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## Electrical activity can impose time-of-day on the circadian transcriptome of pacemaker neurons

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### Summary

**Background**—Circadian (~24hr) rhythms offer one of the best examples of how gene expression is tied to behavior. Circadian pacemaker neurons contain molecular clocks that control ~24hr rhythms in gene expression that in turn regulate electrical activity rhythms to control behavior.

**Results**—Here we demonstrate the inverse relationship: there are broad transcriptional changes in *Drosophila* clock neurons (LN<sub>v</sub>s) in response to altered electrical activity, including a large set of circadian genes. Hyperexciting LN<sub>v</sub>s creates a morning-like expression profile for many circadian genes while hyperpolarization leads to an evening-like transcriptional state. The electrical effects robustly persist in *per<sup>0</sup>* mutant LN<sub>v</sub>s but not in *cyc<sup>0</sup>* mutant LN<sub>v</sub>s suggesting that neuronal activity interacts with the transcriptional activators of the core circadian clock. Bioinformatic and immunocytochemical analyses suggest that CREB family transcription factors link LN<sub>v</sub> electrical state to circadian gene expression.

**Conclusions**—The electrical state of a clock neuron can impose time-of-day to its transcriptional program. We propose that this acts as an internal zeitgeber to add robustness and precision to circadian behavioral rhythms.

### Introduction

Electrical activity can initiate transcriptional changes in neurons that alter their synaptic strength and this has been extensively studied in the context of learning and memory [1]. Hundreds of activity-dependent genes have been identified as have specific transcription factors that regulate gene expression in response to electrical activity e.g.[2–5].

The interplay between electrical activity and transcription is also relevant to circadian pacemaker neurons where ~24hr rhythms in gene expression and neural activity define the functional state of clock neurons [6]. Rather than simply transmitting the state of the molecular clock to downstream neurons to regulate circadian behavior, an emerging view is

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that neural activity supports molecular clock oscillations. Preventing mammalian pacemaker neurons in the Suprachiasmatic Nucleus (SCN) from firing action potentials or reducing extracellular  $K^+$  dampened *mPeriod1* transcriptional rhythms [7, 8]. Furthermore, hyperpolarizing or hyperexciting the master *Drosophila* circadian pacemaker neurons ( $LN_{Vs}$ ) alters rhythmic clock protein accumulation [9–11], although the effects of hyperpolarization are less severe if  $LN_{Vs}$  are only hyperpolarized in adulthood [12].  $Ca^{2+}$  influx has also been implicated in phase-shifting the molluscan retinal clock [13]. While these studies suggest a relationship between membrane excitability and the molecular oscillator in pacemaker neurons, there is relatively little known about the nature of this interaction.

Here, we characterized the transcriptional responses to spatially, temporally and directionally controlled changes to membrane excitability by performing microarrays on circadian pacemaker neurons purified from *Drosophila*. Strikingly, a large set of circadian genes is bi-directionally regulated with reference to the cell's electrical state, implying a potent effect of neuronal excitability on circadian gene expression. Many of these changes correlate with the nature of the alterations such that hyper-excitation created a morning-like expression profile and hyperpolarization created an evening-like expression profile. Hyperexcitation induced a morning-like transcriptional program even in a *per<sup>0</sup>* background whereas the effect of hyperpolarization on circadian gene expression was disrupted in *cyc<sup>0</sup>* mutants, implying that neuronal activity interacts with the core clock transcriptional activators. We establish links between activity-dependent pathways and circadian gene expression and provide evidence that the CREB transcription factor family – integrators of neuronal activity for transcription in learning and memory – are part of the activity-dependent regulatory network in circadian pacemaker neurons.

## Results

### Broad transcriptional response in $LN_{Vs}$ to increased electrical excitability

To test how increased electrical excitability affects gene expression in  $LN_{Vs}$ , brains were dissected from larvae with an  $LN_V$ -specific Gal4 (*Pdf-Gal4*) [14] expressing *UAS-NaChBac* (*NCB*), a low activation threshold voltage-gated  $Na^+$  channel from bacteria, which increases  $LN_V$  excitability in the evening [15]. These *Pdf>NCB* larvae also contained a *Pdf-RFP* transgene to label  $LN_{Vs}$  for flow cytometry. Larval  $LN_{Vs}$  were isolated on day 2 in constant darkness (DD) at CT15 (subjective evening) when expression of core clock genes like *period* (*per*) and *timeless* (*tim*) is high.

We compared expression profiles from  $LN_{Vs}$  expressing *NCB* at CT15 (*NCB-15*) with those from control  $LN_{Vs}$  isolated at CT15 (*WT-15*). We identified 336 mRNAs either up- or down-regulated in *NCB-15* ( $p < .01$ , False Discovery Rate (FDR)  $< 8\%$ , fold change (FC)  $> 1.5$ , Table S1). In separate  $LN_V$  GeneChip experiments, we identified 249 “circadian transcripts” with differential expression in wild type larvae between CT3 (subjective morning, *WT-3*) and CT15 (Table S2). We noticed that 81 of the 336 *NCB*-regulated transcripts show circadian regulation in wild-type  $LN_{Vs}$  (Fig. 1A–B and Table S2). Interestingly, circadian transcripts up-regulated in *NCB-15* are more highly expressed at CT3 than at CT15 in wild type  $LN_{Vs}$ . For example, *Clock* (*Clk*) and *cryptochrome* (*cry*) transcript levels were up-regulated 8-fold ( $p < .001$ ) and 5-fold ( $p < .005$ ) respectively in *NCB-15*. A scatter plot of fold-change versus p-value in the *NCB-15* versus *WT-15* comparison (Fig. 1A) illustrates circadian genes high at *WT-3* (blue circles) or *WT-15* (red circles) among the ~19,000 probesets (grey “+”). Of the 110 circadian mRNAs normally high at *WT-3*, 36 are significantly up-regulated in *NCB-15*, and conversely, 45 of 139 of circadian mRNAs normally high at *WT-15* are significantly down-regulated in *NCB-15* ( $p < .01$ ,  $FC > 1.5$ ). We also noticed a clear split in how expression of the remaining circadian transcripts is affected by *NCB* at CT15. Although many fall below the  $p < .01$  significance

line, genes normally higher at CT3 than CT15 tend to be on the right of the scatter plot (i.e. up-regulated in *NCB-15*) and the converse for genes normally higher at CT15 (Fig. 1A).

To quantify the relationship between *NCB*-regulation and circadian phase for the full set of circadian mRNAs, we plotted wild-type circadian fold differences (*WT-3* vs. *WT-15*) for all 249 circadian mRNAs against their *NCB*-fold differences (*NCB-15* vs. *WT-15*, Fig. 1B). The linear correlations are highly significant ( $R=0.47$ ,  $p<2.1E-7$  and  $R=0.61$ ,  $p<2.0E-15$  for up- and down-regulated mRNAs, respectively), indicating that artificially hyperexciting  $LN_{\nu,s}$  via *NCB* creates a morning-like transcript profile in the evening.

