA patterns. In addition, we found that octopamine receptor mRNAs are preferentially expressed in the l-LNvs. These neurons but not s-LNvs respond robustly to in vitro application of octopamine. All the specific mRNA patterns indicate that the two cell types were successfully purified. We also dissected and purified l-LNvs from flies collected at different circadian times to analyze RNA oscillations within the l-LNvs. The profiles indicate an unusually high amplitude of cycling and an unexpectedly high fraction of oscillating mRNAs compared with all previous assays of

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head RNA. The data suggest that a surprisingly large fraction of specific gene expression as well as RNA cycling is a central feature of clock neuron function.

Results

PDF and Clock Gene mRNAs Are Highly Enriched in Both l-LNvs and s-LNvs. To characterize gene expression within the lateral neurons, we used EGFP expression under PDF-GAL4 control and a modification of the manual purification system of Nelson and coworkers (11, 21) to isolate PDF-containing brain neurons (*Materials and Methods*). Large EGFP-labeled neurons were separately purified from small EGFP-labeled neurons based on size in the dissecting microscope (Fig. 1*A*). As previously described (11), we also used a pan-neuron driver (elav-GAL4) and compared the microarray signal for each mRNA in the l-LNv and s-LNv mRNA populations with that from the ELAV purification (Fig. 1*B*). To avoid complicating these enrichment data with temporal oscillations in mRNA abundance, microarray data were averaged from cells isolated at multiple time points (see below). This strategy results in an enrichment value for each mRNA (*SI Materials and Methods*). We also directly compared each mRNA signal between the s-LNvs and the l-LNvs, resulting in an s/l value.

PDF is the top mRNA by this enrichment criterion in both l-LNv and s-LNv populations (Fig. 1*E*, Tables S1 and S2, and Dataset S1). This is consistent with its reported expression within the brain, namely, nearly exclusive to the LNvs (22, 23). The observed enrichment values from the microarray assay (≈4,000×; Fig. 1*C*) were reproduced by quantitative (q) PCR when comparing PDF transcript abundance in the s-LNv and l-LNv RNA populations vs. the elav RNA population (≈5,000×; Fig. 1*D*). Moreover and consistent with more general clock gene purification data (11), almost all known canonical clock gene mRNAs are highly enriched in both l-LNvs and s-LNvs and are among the top 49 and 57 transcripts in the l-LNvs and the s-LNvs, respectively (57 is <0.5% of the ~19,000 total genes on the microarray; Tables S1 and S2). *Pdp1* mRNA was a modest outlier but is still ranked within the top 2% in both populations (Fig. 1*E*). *Cyc* mRNA is another outlier and is not enriched in either population, consistent with indications that CYC may have a more widespread distribution compared with its partner CLK (24–27).

Between 5 and 10% of all mRNAs (1,000–2,000 mRNAs) are enriched in either the s-LNvs or the l-LNvs, and about 50% of them are enriched in both cell types (Fig. S1*A*). The probability that this is by chance is close to zero, indicating that many of these shared mRNAs probably contribute to adult circadian rhythms and/or other common functions (Fig. S1 *B–D*). The other ≈50% is enriched preferentially only in s-LNvs or only in l-LNvs, suggesting that the two sets of neurons have quite different gene expression profiles. These 500–1,000 distinct mRNAs may have cell-specific circadian roles and/or roles in other behaviors. These mRNAs also suggest that the two neuronal populations have been successfully purified by size and that the overlap of the other 500–1,000 mRNAs is not an artifact.

To verify the successful purification of the two cell types further, we specifically examined four mRNAs encoding proteins recently studied for their apparent s-LNv vs. l-LNv expression patterns. The first two are neuropeptide receptors for PDF and for the diuretic hormone DH31 (Fig. 2*A*). A cAMP FRET sensor was used to study the responsiveness of the s-LNvs and the l-LNvs to application of these neuropeptides. Only the s-LNvs respond robustly to PDF, whereas both the s-LNvs and l-LNvs respond to DH31 (12). Moreover, a GAL4 enhancer trap in the *Pdfr* locus also demonstrates strong preferential expression in the s-LNv (28). Our data fit perfectly with these observations. PDFR mRNA is enriched only in the s-LNvs and has an s/l ranking of no. 88. DH31R mRNA, in contrast, is enriched in both s-LNvs (no. 77) and l-LNvs (no. 33) without a notable s/l ranking.

