Published in final edited form as:
J Neurosci. 2010 April 28; 30(17): 5855–5865. doi:10.1523/JNEUROSCI.2688-09.2010.

The transcription factor Mef2 is required for normal circadian behavior in *Drosophila*

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Abstract

The transcription factor Mef2 has well-established roles in muscle development in *Drosophila* and in the differentiation of many cell types in mammals, including neurons. Here, we describe a role for *Mef2* in the *Drosophila* pacemaker neurons that regulate circadian behavioral rhythms. We found that Mef2 is normally produced in all adult clock neurons and that *Mef2* over-expression in clock neurons leads to long period and complex rhythms of adult locomotor behavior. Knocking down *Mef2* expression via RNAi or expressing a repressor form of Mef2 caused flies to lose circadian behavioral rhythms. These behavioral changes are correlated with altered molecular clocks in pacemaker neurons: *Mef2* over-expression causes the oscillations in individual pacemaker neurons to become desynchronized, while *Mef2* knockdown strongly dampens molecular rhythms. Thus, a normal level of Mef2 activity is required in clock neurons to maintain robust and accurate circadian behavioral rhythms.

Introduction

Circadian rhythms of animal locomotor activity are controlled by molecular clocks located in central brain pacemaker neurons. These endogenous clocks allow animals to anticipate daily environmental changes and, as a result, animals retain behavioral rhythms even without any environmental cues. Genetic studies in *Drosophila* identified a number of core clock genes that form intracellular molecular clocks. In these clocks, two transcription factors, Clock (CLK) and Cycle (CYC) activate expression of *period* (*per*) and *timeless* (*tim*). PER and TIM proteins heterodimerize and then enter the nucleus after a substantial delay, where PER inhibits CLK/CYC activity, thus repressing *per* and *tim* expression. In a second interlocked loop, CLK and CYC activate expression of *vrille* (*vri*) and *Par domain protein 1* (*Pdp1*), whose protein products feed back to regulate expression of *Clk*. Together with abundant post-translational regulation, these transcriptional feedback loops lead to rhythmic clock gene RNA and protein accumulation (reviewed by Hardin, 2005).

Recent studies in *Drosophila* indicate that clock neurons form a neural network that underlies robust circadian behavior (Grima et al., 2004; Stoleru et al., 2004; Stoleru et al., 2005). Two lines of evidence point to the small ventral lateral neurons (s-LN$_v$s), which produce the neuropeptide Pigment Dispersing Factor (PDF), as the most important pacemaker neurons. First, robust behavioral rhythms are lost when LN$_v$s are either ablated or hyperpolarized (Renn et al., 1999; Nitabach et al., 2002). Second, accelerating the clock only in s-LN$_v$s speeds up the clocks in the dorsal lateral neurons (LN$_d$s) and some dorsal neuron (DN) subgroups (Stoleru

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et al., 2005), indicating that s-LN$_v$s can set the pace of other clock neurons. However, other clock neurons signal back to LN$_v$s and can drive rhythmic outputs via LN$_v$s, even if the LN$_v$s lack a functional clock, at least in light:dark (LD) cycles (Stoleru et al., 2004). Indeed, coupling of clock neurons seems essential for robust circadian rhythms in mammals and can even override clock gene mutations that give phenotypes in dissociated cells (Liu et al., 2007). However, the molecular pathways by which fly and mammalian clock neurons communicate with each other to generate robust and self-sustaining behavioral rhythms are unclear.

To identify clock regulatory factors that could either help send or receive signals for intercellular communication, we used a Gal4/UAS mis-expression screen in clock neurons and assayed the behavior of flies in constant darkness (DD). The *Pdf-Gal4* and *tim(UAS)-Gal4* drivers were used to express genes either only in LN$_v$s or in all clock neurons respectively.

Here we describe EP insertion line 1751 which caused long rhythms when expressed in LN$_v$s and long periods or complex rhythms (more than one period) of locomotor activity when expressed in all clock neurons. Line 1751 is inserted in the *Myocyte enhancer factor 2* (*Mef2*) locus, which encodes a transcription factor involved in muscle development in *Drosophila* (Bour et al., 1995; Lilly et al., 1995; Ranganayakulu et al., 1995). In mammals, four *Mef2* genes are expressed in diverse cell types and Mef2 transcriptional activity is regulated by signal transduction pathways to control aspects of cell differentiation (reviewed by Potthoff and Olson, 2007). Of particular relevance, Mef2 regulates synapse development in an activity-dependent manner in mammals (Flavell et al., 2006; Shalizi et al., 2006). Genes involved in synaptic development are among the Mef2 target genes in neurons and include a number of genes mis-regulated in epilepsy and autism spectrum disorder (Flavell et al., 2008). Although Mef2 is also produced in some embryonic and adult *Drosophila* brain neurons (Taylor et al., 1995; Schulz et al., 1996), its function in fly neurons has not been addressed. Here we show that Mef2 is normally produced in all fly clock neurons and that interfering with Mef2 activity in clock neurons causes flies to become behaviorally arrhythmic. At the molecular level, *Mef2* over-expression slows down the molecular clock and causes individual s-LN$_v$s to desynchronize from each other, while knocking down *Mef2* expression via RNAi strongly dampens molecular clock oscillations. These phenotypes are consistent with a role for Mef2 in sustaining circadian rhythms by regulating core clock gene expression and/or by influencing communication between clock neurons.

Materials and Methods

Screen for altered circadian behavior

An EP element (Rorth, 1996) inserted on the X-chromosome (EP55, Bloomington Stock Center) was mobilized to the autosomes using standard procedures to generate novel EP insertions, and the resulting w^+ male progeny were individually crossed to virgin female flies carrying an X-linked *Pdf-Gal4* transgene (Park et al. 2000). The locomotor behavior of six male adult progeny from each cross was assayed in constant darkness at 25°C as described below. Lines were established for EP insertions that altered behavioral rhythms. DNA flanking the insertion was isolated and sequenced using standard procedures to determine their location.

Fly culture and strains

Flies were reared on a standard cornmeal/agar/yeast medium and maintained at 25°C unless otherwise indicated. To make *UAS-Mef2-EnR* flies, DNA encoding the first 128aa of Mef2, which includes the MADS box and *Mef2* domain, was fused by PCR N-terminal to DNA encoding amino acids 2–298 of Engrailed, which includes its repressor domain. This fragment was sequenced, inserted into *pUAST* and transgenic flies made according to standard procedures. Other fly lines have been described previously: *Pdf$_{2.4}$-Gal4* (Renn et al, 1999),

$Pdf_{0.5}$-Gal4 (Park et al., 2000), *tim(UAS)-Gal4* (Blau and Young, 1999b), *Mef2-Gal4* (Ranganayakulu et al., 1998) and *tubulin-$Gal80^{ts}$* (McGuire et al., 2003). Four *UAS-Mef2* constructs were previously described as *UAS-Mef2 I-IV* (Gunthorpe et al 1999), which correspond to *Mef2* isoforms A–D respectively on FlyBase. Here, we refer to *UAS-Mef2 III* as *UAS-Mef2 III(low)* to distinguish it from the *UAS-Mef2* construct of Bour et al. (1995), which we call *UAS-Mef2 III(high)* since it expresses higher levels of Mef2 isoform C (Gunthorpe et al 1999). *UAS-$Mef2^{RNAi}$* (transformant ID 15549) and *UAS-Dicer-2* were described by Dietzl et al. (2007) and obtained from the Vienna *Drosophila* RNAi Center. Clock mutant flies were provided by Mike Young, Michael Rosbash and Ravi Allada. These mutants were crossed to include a *Pdf-RFP* transgene that contains the *Pdf* promoter fused directly to RFP to help visualize LNvs in *cyc^0* and *Clk^{ar}* mutants when endogenous PDF levels are low. This transgene will be described in detail elsewhere (M. Ruben et al., submitted). *UAS-nuclear LacZ* flies were obtained from the Bloomington Stock Center. Control flies were generated by crossing UAS and EP lines to *y w* flies.

Measuring the period of circadian locomotor activity

The locomotor activity of individual flies was recorded at 25°C (except where noted) for between 6 and 12 days in constant darkness (DD) using the TriKinetics infrared beam-crossing system and 5min bins. Raw activity histograms were analyzed for circadian rhythms using Matlab and Clocklab software (Actimetrics). Lomb–Scargle periodograms were constructed according to Van Dongen et al. (1999) and rhythmicity was defined as the presence of a peak above the 0.05 significance line as in Nitabach et al. (2006). A simple and robust rhythm was recorded if Lomb–Scargle analysis revealed one peak above the significance line, while a complex rhythm was defined by a fly having more than one peak above the significance line. Arrhythmicity was defined by the absence of any peak above the significance line. We used chi-squared analysis to measure period length and power for the temperature shift experiment in Figure 5B. Period length and power were calculated for the initial days in DD, for the last 4 days at 31.5°C and the subsequent 4 days at 25°C. We used chi-squared periodograms here since they seemed to more accurately reflect period length over the short timeframes at each temperature.

Immunocytochemistry

Standard immunocytochemistry procedures were used to localize Mef2 protein in the fly brain and to study rhythms of clock proteins in s-LN_vs as in Cyran et al. (2003). For time course experiments, flies were first entrained to a 12:12 light-dark (LD) cycle for 3 days, then shifted to DD and dissected on either the second day (DD2) or the eighth day (DD8). Primary antibodies and their dilutions used were as follows: PER at 1/10,000 (from J. Hall); VRI at 1/10,000 (from P. Hardin); TIM at 1/2,500 (from M. Young); PDP1 at 1/1,000; Mef2 at either 1/200 or 1/1,000 (from B. Patterson and H. Nguyen); mouse anti-β-gal at 1/1,000 (Promega); and mouse anti-PDF at 1/20. The PDF monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa.

Microscopy and image analysis

Images were acquired on a Leica confocal microscope with either a 40× or 20× oil immersion lens by collecting z-sections at ~1 and 2μm intervals respectively and projecting the images onto a single plane for expression pattern studies, or by collecting single z-sections for time course analysis and localization of clock proteins. All samples in one experiment were processed in parallel except where indicated: i.e. they were collected at time points on the same day and then fixed and immuno-stained using master mixes for all solutions. Mef2 levels in Figure 3 were quantified using FIJI software (http://pacific.mpi-cbg.de). Desynchronization was assayed in the following manner: (1) The s-LN$_v$s in a single cluster were scored for either the presence or absence of clock proteins and an s-LN$_v$ cluster was termed desynchronized if at least 1 s-LN$_v$ scored differently from the other s-LN$_v$s. (2) PER levels were quantified for the s-LN$_v$s in a given cluster and plotted to show the standard deviation between s-LN$_v$s in the same hemisphere (Supplementary Figure 1). Statistical analyses were done using Student's t-tests.