### The *NCB* effect persists in the absence of a functional molecular oscillator

A central notion in circadian biology is that a clock cell's circadian transcription follows its molecular clock. However the *NCB* data reveal a potent influence of electrical state on circadian gene expression. The *per<sup>0</sup>* mutation stops circadian rhythms in gene expression since it removes the key transcriptional repressor that inhibits the activity of CLOCK (CLK) and CYCLE (CYC), the transcriptional activators that together activate *per* and *tim* expression [16]. To test whether *NCB* requires an intact molecular oscillator to impose a morning-like transcriptional state in the evening, we performed GeneChips on  $LN_{\nu,s}$  expressing *NCB* in a *per<sup>0</sup>* mutant background isolated at CT15 (*per<sup>0</sup>; NCB-15*).

We identified 246 differentially expressed mRNAs when comparing *per<sup>0</sup>; NCB-15* to *WT-15* (Table S3). Of these, 64 mRNAs are circadian and 44 overlap with the *NCB-15* set (Table S3). Fig. 1C superimposes these 246 mRNAs (colored "+") on the original *NCB-15* versus *WT-15* comparison. There is a clear pattern: mRNAs up-regulated in *per<sup>0</sup>; NCB-15* also tend to be up-regulated in *NCB-15*, and mRNAs down-regulated in *per<sup>0</sup>; NCB-15* tend to be down-regulated in *NCB-15*.

To quantify this relationship, we compared wild-type circadian fold differences for all 249 circadian mRNAs with their *per<sup>0</sup>; NCB*-fold differences. The positive correlation is highly significant ( $R=0.27$ ,  $p<0.005$  and  $R=0.71$ ,  $p<9.0E-23$  for up- and down-regulated mRNAs, respectively, Fig. 1D). Thus hyperexciting  $LN_{\nu,s}$  induces a morning-like transcriptional program even without *per*.

### *NCB*-regulated transcripts are temporally inducible

Altered synaptic activity can disrupt neural network development and lead to compensatory molecular changes [17]. Furthermore, some gene expression changes observed in mutant *Drosophila* with chronic nervous system hyperexcitation were not seen with acute excitation [2]. We tested if inducing *NCB* expression in larval  $LN_{\nu,s}$  after their differentiation recapitulates the effects of constitutive *NCB*-expression. For this, we added a temperature sensitive *tubulin-Gal80<sup>ts</sup>* transgene (*tub-Gal80<sup>ts</sup>*, [18]) into the *Pdf-Gal4; UAS-NCB* background to control *NCB* expression.

Larvae were raised in Light:Dark (LD) cycles at 20°C so that Gal80<sup>ts</sup> repressed Gal4 activity to prevent *NCB*-expression during  $LN_{\nu}$  development and entrainment. Larvae were then shifted to DD at 30°C, inactivating Gal80<sup>ts</sup> to induce *NCB* expression and dissected at CT15 on day 2 in DD (Fig. 1E). We generated expression profiles from  $LN_{\nu,s}$  from *Pdf-RFP/Pdf-Gal4; tub-Gal80<sup>ts</sup>/UAS-NaChBac* larvae (*NCB-ind-15*) and  $LN_{\nu,s}$  from larvae following the same temperature regime without a *NCB* transgene (*WT-ind-15: Pdf-RFP/Pdf-Gal4; tub-Gal80<sup>ts</sup>/+*).

We found 315 mRNAs differentially-expressed between *NCB-ind-15* and *WT-ind-15* (*NCB-ind-15* mRNAs, Table S4). Comparing the overlap between *NCB-ind-15*, *NCB-15*, and circadian datasets, 50 of the 315 *NCB-ind-15* mRNAs overlap with the *NCB-15* dataset,

and 20 of these 50 transcripts are normally expressed with a circadian rhythm (Table S4). We also noted significant positive correlations ( $R=0.49$ ,  $p<7.7E-8$ ;  $R=0.47$ ,  $p<7.7E-9$ ) between levels of the 249 circadian transcripts in the *NCB-15* and *NCB-ind-15* conditions (Fig. 1F). Thus hyper-excitation affects circadian transcripts even when induced post-differentiation. However, 75% of the 50 mRNAs altered by chronic and induced *NCB* show reduced fold-change and statistical significance in the induced condition, which likely reflects a cumulative and/or stronger effect of constitutive *NCB* expression. The mRNAs that only respond to chronic *NCB*-expression may represent homeostatic responses to persistent hyper-excitation. However, the ability of *NCB* to alter circadian gene expression even when more acutely induced is consistent with the idea that neuronal activity may normally help generate circadian rhythms in  $LN_V$  gene expression.

### Hyperpolarizing $LN_V$ s in the morning induces an evening-like circadian expression pattern

If the changes in gene expression induced by *NCB* in  $LN_V$ s are a function of electrical polarity, many could occur with a reciprocal manipulation. To test this, we used the mammalian Inward Rectifier  $K^+$  channel *Kir2.1* (*Kir*) since *Kir*-expressing adult  $LN_V$ s are hyperpolarized and lose spontaneous action potential firing [10]. *Kir*-expressing  $LN_V$ s were isolated at CT3 and CT15 (*Kir-3* and *Kir-15*).

To identify changes in expression caused by decreased  $LN_V$  excitability, *Kir-3* was compared to control *WT-3*. Applying the same cutoffs for differential regulation as for *NCB*, we identified 319 *Kir*-regulated mRNAs (Fig. 2A and Table S5). Many (50/319) *Kir*-regulated mRNAs show circadian oscillations in wild-type  $LN_V$ s, with a correlation between their normal phase (high at *WT-3* or *WT-15*) and the direction of *Kir*-regulation (Fig. 2A and Table S5). To quantify this relationship, we compared wild-type circadian fold differences (*WT-3* vs. *WT-15*) for each of the 249 circadian mRNAs with their *Kir*-fold differences (*Kir-3* vs. *WT-3*) (Fig. 2B). We observed a significant negative correlation, with mRNAs high at *WT-3* down-regulated in *Kir-3* ( $R=-0.36$ ,  $p<9.2E-5$ ) and mRNAs high at *WT-15* up-regulated in *Kir-3* ( $R=-0.39$ ,  $p<2.1E-6$ ).

However, there were less dramatic differences in the *Kir-15* vs. *WT-15* comparison (Fig. 2C–D). *Kir* does alter expression of some circadian genes normally high at *WT-15* ( $R=0.27$ ,  $p<0.005$ ), consistent with the documented effects of *Kir* on some clock protein oscillations [9]. However, *Kir* does not impose an overall morning-like expression profile in the evening, since expression of most circadian genes normally higher at CT3 than CT15 is relatively unchanged in *Kir-15*  $LN_V$ s (Fig. 2D). Thus *Kir* specifically induces an evening-like expression profile in the morning rather than reversing the circadian transcriptional profile independent of sampling time.

