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Effects of TWIN-OF-EYELESS on *Clock* Gene Expression and Central-Pacemaker Neuron Development in *Drosophila*

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Abstract

Circadian oscillators are autonomous molecular rhythms that reside in cells to align whole organism physiology and behaviour to the 24h day. In flies, as in mammals, the oscillator operates in cells that co-express CLOCK (CLK) and CYCLE (CYC). Recent work in *Drosophila* has shown that CLK is unique in its ability to generate heterologous oscillators, indicating that *Clk*-gene-expression defines the circadian-cell-fate. Here, using standard *in vitro* and *in vivo* techniques, we show that TWIN-OF-EYELESS (TOY; dPax6) regulates *Clk*-expression in small ventrolateral neurons (s-LN_vs) that coordinate sleep-wake cycles. Crucially, *toy* binds multiple sites at the *Clk* locus; is expressed independent of CLK-CYC in LN_vs; regulates CLK protein levels under optimal photoperiodic conditions; and, sets clock-speed during endogenous free-run. Furthermore, TOY is necessary for the onset of *Clk*-expression in LN_vs during embryogenesis. We propose that TOY contributes to a transcription complex that functions upstream of the oscillator to promote *Clk*-expression in s-LN_vs.

Keywords

circadian oscillator; drosophila clock; twin-of-eyeless; central pacemaker

Introduction

Circadian oscillators in flies and mammals are driven by a core heterodimer consisting of the bHLH-PAS (basic-Helix-Loop-Helix-Per-Arnt-Sim) transcription factors CLK and CYC/Bmal1 (Brain and Muscle Arnt-Like 1; Hardin 2006; Yu and Hardin 2006; Glossop 2011). In *Drosophila*, the oscillator mechanism functions in diverse cell-types, including photoreceptors and discrete groups of central neurons (Plautz et al. 1997; Kaneko et al, 1997; Siwicki et al. 1998; Shafer et al. 2006). In all of these cells, CLK-CYC binds to E-box

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sequences to activate the transcription of several genes (*period*, *per*, *timeless*, *tim*, *vri*, *par-domain-protein-1epsilon*, *pdp1e*) whose encoded proteins feed-back to, either, inhibit the activity of CLK-CYC (PER-TIM) or, modulate transcriptional activity from the *Clk* locus (VRI/PDP1e; Hardin 2006; Yu and Hardin 2006; Glossop 2011). It is the ~24h molecular rhythm generated by this feedback mechanism that constitutes the core oscillator in clock cells.

Currently, the only factors that have been shown to function as direct regulators of *Clk* are the VRI-repressor and PDP1e-activator. These basic-zipper proteins compete for the same binding sites, V/P-boxes, at the *Clk* locus (Cyran et al. 2003). In mature oscillator cells *vri* and *pdp1e* rely on CLK-CYC for their expression. Hence, early theories posited that daily rhythms in *Clk*-transcription are maintained by sequential feedback, with acute VRI-repression yielding to chronic PDP1e activation (Cyran et al. 2003). However, recent work has questioned whether PDP1e is necessary, and/or sufficient, for *Clk*-expression. First, PDP1e does not induce circadian oscillations in neurons that are transformed by CLK, indicating that PDP1e is not sufficient to activate *Clk* (Kilman and Allada 2009). Second, analysis of head mRNA from *pdp1e*-null, *pdp1³¹³⁵*, flies has shown that the *Clk* mRNA rhythm is essentially normal under optimal light-dark cycles (LD12:12; 12h light: 12h dark). This suggests that in photoreceptors, the predominant oscillator cell-type in the head, *Clk*-expression does not require PDP1e (Zheng et al. 2009). This also appears to be the case in the brain, since CLK protein is present in central oscillator neurons of *pdp1³¹³⁵* mutants (Zheng et al. 2009). Furthermore, a minimal-*Clk* promoter that lacks canonical V/P-sites can still drive expression in most sub-groups of central pacemaker cells, including the s-LN_vs (Gummadova et al. 2009).

To address this PDP1e enigma, we screened the *Clk* locus for known consensus binding sites to identify other factors that might activate *Clk*. One sequence that was present at higher frequency than would be expected by random chance was that of the paired-homeobox-6 (Pax6)-consensus bound by TOY and EY. These Pax6 paralogues are better known for their role in eye development (Punzo et al. 2002). However, both are also expressed in numerous neurons throughout the developing and mature brain. For instance, TOY and EY show extensive overlap in expression amongst Kenyon cells (Kurusu et al. 2000; Furukubo-Tokunaga et al. 2009). Despite this knowledge, the role of TOY, or EY, in central neurons and mature photoreceptors is poorly understood. This is due, primarily, to the fact that they regulate transcription networks via complex context-dependent codes and integrated-feedback (Callaerts et al. 2001; Punzo et al. 2002; Adachi et al. 2003; Kozmik 2005; Furukubo-Tokunaga et al. 2009; Kumar 2009; Blanco et al. 2010).

Using a combination of gel-shift-assay, immunocytochemistry and reporter-gene analysis, we show that; (1) TOY binds multiple sites in the *Clk* locus, (2) *toy* is expressed in all LN oscillator cells of adult brain, (3) TOY-binding-sites enhance expression of a minimal-*Clk* promoter *in vivo*, (4) *toy* is expressed upstream of the clock mechanism in mature LN_vs, and (5) TOY is necessary for *Clk*-expression in presumptive s-LN_vs during embryogenesis. Over-expression of TOY (TOY-OE) increases CLK protein levels in mature LN_vs and accelerates the s-LN_v oscillator, generating short-period locomotor rhythms in DD that reflect CLK-OE behaviour. In contrast, reduction in *toy*-dosage causes long-period

locomotor activity that is comparable, and additive, to the effects of reducing *cyc*. Taken together, these data suggest that TOY functions directly upstream of *Clk* in the s-LN_vs.

Materials and Methods

Electrophoretic Mobility Shift Assay

TOY and EY were synthesised using the rabbit-reticulocyte system (Promega). Full-length TOY was generated using pOT2-*toy* cDNA clone GH14454 from the Drosophila Genomics Resource Center (Bloomington, IN) and full-length EY from pBSIIS-*ey* cDNA (P. Callaerts, personal communication). Lysate protein was incubated in TGKD (10mM Tris, 50mM KCl, 4mM DTT, 4% glycerol [v/v]) for 20min prior to addition of non-specific competitor (100ng/μl salmon testes DNA; Ambion), cold-competitor (10-fold) and radiolabelled-probe (10k cpm). All probes contained the 17bp paired-site and flanking bases (10 bp 5'; 7 bp 3') with exception of +681/2, which was 5' - gaattcgATTAgCTCATGCAATAATTAATttcac-3' (sense). CD19-2Ains was 5' - gaaggcgggtgGTCA CGCCTCAGTGCCCCattctc-3' (sense). Probes were made as described in Glossop et al (2003). For competition assays, DIG-labelled probe was used following manufacturers guidelines (Roche). Antibodies were added post-probe at 1:20 dilution. Reaction products were resolved at 22°C on 6% native polyacrylamide gels.