Results

Isolation of *Mef2* in a circadian mis-expression screen

To identify novel genes involved in *Drosophila* circadian behavior, approximately 2000 novel lines containing random EP insertions on either the second or third chromosome were generated and screened for altered behavioral rhythms in the presence of the *Pdf-Gal4* driver. In adult flies, this construct drives expression in: (i) the 8 pacemaker s-LN$_v$s that are crucial for circadian rhythms; (ii) the 8 large LN$_v$s (l-LN$_v$s) that are probably more important for regulating arousal and sleep than circadian timing (Chung et al, 2009; Donlea et al, 2009; Parisky et al, 2008; Shang et al, 2008; Sheeba et al, 2008); and (iii) in 4–6 cells of unknown function at the tip of the abdominal ganglion (Park et al., 2000). In our behavioral screen, we identified five lines with long-period rhythms, and the characterization of one of these is described here. Flies with both *Pdf-Gal4* and EP insertion 1751 had a 24.7hr period, while flies containing EP1751 alone had a 23.9hr period (Fig. 1A, Table 1). Sequencing the region of insertion of the EP revealed that EP1751 is inserted on the 2nd chromosome, 107bp upstream of the start site of transcription of *Mef2* isoform F according to FlyBase. We refer to this allele hereafter as $Mef2^{EP1751}$.

To test whether expressing *Mef2* in all clock cells would affect circadian rhythms more strongly than expression only in LN$_v$s, we assayed the locomotor activity of flies in which the *tim(UAS)-Gal4* driver was crossed to $Mef2^{EP1751}$. Visual inspection of the actograms of *tim(UAS)-Gal4/*$Mef2^{EP1751}$ flies showed weak rhythms for the first three days in DD followed by superimposed short and long rhythms for days 4–12 (Fig. 1A). To quantify this phenomenon, we used Lomb Scargle analysis of periodograms (see Methods). This analysis identified that 32% of *tim(UAS)-Gal4/*$Mef2^{EP1751}$ flies had more than one rhythmic period detectable above the 95% significance line and we classify these flies as having complex rhythms. Additionally, 6% of *tim(UAS)-Gal4/*$Mef2^{EP1751}$ flies were arrhythmic. In our opinion, Lomb-Scargle analysis is a conservative estimate of complex rhythms since very few *tim(UAS)-Gal4/*$Mef2^{EP1751}$ flies had the normal single period circadian rhythm characteristic of wild type flies when visually inspecting the actograms. In contrast, very few control flies exhibited complex rhythms (8%) and only 3% were arrhythmic. The average period of the most significant peaks for the rhythmic *tim(UAS)-Gal4/*$Mef2^{EP1751}$ flies was 25.2hr, which is slightly longer than when $Mef2^{EP1751}$ was activated only in LN$_v$s by $Pdf_{0.5}$*-Gal4* (Fig. 1B table 1). These phenotypes were dependent on a Gal4 driver since $Mef2^{EP1751}$ flies show normal rhythms without a *Gal4* driver (Fig. 1A, Table 1), as do *tim(UAS)-Gal4* flies without an EP element (Blau and Young, 1999a).

Multiple *Mef2* isoforms recapitulate the $Mef2^{EP1751}$ phenotypes

Mef2 encodes a transcription factor required in *Drosophila* embryos for the development of muscle precursor cells. The single *Drosophila Mef2* gene spans 45 kb. Five different transcripts have been described, each encoding distinct protein isoforms, which share the same DNA binding and dimerization domains (MADS and Mef2 box domains), but differing slightly in their C-terminal activation domains (Taylor et al., 1995; Schulz et al., 1996; Gunthorpe et al., 1999; FlyBase). Four of these isoforms have been shown to rescue the *Mef2* mutant phenotype in embryonic muscle precursor cells with the level of expression of these isoforms being the most important factor in determining the extent of rescue (Gunthorpe et al., 1999). We used

UAS constructs for each of these four Mef2 isoforms to test if expression of different *Mef2* isoforms in clock neurons would recapitulate the phenotypes seen with expressing *Mef2* via the *Mef2*^{EP1751} insertion, or whether functional differences exist between *Mef2* isoforms in adult flies.

Flies containing UAS transgenes for *Mef2* isoforms A–D (called *UAS-Mef2 I–IV* respectively) were first crossed to flies with a *Pdf-Gal4* driver. We found that expression of *UAS-Mef2* transgenes *I-IV* only in LN$_v$s caused long rhythms with periods ranging between 25.1 and 25.5hr (Fig. 1, table 1), which is slightly longer than the periods observed with *Mef2*^{EP1751}. High-level expression of *Mef2* isoform *C* via the *UAS-Mef2 III(high)* transgene caused ~27% of the flies to exhibit complex rhythms, much higher than observed for the other isoforms or for control flies without a *Gal4* driver (Fig. 1B). Gunthorpe et al (1999) estimated that the *UAS-Mef2 III(high)* transgene produces ~10-fold more Mef2 protein than *UAS-Mef2 III(low)* when expressed via an embryonic Gal4 driver, and this higher level of *Mef2* expression correlates with the increased number of flies having complex rhythms in our assay.

When expressed in all clock cells using the *tim(UAS)-Gal4* driver, *UAS-Mef2 I, II, III(low)* and *IV* produced similar phenotypes to *tim(UAS)-Gal4/Mef2*^{EP1751} flies with between 23 and 44% of flies showing complex rhythms, depending on the transgene. Flies expressing *UAS-Mef2 II* and *UAS-Mef2 III(low)* also increased the frequency of arrhythmicity (Figure 1B, Table 1). Strikingly, 82% of flies expressing *UAS-Mef2 III(high)* in all clock neurons were arrhythmic, which supports the idea that the more strongly *Mef2* is expressed, the greater the disruption to circadian rhythms. The most significant period of the rhythmic flies with the *tim(UAS)-Gal4* driver was longer than in wild type flies (25.1–25.6hr, Table 1), except for *UAS-Mef2 III (high)* where the high incidence of arrhythmicity and weak power rhythms prevented a reliable assessment of period length. In summary, expression of individual *Mef2* isoforms in clock neurons causes phenotypes similar to those caused by *Mef2*^{EP1751} and there were no clear differences between the four isoforms used, with the differences in phenotypes likely attributable to different levels of expression of *Mef2* in clock neurons.

***Mef2* is normally expressed in clock neurons**

Although *Mef2* is expressed in vertebrate and fly brains (Leifer et al., 1994; Ikeshima et al., 1995; Lyons et al., 1995; Lin et al., 1996; Schulz et al., 1996), its expression has not previously been reported in clock neurons. To test if the phenotypes described above arise from altering normal Mef2 levels in clock neurons, or from ectopic expression, we used a previously described Mef2 antibody (Lilly et al., 1995) to test if clock neurons normally produce Mef2.

Clock neurons were visualized in wild type adult fly brains using antibodies to PDF and TIM. These experiments were performed at ZT17 (ZT = Zeitgeber time, time in a 12hr:12hr LD cycle) when TIM is mainly cytoplasmic in clock neurons. Mef2 was clearly detected in the nucleus of all of the adult clock neuron groups: the LN$_v$ principal pacemaker neurons, the LN$_d$s and the DN1-3 subgroups (Fig. 2A). There were no obvious differences in Mef2 levels between the different clock neuron groups, although Mef2 levels were lower in clock neurons than in mushroom bodies. We also detected Mef2 staining in photoreceptor cells and many non-clock containing neurons in agreement with previous reports (Schulz et al 1996).

To test which regulatory regions direct *Mef2* expression to clock neurons, we used a *Mef2-Gal4* line (Ranganayakulu et al. 1998) to express a UAS nuclear LacZ transgene. LacZ was detected in the PDF-expressing LN$_v$s and other brain cells, but overall LacZ was present in a much narrower range of cells than Mef2 protein (Fig. 2B). LacZ was not detected in the mushroom bodies since a more distal *Mef2* enhancer is responsible for mushroom body expression (Schulz et al., 1996). Therefore *Mef2-Gal4* includes regulatory information for expression in clock neurons in addition to the previously characterized enhancers that give expression in muscle and cardiac precursor cells (Gajewski et al., 1997; Cripps et al., 1998).

Next we tested whether Mef2 levels are constant or show time-dependence in clock neurons. Focusing on the master s-LN$_v$ pacemaker neurons, we found significantly higher Mef2 levels towards the end of the night (ZT21) than the end of the day (ZT9, $p<0.0001$, Fig. 3A, B). To test for any regulation of Mef2 by the core clock, we measured Mef2 levels in clock gene mutants. We found that Mef2 levels were lower in the s-LN$_v$s of *Clk*$^{a r}$ and *cyc*^{0} mutants than in *per*^{0} and *tim*^{01} mutants (Fig. 3A, B). We also found regulation of Mef2 levels by light and by clock genes in larval LN$_v$s (Fig. 3C, D).

Rhythms in LD cycles and regulation by the clock suggested that Mef2 levels would also be rhythmic in DD like PER, TIM and other clock proteins. Surprisingly, we found that Mef2 levels were similar at CT9 and CT21, suggesting that *Mef2* expression is inhibited by light and/or that Mef2 protein is unstable in the presence of light (Fig. 3A, B). Therefore although Mef2 levels are altered by core gene mutations, this is non-circadian regulation. We also noticed variation in Mef2 levels between s-LN$_v$s in different brains, which could reflect a difference in exposure to light and/or other stimuli that regulate Mef2 levels. Since Mef2 levels are reduced in response to light independent of the circadian clock, Mef2 could provide a novel mechanism by which environmental information feeds into the molecular clock.

The experiments described here indicate that *Mef2* is normally expressed in clock neurons. We quantified Mef2 levels in *tim(UAS)-Gal4/Mef2*^{EP1751} larval s-LN$_v$s and found that Mef2 levels at ZT21 are approximately twice the levels in wild type LN$_v$s (Fig. 3E, F). Thus the altered behavioral rhythms seen in Figure 1 presumably result from over-expression of *Mef2* in clock neurons rather than from ectopic expression. Since this over-expression of *Mef2* alters behavioral rhythms, we propose that 24hr behavioral rhythms in wild type flies require normal levels of Mef2 activity.