The resting membrane potential of s- $LN_V$ s (the adult version of larval  $LN_V$ s) shows diurnal variations, with increased excitability around dawn [19]. Strikingly, we found that hyperexciting  $LN_V$ s via *NCB* in the evening induced a morning-like transcript profile, while hyperpolarizing  $LN_V$ s via *Kir* in the morning created an evening-like profile. The traditional view has been that the molecular clock encodes time-of-day by driving the activity of properly timed neural outputs. The data here indicate the inverse relationship: neural activity can bi-directionally regulate circadian gene expression.

### Electrical activity interacts with the core transcriptional activators

*NCB* up-regulation of morning genes and down-regulation of evening genes persisted in *per<sup>0</sup>* mutant  $LN_V$ s, indicating that electrical activity can circumvent the repression step in the molecular clock. We next examined if electrical activity interacts with the clock transcriptional activators. For this, we generated expression profiles of  $LN_V$ s isolated at CT3

expressing *Kir* in a *cyc<sup>0</sup>* mutant background (*cyc<sup>0</sup>; Kir-3*). We chose this genotype because CLK/CYC activates evening genes including *per*, *tim*, *PAR-domain protein 1 (Pdp1)*, and *vri* (*vri*), while morning genes such as *Clk* and *cry* are highly expressed in *cyc<sup>0</sup>* mutants [20, 21]. However, evening genes are up-regulated while morning genes are down-regulated in *Kir-3* LN<sub>v</sub>s (Fig. 2A–B). Thus it seemed informative to assay circadian gene expression with two conflicting manipulations (*Kir* and *cyc<sup>0</sup>*).

Fig. 2E shows that *Kir* expression does not up-regulate mRNAs normally high in the evening in a *cyc<sup>0</sup>* mutant background (*cyc<sup>0</sup>; Kir-3* vs. *WT-3*,  $R=0.003$ ,  $p>0.9$ ). However, *cyc* was not essential for the down-regulation of the mRNAs high at *WT-3* ( $R=-0.30$ ,  $p<0.01$ ), raising the possibility that some mRNAs high at *WT-3* are controlled by activity-dependent transcription factors.

The core molecular clock drives rhythmic electrical activity in clock neurons [19, 22–24]. The data here reveal that electrical activity cooperates with the core clock transcriptional activators to regulate circadian transcription (Fig. 2E). We propose that this mutually dependent interaction helps determine the phase and/or amplitude of circadian oscillations for many mRNAs.

Expression of *Kir* or *NCB* in LN<sub>v</sub>s affects behavioral rhythms and can change molecular clock rhythms [9, 11]. However, this has only been characterized at the protein level for TIM, PER and PDP1. To understand the extent of electrical effects on core clock gene expression, we examined which are regulated by neuronal excitability. A heat map showing expression of the core *Drosophila* clock genes across genomic experiments (Fig. 2F) revealed that some core clock mRNAs are sensitive to altered neuronal activity ( $FC>1.5$ ,  $p<0.01$ ). The normally low *Clk* and *cry* RNA levels at CT15 were up-regulated by *NCB* whereas *cyc* was up-regulated by *Kir* at CT3. *tim* mRNA levels were slightly down-regulated in *NCB-15* but not in *Kir-3*. *Pdp1* mRNA levels were not affected by *Kir* or *NCB* expression and *per* mRNA levels are only down-regulated ~3-fold when comparing *Kir-15* vs *WT-15*. These modest effects on *per*, *tim* and *Pdp1* RNA levels suggest that post-transcriptional regulation also contributes to the effects of electrical activity on clock protein abundance [9, 11]. Overall, Fig. 2F indicates that altering LN<sub>v</sub> excitability fine-tunes core clock mRNA levels. Fig. 2F is also in line with the modest effects on PER protein rhythms with inducible *Kir* expression in LN<sub>v</sub>s [12]. Thus the most dramatic effects of electrical activity are not on core clock genes (see Tables S1 and S4).

### Interactions between electrical activity and circadian transcription

The data so far has demonstrated bi-directional regulation of the circadian transcriptional program with reference to the electrical state of LN<sub>v</sub>s. Since the electrical activity in wild-type LN<sub>v</sub>s shows daily changes [19, 24], electrically regulated mRNAs could have circadian expression as a result of rhythmic LN<sub>v</sub> excitability. To search for evidence of this, we measured the circadian changes in expression for all 336 *NCB*-regulated mRNAs (the inverse of Fig. 1 & 2). We plotted the *NCB*-fold differences for these mRNAs (*NCB-15* vs. *WT-15*) against their circadian fold differences (*WT-3* vs. *WT-15*). The linear correlations are highly significant ( $R=0.47$ ,  $p<6.7E-11$  and  $R=0.52$ ,  $p<1.3E-12$  for up- and down-regulated mRNAs, respectively, Fig. 3A). Importantly, the mRNAs up-regulated by *NCB* are normally higher in the morning while the down-regulated *NCB* transcripts are normally higher in the evening. These data indicate many of the transcripts regulated by *NCB* likely normally exhibit circadian changes in expression in wild-type LN<sub>v</sub>s.

Next we plotted *Kir* fold differences for all 319 *Kir* regulated mRNAs (*Kir-3* vs. *WT-3*) against their circadian fold differences (*WT-3* vs. *WT-15*). Here too, we found a significant negative correlation, with mRNAs up-regulated by *Kir* normally higher at *WT-15* ( $R=-0.36$ ,

$p < 9.2E-7$ ) and mRNAs down-regulated by *Kir* normally higher at *WT-3* ( $R = -0.39$ ,  $p < 1.6E-6$ , Fig. 3B). Together, these data indicate that a significant fraction of electrically regulated mRNAs exhibit circadian changes in  $LN_v$ s: i.e. genes activated by increased electrical activity tend to be higher in the subjective morning and mRNAs up-regulated by hyper-polarization tend to be higher in the evening.

However, we had found that only 111 of 616 (18%) of the *NCB* and/or *Kir* regulated mRNAs are under circadian control with  $p < 0.01$  (Fig. 3C). To evaluate the relationship between these high confidence activity-dependent transcripts and circadian phase, we measured the p-values for rhythmic expression (different between *WT-3* vs. *WT-15*) for all 336 *NCB*- and 319 *Kir*-regulated mRNAs. We found that 44.3% of *NCB*-regulated mRNAs (149/336) and 39.2% of *Kir*-regulated mRNAs (125/319) show circadian regulation with  $p < 0.05$  (Fig. 3D). Thus a large fraction of electrically regulated mRNAs display circadian expression, but some were filtered out using our original cutoffs ( $FC > 1.5$ ,  $p < 0.01$ , Fig. 3C). Our analysis may also underestimate the extent of circadian regulation in the *NCB* and *Kir* regulated mRNAs since we only sampled two timepoints per day and may have missed genes whose expression peaks with different phases.