Western Blot

TnT-TOY, TnT-EY, and controls, were run on 8% SDS-polyacrylamide gels; transferred to nitrocellulose (Hybond-ECL; Amersham); blocked in milk (140mM NaCl; 10mM Tris; 0.05% Tween-20 [v/v]; 0.05% Thimerosol [w/v]; 5% milk powder) and probed for TOY or EY. Antibody combinations were; guinea-pig-α-TOY (1:5000) with goat-α-guinea-pig-peroxidase (1:1000; Sigma-Aldrich); rat-α-EY (1:5000) with goat-α-rat-peroxidase (1:1000; Sigma-Aldrich). ECL detection was used (Amersham). α-TOY and α-EY antibodies were generated against the entire C-terminal domain of TOY and EY, respectively. Hence, the N-term starts 2 amino acids after the KWREE residues of the homeodomain (U. Walldorf, personal communication).

Drosophila Stocks and Transgenics

For mis-expression, fly lines were: w;UAS-Gal4/UAS-*toy*; *pdf*-Gal4/UAS-cGFP (TOY-OE); w;UAS-Gal4/+; *pdf*-Gal4/UAS-cGFP (WT) and w;UAS-Gal4/UAS-*toy*; +/UAS-cGFP (*toy*-con), or non-UAS-cGFP equivalents. For gene-dosage experiments, *cyc*⁰¹ and *toy*Pw⁺[LacZ] were crossed into isogenic w;+;+;+. Further details are in supplemental Fig. S5C. -206*Clk*:*Gal4*^[TEmut] lines were generated as described previously for -206*Clk*:*Gal4*^[WT] (Gummadova et al. 2009). Sixteen lines were generated (1 on X; 5 on 2; 10 on 3) and crossed into w;+;+;+ prior to analysis. Transgene origins were as follows; *pdf*-Gal4 (Renn et al. 1999); UAS-Gal4 (lines 7, 12b & 22; Hassan et al. 2000); UAS-*toy* (4 & 14; Czerny et al. 1999); UAS-cGFP (Bloomington, IN); *toy*Pw⁺[LacZ]/*cP* (P. Callaerts and U. Walldorf, personal communication); -206*Clk*:*Gal4*^[WT] (Gummadova et al. 2009). All animals were reared at 25°C in LD12:12.

Co-immunofluorescence

Brains were processed as described in Gummadova et al. (2009). Primary antibodies were: guinea-pig- α -VRI (1:2000; Glossop et al. 2003), guinea-pig- α -CLK (GP50; 1:2000; Houl et al. 2008), guinea-pig- α -TOY and rat- α -EY (1:100; U. Walldorf, personal communication), rat- α -TIM (UPR41; 1:1000; Shafer et al. 2006) and rabbit- α - β GAL (1:1000; Cappell). Secondary antibodies were Cy5- α -guinea-pig, Cy3- α -rat and [FITC;Cy3;Cy5]- α -rabbit; generated in donkey (1:200; Jackson ImmunoResearch). Fluorescence was visualized on a Leica sp5 microscope with the following excitation/[emission] parameters (GFP/FITC, 488/[500-535]nm; Cy3, 543/[567-610]nm; Cy5, 633/[650-750]nm). Images were captured as sequential scans (z-sections; 0.5-1 μ m thick) and processed using LAS-AF (Leica) or ImageJ (<http://rsb.info.nih.gov/ij/>) software. For comparison between -206*Clk:Gal4*^{WT} and -206*Clk:Gal4*^{TEmut} all lines were raised, and brains dissected, in parallel before imaging with a fixed laser setting; with exception of Fig. 3C where the gain was increased for TE^{mut} to reveal cells with basal GFP signal.

Quantification of protein levels in single neurons

To quantify CLK levels, WT and TOY-OE brains were processed in parallel and imaged using the same laser settings. LN_v5 was discernable as a GFP^{-ve}/CLK^{+ve} nucleus amongst *pdf>GFP^{+ve}/CLK^{+ve}* LN_vs. All LN_v nuclei used for quantification were within 15 z-sections of LN_v5 (note: minimal bleaching occurred over ± 20 z-sections). Values were averaged from eight WT and four TOY-OE brains. For each nucleus, CLK-levels were calculated as mean pixel density minus background. For VRI and TIM signal in LN_vs, images were collected in parallel using fixed settings and scan number. Channels were converted to grayscale and number of *pdf>GFP^{+ve}* LN_v neurons recorded. VRI and TIM were scored using the GFP channel as a guide, with LN_v5 distinguished as a GFP^{-ve} neuron amongst the GFP^{+ve} LN_vs.

Locomotor Activity Rhythms

Locomotor activity of male flies (1-4 day old) was measured using Trikinetics Drosophila activity monitors (Waltham, MA) for 3-5 days in LD12:12 followed by constant darkness (DD) at 25°C. χ^2 periodogram analysis was performed on individual flies for LD or DD, based on 30min time bins. Animals with power ≥ 10 and width ≥ 2 at 95% confidence were deemed rhythmic. Activity plots show normalized data of rhythmic flies, grouped by genotype.

Statistical Analysis

Analysis was one-way-ANOVA with Tukey HSD post hoc. Locomotor groups that failed homoscedasticity ($p < 0.05$; Levene) were analysed using Welch ANOVA with Games-Howell post hoc. Statistics were done using SPSS (www.spss.com) software.

Results

TOY and EY bind multiple sites at the *Clock* locus

An initial screen of the *Clk* locus identified over 20 sequences with high similarity to the paired-domain (PD) consensus bound by vertebrate Pax6 and its fly orthologues TOY and

EY (Epstein et al. 1994; Czerny and Busslinger 1995; Callaerts et al. 1997; Czerny et al. 1999; Punzo et al. 2002; Kozmik 2005). Electrophoretic mobility shift assays (EMSAs) were used to determine if TOY, and/or EY, could form protein-DNA complexes with these sequences. A probe based on the *Clk*+46 sequence showed a clear shifted complex when it was incubated with rabbit-lysate that had been primed to synthesize, either, TOY, or, EY (Fig. 1A). In contrast, no shift was observed when the probe was incubated with relevant lysate controls (Fig. 1A). Each shifted-complex migrated identically to a CD19-2-Ains positive control probe (Fig. 1A; Czerny et al. 1999). Mutating four bases within the paired-site of the *Clk*+46 probe eliminated the shift produced by either protein, whilst self-competition with cold competitor reduced both shifted bands >20-fold (Fig. 1A). The TOY-complex was super-shifted by α -TOY antiserum, whilst the EY-complex disappeared in the presence of α -EY antiserum (Fig. 1A). In contrast, neither antiserum showed cross-reactivity between these protein paralogues (Fig. 1A).