Mef2 regulates *Drosophila* circadian locomotor activity

Since *Mef2* mutants affect many tissues and since many also cause embryonic lethality, we used the Gal4 / UAS system to interfere with Mef2 function only in clock neurons in two mechanistically distinct ways. First, we used a Mef2-EnR construct, which has the MADS box and Mef2 DNA-binding and dimerization domains fused to the Engrailed transcriptional repression domain (EnR). Replacing the activation domain of transcription factors has been widely used to dominantly interfere with endogenous transcription factor function both in *Drosophila* (Han et al., 2002) and in mammals, including investigations of mammalian Mef2 function (Karamboulas et al., 2006; Arnold et al., 2007).

We found that 39% of flies in which *UAS-Mef2-EnR* was expressed via *Pdf-Gal4* were arrhythmic and the remainder exhibited weaker rhythms than control flies (Table 2, Fig. 4). When *UAS-Mef2-EnR* was expressed using *tim(UAS)-Gal4*, flies had a ~25.4hr period, although half of these flies died within the first 5 days of the assay. The high incidence of lethality is unusual in our experience and probably reflects a small amount of non-clock cell expression of the *tim(UAS)-Gal4* driver. Overall, the results with *UAS-Mef2-EnR* indicate that down-regulation of *Mef2* target genes in clock neurons prevents robust circadian behavior.

For a second and independent way to interfere with Mef2 function, we used transgenic RNA interference (RNAi) to reduce *Mef2* RNA levels and thus Mef2 activity. We used a *UAS-Mef2*^{RNAi} construct in combination with *UAS-Dicer-2 (UAS-Dcr-2*) to knock down *Mef2* expression and we confirmed that this reduced Mef2 protein levels in LN$_v$s (Fig. 3E, F).

Pdf-Gal4 / +; UAS-Dcr-2 / UAS-Mef2^{RNAi} flies have ~25hr rhythms, with a much lower power than control flies (32.6 vs. 80.7, Fig. 4A, Table 2). Expression of *UAS-Mef2*^{RNAi} and *UAS-Dcr-2* in all clock neurons via *tim(UAS)-Gal4* driver made 70% of flies become progressively arrhythmic over a 12 day period (Fig. 4). Similar, but weaker, phenotypes were seen without *UAS-Dcr-2* (data not shown). In summary, the results with expression of *Mef2-EnR* and *Mef2*^{RNAi} in clock neurons indicate that normal levels of Mef2 activity are required to sustain circadian behavioral rhythms in DD. Although *Mef2*^{RNAi} might have been expected to give the opposite of *Mef2* over-expression (i.e. short period behavioral rhythms), the long period seen here may be the result of weak rhythms, which is the true phenotype. Together with the results of *Mef2* over-expression described in Figure 1, and the detection of Mef2 protein in adult clock neurons in Figure 2 and Figure 3, we conclude that normal *Mef2* activity is essential for the maintenance of robust 24hr rhythms in DD.

***Mef2* has a functional role in adult clock neurons**

Alterations to s-LN$_v$ morphology have previously been associated with a change in the period of behavioral rhythms (Berni et al., 2008). Given the widespread roles of *Mef2* in development and differentiation, we tested if LN$_v$ morphology is altered in flies with altered Mef2 activity levels. We used antibodies to PDF to label the LN$_v$ cell bodies and projections. The results in Fig. 5A indicate that adult LN$_v$s in flies are present and project normally when *tim(UAS)-Gal4* was used to express either *Mef2*^{EP1751}, *Mef2-EnR* or *Mef2*^{RNAi}. Therefore normal levels of *Mef2* are not required for correct LN$_v$ morphology at least at the level measured here. However, we did notice increased PDF levels in when *tim(UAS)-Gal4* was used to express *Mef2*^{EP1751} (discussed later).

Next we tested if over-expressing *Mef2* in clock neurons in adulthood is sufficient to alter circadian behavioral rhythms. For this experiment, we used flies with a *tubulin-Gal80*^{ts} transgene *(tub-Gal80*^{ts}, McGuire et al. 2003) in addition to *tim-(UAS)-Gal4* and *Mef2*^{EP1751} transgenes. *tub-Gal80*^{ts} gives ubiquitous expression of a temperature-sensitive Gal80 protein, which represses Gal4 activity at permissive temperatures (McGuire et al., 2003). These flies were raised at the permissive temperature of 25°C and locomotor activity of adult flies was then assayed in DD first at 25°C and then at the restrictive temperature of 31.5°C (Fig. 5B). Since the first time that flies were shifted to the restrictive temperature was during the locomotor assay, Gal4 activity and thus *Mef2* over-expression in clock neurons would only be initiated in adulthood after clock neurons are functional.

The representative actogram in Fig. 5B shows that at 25°C, *tim(UAS)-Gal4/Mef2*^{EP1751} flies with *tub-Gal80*^{ts} had normal rhythms, consistent with no *Mef2* over-expression. After shifting to 31.5°C, their periods lengthened, reaching an average of 26.0 ± 0.8 hr. Importantly after returning to 25°C, all of the flies remained rhythmic (n=15) and their periods shortened again, consistent with Gal80 re-activation preventing *Mef2* over-expression. However, there was a residual long-period (average 24.6 ± 0.6 hr), presumably due to persistence of *Mef2* RNA and/or protein.

As a control, we also assayed *tim(UAS)-Gal4/Mef2*^{EP1751} flies without *tub-Gal80*^{ts} with the same temperature shifts. On shifting to 31.5°C, these flies also had long periods (26.1± 0.7 hr), but these degenerated into very weak rhythms, complex rhythms or arrhythmicity. After returning to 25°C, these flies had significantly weaker power rhythms than flies with the *tub-Gal80*^{ts} transgene ($p<0.004$). The periods of *tim(UAS)-Gal4/Mef2*^{EP1751} flies are longer at 31.5°C than at 25°C (Fig. 1A), whether or not they have the *tub-Gal80*^{ts} transgene and this is probably due to increased Gal4 activity at higher temperatures (Duffy, 2002). Control flies with *tim(UAS)-Gal4*, *tub-Gal80*^{ts} and UAS-nuclear LacZ had normal period lengths at both 25°C and 31.5°C (data not shown). In summary, the ability of *Mef2* to lengthen period when over-expressed only in adulthood indicates that normal Mef2 levels are required in adult clock neurons for 24hr rhythms.

Altered s-LN$_v$ molecular clock oscillations in flies over-expressing *Mef2*

Since *Mef2* affects circadian behavior, we expected changes in the molecular clocks of their master pacemaker neurons, the s-LN$_v$s. We examined the s-LN$_v$ molecular clocks at different times of day on either the second day (DD2) or the eighth day (DD8) in DD in flies that were previously entrained to LD cycles.

In control flies, the s-LN$_v$ molecular clocks show robust oscillations at DD2 with PER at high levels at CT23 and CT7 (Fig. 6A), VRI at high levels at CT11 and CT18 (Fig. 6A), and PDP1 and TIM at high levels at CT18 and CT23 (Fig.6B). On DD2, PER, TIM, PDP1 and VRI all show clear oscillations in the s-LN$_v$s of *tim(UAS)-Gal4/Mef2*^{EP1751} flies, since there were always one or two timepoints at which a particular clock protein was undetectable (Fig. 6A, B). However, VRI was detected in all four s-LN$_v$s in *tim(UAS)-Gal4/Mef2*^{EP1751} flies at CT23 as opposed to being undetectable in control flies at that time. Similarly PER was still detectable at CT11 in *tim(UAS)-Gal4/Mef2*^{EP1751} flies, but not in control flies. Therefore, PER and VRI disappearance is slightly delayed in *tim(UAS)-Gal4/Mef2*^{EP1751} s-LN$_v$s relative to controls. The timing of TIM and PDP1 accumulation was similar in both *tim(UAS)-Gal4/Mef2*^{EP1751} and control flies on day 2 in DD (Fig. 6B), although levels may have been slightly lower in the latter. Overall, these data indicate that the s-LN$_v$ molecular clocks in *tim(UAS)-Gal4/Mef2*^{EP1751} flies are functional but are slightly delayed in phase compared to wild type flies.

We also noted that levels of PDF are ~2-fold higher in the cell bodies of *tim(UAS)-Gal4/Mef2*^{EP1751} s-LN$_v$s compared to control flies at both CT6/7 and CT18 on DD2 (Fig. 6C). PDF levels are also higher in the dorsal projections of *tim(UAS)-Gal4/Mef2*^{EP1751} s-LN$_v$s (data not shown). Previous studies have shown that overexpressing *Pdf* in the dorsal part of the brain causes complex rhythms in flies (Helfrich-Forster et al., 2000), therefore increased PDF levels in s-LN$_v$s could contribute to the complex rhythms found in *tim(UAS)-Gal4/Mef2*^{EP1751} flies.

The timing of PER and VRI accumulation and turnover in *tim(UAS)-Gal4/Mef2*^{EP1751} s-LN$_v$s on day 8 in DD was also delayed compared to control flies (Fig. 7A). PER and VRI levels were either very low or undetectable at one time point (CT17 and CT11.5 respectively) but present at other time points, indicating that the molecular clocks are still oscillating. However, on closer inspection we noticed different numbers of s-LN$_v$s with high levels of VRI staining at CT5 and CT23 in *tim(UAS)-Gal4/Mef2*^{EP1751} flies. A higher power magnification of the three s-LN$_v$s shown at CT23 in Fig. 7B revealed that (from left to right) one s-LN$_v$ had neither PER nor VRI, another s-LN$_v$ had high levels of PER (red) but no VRI, while the third had high levels of VRI (blue) and low levels of PER. Thus the molecular clocks in the s-LN$_v$s in *tim(UAS)-Gal4/Mef2*^{EP1751} flies have different phases from each other and/or are running at different speeds. Desynchrony within one s-LN$_v$ cluster was detected in one third of the *tim(UAS)-Gal4/Mef2*^{EP1751} brains examined across all time points (18 / 54), but this was very rarely seen in control brains (2 / 43). To measure s-LN$_v$ synchrony more precisely, we quantified PER levels in individual in one cluster and plotted the average and the distribution of a single cluster (Fig. S1). We found that PER levels in the s-LN$_v$s of *tim(UAS)-Gal4/Mef2*^{EP1751} flies tend to have a much larger standard deviation than in control flies. These data support the conclusion that the molecular clocks in *tim(UAS)-Gal4/Mef2*^{EP1751} s-LN$_v$s have a higher incidence of desynchrony than in control flies and this could even underlie the complex rhythms that appear in DD. The overall delayed appearance of VRI and PER in *tim(UAS)-Gal4/Mef2*^{EP1751} s-LN$_v$s is consistent with the ~25hr period rhythm component seen in *tim(UAS)-Gal4/Mef2*^{EP1751} flies.