The third mRNA encodes the neuropeptide sNPF (Fig. 2*A*). Although not strongly enriched in either PDF cell type relative to the ELAV transcript population, this mRNA is no. 1 on the s/l ranking. Consistent with these data, sNPF has very recently been shown by immunohistochemistry to be present in s-LNvs but not in l-LNvs (29). The lack of strong enrichment is almost certainly because there are many other neurons that express sNPF and/or because these other neurons have much higher sNPF mRNA levels than s-LNvs. There is good evidence in favor of the first possibility (29).

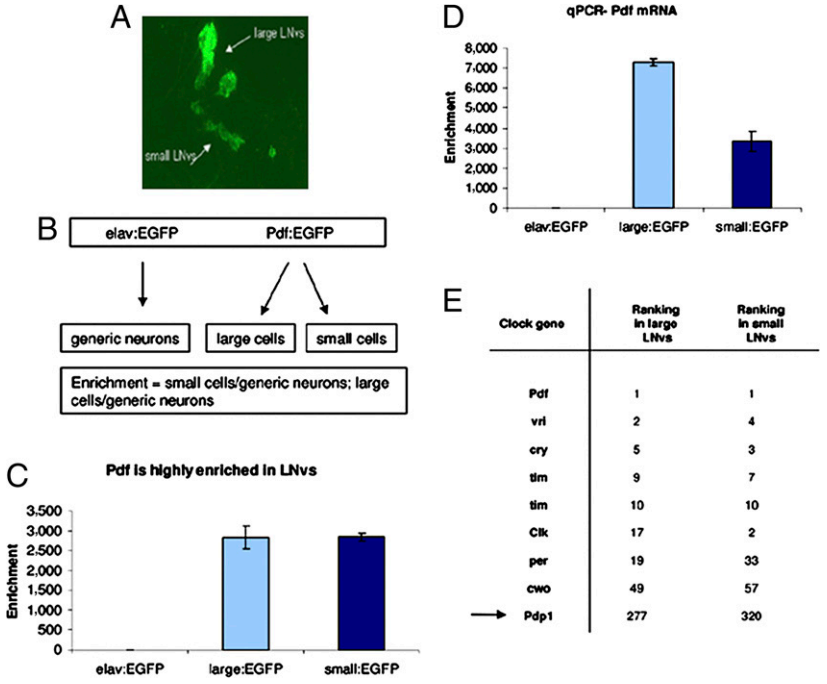

Fig. 1. Gene expression can be directly assessed in the purified clock neurons. (*A*) l-LNvs and s-LNvs can be identified by size differences of their somas. (*B*) Experimental overview. l-LNvs and s-LNvs were purified separately. To identify circadian genes, we used a panneuronal driver, elav-GAL4. Enrichment analysis was performed relative to elav/EGFP neurons. (*C*) *Pdf* mRNA is highly enriched in both l-LNvs and s-LNvs relative to generic neurons (elav-GAL4 driver in combination with UAS-EGFP). All comparisons are based on chip data that were assayed in triplicate (elav/EGFP, large/EGFP cells) or duplicate (small/EGFP cells). (*D*) qPCR validation of *Pdf* mRNA enrichment in the l-LNvs and s-LNvs. Gene expression was assayed in triplicate and normalized to *calmodulin* (11). Enrichment is shown as fold-change relative to ELAV cells. Error bars represent SEM. (*E*) Clock genes are highly enriched in LNvs. Enrichment analysis was performed relative to elav-GAL4/EGFP neurons. Clock gene mRNAs rank high in both l-LNvs and s-LNvs and essentially show similar enrichment in these two cell populations (1 indicates top enriched, 2 indicates next most enriched, etc.). Essentially identical results were obtained when the chips were divided in half and analyzed separately. An arrow points to *Pdp1*, an outlier among clock genes.