To confirm the presence of TOY/EY in reactions, samples of each lysate were immunoblotted and probed for α -TOY or α -EY immuno-reactivity (IR). The α -TOY antiserum recognized a ~60kDa protein (predicted = 60kDa) present in TOY-lysate but absent in control (T7) or EY-lysate (Fig. 1B). The α -EY antiserum recognized a ~100kDa protein (predicted = 94kDa) present in EY-lysate and absent in control (T3) or TOY-lysate (Fig. 1B). These data confirm the presence of TOY/EY in EMSA reactions. In addition, the EMSA and western blot data show that neither antibody cross-reacts with native, or denatured, forms of their respective paralogue.

An additional site bound by TOY and EY was identified in intron 1, and six further sites (upstream, in 5'UTR and intron 1) were found to be targeted by TOY. The relative affinity of all sites for TOY-binding was then determined by their ability to outcompete labelled CD19-2-Ains (Fig. 1C) and an updated map of binding sites in the *Clk* locus is shown in figure 1D. In addition to these novel TOY-sites 14 other putative sites were screened and found to have weak, or no, affinity for TOY (Fig. 1C; supplemental Fig. S1). TOY contains three separate DNA-binding domains, the homeo-domain (HD) and bipartite paired-domain (PD) comprising PAI and RED domains (Kozmik 2005). Comparison between strong and weak targets suggests TOY only binds PD-sites in *Clk* that also contain a 5'-HD half-site, or ATTA, sequence (supplemental Fig. S1; Kozmik 2005). In addition, all poor-affinity sequences contain at least one, of three, bases in the PD that is not present in any of the strong targets (Fig. 1C; supplemental Fig. S1). This suggests these bases disrupt TOY-binding and may, instead, facilitate binding by other members of the Pax family. In summary, these data show that TOY can bind a specific subset of Pax6-PD sites at the *Clk* locus, and, EY binds a subset of these sites.

toy is expressed in all lateral neuron pacemaker cells

The presence of TOY/EY binding-sites at the *Clk* locus suggests both proteins regulate *Clk* expression. In adult brain, TOY and EY are expressed in many neurons that do not contain oscillators (e.g. Kenyon cells) suggesting both may be dedicated repressors of *Clk* (Kaneko et al. 1997; Adachi et al. 2003; Furukubo-Tokunaga et al. 2009). However, microarray analysis has shown that *toy* mRNA is enriched in LN_vs (Nagoshi et al. 2010; Kula-Eversole

et al. 2011). Hence, we wanted to determine if TOY protein is present in the nucleus of these, and other, central pacemaker neurons at times when the *Clk* promoter is active: between zeitgeber times (ZT) 16 and 4 ([ZT0-12 = lights on; ZT12-24/0 = lights off]; Kula-Eversole et al. 2011).

Initially, specificity of α -TOY antiserum was confirmed by the co-immunosignal for TOY and β -galactosidase (β GAL) in stage 16-17 embryos (E16-17) containing a *toyP*[lacZ] transgene (*toy*> β GAL; U Walldorf and P Callaerts, personal communication). This analysis also confirmed that *toyP* is a severe hypomorph, or null, insertion (supplemental Fig. S2A). Next, to determine if TOY is in the nucleus of LN_vs at times when *Clk* is being expressed, flies containing an LN_v marker (*pdf*>cGFP) were entrained to LD cycles and brains of 4-day-old adults processed for α -TOY/ α -TIM-IR at ZT16, 22 and 4. TOY was present in the nucleus of all *pdf*>GFP⁺ LN_v neurons at all three time-points (Fig. 2A; supplemental Fig. S2B). Furthermore, TOY was also evident in GFP^{-ve} LN_ds at times when TIM-IR made it possible to discern these neurons (Fig. 2A; supplemental Fig. S2B). Intriguingly, TOY⁺ oscillator cells were often distinct from TOY⁺ non-oscillator cells by virtue of nuclear foci (Fig. 2A). Whilst this was not investigated further, it seems likely that these foci represent TOY aggregates in transcription complexes or proteasomes. Irrespective, it suggests that TOY may have some unique property in oscillator-cell-nuclei. In all LNs, TIM was predominantly cytoplasmic at ZT16; nuclear at ZT22; and, undetectable at ZT4, indicating normal functionality of the oscillator mechanism (Fig. 2A; Myers et al. 1996; Shafer et al. 2002). These data show that TOY is present in the nucleus of LNs at times when *Clk* is being transcribed.

The spatial complement of TOY⁺ LNs was verified using *toyP*, since this transgene marks the nuclei of *toy*-expressing cells with β GAL. Flies containing *toyP*, and an *8.0Clk*>cGFP reporter, were entrained to LD and 6-day-old adults processed for β GAL-/TIM-IR (Glossop et al. 2003). Strong *toy*> β GAL-IR was evident in all s-LN_v and l-LN_v neurons (Fig. 2B; negative control for β GAL-IR in supplemental Fig. S2C). *toy*> β GAL was also evident in LN_ds and the solitary PDF^{-ve} LN_v neuron, LN_v5 (Fig. 2B; supplemental Fig. S2D). However, in these neurons the signal was qualitatively weaker than in PDF⁺ LN_vs. For LN_vs, *toy*-expression occurs independent of the circadian mechanism, since *toy*> β GAL-IR was evident even in a *cyc*⁰ mutant background (Fig. 2C; later discussed). These results confirm that *toy* is expressed in s-LN_v and l-LN_v clusters and, to a lesser extent, LN_ds and LN_v5.

TOY functions independently of EY in lateral neuron pacemaker cells

EMSA analysis indicated that EY can bind the *Clk* locus, raising the possibility that EY may regulate *Clk* in LNs. To determine if EY is present in these neurons, *pdf*>cGFP adults were processed for EY- and CLK-IR at ZT16, 22 and 4 (Houl et al. 2008). Using the Kenyon cells as an internal control for EY-IR (not shown) we were unable to detect EY in any central oscillator neurons, including s-LN_vs (supplemental Fig. S2E).

TOY-sites enhance expression of a minimal-*Clk* promoter *in vivo*

The -206*Clk:Gal4* transgene, comprising *Clk*[-206 to +1485], contains several TOY-binding-sites and drives expression in type-A oscillator cells: which includes all s-LN_v, l-LN_v, LN_d, DN1a and DN2 neurons (Fig. 2A; Gummadova et al. 2009). To determine if the TOY-sites contribute to this expression the highest-affinity sites were mutated. EMSA was first used to identify minimal mutations that disrupted TOY-binding (Fig. 3A). These mutations were then incorporated into -206*Clk:Gal4*^{WT} to produce -206*Clk:Gal4*^{TEmut} and 16 independent transgenic lines were generated. Each line was crossed to UAS-cGFP and a preliminary screen of 3-day-old females in LD (and LL3; constant light from eclosion, to minimize circadian fluctuation) showed a complete lack of *Clk*>GFP fluorescence in 11 lines and weak, but consistent, signal in 5 lines (1 on chromosome X; 4 on c2; n>4 brains per line, per condition). The few positive lines, and controls, were rescreened as 1-day-old females at ZT7 (n>5 brains per line). In all TEmut lines weak GFP signal was evident in two cells in the region of the s-LN_vs. In one exceptional line, 16/2, weaker signal was occasionally evident in two additional cells in this region (Fig. 3B; supplemental Fig. S3). Weak signal was also evident in solitary cells in the region of the LN_d and DN2 clusters, but was inconsistent even between hemispheres of the same brain (Fig. 3B; supplemental Fig. S3). As expected, control lines showed robust signal in all type-A oscillator cells (Fig. 3B).