Altered molecular clock oscillations in s-LN$_v$s of flies expressing *Mef2*^{RNAi}

We also examined the effect of knocking down *Mef2* expression on the s-LN$_v$ molecular clock by assaying clock protein oscillations in *tim(UAS)-Gal4/+; UAS-Mef2*^{RNAi}*/UAS-Dcr-2* flies. Fig. 8A shows that oscillations of PER and VRI were detected in both control and *tim(UAS)-Gal4/+;UAS-Mef2*^{RNAi}*/UAS-Dcr-2* s-LN$_v$s on DD2. However, VRI was not detectable at CT12, indicating that VRI accumulation is delayed in these flies relative to control flies. By DD8, PER was barely detectable in *tim(UAS)-Gal4/+;UAS-Mef2*^{RNAi}*/UAS-Dcr-2* s-LN$_v$s at any time point, and VRI was only detected at high levels at one timepoint (CT17, Fig. 8B). *Mef2* knock down had no obvious effect on PDF levels in s-LN$_v$s. Thus expression of *Mef2*^{RNAi} in all clock cells dampens the molecular oscillator in the s-LN$_v$s, and this parallels the behavior of these flies in which flies become arrhythmic during DD (Fig. 4).

Discussion

Mef2 is required for robust circadian rhythms

This study establishes that normal Mef2 levels and transcriptional activity are required for the maintenance of robust and accurate *Drosophila* circadian locomotor rhythms. This conclusion is based on: (i) flies in which *Mef2* was over-expressed in clock neurons via *Mef2*^{EP1751} or *UAS-Mef2* transgenes; and (ii) flies with a repressor form of Mef2 (Mef2-EnR) or RNAi to knockdown *Mef2* expression. All of these manipulations altered normal rhythms, and some had strong effects when targeted only to the PDF-producing LN$_v$s. Interestingly, some of the behavioral phenotypes developed over several days e.g. the appearance of complex rhythms in *Mef2*^{EP1751} flies and the loss of rhythms in *Mef2*^{RNAi} flies.

Molecular correlates of the adult behavioral alterations also developed after multiple days in DD. *Mef2* over-expression in all clock neurons dramatically increased the incidence of desynchronized s-LN$_v$ molecular clocks by day 8 in DD. Since there were always time points when a particular clock protein was absent, we conclude that the s-LN$_v$ clocks in *Mef2*^{EP1751} flies still oscillate but have lost their normal tight coupling to one another. Flies in which *Mef2*^{RNAi} was expressed in all clock neurons had relatively normal molecular rhythms on day 2 in DD, but strongly dampened oscillations in their s-LN$_v$ molecular clocks by day 8. These results indicate that *Mef2* is probably not a typical core clock gene in which the behavioral and molecular phenotypes are obvious immediately on transferring flies to DD (e.g. Price et al., 1998). Instead, *Mef2* is required to maintain robust and synchronized molecular and behavioral circadian rhythms.

A functional role for Mef2 in clock neurons

Our results show that Mef2 is normally expressed in clock neurons and its levels are regulated by both light and clock genes. Importantly, the behavioral alterations seen with altered Mef2 activity only in clock neurons indicates that wild type Mef2 activity is required for their normal function in circadian rhythms. Although we cannot rule out subtle alterations to s-LN$_v$ morphology with manipulation of Mef2 activity, the persistence of molecular oscillations in the s-LN$_v$s on day 2 in DD in flies in which *Mef2* expression was knocked down via *Mef2*^{RNAi} indicates that these cells are largely functional. Furthermore, since over-expressing *Mef2* in adult clock neurons after they have developed was sufficient to lengthen period, we conclude that Mef2 has an important regulatory role in adult pacemaker neurons. Below, we describe three possibilities for the normal role of Mef2 in clock neurons that are not mutually exclusive.

One explanation for the period alterations seen with *Mef2* over-expression is that Mef2 direct target genes include one or more of the core clock genes whose altered expression can change period length. Mef2 has been reported to work in concert with basic Helix-Loop-Helix (bHLH) transcription factors in muscle development (Molkentin and Olson, 1996). This is intriguing because the core clock transcriptional regulators CLK and CYC are bHLH transcription factors. Indeed the low levels of VRI and PER seen when *Mef2* expression was targeted by RNAi are consistent with reduced expression of CLK/CYC-activated genes.

It is also possible that Mef2 regulates PDF synthesis, maturation and/or release, which could in turn affect period length and/or the coherence of rhythms. Although simple over-expression of PDF in LN_vs does not affect the overall strength of behavioral rhythms (Helfrich-Forster et al., 2000), complex rhythms have been observed in *sine oculis* mutants in which PDF levels are increased in LN_vs (Wulbeck et al., 2008). Complex rhythms were also observed by Helfrich-Forster et al. (2000) when *Pdf* was ectopically expressed in the dorsal part of the brain, although this is likely from non-clock neurons that continuously release PDF and therefore disrupt the normal s-LN_v-mediated rhythm in PDF signaling. The desynchrony of the s-LN_v molecular clocks when *Mef2* is over-expressed could also arise from altered PDF levels since PDF is required for s-LN_vs to maintain synchrony (Lin et al., 2004).

A third possibility is that Mef2 regulates period length and rhythm coherence by altering the excitability of clock neurons and/or by affecting communication between clock neurons. This idea is based on the similar behavioral phenotypes and increased PDF levels that we observe with *Mef2* over-expression in all clock neurons and that Nitabach et al. (2006) observed with LN_v hyper-excitation via NaChBac, a bacterial voltage-gated Na+ channel. Indeed, a growing body of evidence indicates that events at the membrane of pacemaker neurons in both flies and mammals are intimately tied to their molecular clocks (Harmar et al., 2002; Nitabach et al. 2002; Lundkvist et al., 2005; Liu et al. 2007). A role for Mef2 in cellular communication in *Drosophila* is consistent with Mef2 regulating synapse formation in rat hippocampal and cerebellar neurons (Flavell et al., 2006; Shalizi et al., 2006). In hippocampal neurons, Ca^{2+} influxes in response to neuronal activity activate Calcineurin, which dephosphorylates and activates Mef2. In turn, increased Mef2 activity leads to reduced synapse numbers (Flavell et al., 2006). Although it is not known if *Drosophila* Mef2 activity can be regulated in this manner, normal Ca^{2+} levels in LN_vs are required for 24hr rhythms (Harrisingh et al., 2007). Since mammalian circadian pacemaker neurons show daily changes in Ca^{2+} levels (Colwell, 2000; Ikeda et al., 2003), it is conceivable that daily changes in Ca^{2+} levels in LN_vs could influence Mef2 activity. Mef2 could even contribute to the recent description of circadian rhythms in the branching patterns of s-LN_vs (Fernandez et al., 2008). In the long-run, it will be important to identify the set of genes regulated by Mef2 in s-LN_vs, uncover their function, and thus to understand how Mef2 contributes to robust circadian behavior. It will also be interesting to test a role for mammalian Mef2 in pacemaker neurons in the SCN.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are very grateful to Cyrille Alexandre, Jeff Hall, Zhe Han, Paul Hardin, Javier Morante, Hanh Nguyen, Eric Olson, Jae Park, Bruce Paterson, Mike Young, the Bloomington Stock Center, the Vienna *Drosophila* RNAi Center and the Developmental Studies Hybridoma Bank at the University of Iowa for supplying us with DNA, antibodies and fly strains. We thank Meg Younger and Monica Dandapani for their help with the genetic screen. We also thank Rich Cripps, Claude Desplan and Hanh Nguyen for helpful discussions and David Dahdal, Marc Ruben and Javier Morante for comments on the manuscript. This investigation was conducted in a facility constructed with support from Research Facilities Improvement Grant Number C06 RR-15518-01 from NCRR, NIH, and the work was supported by NIH grant GM063911 to JB.