A

Gene	Enrichment ranking in large LNvs	Enrichment ranking in small LNvs	Enrichment ranking in small/large LNvs (l/s LNvs)
DH31R	33	77	14,456 (4,497)
PDFR	12,895	131	88 (18,865)
sNPF	18,937	17,070	1 (18,952)
Dimmed	1,506	17,663	18,774 (209)
Oamb	91	5,527	18,868 (60)
Oa2	2,395	18,325	18,834 (119)

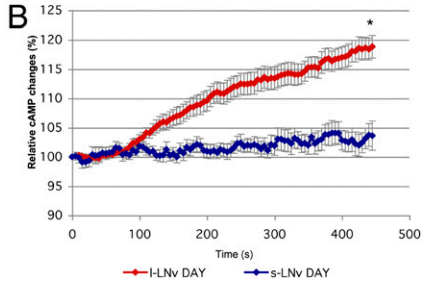

Fig. 2. Number of l-LNv– and s-LNv–specific mRNAs identified from purified clock neurons. (*A*) Examples of candidate genes showing high enrichment in both l-LNvs and s-LNvs (DH31R) and enrichment in s-LNvs but not in large cells (PDFR, sNPF) as well as two octopamine receptor mRNAs (*Oa2* and *Oamb*) specifically enriched in l-LNvs but not in small cells; ranking in l-LNvs and s-LNvs is relative to ELAV cells, whereas ranking in s-LNvs and L-LNvs directly compares the expression levels in small vs. large PDF cells [in parentheses, a reverse ranking (i.e., l-LNvs/s-LNvs)] (*SI Materials and Methods*). (*B*) l-LNvs but not s-LNvs respond potently to octopamine. A star indicates statistically significant differences (*t* test, $P < 0.05$).

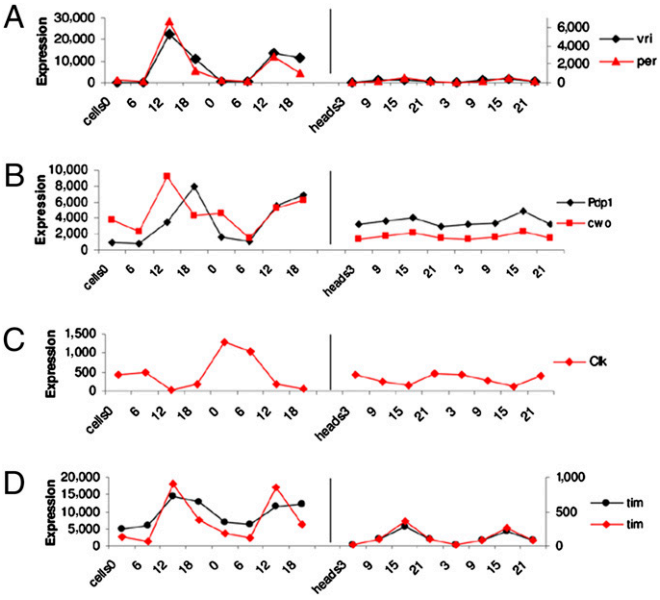

Fig. 3. Most clock gene mRNAs cycle with much higher amplitude in pacemaker cells than in heads. *per* and *vri* mRNA (*A*), *Pdp1* and *cwo* (*B*), *Clk* (*C*), and *tim* probes (*D*) are shown across four time points in clock neurons (ZT0, ZT6, ZT12, and ZT18) and in heads (ZT3, ZT9, ZT15, and ZT21). Cell and head data represent independent samples [GeneSpring (Agilent Technologies); one-way ANOVA at $P < 0.05$, 5% false discovery rate, and fold change = at least 1.8; *SI Text*].

The fourth mRNA encodes the bHLH transcription factor Dimmed. It regulates neuroendocrine cell differentiation and activates the expression of peptidylglycine α-monooxygenase, a key enzyme in neuropeptide amidation (30). Previous observations suggest restricted expression of Dimmed to l-LNvs but not s-LNvs, because only l-LNvs appear to synthesize amidated PDF (31). Although only somewhat enriched in l-LNvs relative to generic neurons (no. 1,506), *dim* mRNA has an impressive l/s ranking of no. 209 (Fig. 2*A*).