Previous work has shown that removing the -206/-84 region from -206*Clk:Gal4*^{WT} attenuates expression in all type-A clock cells of adults. Surprisingly, weak signal in the region of the s-LN_vs was due to ectopic expression (Gummadova et al. 2009). To determine if -206*Clk:Gal4*^{TEmut}, likewise, drives ectopic expression, TEmut[16/2] and WT[9/2] were crossed to an independent UAS-cGFP reporter and brains of 4-day-old females processed at ZT15 using VRI-IR to mark oscillator cell nuclei (n>4 brains per line; Glossop et al. 2003). In TEmut, GFP was evident in two cells in the region of the s-LN_vs. However, only one of these was an oscillator cell as the other was VRI^{-ve} (Fig. 3C). Increasing the gain revealed two further GFP^{+ve}/VRI^{+ve} s-LN_v clock cells and 1 (of 4) l-LN_v neurons (Fig. 3C). In addition, faint GFP signal was detected in the DN1a pair, and, solitary neurons of the LN_d and DN2 clusters. However, the gain level required to visualise these fainter GFP^{+ve} cells also revealed ectopic expression scattered throughout the brain. This was in marked contrast to WT, which showed robust signal in all type-A neurons (Fig. 3C).

Taken together, these data show that activity of the TEmut transgene is severely crippled in 16 independent reporter lines. The fact that a few lines show expression demonstrates that TEmut is only able to drive weak expression even when the insert is in a region of accessible chromatin. This indicates that one, or more, of the TE-sites is necessary to enhance expression of the minimal-*Clk* promoter.

Over-expression of TOY enhances CLK protein levels in the LN_vs and selectively speeds up the s-LN_v oscillator to generate short-period locomotor rhythms

Attenuated expression of the -206*Clk:Gal4*^{TEmut} transgene suggests that TOY enhances *Clk*-expression in type-A oscillator cells. To determine if TOY promotes expression from the genomic *Clk* locus we turned to over-expression in the context of LN_vs. Initially, over-expression was confirmed by driving UAS-*toy* in all oscillator cells and comparing against

controls on western blot (supplemental Fig. S4A). Subsequently, TOY was over-expressed specifically in the LN_vs using the *pdf*-Gal4 driver: hereafter referred to as *pdf*>TOY or TOY-OE (Renn et al. 1999). These flies also contained a UAS-cGFP reporter to co-mark TOY-OE cells and facilitate their morphological analysis. As expected, *pdf*>GFP was restricted to LN_vs, which showed enhanced TOY-IR compared to all other TOY⁺ neurons in the brain. Importantly, TOY-OE did not affect gross morphology of the LN_vs (supplemental Fig. S4B). Additionally, since TOY can activate *ey* (Czerny et al. 1999) and EY can regulate *Clk* (Fig. 1) we also confirmed TOY-OE does not induce ectopic *ey* (supplemental Fig. S4B). Next, brains of TOY-OE were processed to determine if CLK protein levels were elevated in *pdf*>TOY nuclei relative to an internal control neuron: the PDF^{-ve}/CLK⁺ oscillator cell LN_v5. Brains were processed at ZT3 since this is the time in a standard LD cycle when *Clk* mRNA levels peak in s-LN_vs (Kula-Eversole et al. 2011). CLK protein levels were statistically significantly higher in s-LN_v and l-LN_v nuclei in TOY-OE compared to control (Fig. 4A; one-way-ANOVA [F(3,78)=29.93, p<0.001). Hence, elevated levels of TOY lead to increased levels of CLK in both sub-groups of LN_v neurons.

Output rhythms in locomotor activity are driven by the s-LN_vs in DD (Rieger et al. 2006). Previous work has shown that over-expression of *Clk* leads to ~1hr shortening of locomotor period under DD conditions due to acceleration of the s-LN_v oscillator (Kadener et al. 2008). Hence, to confirm that TOY-OE is increasing CLK protein levels in s-LN_vs, *pdf*>TOY flies were tested to see if they had a short-period locomotor rhythm consistent with CLK-OE. Indeed, TOY-OE flies showed wild-type entrainment during LD, but a statistically significant 0.5-1hr shortened period in DD compared to controls (Table 1; supplemental Figs. S4C-D). Locomotor activity plots confirmed the daily advance in morning and evening activity peaks (Fig. 4B; supplemental Fig. S4C). Hence, over-expressing TOY in s-LN_vs generates a CLK-OE behavioural phenocopy. Furthermore, the persistence of the short-period rhythm in DD demonstrates that *toy* interacts with the core oscillator mechanism in the s-LN_vs even after several days of constant darkness.

To confirm the acceleration of the molecular oscillator in s-LN_vs, brains of 13-day-old TOY-OE, and control, were dissected across LD8 and DD8 and processed for VRI-/TIM-IR. In s-LN_vs the protein rhythms for VRI and TIM persist during DD, so monitoring both proteins provides better temporal resolution of oscillator status. In brief, VRI is nuclear-localized and present between ~ZT/CT9-22 (peak=ZT/CT15; CT = circadian time in DD, based on previous ZT cycle in LD), whilst TIM accumulates in the cytoplasm between ~ZT/CT15-21 then enters the nucleus and degrades by ~ZT/CT24 (Glossop et al. 2003; Shafer et al. 2002; N Glossop, unpublished data). For this analysis it was necessary to distinguish s-LN_vs at all times, so the TOY-OE line containing UAS-cGFP with a ~22.5hr locomotor period was used.

During LD8, VRI and TIM protein rhythms were in phase in s-LN_vs of WT and TOY-OE, with both proteins being undetectable at ZT4, 8 and readily detected at ZT16, 20 (Table 2; supplemental Fig. S4E). By DD8, VRI and TIM profiles were unchanged in WT, whereas in TOY-OE both proteins were present in subjective day (CT4, 8) and undetectable in the majority of s-LN_vs during subjective night (CT16, 20; Table 2; Fig. 4C). Hence, in TOY-OE

the molecular oscillator in s-LN_vs is in anti-phase to that of WT, due to a ~12hr advance over 8 days of DD.