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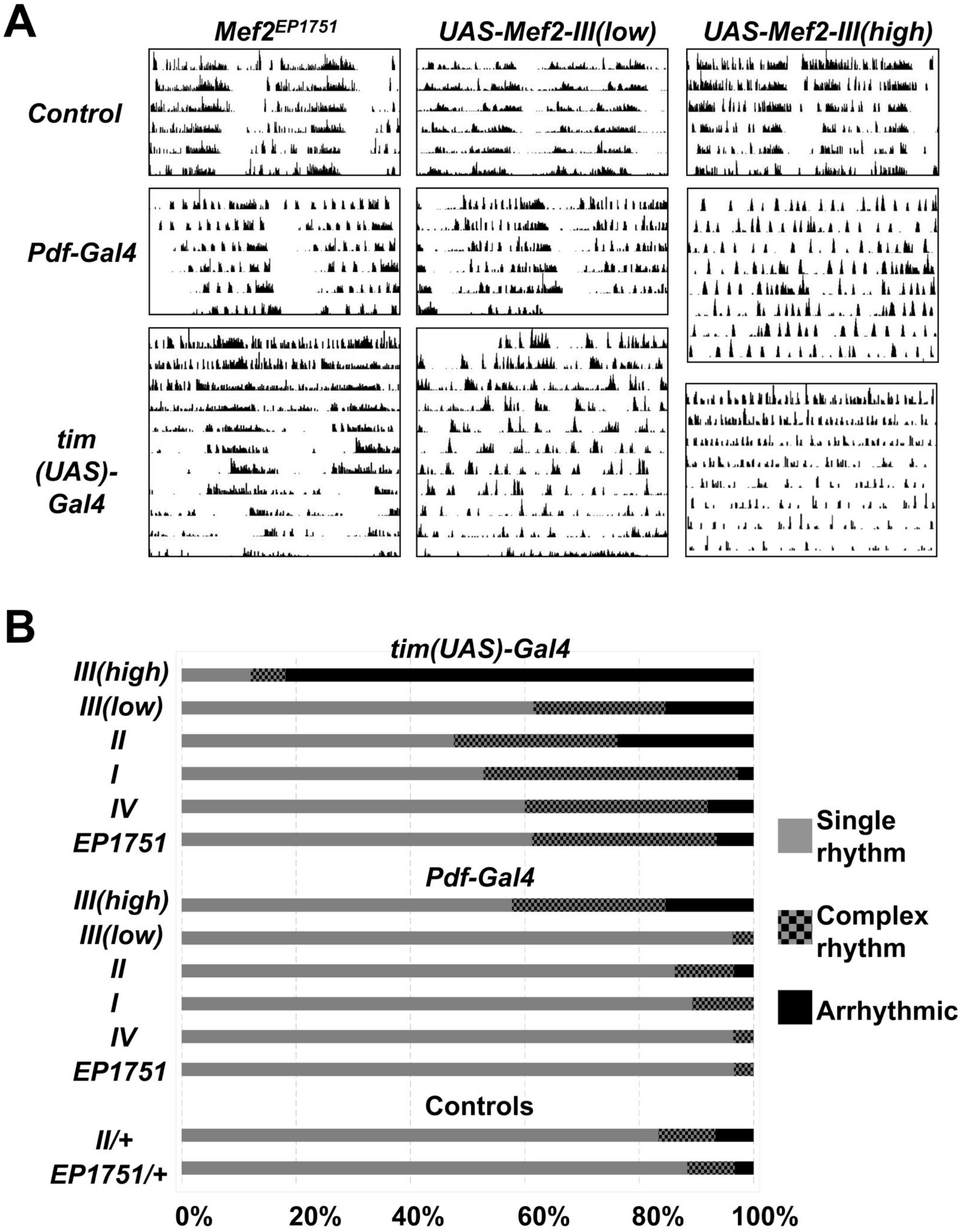
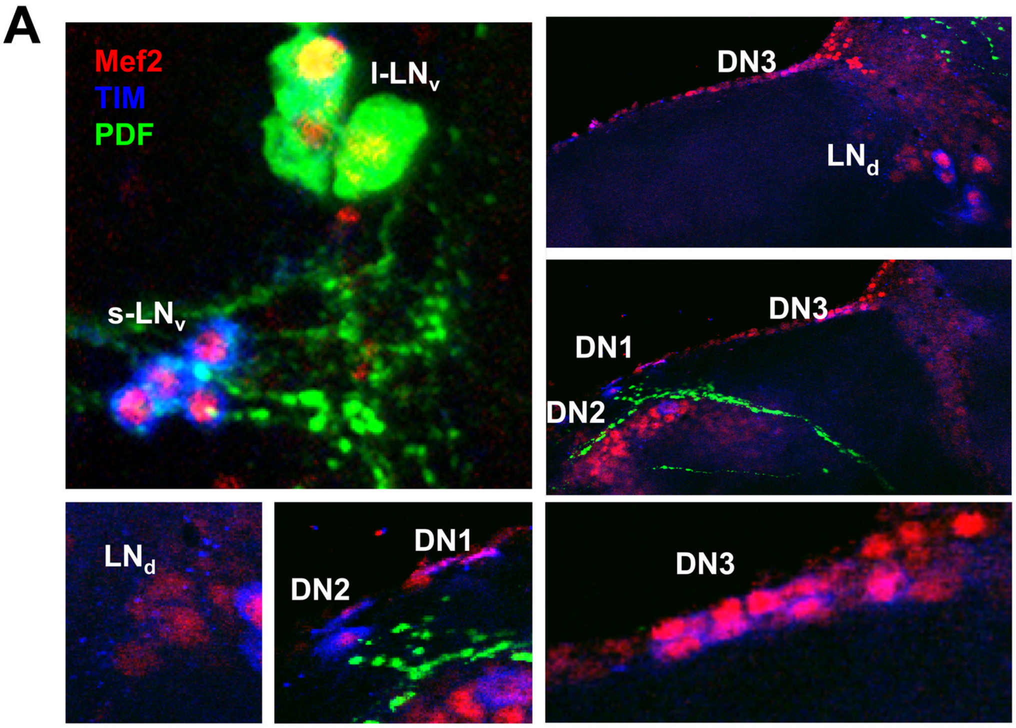


Figure 1. Over-expressing *Mef2* in clock neurons disrupts normal circadian locomotor activity

(A) Representative double-plotted actograms in DD from *Mef2*^{EP1751} flies (left column), *UAS-Mef2 III(low)* flies (center column), and *UAS-Mef2 III(high*) flies (right column). Actograms are shown for these flies crossed to *y w* flies (top row), *Pdf-Gal4* (center row) and *tim(UAS)-Gal4* flies (bottom row). **(B)** Graph shows percentage of flies exhibiting either a single rhythm (gray), complex rhythms (checked) or arrhythmicity (black). A complex rhythm is defined as a rhythm exhibiting more than one significant period above the significance level at 95% confidence when analyzed with Lomb-Scargle analysis. Single rhythm and arrhythmicity are defined by the presence or the absence of a peak above the significance level respectively. *I, II and IV* refer to *UAS-Mef2* constructs for isoforms A, B and D respectively in. *III(low)* and

III(high) refer to two different *UAS-Mef2* transgenes for *Mef2* isoform C that express low and high levels of Mef2 protein respectively.



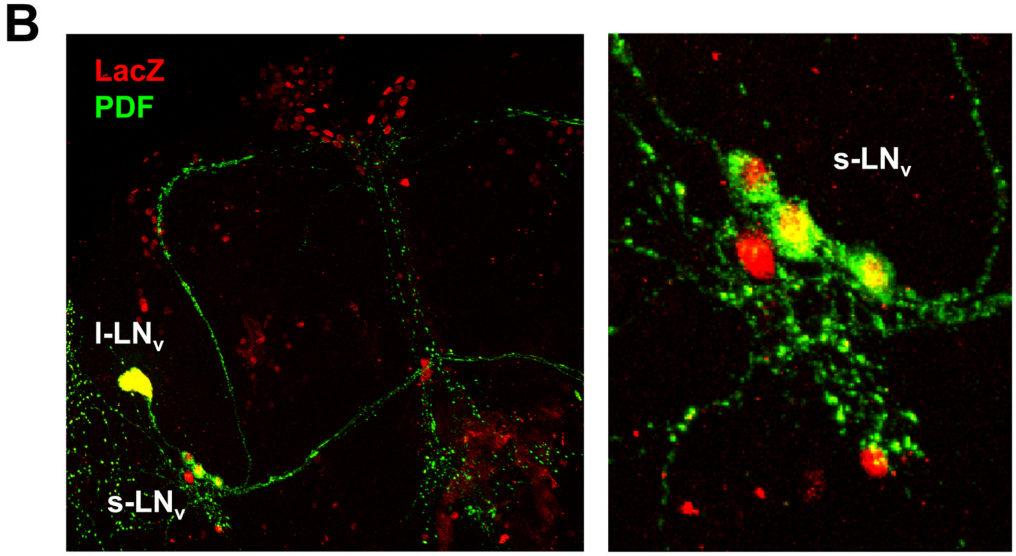


Figure 2. *Mef2* is normally expressed in clock neurons

(A) Whole-mount brains from *y w* flies at ZT17 stained with antibodies to Mef2 (red), TIM (blue, mainly cytoplasmic at ZT17) and PDF (green). Mef2 is detectable in the nucleus of all clock neurons groups at ZT17. **(B)** Whole-mount brains from flies with *Mef2-Gal4* and *UAS-nLacZ* transgenes stained with antibodies to LacZ (red) and PDF (green). The right-hand panel shows a close-up of the s-LN$_v$s in the left panel.

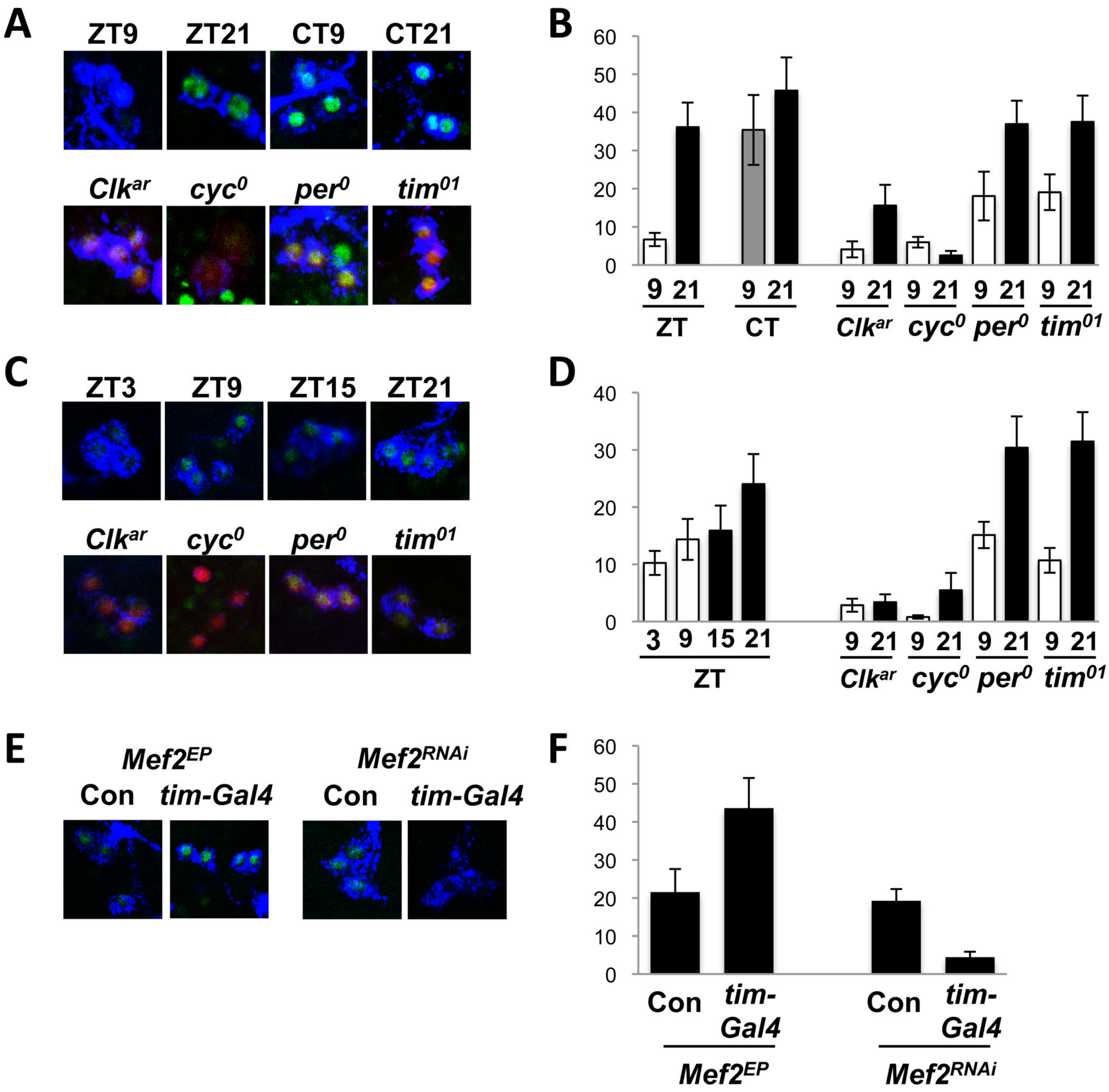


Figure 3. *Mef2* expression in LN$_v$s is regulated by light and by clock genes

(A) Representative images and **(B)** quantification of Mef2 levels in adult s-LN$_v$s. *y w* control brains were stained with antibodies to Mef2 (green) and PDF (blue) at ZT9 or ZT21 in LD and CT9 or CT21 on the second day of DD. *Clk*^{ar}, *cyc*^{0}, *per*^{0} and *tim*^{01} brains were also stained with antibodies to Mef2 at ZT9 and ZT21. The clock mutant images are from ZT21. A *Pdf-RFP* transgene was included to help mark LN$_v$s in clock mutants and the red channel was used to record RFP fluorescence since endogenous PDF levels are low in *Clk*^{ar} and *cyc*^{0} mutants. The strongly Mef2-staining cells visible in *cyc*^{0} mutants are not LN$_v$s. Mef2 levels in adult s-LN$_v$s were higher at ZT21 than at ZT9 ($p<0.0001$) but not different between CT9 and CT21 ($p=0.42$). Mef2 levels at ZT21 are higher in *per*^{0} and *tim*^{01} mutants than in *Clk*^{ar} and *cyc*^{0}