As an additional indication of specificity, we examined the distribution of octopamine receptor mRNAs between l-LNvs and s-LNvs. This neurotransmitter is the insect equivalent of noradrenaline (32) and has been implicated in *Drosophila* sleep regulation (33). Because l-LNvs are light-mediated arousal neurons (17, 19), we considered that they might also be sensitive to octopamine. mRNAs encoding the two major octopamine GPC receptors, OA2 and OAMB, are much more abundant in l-LNvs than in s-LNvs, and OAMB is even highly enriched in l-LNvs relative to ELAV neurons (Fig. 2*A*). To verify that this l-LNv expression is reflected at the level of the neuronal response, we used a cAMP FRET reporter and neuronal imaging (12) to assay the in vitro LNv response to exogenously applied octopamine. The l-LNvs respond, whereas the s-LNvs do not respond or respond only much less well (Fig. 2*B*). Taken together, the data indicate successful purification of l-LNvs vs. s-LNvs.

We then asked whether the circadian regulation of gene expression within l-LNvs was different from what has been previously reported from fly heads (*SI Materials and Methods*). We initially compared *tim* mRNA levels between ZT12 and ZT0 (ZT12, lights off; ZT0, lights on) from l-LNv RNA by qPCR and found levels at ZT12 to be about 4-fold more abundant than those at ZT0, consistent with published head RNA data (see below) (34, 35). We then extended the analysis to four time points (ZT0, ZT6, ZT12, and ZT18) and performed whole-genome microarray analysis of two independent time-point series. (Two time points were in triplicate, including ZT0 and ZT12.) Head RNA was assayed in parallel (Fig. 3).

The cycling of all clock gene mRNAs within the l-LNvs has a similar phase to that from head mRNA (Fig. 3; first set of eight data points represents 2 d of l-LNv data, each point represents every 6 h, and the second set of eight data points represents 2 d of head data). Note that *clk* mRNA cycling within the l-LNvs has a phase opposite to that of most other clock mRNAs (Fig. 3*C*), consistent with the literature as well as with the head data (35–37). However, the cycling amplitude of most clock mRNAs, including *vri*, *per*, and *Clk*, is much higher in l-LNvs than in heads (Fig. 3 *A*–*C*). The exception is *tim* mRNA because its cycling amplitude was comparable between heads and l-LNvs or perhaps even a little higher in heads (Fig. 3*D*), consistent with the qPCR analysis mentioned above. Clock gene mRNA cycling amplitude appeared similar in s-LNvs, although only two time points were collected from this more difficult preparation (ZT0 and ZT12). The cycling amplitudes of these clock mRNAs were verified by qPCR analysis on independent RNA preparations from purified l-LNvs and heads (Fig. S2). The data indicate that most clock gene mRNAs cycle more robustly in circadian neurons than previously estimated from head RNA (*Discussion*).

Many More Cycling Genes Identified from Purified Clock Neurons Than Heads. What about previously identified cycling head mRNAs that do not encode core clock machinery components? Microarray studies identified about 100–200 cycling *Drosophila* mRNAs in head RNA, (i.e., about 1% of the genome) (35, 38–43). In two of these studies (35, 38), 36 cycling mRNAs were in common, including 5 core clock mRNAs. Only 5 of these 31 noncore clock head mRNAs manifest clear cycling in l-LNvs by visual inspection of the circadian time-point graphs (Figs. 3 and 4). This is because most of the other 26 head cycling mRNAs are very poorly expressed and probably not expressed in the clock neurons (Fig. 4). Little or no expression is also the case in RNA from ELAV neurons, indicating that most head cycling RNAs are probably only expressed in the eye or fat body and not in neurons. Two