We also re-examined how many circadian transcripts are altered by *NCB* and *Kir* with  $p < 0.05$  by plotting the p-values of all 249 circadian transcripts (*WT-3* vs. *WT-15*,  $FC > 1.5$ ,  $p < 0.01$ ) from *NCB-15* vs. *WT-15* and *Kir-3* vs. *WT-3* comparisons (Fig. S1A and S1B). 33% and 20% of the 249 circadian mRNAs are *NCB*- and *Kir*-regulated with a cut-off of  $p < 0.01$  (Fig. 3C). However, these numbers rise to 52% for *NCB* and 40% for *Kir* when the p-value is relaxed to 0.05 (Fig. S1). Overall these analyses indicate a major overlap between activity-dependent and circadian gene expression in  $LN_v$ s.

To better understand the directionality of electrical effects on circadian transcripts, we plotted the fold change differences of the circadian transcripts in response to *NCB* or *Kir* without filtering by p-value or fold-change cutoffs. *Kir* down-regulates 92% (101/110) of the transcripts normally high at CT3 (*Kir-3* vs *WT-3*) while *NCB* up-regulates 85% (94/110) of these transcripts (*NCB-15* vs *WT-15*) (Fig. S1C). Conversely, *Kir* up-regulates 84% (117/139) of the transcripts normally high at CT15 (*Kir-3* vs *WT-3*) while *NCB* down-regulates 91% (126/139) of these transcripts (*NCB-15* vs *WT-15*) (Fig. S1D). These data further demonstrate the dramatic reorganization of  $LN_v$  circadian gene expression by *Kir* and *NCB*.

### Validating the GeneChip data

We used qPCR to validate rhythmic expression of several transcripts and the effects of neuronal activity. To provide higher resolution, larval  $LN_v$ s were isolated at four time-points on day 2 in DD. Initially, we measured the levels of 4 core clock transcripts (*per*, *tim*, *Clk* and *cry*) and confirmed circadian regulation (Fig. 4A,  $p < 0.01$ , one-way ANOVA for *per* and *tim*,  $p < 0.05$  for *cry*, and  $p = 0.05$ , t-test for *Clk*, CT5 vs CT11).

According to our GeneChip experiments, *NAD-dependent methylenetetrahydrofolate dehydrogenase* (*Nmdmc*), *Calbindin 53E* (*Cbp53E*), *TAK1-associated binding protein 2* (*Tab2*) and *Pka-C1* mRNAs exhibited strong circadian changes with higher levels in the subjective morning (*WT-3*) than evening (*WT-15*) and were electrically regulated. The additional time-points for qPCR in Fig. 4B give more accurate circadian expression profiles: *Nmdmc* peaked at CT5 with a robust oscillation ( $p < 0.0001$ , one-way ANOVA); *Tab2* had a sharp peak at CT11 ( $p < 0.05$ ); *Cbp53E* showed circadian variation with higher levels at CT23 than CT11 and CT17 ( $p < 0.05$ ); and *Pka-C1* had higher levels during the subjective day than at CT17 ( $p < 0.05$ , t-test). In spite of some differences between their profiles, all

these transcripts exhibit circadian profiles with higher levels in the subjective day than early evening (CT17), coinciding with their GeneChip values ( $WT-3 > WT-15$ ).

We also measured levels of these 8 transcripts in *Kir* or *NCB* expressing  $LN_v$ s isolated at two time-points (CT11 and CT23) on day 2 in DD. The GeneChip data showed that, of the core clock regulators, expression of *Clk*, *cry*, *cyc*, *tim* and *per* is altered by neuronal excitability with  $FC > 1.5$ ,  $p < 0.01$ . qPCR revealed that *per* is *Kir* and *NCB* sensitive at both time-points (t-test,  $p < 0.05$ ) and that *tim* abundance at CT11 was decreased by both *Kir* and *NCB* ( $p < 0.01$ ). *Clk* and *cry* expression was up-regulated by *NCB* expression ( $p < 0.05$ ). Of the electrically-sensitive transcripts, *Nmdmc* was bi-directionally regulated by *Kir* and *NCB* (t-test,  $p < 0.05$ ) and *Cbp53E* and *Pka-C1* lost their time-dependent regulation in *NCB*-expressing  $LN_v$ s ( $p > 0.1$ ). Finally, *Tab2* levels were reduced by *Kir* expression ( $p < 0.01$ ) and up-regulated by *NCB* at CT23 ( $p < 0.05$ ).

These findings validate that electrical activity regulates the levels of some circadian transcripts, indicating a central role for neuronal excitability in regulating  $LN_v$  gene expression. However, changes in  $LN_v$  electrical state do not completely activate or shutdown core clock transcripts, but rather fine-tune their levels.

### A role for activity-dependent genes in circadian behavior

Activity-dependent genes regulate cellular processes that alter neuronal function and behavior e.g. [5, 25]. To test if electrically regulated transcripts affect circadian behavior, we used RNAi to knockdown their expression in clock neurons *in vivo* and measured adult locomotor rhythms.

Focusing on genes with known functions, we screened 41 transgenic lines that target 31 genes. We identified 19 RNAi lines (14 genes) whose expression in clock neurons altered the period of locomotor rhythms ( $p < 0.05$ , one-way ANOVA with post-hoc Tukey-Kramer multiple-comparison, Fig. 4C–D; Table S6). RNAi transgenes that target 4 genes in the inositol phosphate/diacylglycerol pathway [*CDP diglyceride synthetase (CdsA)*, *Protein C kinase 53E (Pkc53E)*, *Diacyl glycerol kinase (Dgk)* and *retinal degeneration A (rdgA)*] altered circadian period. The most dramatic period-altering phenotype (26hr) came from targeting *Cbp53E*, a  $Ca^{2+}$ -buffering protein, highly related to mammalian Calbindin, implicated in circadian rhythms and light entrainment of the SCN [26]. Finally, an RNAi targeting a regulator of the CREB pathway, *Pka-C1*, made flies arrhythmic. The phenotypes results from this screen indicate that electrically regulated genes are important in generating 24hr rhythms and provide insight into electrically sensitive signaling pathways that regulate circadian behavior.

### CrebA and CrebB protein levels are electrically sensitive and are predicted to bind many *NCB* and *Kir*-targets

To identify transcription factors (TFs) that bind sequences common to co-regulated genes, we used REDUCE to search for novel n-mers over-represented among genes sharing similar fold-change responses within each condition [27]. We then used TESS to search TF binding site databases to identify factors known to interact with these motifs [28]. REDUCE found several motifs (Table S7), including an 8-mer perfect match to the mammalian CRE-motif (TGACGTCA), which is bound by the ATF/CREB family of TFs. Occurrence of this motif correlates with a set of 31 mRNAs down-regulated in *Kir-3* compared to *WT-3* ( $p < 5.0E-5$ , Table S7). Using mammalian CREB positional weight matrices (PWMs) as inputs, we also tested if CRE-containing genes are enriched among the *NCB-15* and *Kir-3* datasets. We scanned the promoters (3kb) and first introns of all genes in the genome, and identified significant enrichment of putative CRE-targets among the differentially expressed genes for

both *NCB-15* vs. *WT-15* and *Kir-3* vs. *WT-3* comparisons (Hypergeometric test,  $p < .005$ , Table S7).