CLK protein levels in LN_v5 are wild-type in *pdf>TOY* flies (Fig. 4A). As such, LN_v5 provides an internal control to determine if TOY-OE is causing selective acceleration of s-LN_vs. Analysis of VRI and TIM during LD8 showed that LN_v5 was in-phase with s-LN_vs in WT and TOY-OE (Table 2; supplemental Fig. S4E). By DD8, LN_v5 remained in-phase with s-LN_vs in WT, whilst in TOY-OE it phase-lagged the s-LN_vs by ~4-8hrs (Table 2; Fig. 4C). Hence, TOY-OE is accelerating the oscillator in s-LN_vs even when compared to non-TOY-OE oscillator cells in the same brain.

Reduction in *toy* and *cyc* dosage cause additive lengthening of locomotor period

Due to recessive lethality of the *toyP* transgene, and resistance of chromosome 4 to recombination-based mosaic analysis, it is not possible to monitor the effect a *toy*-null phenotype has on *Clk*-expression in mature pacemaker cells (Furukubo-Tokunaga et al. 2009; Blair 2003). However, if the *toy-Clk* interaction has physiological relevance then a reduction in TOY levels should impair CLK-CYC function; slow down the s-LN_v oscillator; and, lengthen the locomotor rhythm in a manner consistent with perturbations in *Clk* or *cyc* (Allada et al. 1998; Rutila et al. 1998). Initial attempts to reduce TOY levels using *pdf>toyRNAi* proved inefficient (<20% reduction in LN_vs) or, lethal in combination with UAS-*Dicer*. Hence, to reduce TOY levels a gene-dosage approach was used.

Halving *toy* copy number had no effect on period length during LD entrainment, but lengthened the free-running period of locomotor activity by a modest, yet statistically significant, ~0.4hr compared to background (Table 3; Fig. 5). This was comparable to a ~0.6hr lengthened period when a null-copy of *cyc* was tested in the same isogenic background (Table 3; Fig. 5; supplemental Fig. S5). More compelling, the effects of *toy* and *cyc* were additive, since double heterozygotes had a ~1hr lengthened period (Table 3; Fig. 5; supplemental Fig. S5). This confirms there is a genetic interaction between *toy* and *cyc* in the context of the circadian mechanism and is consistent with TOY promoting CLK-function in pacemaker cells that regulate locomotor activity. Furthermore, this shows that physiological levels of TOY have rate-determining effects on the oscillator mechanism during DD.

TOY is necessary for *Clk*-expression during s-LN_v development

The onset of a functional circadian system occurs during embryonic stage 16 when a handful of neurons first begin to express CLK (Houl et al. 2008). These neurons persist through to adulthood to become the s-LN_v and DN1a/2 clusters. Since *toyP* homozygotes progress to larval stages it is possible to determine if TOY is necessary for this onset of *Clk*-expression in the s-LN_vs.

Analysis of *toy>βGAL* and CLK in heterozygous wild-type (*toyP/c^D*) embryos confirmed that *toy* is expressed in s-LN_vs when the *Clk* promoter first becomes active (Fig. 6A; n=9 embryos/hemispheres). This analysis also revealed that the DN clusters consist of *toy>βGAL^{+ve}* and *βGAL^{-ve}* nuclei at this time (Fig. 6A; arrowheads indicate CLK^{+ve}/*toy>βGAL^{-ve}*). In stark contrast to wild-type, CLK-IR was only evident in a pair of neurons

in dorsal lobe of *toy*-null (*toyP/toyP*) embryos (Fig. 6B; n=6). One of these CLK^{ve} neurons was βGAL^{ve}. Hence, this is one of the DN pairs (Fig. 6B). The lack of CLK-IR elsewhere in the brain shows that the onset of *Clk*-expression in presumptive s-LN_vs is severely attenuated in the absence of TOY.

***toy* is expressed upstream of CLK-CYC in mature LN_vs**

Current knowledge of the oscillator mechanism is restricted to regulation that occurs downstream of CLK-CYC. Since TOY is necessary for *Clk*-expression in presumptive s-LN_vs it seems likely this regulation normally persists to maintain the oscillator. If true, this would place TOY in a unique position with regard to oscillator function, as it would provide input rather than PDP1ε-like feedback. To address this, the core oscillator was knocked-out by crossing the *cyc*⁰ mutation into flies containing the *8.0Clk>cGFP* and *toy>βGAL* reporters (Rutila et al. 1998; Glossop et al. 2003). Despite the lack of CLK-CYC in *cyc*-null flies, basal levels of TIM are still detectable in LN_vs towards the end of the night. Hence, TIM-IR was used in conjunction with *Clk>cGFP* as a dual marker of LN_v neurons.

Analysis of adult brains at ZT22 and ZT4 did not reveal any gross difference in the spatial pattern of *toy>βGAL*^{ve} cells in *cyc*⁰; *toyP/+*. The level of *8.0Clk>cGFP* was predictably lower than in wild-type. Nevertheless, *toy>βGAL* co-localized with TIM and/or *Clk>cGFP* in three s-LN_v and three l-LN_v nuclei (Fig. 2C). Hence, *toy*-expression persists in LN_vs in the absence of functional CLK-CYC. This suggests that the role of TOY, in relation to *Clk*, is one of input rather than feedback maintenance.

Discussion

Novel TOY- and EY-binding sites in the *Clk* promoter

Our analysis has revealed that TOY binds multiple sites in the *Clk* locus and that EY binds a subset of these sites. Previous work has shown that TOY binds PD-sites at the *eyeless* (*ey*) and *sine oculis* (*so*) loci, whilst EY binds a subset of the TOY-sites in *so* (Halder et al., 1998; Punzo et al. 2002). A common feature of the binding sites at the *Clk* locus is that they all contain a 5'-HD half-site. This suggests TOY (and EY) bind in a different configuration at the *Clk* locus to that at *ey* and *so*. Whilst it is known that DNA-binding configuration can change the functional properties of Pax factors, it is currently not known why this is (Jun and Desplan. 1996; Yamaguchi et al. 1997; Punzo et al. 2001; Kozmik 2005). This mechanistic nuance can now be tested *in vivo* by comparing the regulatory complex/es TOY forms at the *Clk* locus with those at *ey* and *so*.

In addition to the TOY-sites, we identified PD-sequences in *Clk* that are poor targets for TOY. This has revealed three bases in the paired-box that correlate with poor binding. We suspect these sites are targeted by different Pax factors and, hence, these three bases represent key nucleotides for Pax-factor discrimination. Thus, other members of the Pax-family may target these sites to regulate *Clk*-expression in TOY^{ve} cell-types.

In the brain, TOY and EY function in parallel and independent regulatory cascades (Kammermeier et al. 2001; Adachi et al. 2003; Furukubo-Tokunaga et al. 2009). There are four predicted isoforms of EY, all of which contain a common C-terminal domain. Using a

polyclonal antibody generated against the entire C-terminal domain we did not detect EY in any central clock neurons, indicating that TOY probably functions independently of EY in central pacemaker cells.