mutants ($p<0.01$). **(C)** Representative images and **(D)** quantification of Mef2 levels in larval LNs. *y w* (control) and clock mutant brains were stained as in A. The clock mutant images are from ZT21. The strongly Mef2+ cells visible in cyc^0 mutants are not LN_vs. Mef2 levels at ZT21 are higher than ZT3 ($p<0.01$). Mef2 levels are higher in per^0 and tim^{01} mutants than in Clk^{ar} and cyc^0 mutants ($p<0.002$) and show time-dependency in per^0 and tim^{01} mutants ($p<0.05$). **(E)** Representative images and **(F)** quantification of Mef2 levels in larval LNs at ZT21. *tim(UAS)-Gal4/*$Mef2^{EP1751}$ had higher Mef2 levels than $Mef2^{EP1751}$/+ controls (Con, $p<0.05$). *tim(UAS)-Gal4/+; UAS-*$Mef2^{RNAi}$*/ UAS-Dcr-2* larvae had lower Mef2 levels than *UAS-*$Mef2^{RNAi}$/+ control larvae ($p<0.0005$).

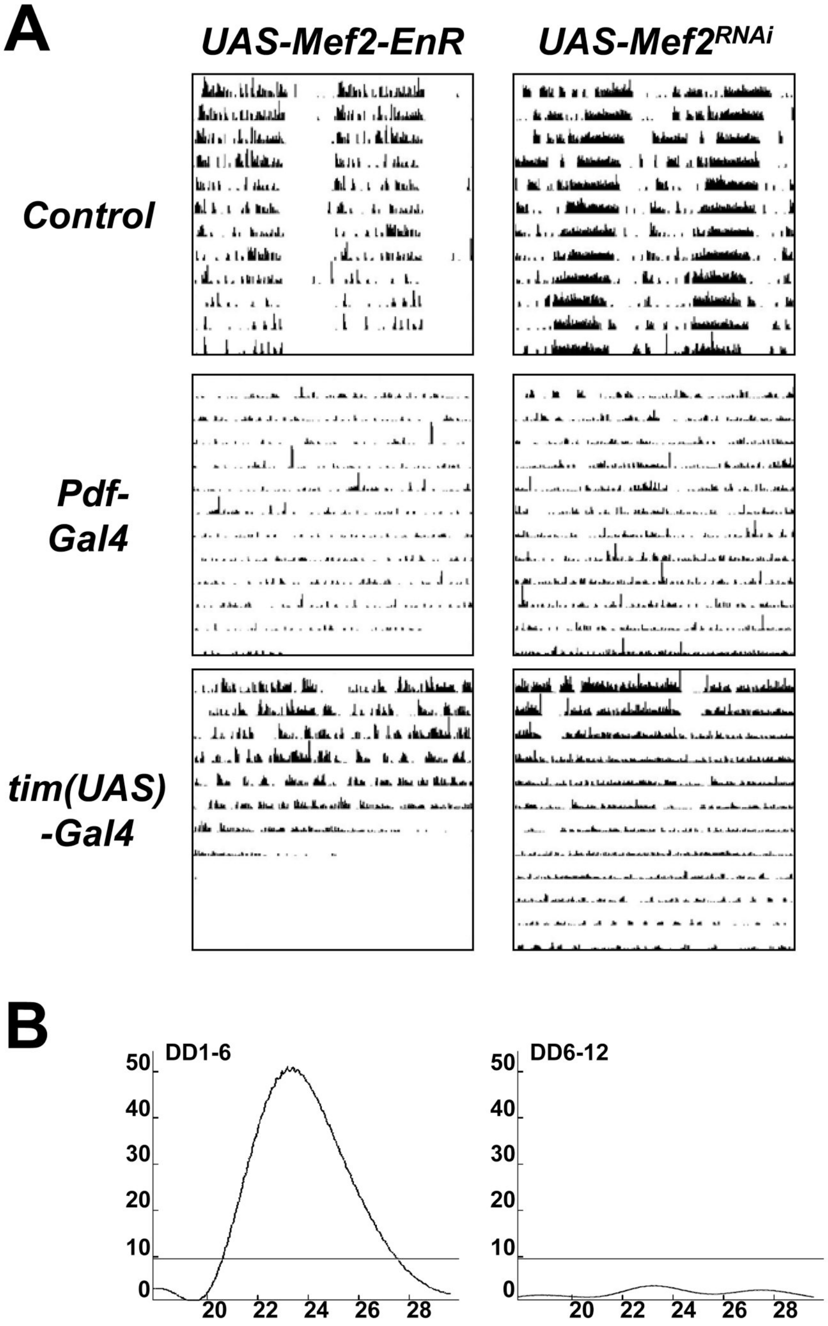
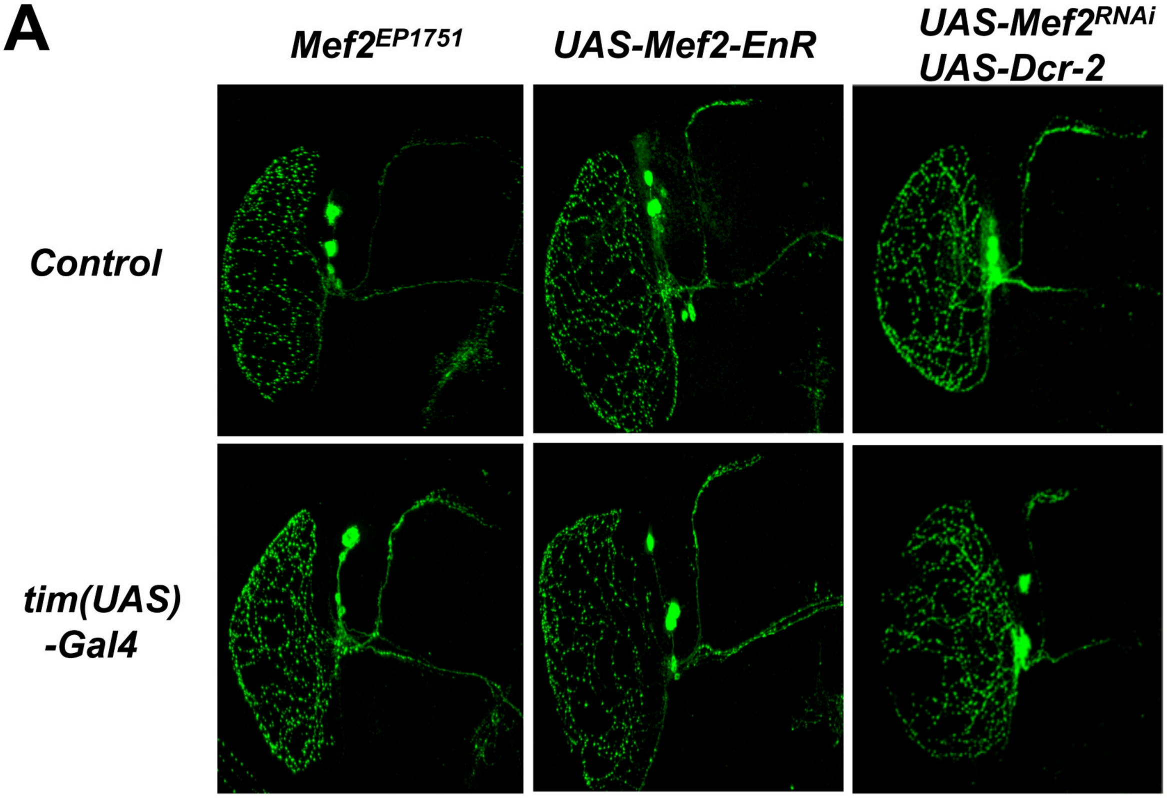


Figure 4. Expression of a Mef2 repressor or knocking down *Mef2* expression in clock neurons disrupts circadian behavioral rhythms

(A) Representative double-plotted actograms from *UAS-Mef2-EnR* (left panels) and *UAS-Mef2*^{RNAi} (right panels) flies in DD crossed to either *y w* control flies (top row), *Pdf-Gal4* (center panel) and *tim(UAS)-Gal4* flies (bottom panels). The *Pdf-Gal4* line crossed to *UAS-Mef2-EnR* had two copies of the *Pdf*$_{0.5}$*-Gal4* driver. The *Pdf-Gal4* and *tim(UAS)-Gal4* flies crossed to *UAS-Mef2*^{RNAi} flies also had a *UAS-Dcr-2* transgene. **(B)** Representative Lomb-Scargle periodograms for *tim(UAS)-Gal4/+; UAS-Dcr-2/UAS-Mef2*^{RNAi} flies from days 1–6 (left) and from days 6–12 (right) show the decrease in power of the rhythm in the second half of the assay.