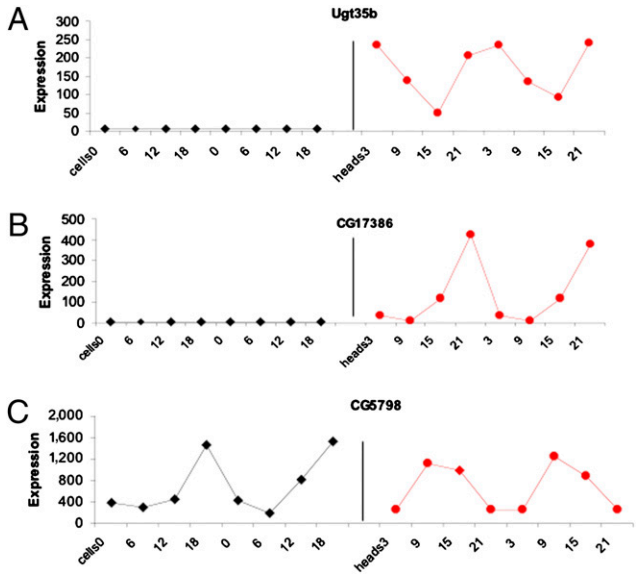

Fig. 4. Assay of previously identified cycling head genes in clock neurons. *Ugt35b* (*A*) and *CG17386* (*B*) are examples of genes cycling robustly in heads but not in the clock neurons. (*C*) *CG5798* is the only exception because it shows robust cycling in clock neurons. Cell and head data are from independent samples.

examples are shown as well as an exception (Fig. 4 *A*–*C*). The exception is CG5798 mRNA, which cycles very well in l-LNvs as well as in heads but with an apparent phase difference between the two sources of RNA (Fig. 4*C*). The data suggest that cell-type specificity of gene expression underlies a substantial fraction of tissue- and cell-type specificity of circadian mRNA cycling (*Discussion*).

Despite this failure to observe l-LNv cycling of most previously observed oscillating head mRNAs, the identical criterion indicates at least 10-fold more cycling mRNAs (>2,000 transcripts) in l-LNvs than in heads (Fig. 5 *A* and *B*, Fig. S3 *B* and *C*, and Table S3). This dramatic difference was verified by comparing two time points, ZT12 and ZT0, with the same criterion and on parallel microarrays from additional head RNA and l-LNv preparations; 13 times fewer cycling mRNAs were identified from the head RNA than from l-LNv RNA (Fig. 5*B*). Because the identical criterion identified less than 10% as many cycling mRNAs from l-LNv RNA isolated from the arrhythmic per^{01} strain (Fig. S4), most l-LNv cycling mRNAs are under clock control. qPCR analysis of a small subset of these cycling transcripts validated the microarray data (Fig. S5).

Although there are a small number of cycling l-LNv mRNAs that are poorly expressed in heads, and may therefore be clock cell-specific (e.g., *CG4726*; Fig. 5*C*), most cycling l-LNv mRNAs are well expressed in heads. Three examples are shown: *PKA-R1*, *PKA-R2*, and *Ir* (Fig. 5 *D*–*F*). These transcripts and many other l-LNv oscillating mRNAs do not cycle or do so only with much lower amplitude in other head cells and tissues (35, 38–43) (Fig. S3 *B* and *C*). This indicates that a large fraction of mRNA cycling is qualitatively or quantitatively restricted to l-LNvs or to clock neurons more generally.

Discussion

In this study, we separately profiled the mRNA populations from generic adult neurons as well as from two sets of *Drosophila* circadian neurons, the l-LNvs and s-LNvs. These two adjacent cell groups are the only documented neuronal sites of PDF expression within the adult fly brain, and the enrichment data strongly indicate that PDF is the only mRNA restricted to the LNvs. This is because the l-LNv– and s-LNv–enriched mRNAs in second place are about 10-fold less enriched than PDF. Other canonical clock mRNAs are also highly enriched but ≈10- to 100-fold less so than PDF mRNA. This is consistent with more widespread brain expression patterns, at least throughout the circadian network (Tables S1 and S2). The definition of a canonical clock protein, important only for circadian rhythms, does not apply to circadian kinases, most of which are also essential for development and other physiological processes. Not surprisingly therefore, clock kinase mRNAs are neither enriched nor cycling in LNvs (Fig. S6).