These data were striking because our cluster analysis (Fig. S2) had revealed that *cAMP response element binding protein A (CrebA)* and *Activating transcription factor-2 (Atf-2)*, two members of the ATF/CREB-family of transcription factors, exhibited circadian regulation (higher in *WT-3* than *WT-15*) and were bi-directionally regulated by electrical activity (high in *NCB-15* and low in *Kir-3*). Expression of *CrebB*, a cAMP-responsive member of this family, is also up-regulated in *NCB-15* (~8-fold,  $p < 0.05$ ). Expression of *Epac* and *cAMP-dependent protein kinase 1 (Pka-C1)*, key components of cAMP signaling, also cluster with *CrebA* and *Atf-2* respectively (Fig. S2). The coherent expression pattern of cAMP- and CREB-related genes suggests the importance of transcriptional regulation of this pathway in  $LN_v$ s in response to *NCB* and *Kir*.

Based on these bioinformatic and expression data, we tested if Creb protein levels follow transcript levels in larval  $LN_v$ s. We measured CrebA and CrebB levels in LD cycles and detected rhythms in their abundance: CrebA levels were higher during the day (ZT4 and ZT10) than at night (ZT16 and ZT22) and CrebB levels were highest at ZT11 (Fig. 5A–D,  $p < 0.01$ , one way ANOVA followed by Tukey comparison). Next, we tested whether altering  $LN_v$  neuronal activity changes CrebA and CrebB protein levels. *NCB* increased CrebA and CrebB levels at the end of the night, when their levels are normally low, while *Kir* decreased CrebA and CrebB abundance at the end of the day, when their levels are normally high ( $p < 0.01$ , Fig. 5A–D). Together, these data further validate the GeneChip expression data by showing that electrical alteration mis-regulates CrebA and CrebB protein levels.

Mammalian CREB plays a role in responding to neuronal activity e.g. [4] and in light-resetting of SCN pacemaker neurons [29]. Furthermore, *CrebB* mutant flies have either ~1hr shorter periods than wild type flies or are arrhythmic [30]. We tested the behavioral role of *CrebA* and *CrebB* in  $LN_v$ s by over-expression and found that this lengthened the circadian period to 24.9hr and 25.0hr respectively (Fig. 5E, Table S8). *UAS-CrebA* resulted in even longer rhythms (25.6hr) when expressed with *tim-Gal4*, a stronger and broader clock neuron driver [31]. Flies had normal period rhythms (23.8hr) when *UAS-CrebA* was expressed in all clock neurons except  $LN_v$ s (using *tim-Gal4* and *Pdf-Gal80*), indicating that the long period rhythm requires *CrebA* over-expression in  $LN_v$ s (Table S8). Shimizu et al. [32] detected ATF-in adult  $LN_v$ s and suggested that *Atf-2* regulates sleep. We found that over-expressing *Atf-2* either in  $LN_v$ s or in all clock neurons gave a period-lengthening similar to *CrebA* and *CrebB* over-expression (Table S8).

Starting by identifying a set of co-regulated genes, our analyses revealed that members of the *Drosophila* Creb/ATF family likely regulate a set of electrically sensitive circadian genes. Since *CrebA*, *CrebB* and *Atf-2* RNA and/or protein levels are regulated by neuronal activity in  $LN_v$ s, and since normal levels of these proteins are required for 24hr rhythms, they are strong candidates for mediating the effect of electrical activity on circadian gene expression.

## Discussion

### Pacemaker neuron excitability can regulate circadian gene expression

By analyzing the transcriptome of a single pacemaker cell type to spatially, temporally, and directionally controlled changes to its excitability, we identified large sets of mRNAs whose levels can be regulated by the electrical state of  $LN_v$ s. Strikingly, expression of circadian genes in  $LN_v$ s correlate with the nature of the alterations: hyperexcitation triggers a morning-like expression profile and hyperpolarization an evening-like profile. These results



demonstrate the ability of neural activity to reprogram the transcriptional oscillations in  $LN_V$ s. Some of this reprogramming occurs through an interaction with the core clock activators but many mRNAs normally high in the morning likely use additional transcriptional mechanism(s) for rhythmic expression. Although constitutively manipulating the electrical state of a neuron is not physiological, the potent transgenes used here allowed us to identify how strongly and broadly electrical activity can affect  $LN_V$  gene expression. Whether the physiological changes in  $LN_V$  electrical activity between morning and evening influence  $LN_V$  gene expression so profoundly remains to be tested. Zeitgebers, such as light:dark cycles, can stabilize the phase and strengthen the amplitude of molecular oscillations [33, 34]. Our data suggest that electrical activity acts as an endogenous zeitgeber to help drive robust molecular oscillations in  $LN_V$ s.

Given that many synchronizing factors affect the electrical properties of clock neurons [35], a circadian oscillator that incorporates electrical state by adjusting its circadian transcriptome is a potential mechanism for intercellular coupling in circadian networks. Coupling between pacemaker neurons in the mammalian SCN can rescue circadian oscillations in core clock gene mutants [36], although the molecular mechanism is unclear. Our data showing that hyperexcitation of  $LN_V$ s imposes a morning-like transcriptional program even in *per<sup>0</sup>* mutants, can be viewed as an extreme example of how electrical state can impose time-of-day onto an oscillator.

### Activity-dependent expression of a signal transduction pathway and its implications for circadian network synchrony

In the SCN, the circadian transmitter vasointestinal peptide (VIP) activates the CREB-pathway which may help synchronize individual pacemakers [37]. Bi-directionally regulated cAMP/CREB-related genes and the behavioral phenotypes of mis-expressing Creb family members indicate their importance in  $LN_V$ s. Circadian variations in the availability of CREB-pathway components suggests a signaling pathway with time-of-day representation which could rhythmically regulate  $LN_V$ s' response to synchronizing factors in the clock network and/or to entrainment cues. Thus this pathway could admit correctly timed circadian cues and exclude mistimed inputs. The response of the CREB pathway to electrical activity suggests a positive feedback loop where membrane excitability increases pathway activity, which in turn sustains the pathway's responsiveness to membrane excitability. An autoregulatory role for CREB has been documented in other systems [38] and we noticed the presence of CREs within the *CrebA* regulatory region (Table S8). Thus we suggest that the CREB-pathway not only relays  $LN_V$  membrane activity, but also *gates*  $LN_V$  responsiveness to membrane activity as a function of time.

Activity-dependent gene expression is critical in synaptic plasticity [1] and even in Alzheimer's disease [39]. However, activity-dependent transcription in circadian rhythms has been largely unexplored outside of clock resetting, arguably because the CLK/CYC/PER intracellular feedback loop has been viewed as the primary determinant of rhythmic transcription in *Drosophila* pacemaker neurons. The work here leads us to propose that neural activity acts as an internal zeitgeber and that activity-dependent transcription is a core feature of the multi-oscillator circadian network.