TOY is an enhancer of *Clk* in lateral neuron pacemaker cells

Using standard approaches we have shown that TOY is a positive regulator of *Clk* in pacemaker neurons that coordinate daily sleep-wake cycles. The *-206Clk^{TEmut}* analysis shows that TOY-sites enhance expression in type-A oscillator neurons, including the s-LN_vs (Fig. 7A). Importantly, two-thirds of lines containing this reporter showed a complete lack of *Clk*>GFP fluorescence in adult brain, while a quarter showed weak signal in one s-LN_v neuron (note: a 2nd putative GFP⁺ s-LN_v neuron was VRI^{-ve} and, thus, ectopic). In one exceptional line, 16/2, *Clk*>GFP was evident in additional type-A neurons. However, even in this line expression was weaker than in *-206Clk^{WT}* controls. Critically, the *-206Clk^{TEmut}* transgene still contains low-affinity TOY-sites. Hence, even this residual expression may be dependent on TOY.

Focussing on the LN_vs, the over-expression data show that TOY enhances CLK levels, demonstrating relevance of the *toy*>*Clk* link in the context of genomic-*Clk*-gene-expression. Furthermore, the *toy*-manipulation data show that physiological levels of TOY interact with the core oscillator mechanism in s-LN_vs, with rate-determining effects during DD. This contrasts with results from *pdp1e*-manipulation, which has minimal effect on the free-running period of locomotor activity (Benito et al. 2007; Zheng et al. 2009). Hence, even during constant darkness TOY appears to be more critical than PDP1e when it comes to setting clock-speed in these neurons.

Naturally, the most direct way to show the necessity of TOY for *Clk*-gene-expression would be the complete lack of *Clk*/CLK in *toy*-null oscillator cells. Unfortunately, such analysis is not possible in adult. However, we have shown that *toy* is expressed in presumptive s-LN_vs at the time CLK protein first becomes detectable, and, that CLK is undetectable in these neurons in *toy*-null embryos. Hence, TOY appears to be necessary for the onset of *Clk*-expression in the s-LN_vs. The loss of TOY also affects *Clk*-expression in some, but not all, of the early-born DNs. This conflicts with the *-206 Clk^{TEmut}* data, which shows attenuated expression in DN1a and DN2 neurons in adult brain. A possible explanation for this is that TOY may be redundant during the onset of *Clk*-expression in certain DN neurons but becomes necessary, later, to ensure clock-maintenance. Alternatively, it may be that some other Pax-factor binds the TE-site(s) to drive expression in these DNs. Nevertheless, the combined data from embryo and adult suggest that TOY positively regulates *Clk*-expression throughout the life of the s-LN_vs.

Regulatory hierarchy between *toy*, *Clk* and *pdp1e* in LN_vs

One of the main drives behind this research was the discrepancy in the current model of the oscillator mechanism due to mounting evidence that PDP1e is not necessary for *Clk* expression. Here we have shown that TOY functions upstream of CLK-CYC in mature LN_vs. We propose that TOY combines with an unknown factor (Z; that binds the *Clk*-206/-84 region) and this probably forms the primary mechanism that drives *Clk*-

expression in LN_{vs} (Fig. 7A). Hence, TOY+Z generate sufficient levels of *Clk* for CLK-CYC to activate the core oscillator genes, including *pdp1e*. PDP1e then feeds-back to the *Clk* locus to help maintain expression via a semi-redundant mechanism based on co-operative, or additive, regulation (Fig. 7B).

So what is factor Z? Previous work has shown that CLK levels are attenuated in the LN_{vs} of *48-related-2* (*Fer2*)-null adults. However, it was not determined if this is due to a specific loss of *Clk*-expression, or, an indirect effect (Nagoshi et al. 2010). FER2 is a bHLH factor so presumably targets E-box (CANNTG) sequences as a dimer. The *Clk*-206/-84 region contains three putative E-boxes (e.g. CAACTGCACTTG). Whilst these sites necessarily differ from the canonical circadian-E-box bound by CLK-CYC (CACGTG) they may, nevertheless, be targeted by a FER2-containing dimer. Future work will be required to determine the link between *Fer2* and *toy* with regard to *Clk*-expression in the s-LN_{vs}.

Differentiating between TOY^{+ve} oscillator and non-oscillator cells

The majority of TOY^{+ve} neurons in the adult brain do not express *Clk* and are, therefore, not oscillator cells. For example, Kenyon cells are not oscillator cells. So why doesn't TOY activate *Clk* in these neurons? TOY function is highly context-dependent (Blanco et al. 2010). Hence, TOY may only activate *Clk* in oscillator cells because these are the only TOY^{+ve} cells that also contain factor Z. These cofactors may form stable complexes on the *Clk* locus - which might explain the unique TOY-foci evident in clock cells. Irrespective, previous work suggests that repression is also necessary to prevent *Clk*-expression in certain groups of TOY^{+ve} non-oscillator cells. For example, the -1982*Clk:Gal4* transgene drives expression in Kenyon cells (Gummadova et al. 2009). This transgene is a 5'-extended version of -206*Clk:Gal4* and, therefore, contains additional TOY-sites at -1363, -1153 and -656. Thus, in different cell-types TOY may combine with different cofactors to form alternative enhancer complexes at the *Clk* locus. Hence, in Kenyon cells TOY may bind sites in the -1982/-207 region to co-activate with factor Y, whilst in type-A clock neurons TOY binds the -206/+1485 region to co-activate with factor Z. This potential expression in Kenyon cells is blocked by repressors that bind further upstream in the *Clk* locus (Gummadova et al. 2009). However, in addition to expression in Kenyon cells the -1982*Clk:Gal4* transgene also supports expression in the visual system (Gummadova et al. 2009). Hence, it seems plausible that in photoreceptors and Kenyon cells a common enhancer complex may form within the *Clk*-1982/-207 region, but unique repressors block this in the latter cell-type to prevent them from becoming oscillator cells. It will now be important to determine if TOY-sites in the *Clk*-1982/-207 region contribute to expression in retinal photoreceptors, and, if this also requires the *Clk*-206/+1485 region.

Novel insights into a potential role for Pax6 (mTOY) in the mammalian oscillator

The circadian mechanism is highly conserved from flies to mammals. Hence, the core drive for the mammalian oscillator is provided by the homologues of CLK (Clock, or its paralogue, *Npas2*) and CYC (*Bmal1*). In contrast to flies, mammalian homologues of VRI/PDP1e do not appear to feed-back to regulate *Clock/Npas2* and it is currently not known what activates either of these mammalian *Clock* paralogues (Glossop 2011). It has been shown that *Otx2* and *Clock* function in an interdependent network during neural plate

formation (Green et al. 2001; Morgan 2002). However, it has not yet been shown if *Otx2* is a direct regulator of *Clock*, or, if such regulation also applies in the context of mature clock neurons. Intriguingly, *Clock-Bmal1* can activate *Pax6*, the mammalian homologue of *toy* (Morgan 2004). In light of our data, we propose that *Pax6* may also function upstream of *Clock*. Hence, *Pax6* may combine with co-activators to drive the expression of *Clock*, with *Clock-Bmal1* then establishing positive-feedback by up-regulating *Pax6*. Hypothetically, such a mechanism may be relevant in master pacemaker cells of the suprachiasmatic nuclei (SCN) that modulate sleep-wake behaviour in mammals (DeBruyne et al. 2007). Alternatively, other members of the Pax-family may have evolved to fulfil this function in the SCN whilst *Pax6*, instead, regulates *Clock* (or *Npas2*) expression in other oscillator cell-types, such as retinal photoreceptors. Indeed, the link between *toy* and *Clk* in flies suggests that visual photoreception evolved from the genetic machinery whose primitive function was to establish circadian molecular time-keeping.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