Because there are only five l-LNvs and four s-LNvs on each side of the adult brain, these are probably the most specific sets of neurons ever studied by gene expression profiling. Given that both groups are circadian neurons, express PDF, and are adjacent, it is not surprising that about half of the two enriched mRNA populations overlap. These 500–1,000 transcripts presumably underlie their shared functions, including circadian rhythms. Interestingly, this overlap is more extensive than that between enriched s-LNv transcripts and larval LNv transcripts (11). Larvae do not have l-LNvs, and the four PDF-expressing larval LNvs are functional circadian cells as well as precursors of the four adult s-LNvs (14). This makes the more extensive overlap between the two sets of adult LNvs surprising and suggests that most of these transcripts contribute to shared adult functions rather than to core circadian timekeeping. It also suggests that many of the enriched s-LNv larval transcripts (11) are dedicated to larval or perhaps developmental functions.

The other 500–1,000 enriched transcripts are adult s-LNv– or l-LNv–specific, and they include the two specific mRNAs described above, PDF-R and Oamb. This large number of transcripts presumably underlies the different functions and/or connectivity of the s-LNvs and the l-LNvs.

In addition to enrichment, the data provide the criterion of s/l ranking. For example, sNPF mRNA is substantially more abundant in s-LNvs than in l-LNvs without being enriched (Fig. 2*A*), consistent with recent immunohistochemical data (29). The transcription factor Dimmed and the octopamine receptor Oamb are only marginally enriched in l-LNvs but have very low expression in s-LNvs. The imaging data with octopamine are consistent with the s/l rankings of Oamb and Oa2 (Fig. 2*B*), which broadens the potential arousal signals influencing l-LNvs from just the external signal light to the internal signal octopamine. The s/l ranking data therefore complement the enrichment data by providing many additional candidate genes for s-LNv– and l-LNv–specific functions.

A third criterion is mRNA cycling, which has never been quantitatively addressed in large-scale expression profiling studies within specific neurons in any system (11). Many mRNAs manifest surprisingly robust oscillations within LNvs (Table S3), and most canonical clock mRNAs show a much higher cycling amplitude in l-LNvs than in heads. (It is also higher in s-LNvs based on the two-time point s-LNv data). Because the stronger cycling amplitude has been validated by qPCR, the result is unlikely to be a microarray artifact. Moreover, the amplitude of *tim* mRNA cycling is not substantially higher in l-LNvs than in heads (Fig. S2*C*), further supporting the higher cycling amplitude of most other clock mRNAs.

A rough minimal estimate of cycling mRNAs in l-LNvs is 10% of the genome. Most cycling disappears in a per^{01} strain, indicating that it is largely under clock control rather than driven by the light/dark (LD) cycle. Although this difference is based on only two time-point data from the per^{01} strain (Fig. S4), 10% is much greater than all previous estimates from six time-point data from fly head RNA, which were all about 1% or less. Moreover, most of these head cycling data were gathered under identical LD conditions to those used in this study (35, 38–43). Although the number of identified cycling transcripts is somewhat arbitrary and depends strongly on the stringency cutoff, the identical cri-