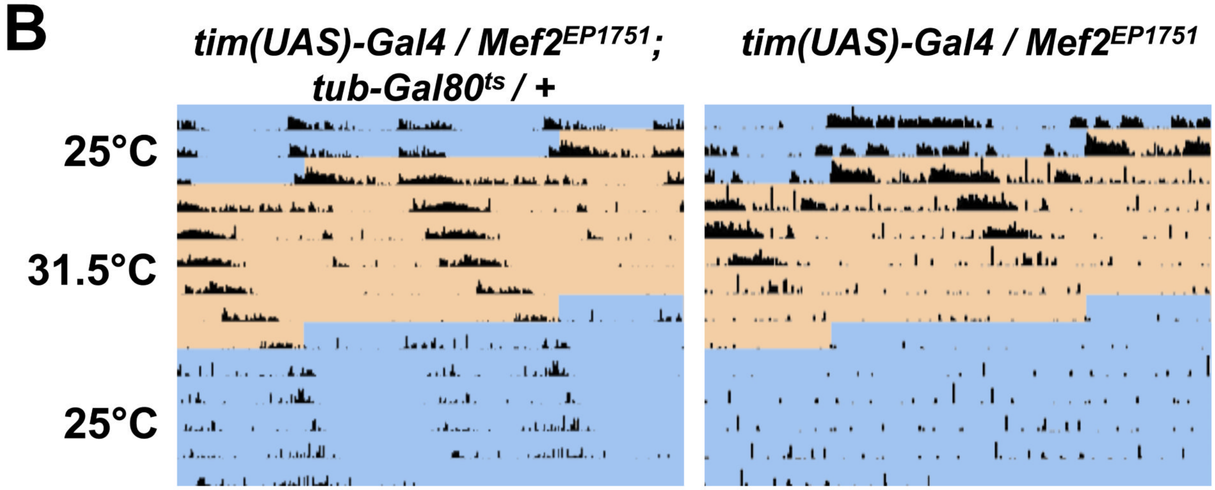


Figure 5.

(A) ***Mef2*** **is not required for the normal development of LN$_v$s**. Comparison of LN$_v$s labeled with an antibody against PDF for *Mef2^{EP1751}*flies (left column), *UAS-Mef2-EnR* flies (center) and *UAS-Mef2^{RNAi}* and *UAS-Dcr-2* flies (right column) crossed to either *y w* (top row) or *tim (UAS)-Gal4* (bottom row). **(B) Circadian phenotypes can be induced when** ***Mef2*** **over-expression is restricted to adult clock neurons**. Representative actograms of *tim(UAS)-Gal4/ Mef2^{EP1751}* flies either with a *tubulin-Gal80^{ts}* transgene (left) or without this transgene (right). Flies were maintained at 25°C until after eclosion and for the first 2.5 days in DD (shown in blue on the actogram), before shifting to the restrictive temperature for Gal80^{ts} (31.5°C, shown in orange) for 6 days before returning to 25°C for 5 days. These results are representative of

15 flies assayed with or without the *tubulin-Gal80^{ts}* transgene respectively. Average periods for *tim(UAS)-Gal4/Mef2^{EP1751}*; *tubulin-Gal80^{ts}* / + flies were 26.0 ± 0.8 hr and 24.6 ± 0.6 hr for the last 4 days at 31.5°C and the next 4 days at 25°C respectively. Average periods for *tim(UAS)-Gal4/Mef2^{EP1751}* flies were 26.1± 0.7 hr for the last 4 days at 31.5°C. On returning to 25°C, the average power of rhythms was higher for *tim(UAS)-Gal4/Mef2^{EP1751}*; *tubulin-Gal80^{ts}* / + flies than for *tim(UAS)-Gal4/Mef2^{EP1751}* flies ($p<0.004$), making period estimates very unreliable.

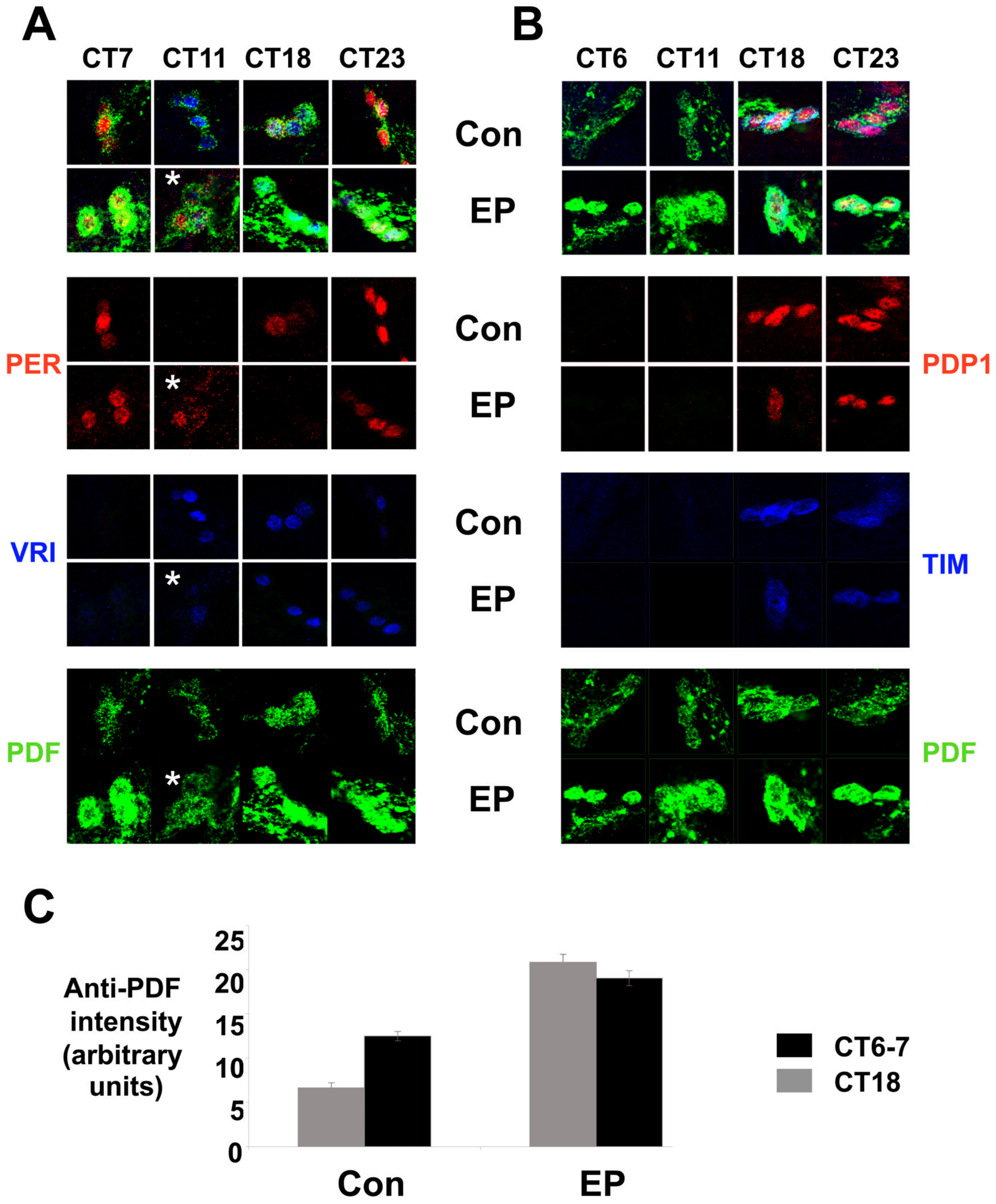


Figure 6. Molecular clock oscillations in s-LN$_v$s of flies over-expressing *Mef2* on day 2 in DD

Time series immunocytochemistry on whole mount brains from either *Mef2*^{EP1751}/+ control flies (labeled **Con**) or *tim(UAS)-Gal4/Mef2*^{EP1751} flies (**EP**) showing s-LN$_v$s stained with antibodies to PER (red), VRI (blue) and PDF (green) in **(A)** or PDP1 (red), TIM (blue), and PDF (green) in **(B)**. The asterisk indicates one image taken from an independent experiment from a different day. These images are representative of at least 10 brains stained in each experiment. Each experiment was performed 3 times with very similar results. **(C)** Quantification of anti-PDF staining in s-LNv cell bodies performed at CT6 or CT7 and CT18.

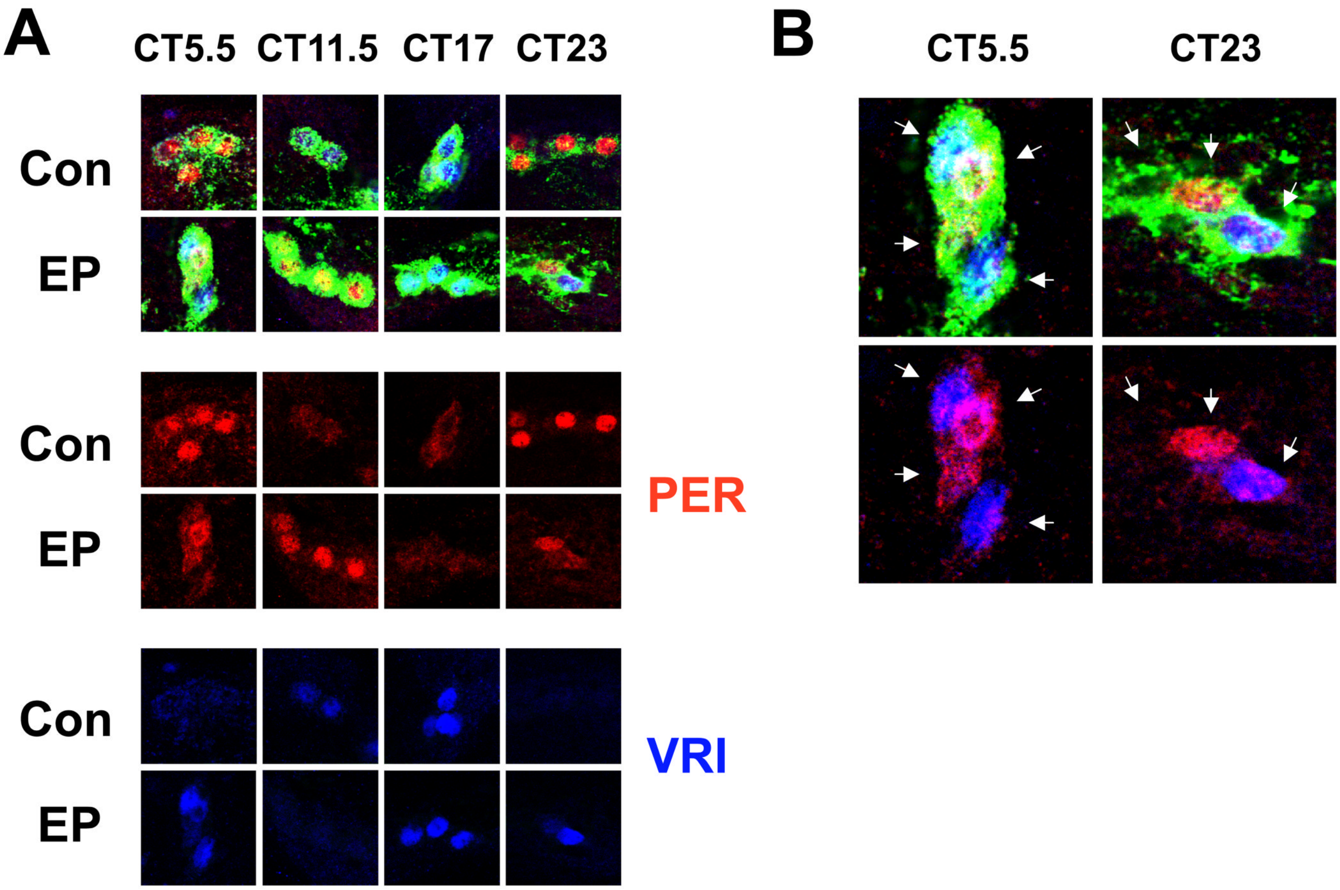


Figure 7. Desynchronized molecular clock oscillations in s-LN$_V$s of flies over-expressing *Mef2* on day 8 in DD