TOY and EY sites in *Clk*. (A) EMSAs showing TOY (left) and EY (right) bind *Clk*+46. Probes were wild-type (+), mutant (M) and CD19-2-Ains control (C). Proteins were full-length TOY (T) or EY (E) generated in TnT-rabbit-lysates, or, unprimed-lysate control (C). Antibodies were anti-TOY (αT) and anti-EY (αE). Cold-competitor was 10-fold excess of *Clk*+46 (+). Arrows show TOY (left) and EY (right) shifted bands, arrowhead marks TOY super-shift. Experiments were repeated three times with similar results. (B) Western blots of protein input for EMSA. Key: TOY; TnT-TOY, T7; lysate control for TnT-TOY, EY; TnT-EY, T3; lysate control for TnT-EY, arrow; full-length TOY, arrowhead; full-length EY. Blots were repeated twice with similar results. (C) Competition assay (left) showing how equimolar concentrations of cold *Clk* dsDNA compete with labelled CD19-2-Ains for TOY-binding. Key: TnT mock; unprimed lysate with labelled CD19-2-Ains, TOY+hot probe; toy-

primed lysate with labelled CD19-2-Ains. This experiment was repeated twice with similar results. Sequences (*right*) show paired-domain (PD) consensus bases in bold and homeo-domain (HD) half-site underlined. Asterisk marks reference base. (*D*) Schematic of *Clk* locus showing positions of TOY/EY binding sites relative to transcriptional start site at +1. Numbers in ovals indicate relative affinity for TOY (4 = strongest). Also shown are 100%-consensus V/P-binding-sites previously identified (Glossop et al. 2003). Key: open boxes; non-coding, filled boxes; coding.

Figure 2.

toy-expression in LN oscillator cells of adult brain. In all panels, dorsal is up and medial left; Scale = 10 μ m. (A) Schematic of right-hemisphere of a fly brain (left) showing central oscillator subgroups, type-A/B division, and PDF⁺ve cells. Key: LN; lateral neuron, DN; dorsal neuron, v; ventral, d; dorsal, s; small, l; large, 1a; 1 anterior, 1p; 1 posterior, LN_v5; PDF⁻ve LN_v, LPN; lateral posterior neuron. Confocal images (right) show TOY-IR in LNs marked with TIM and/or *pdf*>cGFP at indicated zeitgeber times (ZT). Key: arrows; TOY⁺ve LN nuclei, open arrowheads and insets; nuclear foci. Images are compressed stacks of 0.5 μ m z-sections through the LNs; n>10 hemispheres from 6 brains. (B, C) Confocal images of LNs in *toy*P[LacZ]/+ adults showing *toy*> β GAL, TIM and *8.0Cik*>cGFP in *cyc*⁺ (B) and *cyc*⁰ (C) backgrounds at indicated ZT. Images are compressed stacks of 0.5 μ m z-sections; n>8 hemispheres from 6 brains. Arrows show *toy*> β GAL signal in TIM⁺ve and/or

Clk>cGFP^{+ve} LNs. Double arrowhead denotes LN_v5. Additional images in supplemental Fig. S2.

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TOY-sites enhance expression of the -206*Clk:Gal4*, minimal-*Clk*-promoter. (A) EMSA showing mutations that disrupt TOY binding to the *Clk* [+46; +107; +681] sites. (B) Confocal images showing UAS-cGFP reporter signal in the right hemisphere of WT and TEmut. Two WT (8/1; 9/2) and the strongest TEmut inserts on chromosome X (9/1) and 2 (16/2) are shown. Abbreviations are as Fig. 2 with number of GFP⁺ve neurons per cluster shown in brackets. Brains were processed in parallel at ZT7 (n=5 brains/line). Similar results were obtained when processed at ZT2. Scale = 100μm. (C) Representative images for WT[9/2] and TEmut[16/2] showing *Clk*>cGFP and VRI-IR in brains processed at ZT15. Arrows show GFP⁺ve/VRI⁻ve non-oscillator cell. Inset in TEmut shows GFP signal in s-LN_{vs} when gain is set to same level as WT. Scale = 10μm.

Figure 4.

Over-expression of TOY increases CLK protein levels and accelerates the *s-LN_v* molecular oscillator. (A, C) For hemisphere images dorsal is up and medial left. (A) Comparison between WT and TOY-OE showing quantitative increase of CLK levels in TOY-OE neurons (*s-LN_v* and *I-LN_v*) compared to a *PDF^{ve}/LN_v5* internal control neuron (arrows). Images for WT and TOY-OE are shown (*left*) with quantitative analysis of CLK levels in *LN_v* neurons relative to *LN_v5* (*right*). Key: scale bar = 10 μ m; error bars = SEM; a-b (p < 0.001; Tukey HSD). (B) Normalized group locomotor activity plots for TOY-OE, driver control (WT) and responder control (*toy-con*). LD shows synchronized morning (M) and evening (E) peaks of activity during entrainment and arrowhead denotes time at which samples were taken for A. DD shows advanced morning (M) and evening (E) activity peaks for TOY-OE flies, leading to ~12h advance in M9 (arrow; compare with WT; E8) and E9 (solid arrowhead; compare

with WT; M9), which now occur during DD8. LD background indicates day (white; ZT0-12) and night (gray; ZT12-24/0); DD background indicates subjective day (hashed; CT0-12) and subjective night (gray; CT12-24/0). x-axes = 30min time bins; y-axes = average activity per 30min $\pm$ SEM. Numbers in top right show raw activity average per 30min. Open arrowheads indicate times at which brains were processed for C. *toy-con* is not shown in DD to make comparison of the anti-phase rhythms for TOY-OE and WT clearer. (C) TOY-OE selectively advances the s-LN_v oscillator in DD. Confocal images of WT and TOY-OE brains in the region of the LN_vs taken at indicated time-points (CT4, 8, 16, 20; open arrowheads in B) during DD8 and processed for VRI-/TIM-IR. Images show compressed z-stacks (0.5 μ m). Dashed boxes outline s-LN_vs, while arrows and insets indicate LN_v5. n>5 hemispheres from 3 brains per time-point. Images for LD are shown in supplemental Fig. S4E.

Figure 5.

Normalized group locomotor activity plots in LD (A) and DD (B) showing effects of reduced *toy* and *cyc* dosage on free-running period. Key: LD and DD background as in Fig. 4C, x-axes; 30min time bins, y-axes; average activity per 30min $\pm$ SEM. Numbers in the top right show raw activity average per 30min.