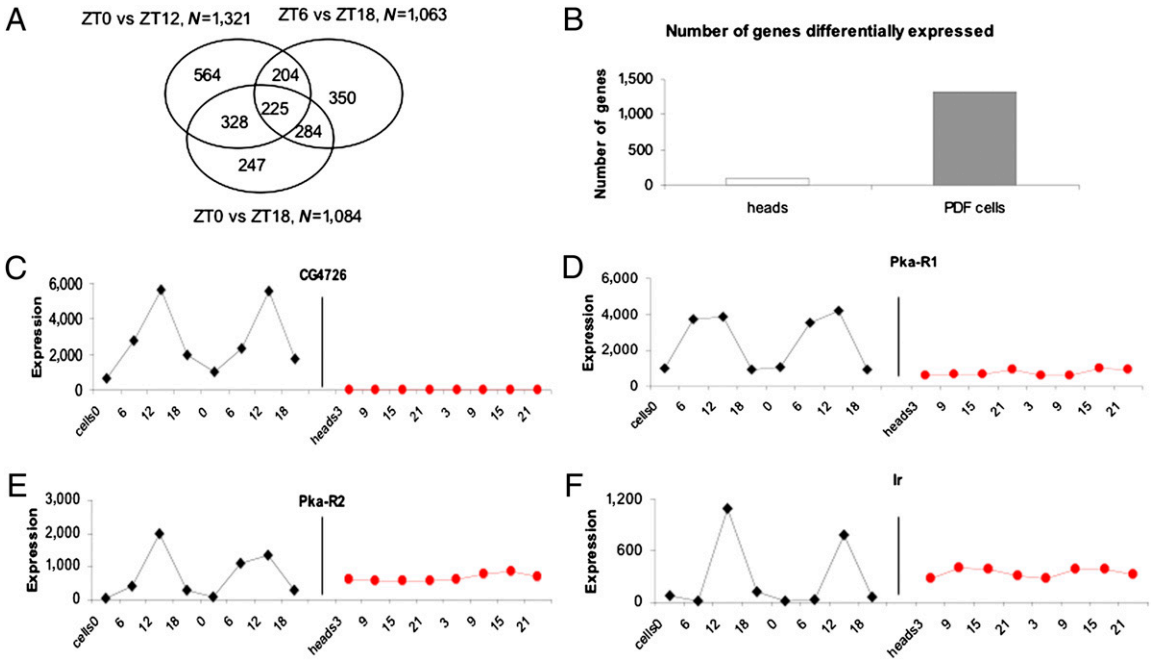

Fig. 5. Many more cycling genes identified in clock neurons than in heads. (*A*) Microarray data collected from large PDF neurons at the indicated time points are shown. GeneSpring (Agilent Technologies) analysis was applied to identify cycling transcripts: one-way ANOVA at $P < 0.05$, 5% false discovery rate (FDR), and fold change = at least 1.8. To analyze circadian gene expression, we looked at four time points and compared gene expression levels between three pairs: ZT0 vs. ZT12, ZT6 vs. ZT18, and ZT0 vs. ZT18. Each comparison yields a set of genes (e.g., ANOVA showed that there were 1,321 differentially expressed genes between ZT0 vs. ZT12). To identify genes overlapping between different time point comparisons, we cross-referenced the datasets. This analysis showed, for example, that there were 225 mRNAs that were identified as differentially expressed between the above time points. Also, 564 genes were only significant between ZT0 vs. ZT12. (*B*) Data analysis on head mRNA at two time points revealed many fewer cycling genes than the same comparison on clock neuron mRNA (two time points examined: ZT0 and ZT12). After normalization, microarray data were analyzed to determine the number of genes differentially expressed in heads and PDF neurons (GeneSpring; one-way ANOVA at $P < 0.05$, 5% FDR, and FC = at least 1.8). The bars represent the number of differentially expressed genes between ZT0 and ZT12 in heads and cells. There are about 13 times more differentially expressed mRNAs in PDF neurons than in heads: *CG4726* (*C*), *Pka-R1* (*D*), *Pka-R2* (*E*), and *Ir* (*F*). (*C–F*) Examples of genes cycling only in the clock neurons.

teria were used here in a side-by-side comparison of l-LNvs and heads under LD conditions (Fig. 5*B*), with considerably more than an order of magnitude difference. Moreover, this value of 10% resembles more closely that from homogenous mammalian tissue (44–46). The eye and fat body clocks may generate lower cycling amplitudes and/or may engage fewer genes than the circadian neurons. Another possibility is that the mixed nature of head RNA obscures transcript cycling (i.e., noncycling mRNAs in some cells obscure quantitatively or qualitatively the cycling from clock neuron RNA). Because head RNA obviously includes all l-LNv RNAs, most l-LNv cycling transcripts are probably expressed in other locations (other neurons, glia, eyes, and/or the fat body) but with little or no cycling in these locations. This interpretation agrees with manual inspection of many individual l-LNv cycling transcripts within the head microarray data (35, 47) as well as with qPCR analysis of some examples (Fig. S5). Consistent with this conclusion, many l-LNv cycling transcripts are expressed robustly in ELAV neurons but cycle there poorly if at all; this conclusion is based on ELAV RNA from two time points (Fig. S3*A*). Taken together, the data indicate that mRNA cycling, including that of most core clock mRNAs, is more potent within the circadian network than elsewhere within the brain or head.