Time series immunocytochemistry on whole mount brains from either *Mef2*^{EP1751}/+ control flies (labeled **Con**) or *tim(UAS)-Gal4/Mef2*^{EP1751} flies (**EP**). Images show s-LN$_V$s stained with antibodies to PER (red), VRI (blue) and PDF (green). **(A)** shows stainings across 4 time points from both genotypes. **(B)** shows higher power magnification of s-LN$_V$s from *tim(UAS)-Gal4/Mef2*^{EP1751} flies at CT5.5 (left panels) and CT23 (right panels). The top panels show staining for PER, VRI and PDF, while the green PDF channel has been removed from these same images for the bottom panels. Arrowheads point to the same individual s-LN$_V$s on the top and bottom. Across all time points, desynchrony within an s-LN$_V$ group was detected in 18/54 *tim(UAS)-Gal4/Mef2*^{EP1751} brains, versus 2/43 control brains. These images are representative of at least 10 brains stained in each experiment. Each experiment was performed 3 times with very similar results.

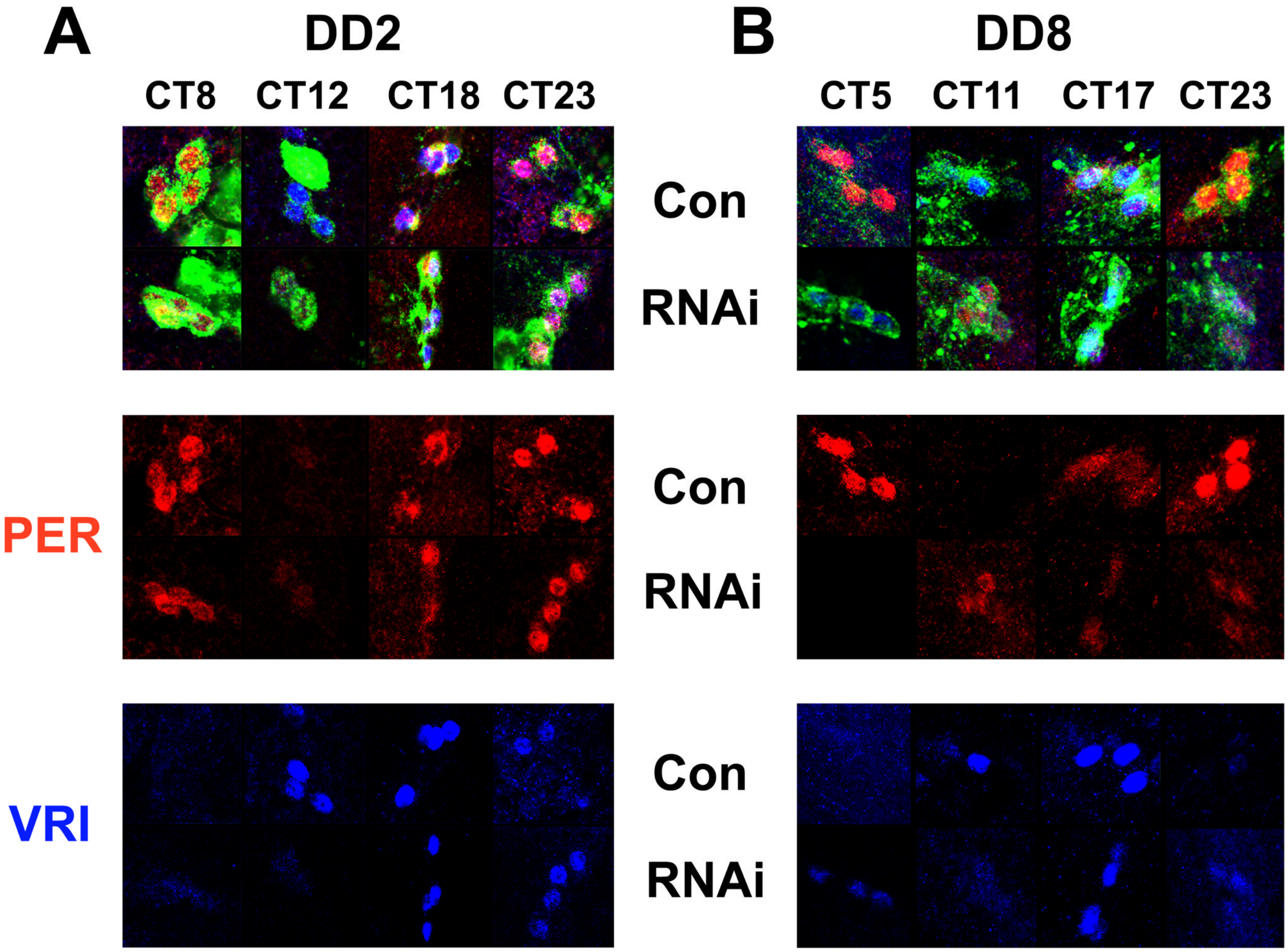


Figure 8. Knocking down *Mef2* in clock neurons dampens molecular clock oscillations in s-LN$_v$s

Time series immunocytochemistry on whole mount brains from either *UAS-Mef2^{RNAi}/+* control flies (labeled **Con**) or *tim(UAS)-Gal4/+; UAS-Mef2^{RNAi}/UAS-Dcr-2-2* flies (**RNAi**). Images show s-LN$_v$s stained with antibodies to PER (red), VRI (blue) and PDF (green). Time series are shown from either the second day (DD2 in **A**) or the eighth day in DD (DD8, **B**). These images are representative of at least 10 brains stained in each experiment. Each experiment was performed 3 times with very similar results.

Table 1

Adult locomotor activity rhythms with *Mef2* over-expression

Lomb-Scargle analysis of adult locomotor activity showing period (in hr) with standard error of the mean (SEM), power (to indicate the strength of the rhythm), number of flies analyzed (n), and the percentages exhibiting either a single rhythm, complex rhythm or arrhythmicity (AR). The period is the average of single rhythmic flies and the highest peak detected (i.e. the dominant period) for flies with complex rhythms.

Genotype	Period (hr)	Power	n	Single rhythm (%)	Complex rhythm (%)	AR (%)
Pdf-Gal4 / UAS-Mef2-III(high)	24.3 ± 0.6	24.8 ± 3.7	26	57.8	26.9	7.7
tim(UAS)-Gal4 / +; UAS-Mef2-III(high) / +	22.4 ± 1.4	11.5 ± 0.7	33	12.1	6.1	81.8
Pdf-Gal4 / UAS-Mef2-III(low)	25.1 ± 0.1	57.2 ± 5.9	27	96.3	3.7	0.0
tim(UAS)-Gal4 / +; UAS-Mef2-III(low) / +	24.6 ± 0.3	28.5 ± 4.7	26	61.5	23.1	15.4
Pdf-Gal4 / UAS-Mef2-II	25.1 ± 0.1	58.1 ± 8.7	29	86.2	10.3	3.5
tim(UAS)-Gal4 / +; UAS-Mef2-II / +	25.6 ± 0.3	21.5 ± 2.1	21	47.6	28.6	23.8
Pdf-Gal4 / UAS-Mef2-I	25.5 ± 0.1	58.3 ± 6.6	28	89.3	10.7	0.0
tim(UAS)-Gal4 / +; UAS-Mef2-I / +	25.4 ± 0.5	37.4 ± 6.7	18	52.8	44.4	2.8
Pdf-Gal4 / UAS-Mef2-IV	25.5 ± 0.1	69.4 ± 9.7	28	96.4	3.6	0.0
tim(UAS)-Gal4 / +; UAS-Mef2-IV / +	25.1 ± 0.1	69.0 ± 10.1	25	60.0	32.0	8.0
Pdf-Gal4 / Mef2^{EP1751}*; Pdf-Gal4 / +*	24.7 ± 0.1	80.8 ± 4.9	29	96.5	3.5	0.0
tim(UAS)-Gal4 / Mef2^{EP1751}	25.2 ± 0.2	47.0 ± 5.2	31	61.3	32.3	6.5
UAS-Mef2-II / +	23.3 ± 0.2	53.5 ± 7.3	30	83.3	10.0	6.7
Mef2^{EP1751} / +	23.9 ± 0.1	51.8 ± 6.2	24	88.4	8.3	3.3

Table 2

Adult locomotor activity rhythms with *UAS Mef2-EnR* and *UAS-Mef2^{RNAi}*

Lomb-Scargle analysis of adult locomotor activity showing period (in hr) with SEM, power (to indicate the strength of the rhythm), the number of flies analyzed (n), and the numbers of either arrhythmic flies (AR, top) or the numbers of flies that were arrhythmic from days 7–12 in the assay despite starting off as rhythmic (bottom).

Genotype	**Period (hr)**	**Power**	**n**	**AR**
UAS-Mef2-EnR / +	23.7 ± 0.1	52.4 ± 5.7	33	1
Pdf-Gal4 / +; Pdf-Gal4 / UAS-Mef2-EnR	23.6 ± 0.4	32.9 ± 6.9	18	7
tim(UAS)-Gal4 / +; UAS-Mef2-EnR / +	25.4 ± 0.7	29.2 ± 7.4	14	5
	Period (hr)	**Power**	**n**	**Loss of rhythms**
UAS-Mef2^{RNAi} / +	23.8 ± 0.2	80.7 ± 16.9	23	0
Pdf-Gal4 / +; UAS-dcr-2 / UAS-Mef2^{RNAi}	25.0 ± 0.4	32.6 ± 5.2	25	3
tim(UAS)-Gal4 / +; UAS-dcr-2 / UAS-Mef2^{RNAi}	23.7 ± 0.5	50 ± 4.7	36	25