## Experimental Procedures

### $LN_V$ isolation, RNA amplification and analysis

3<sup>rd</sup> instar larvae were kept in standard LD cycles, transferred to DD and 50 brains dissected centered at either CT3 or CT15, which took ~30 minutes. Dissected brains were dissociated and sorted by flow cytometry as described [40]. mRNA from 150–300 RFP+ cells was

amplified using the NuGen WT-Ovation™ Pico System. The resulting single-stranded DNA was either labeled and hybridized to Affymetrix *Drosophila* 2.0 GeneChips, with 3 biological replicates for each timepoint, or qPCR was performed on 30ng amplified LN<sub>v</sub> samples with 2–3 biological replicates per timepoint.

Additional experimental procedures including fly stocks, GeneChip data analysis, BioInformatic analyses, qPCR primer sequences, behavioral analyses are described in the Supplementary information online.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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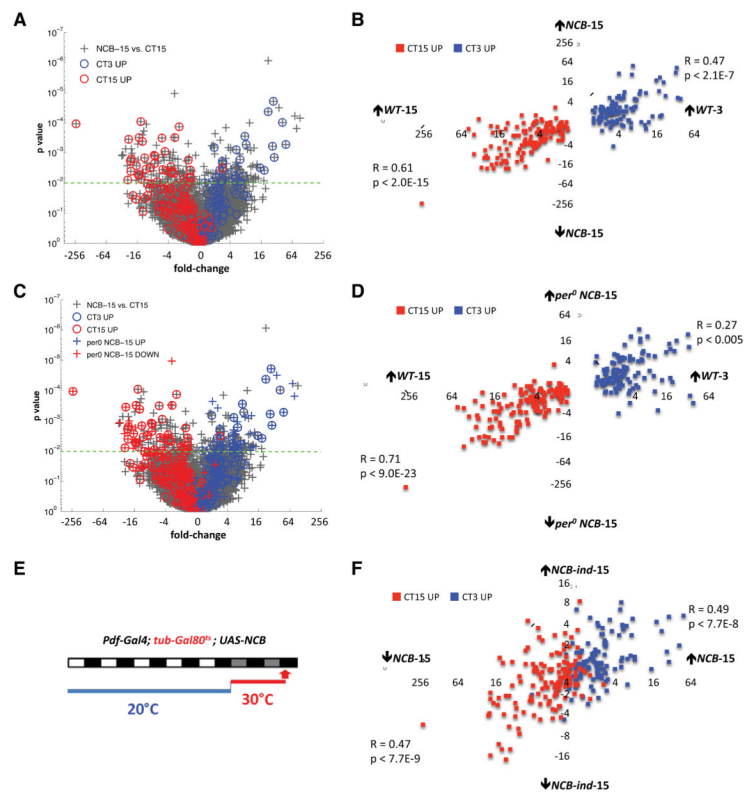
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### Highlights

Pacemaker neuron electrical activity can switch circadian gene expression

Many genes expressed with a circadian rhythm are activity-dependent

Creb family members may link pacemaker neuron activity to circadian gene expression



**Figure 1. Hyperexciting LN<sub>v</sub>s creates a morning-like transcript profile in the evening**

(A) Scatter-plot of fold change versus p-value for all mRNAs in the *NCB-15* vs. *WT-15* comparison (grey “+”). We identified 336 *NCB*-regulated mRNAs using  $p < .01$ ,  $FDR < 8$  and  $FC > 1.5$  as cut-offs. The 249 circadian genes are identified with blue (high at CT3) or red (high at CT15) circles.

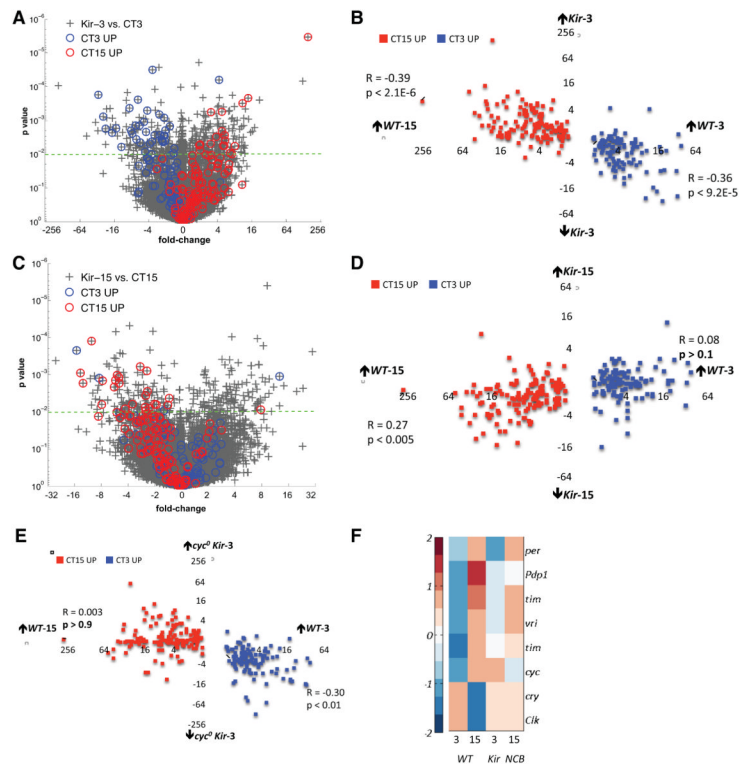
(B) Scatter-plot of wild type circadian fold differences (*WT-3* vs. *WT-15*) for each of the 249 circadian mRNAs against their *NCB*-fold differences (*NCB-15* vs. *WT-15*). Blue boxes: mRNAs normally high at *WT-3*. Red boxes: mRNAs normally high at *WT-15*. Pearson correlation coefficients (*R* values) were transformed to test hypotheses of no correlation (*p* values).

(C) Re-plot of the *NCB-15* versus *WT-15* comparison from Figure 1A with the addition of 246 mRNAs either up- (blue “+”) or down-regulated (red “+”) in the *per<sup>0</sup>; NCB-15* vs. *WT-15* comparison.

(D) Scatter-plot of circadian fold differences (*WT-3* vs. *WT-15*) for all circadian mRNAs against their *per<sup>0</sup>; NCB*-fold differences (*per<sup>0</sup>; NCB-15* vs. *WT-15*).

(E) *Pdf-Gal4>NCB/tub-gal80<sup>S</sup>* larvae were entrained in LD cycles at 20°C, when *Gal80<sup>S</sup>* represses Gal4 activity to prevent *NCB*-expression. Larvae were transferred to DD at 30°C to induce *NCB*-expression (*NCB-ind-15*) and dissected at CT15 on day 2 in DD. Control larvae without a *NCB* transgene went through the same temperature shifts (*Pdf-Gal4>+/tub-gal80<sup>S</sup>*, *WT-ind-15*).

(F) Scatter-plot of fold differences for each of the 249 circadian mRNAs between the *NCB-15* vs. *WT-15* and *NCB-ind-15* vs. *WT-ind-15* comparisons.