The opposite comparison indicates that rather few previously identified cycling head transcripts, other than core clock mRNAs, also cycle in l-LNvs (Fig. 5 *C–F* and Fig. S3 *B* and *C*). Expression of many, and perhaps most, of these cycling head transcripts is probably specific to the eye and/or the fat body. A corollary is that ubiquitous cycling mRNAs are rather rare. Most mRNA cycling is therefore tissue- or cell type-specific, which agrees with the mammalian system (44–46). Some tissue-specific cycling mRNAs are not expressed in the second tissue, whereas others are expressed in both but only cycle in one (44). This too appears to apply to many s-LNv and l-LNv cycling transcripts: some strongly cycling mRNAs like sNPF appear to be poorly expressed in l-LNvs, whereas others are expressed in both l-LNvs and s-LNvs, with prominent cycling in only one or the other set of neurons.

However, some mRNAs cycle robustly in both sets of neurons. The ion channel Ir mRNA is one of the most potent cyclers in both l-LNvs and s-LNvs (Fig. 5*F*) and has a comparable 55× peak-to-trough amplitude in both locations (Fig. 5*F*). Although it is also highly enriched in both sets of neurons (Tables S1 and S2), Ir mRNA must be expressed elsewhere in heads. This is because it does not cycle or cycles only with a much lower amplitude in head RNA (Fig. 5*F*). There is a more general correlation between enrichment and cycling (i.e., l-LNv–enriched mRNAs have a relatively high cycling amplitude). As is the case for Ir mRNA, this reflects the fact that enrichment is quantitative and does not indicate restricted expression (an exception is *pdf* mRNA). Otherwise put, most of these transcripts are also expressed elsewhere, probably in the eyes and the fat body, despite considerable neuronal "enrichment" within LNvs.

The potent oscillations of most core circadian mRNAs and many enriched mRNAs like Ir within LNvs suggest that mRNA cycling is especially important for circadian neuron functions. They might even include rhythms of neuronal firing and/or electrical excitability. Testing the contribution of RNA cycling to circadian neuron function, including firing rhythms, is an important future goal.

Materials and Methods

Fly Stocks. All flies were entrained for at least 3 d under LD (12 h light/12 h dark) conditions. Adult PDF neurons were labeled as described previously (11). Adult ELAV neurons were labeled by crossing elav-GAL4 (C155-GAL4; Bloomington Stock Center) virgin female flies to UAS-EGFP (cytoplasmic signal) male flies. Adult per^{01}:l-LNvs were labeled by crossing per^{01};pdf-GAL4 virgin female flies to UAS-EGFP male flies. Male progeny of this cross were used for subsequent cell purifications. The UAS-Epac1-camps (50A) flies were kindly provided by Paul Taghert (Washington University, St. Louis, MO). The pdf-GAL4 was used to express the Epac sensors in the l-LNvs and the s-LNvs.

Purification of Cells, RNA, and Probe Preparation for Microarrays. About 50–60 adult brains of a given genotype were dissected per time point. Neurons were purified, RNA was extracted, and probes were prepared exactly as previously described (11). l-LNv ZT0 and ZT12 samples were done in triplicate, whereas ZT6 and ZT18 samples were done in duplicate. per^{01} and s-LNv samples were done in duplicate. We collected about 50–60 l-LNvs, about 80–90 s-LNvs, and 100 ELAV cells per sample. Affymetrix high-density arrays for *Drosophila melanogaster* were probed, hybridized, stained, and washed according to the manufacturer's protocol. The number of present calls in all cases ranged from 37 to 50% (details regarding microarray data analysis are provided in *SI Text*).

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