Figure 6.

TOY is necessary for the onset of *Clk* expression in s-LN_vs. Images show stage 17 embryos (E17) processed for CLK- and *toy>βGAL*-IR. Upper panels show embryos and dashed box indicates optic lobe shown in detail in lower panels. Scale = 100μm. (A) heterozygous wild-type (*toyP/c^P*) showing CLK-IR in oscillator neurons and co-IR with *toy>βGAL* in s-LN_vs (bottom inset). DN pairs contain βGAL^{-ve} nuclei (upper insets; arrowheads). (B) *toy*-null (*toyP* homozygote) showing CLK-IR restricted to a pair of DNs, one of which is βGAL^{-ve} (arrowhead).

Figure 7.

(A) Schematic showing key regions of the *Clk*-promoter that lack cononical VRI/PDP1ε-sites but support expression in type-A oscillator cells. The -206/+1485 region drives expression in type-A neurons due to additive, or cooperative, interaction between TOY and unknown factor/s that bind the -206/-84 region (Gummadova et al. 2009). (B) Model showing *Clk*-gene-expression in s-LN_vs. Circles denote an s-LN_v nucleus with block arrows indicating progressive steps. *Clk*-expression is driven by TOY and factor Z. This mechanism probably initiates expression and explains why *Clk* is expressed in a CLK-CYC-, or, PDP1ε-null background (*upper left*). In wild-type, CLK-CYC activates *vri* and *pdp1ε*. VRI then binds V/P-sites to suppress *Clk*-expression, either, by inhibiting TOY-Z, or, by independent suppression of the basal promoter machinery (*upper right*). VRI-suppression of *Clk* leads to falling levels of CLK-CYC and subsequent decline in VRI. This allows PDP1ε to bind V/P-boxes and enhance *Clk*-expression, either, via TOY-Z, or, an alternative mechanism (*lower*).

Key: solid arrows; activation, dashed arrow; possible mode of enhancement, dashed blunt-ended line; possible mode of suppression, gray arrows; transcription/translation.

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Table 1

Over-expression of TOY in *LN_vs* causes short-period locomotor rhythms. *n* = number of flies; %R = percent rhythmic through LD and DD; period is mean ± SEM. Data from different transgene combinations are pooled relative to ±UAS-GFP. Statistical significance values for period versus genotype: LD (-GFP:[F(2,13.96)=1.97, p=0.18]; +GFP:[F(2,73.70)=1.50, p=0.23]) and DD (-GFP:[F(2,36)]=53.01, p<0.001]; +GFP:[F(2,63.47)=200.01, p<0.001]). Groupings in DD are: a-b (p<0.001; Tukey HSD), c-d, c-e and d-e (p<0.001; Games-Howell). For representative actograms and power/width values see supplemental Fig. S4.

<i>pdf-Gal4</i>	UAS-Gal4	UAS- <i>toy</i>	UAS-cGFP	n	%R	LD		DD	
						period (h)		period (h)	
+	+	+	-	23	78	24.03 ±0.03		22.94 ±0.06 ^a	TOY-OE
-	+	+	-	17	82	23.96 ±0.04		23.79 ±0.09 ^b	<i>toy</i> -con
+	+	-	-	7	100	24.14 ±0.09		24.07 ±0.13 ^b	WT
+	+	+	+	57	91	23.89 ±0.03		22.49 ±0.03 ^c	TOY-OE
-	+	+	+	41	85	23.96 ±0.02		23.04 ±0.05 ^d	<i>toy</i> -con
+	+	-	+	36	100	23.94 ±0.04		23.83 ±0.06 ^e	WT

Table 2

Over-expression of TOY selectively accelerates the s-LN_v molecular oscillator.

n = number of brain hemispheres scored per time-point; ZT = zeitgeber time; CT = circadian time. Columns show number of *pdf*>GFP⁺ cells and whether these were VRI⁺/TIM⁺ as indicated on left; boxes highlight peak VRI/TIM signal in s-LN_vs (clear) or LN_v5 (gray). Images for all 16 data points are in supplemental Fig. S4.

		LD8 (ZT)				DD8 (CT)			
		4	8	16	20	4	8	16	20
WT	n	2	2	5	6	5	10	5	12
	s-LN _v <i>pdf</i> >GFP	8	6	19	20	17	37	18	47
	VRI	0	0	19	20	1	0	18	40
	TIM	0	0	19	20	3	0	18	40
	I-LN _v <i>pdf</i> >GFP	8	8	20	22	20	33	19	48
	VRI	0	0	20	22	0	0	6	0
	TIM	0	0	20	22	0	0	2	0
	LN _v 5 <i>pdf</i> >GFP	0	0	0	0	0	0	0	0
	VRI	0	0	5	5	0	0	4	10
	TIM	0	0	5	5	0	0	4	10
	n	6	6	10	6	7	7	9	9
	s-LN _v <i>pdf</i> >GFP	23	23	38	23	24	25	35	32
	VRI	0	0	37	23	22	23	1	0
	TIM	0	0	36	23	16	16	3	1
TOY OE	I-LN _v <i>pdf</i> >GFP	24	23	37	18	25	28	26	24
	VRI	0	0	37	18	4	0	7	0
	TIM	0	0	37	18	5	0	0	1
	LN _v 5 <i>pdf</i> >GFP	0	0	0	0	0	0	0	0
	VRI	0	0	10	4	0	6	4	0
	TIM	0	0	10	4	0	0	7	4

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Table 3

Reduction in *toy* and *cyc* dosage cause additive lengthening of locomotor period.
n = number of flies; %R = percent rhythmic; period is mean $\pm$ SEM. Statistical significance values for period versus genotype: LD one-way-ANOVA [F(3,109)=1.38, p=0.25]; DD one-way-ANOVA [F(3,109)=44.33, p<0.001]. Groupings in DD are: a-b (p<0.01); a-c and b-c (p<0.001); Tukey HSD. Additional controls in supplemental Fig. S5.

	LD				DD				
	n	%R	period (h)	power	width	%R	period (h)	power	width
+ ; +	18	100	24.03 ±0.03	91.6 ±8.6	4.4 ±0.3	100	23.44 ±0.07 ^a	90.6 ±12.1	4.9 ±0.2
+ ; <i>toyP</i> /+	31	100	24.02 ±0.02	102.2 ±6.2	5.7 ±0.3	100	23.81 ±0.05 ^b	100.8 ± 7.7	5.7 ±0.3
<i>cyc</i> ⁰ /+ ; +	35	97	24.00 ±0.00	84.9 ±4.0	4.6 ±0.2	91	24.00 ±0.06 ^b	120.5 ±12.5	4.7 ±0.2
<i>cyc</i> ⁰ /+ ; <i>toyP</i> +	34	100	23.99 ±0.02	65.9 ±3.8	4.6 ±0.2	97	24.42 ±0.06 ^c	139.5 ±11.8	5.3 ±0.1