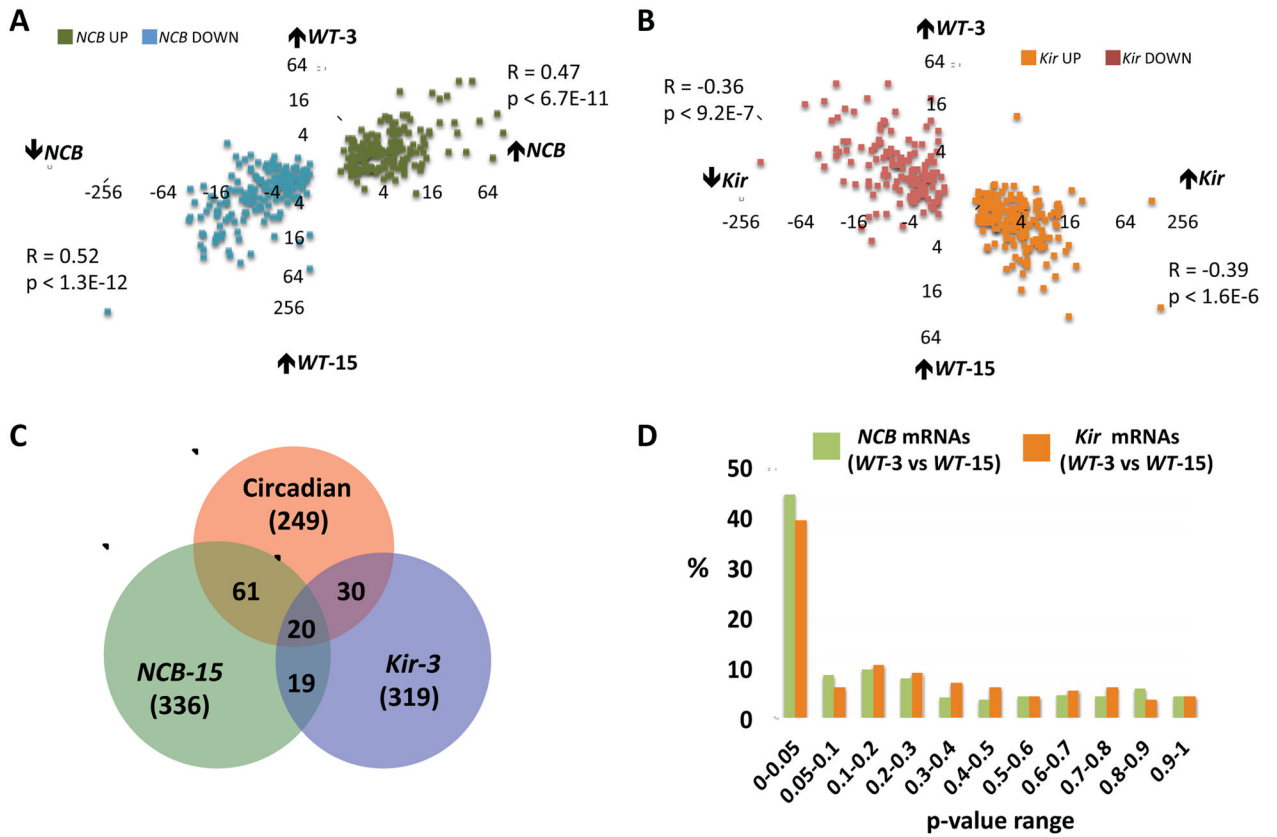


**Figure 2. Hyperpolarizing  $LN_v$ s at morning induces an evening-like circadian expression pattern (A&C)** Scatter-plot of fold change versus p-value for all mRNAs in the *Kir-3* vs. *WT-3* (A) and *Kir-15* vs. *WT-15* (C) comparisons. Circadian genes (249) are identified with blue (high at *WT-3*) and red (high at *WT-15*) circles.

**(B&D)** Scatter-plot of wild type circadian fold differences (*WT-3* vs. *WT-15*) for each of the 249 circadian mRNAs against their *Kir-3* fold differences (*Kir-3* vs. *WT-3*, B) and *Kir-15*-fold differences (*Kir-15* vs. *WT-15*, D).

**(E)** Scatter-plot of wild type circadian fold differences (*WT-3* vs. *WT-15*) for all circadian mRNAs against their *cyc<sup>0</sup>; Kir-3* fold differences (*cyc<sup>0</sup>; Kir-3* vs. *WT-3*).

**(F)** Expression values for core clock mRNAs (row) across four genomic conditions (columns) standardized by mean centering (row mean = 0, row standard deviation = 1) and assigned color-map values based on their standard deviation from the row mean.



**Figure 3. A strong relationship between electrical activity and circadian gene expression in  $LN_v$ s** (A) Scatter-plot of *NCB* fold differences (*NCB*-15 vs. *WT*-15) for all 336 *NCB*-regulated mRNAs against their circadian fold differences (*WT*-3 vs. *WT*-15). mRNAs up- and down-regulated by *NCB* are in green and blue respectively.

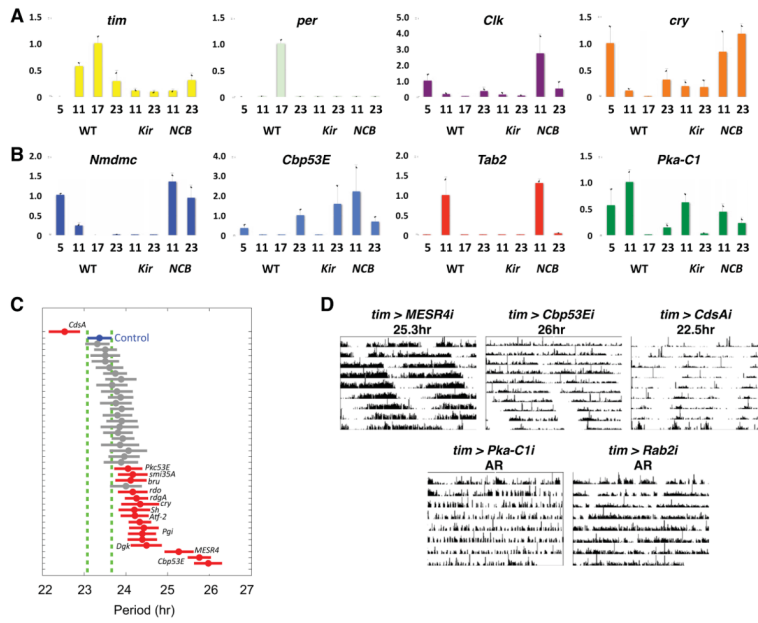
(B) Scatter-plot of *Kir* fold differences (*Kir*-3 vs. *WT*-3) for all 319 *Kir*-regulated mRNAs against their circadian fold differences (*WT*-3 vs. *WT*-15). mRNAs up- and down-regulated by *Kir* are in orange red respectively.

(C) Overlap of significantly regulated genes ( $p < 0.01$ ;  $FC > 1.5$ ) from 3 independent genomic experiments: *WT*-3 vs. *WT*-15 (red); *NCB*-15 vs. *WT*-15 (green); and *Kir*-3 vs. *WT*-3 (blue).

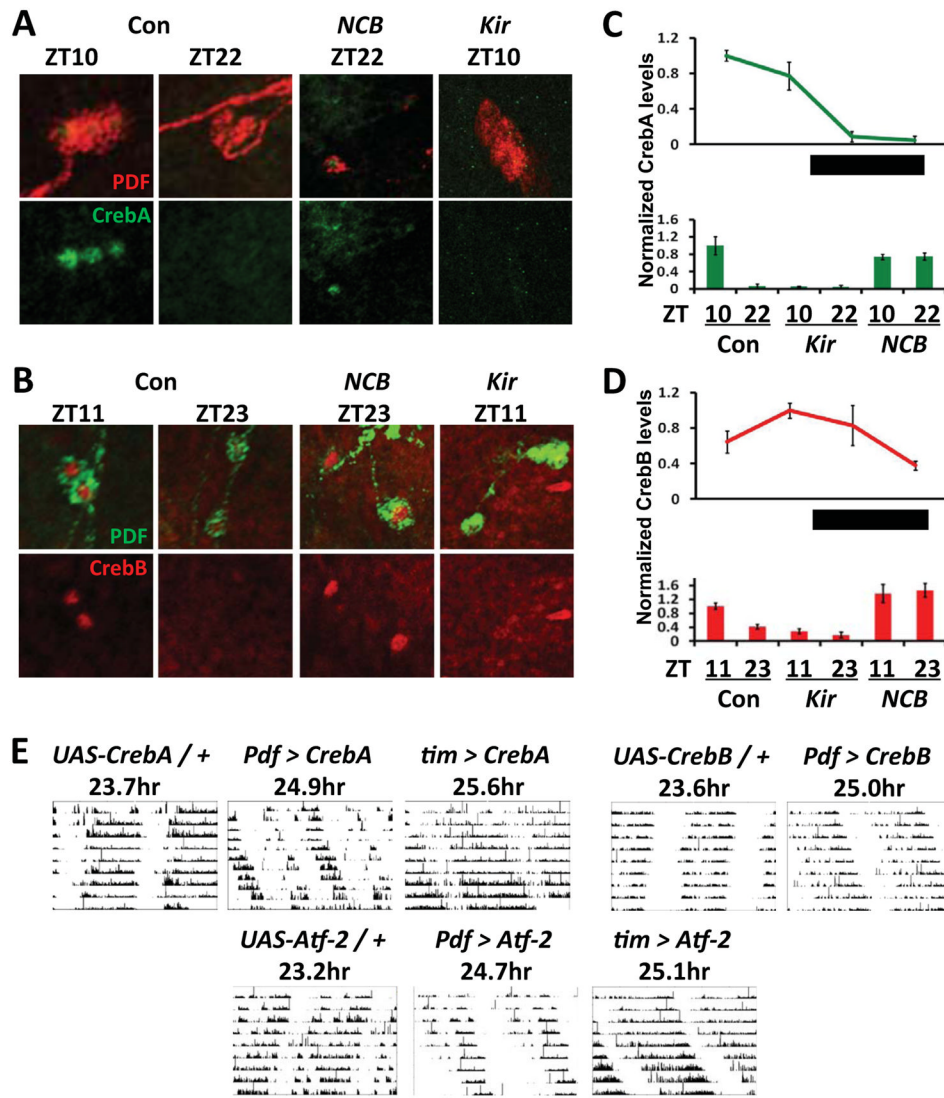
(D) p-values of 336 *NCB*-regulated mRNAs (green) or 319 *Kir*-regulated mRNAs (orange) from the *WT*-3 vs. *WT*-15 comparison.

See also Figure S1.





**Figure 4. Molecular and circadian behavioral validation of electrically sensitive genes**  
**(A)** Wild-type larval LN<sub>v</sub>s were isolated at CT5, CT11, CT17 and CT23 and *Kir* or *NCB* expressing LN<sub>v</sub>s at CT11 and CT23 on day 2 in DD. RNA amplification and qPCR are described in Experimental Procedures, with 3 replicates for each condition except *WT-17* (2 replicates) to measure expression of *tim*, *per*, *Clk* and *cry*.  
**(B)** qPCR as in A to analyze *Nmdmc*, *Cbp53E*, *Tab2* and *Pka-C1* expression.  
**(C)** *tim(UAS)-Gal4* flies were crossed to 41 *UAS-RNAi* transformants with *UAS-dcr-2* co-expressed to boost RNAi effectiveness. Group mean activity period (circle) with confidence interval are plotted. Non-overlapping intervals (red) are significantly different than control (blue),  $p < .05$  (one-way ANOVA with post-hoc Tukey-Kramer multiple-comparison).  
**(D)** Representative actograms from single RNAi-expressing flies whose activity periods were significantly lengthened (*MESR4* and *Cbp53E*), shortened (*CdsA*), or arrhythmic (AR, *Pka-C1* and *Rab2*). Period shown is the group mean.



**Figure 5. Creb family member protein levels are electrically sensitive and their over-expression alters circadian behavior**

(A) Top: Control *y w* (Con) larval brains were dissected at ZT10 or ZT22. *Pdf* > *NCB* brains were dissected at ZT22 (*NCB* ZT22) and *Pdf* > *Kir* at ZT10 (*Kir* ZT10) and stained using antibodies to CrebA (green) and PDF (to mark LN<sub>v</sub>s, red). Bottom: red channel (PDF) removed from images in top panel.

(B) As in A except that brains were stained with antibodies to CrebB (red) and PDF (green) at ZT11 and ZT23.

(C) Top: quantification of CrebA immuno-staining in wildtype (*y w*) larval brains at four time points in LD. Each data point represents the average CrebA intensity (background corrected) from 5 brain hemispheres, normalized to peak. Bottom: quantification of CrebA in *y w* (Con), *Pdf* > *Kir* (*Kir*) and *Pdf* > *NCB* (*NCB*) larval brains at ZT10 and ZT22.

(D) As in C except that CrebB immuno-staining was quantified.

(E) Top left: Representative actograms of *UAS-CrebA* flies crossed to (from left to right): control *y w* flies (*UAS-CrebA*/+), flies with two copies of *Pdf*<sub>0.5</sub>-*Gal4* (*Pdf* > *CrebA*), or *tim*(*UAS*)-*Gal4* flies (*tim* > *CrebA*). Top right: Representative actograms of *UAS-CrebB* flies crossed to either *y w* flies (*UAS-CrebB*/+) or flies with two copies of *Pdf*<sub>0.5</sub>-*Gal4* (*Pdf*

> *CrebB*). Bottom: Representative actograms of *UAS-Atf-2* flies crossed to either control *y w* flies (*UAS-Atf-2/+*), flies with two copies of *Pdf<sub>0.5</sub>-Gal4* (*Pdf > Atf-2*) or *tim(UAS)-Gal4* flies (*tim > Atf-2*). Periods shown are the group mean. See also Figure S2.