Cellular/Molecular

Drosophila TRPA1 Functions in Temperature Control of Circadian Rhythm in Pacemaker Neurons

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Most animals from flies to humans count on circadian clocks to synchronize their physiology and behaviors. Daily light cycles are well known environmental cues for setting circadian rhythms. Warmer and cooler temperatures that mimic day and night are also effective in entraining circadian activity in most animals. Even vertebrate organisms can be induced to show circadian responses through exposure to temperature cycles. In poikilothermic animals such as *Drosophila*, temperature differences of only 2–3°C are sufficient to synchronize locomotor rhythms. However, the molecular sensors that participate in temperature regulation of circadian activity in fruit flies or other animals are enigmatic. It is also unclear whether such detectors are limited to the periphery or may be in the central brain. Here, we showed that *Drosophila* TRPA1 (transient receptor potential cation channel A1) was necessary for normal activity patterns during temperature cycles. The *trpA1* gene was expressed in a subset of pacemaker neurons in the central brain. In response to temperature entrainment, loss of *trpA1* impaired activity, and altered expression of the circadian clock protein period (Per) in a subset of pacemaker neurons. These findings underscore a role for a thermoTRP in temperature regulation that extends beyond avoidance of noxious or suboptimal temperatures.

Introduction

Circadian rhythms are self-sustained molecular oscillations, which adjust to environmental changes, such as daily oscillations in light intensities (Reppert and Weaver, 2002; Allada and Chung, 2010; Hardin, 2011; Kwon et al., 2011; Peschel and Helfrich-Forster, 2011). Day/night cycles enable animals to regulate innate behaviors, such as activity patterns. In standard laboratory strains of the fruit fly, *Drosophila melanogaster*, the animals display activity peaks at dawn and dusk. Once their activity is entrained by the light fluctuations, the flies show anticipatory behavior, which is increased movement before the times that the lights are turned on and off. Another feature of entrainment is that the animals sustain their cyclic behavior in constant darkness.

Over the last 30 years, there has been enormous progress in unraveling the molecular mechanisms underlying the *Drosophila* light-driven clock (Allada and Chung, 2010; Hardin, 2011; Peschel and Helfrich-Forster, 2011). Light input requires the blue light photoreceptor cryptochrome (Cry), which functions cell-autonomously in pacemaker neurons in the central brain (Emery et al., 1998, 2000; Stanewsky et al., 1998; Krishnan et al., 2001). Changes in circadian activity are controlled by an internal clock that depends on several clock genes and proteins, such as period (Per), which cycle in concentration and in spatial distribution over the circadian cycle in the pacemaker neurons (Konopka and Benzer, 1971; Bargiello et al., 1984; Reddy et al., 1984; Baylies et al., 1987; Siwicki et al., 1988; Zwiebel et al., 1991; Hardin et al., 1992; Curtin et al., 1995).

In addition to light, which is considered to be the strongest Zeitgeber (given time; ZT), it has been long-known that temperature cycles can also synchronize locomotor rhythms in both vertebrate and invertebrate organisms (Roberts, 1962; Helfrich, 1986; Wheeler et al., 1993; Glaser and Stanewsky, 2005, 2007; Boothroyd et al., 2007; Dubruille and Emery, 2008; Buhr et al., 2010; van der Linden et al., 2010). Peripheral neurons participate in temperature entrainment (Glaser and Stanewsky, 2005; Sehadova et al., 2009). In addition, a subset of central pacemaker neurons also contribute to temperature-induced synchronization of locomotor activity and these cells depend on input from the peripheral tissues (Yoshii et al., 2005; Busza et al., 2007; Miyasako et al., 2007; Picot et al., 2009; Sehadova et al., 2009). However, we do not know whether central pacemaker neurons express temperature sensors that function in temperature entrainment.

Members of the TRP family of cations comprise a well established class of temperature detectors (Ramsey et al., 2006;

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Venkatachalam and Montell, 2007; Damann et al., 2008). Among these channels is *Drosophila* TRPA1, which functions in the avoidance of noxious tastants (Kang et al., 2010; Kim et al., 2010), insect repellents (Kim et al., 2010), excessively bright light (Xiang et al., 2010), and uncomfortably warm and slightly suboptimal temperatures (Viswanath et al., 2003; Rosenzweig et al., 2005; Kwon et al., 2008; Neely et al., 2011; Shen et al., 2011; Kang et al., 2012; Zhong et al., 2012). Here, we identified a role for *Drosophila trpA1* in temperature synchronization of circadian activity. *trpA1* was expressed and functioned in a subset of pacemaker neurons in the brain. These data provide evidence that a subset of pacemaker neurons contribute to temperature synchronization of circadian activity through functioning cell-autonomously in temperature sensation.

Materials and Methods

Fly stocks. We previously described the generation of the $trpA1^{1}$ (Kwon et al., 2008) and $trpA1^{GAL4}$ (Kim et al., 2010), which we deposited at the Bloomington Stock Center. Both *trpA1* mutants were backcrossed into a w^{1118} background for five generations. The w^{1118} strain was used as the wild-type control. The *tim-GAL4* and *cry-GAL4* were contributed by A. Seghal (University of Pennsylvania, Philadelphia, PA) and M. Roshbash (Brandeis University, Waltham, MA), respectively. The w^{1118} and the *UAS-mCD8::GFP* flies were from the Bloomington Stock Center.

Immunohistochemistry. Immunostainings of adult brains were performed as previously described (Lee et al., 2009), except for the addition of the pre-fix step to prepare the data shown in Figures 2, 3, and 6. Briefly, we isolated flies at ZT9 or ZT21 after 5 or 6 d of temperature entrainment (18 h 29°C/6 h 18°C) as mentioned in the figure legends, and fixed the whole flies for 1–2 h with 4% paraformaldehyde in phosphate buffer with 0.1% Triton X-100. We dissected the brains and postfixed them on ice for 45 min by inserting them in 24-well cell culture cluster plates (Costar) containing 940 μl of fix buffer (0.1 M PIPES, pH 6.9, 1 mM EGTA, 1% Triton X-100, 2 mM $MgSO_4$, 150 mM NaCl) and 60 μl of 37% formaldehyde. To perform the immunostainings shown in Figure 4, we used 3- to 7-d-old flies that were entrained using light/dark cycles for 3–5 d. We then dissected the brains at ZT23 and fixed them without a pre-fix. The tissues were washed three times (1× PBS, 0.2% saponin) and blocked overnight at 4°C with 1 ml of Blocking Buffer (1× PBS, 0.2% saponin, 5 mg/ml BSA). The tissues were incubated with the primary antibodies for 24 h at 4°C, washed three times, blocked for 15 min and incubated with the secondary antibodies (Alexa Fluor 488 and Alexa Fluor 568, 1:200; Invitrogen) for 4 h at 4°C, and washed three times. The tissues were transferred into 1.25× PDA solution (37.5% glycerol, 187.5 mM NaCl, 62.5 mM Tris, pH 8.8) and viewed by confocal microscopy (Carl Zeiss LSM510). Primary antibodies were used at the following dilutions: mouse anti-GFP (1:1000, Invitrogen), rabbit anti-GFP (1:1000, Santa Cruz Biotechnology), mouse anti-β-GAL (1:1000, Promega), and rat anti-Per (1:1500; gift from A. Seghal and M. Wu).

Behavioral assays. For circadian locomotor behavior, we used the *Drosophila* Activity Monitoring (DAM) system (TriKinetics), which automatically records activity every time a fly crosses an infrared beam. Three- to 7-d-old flies were loaded into tubes that had 1% agarose and 5% sucrose food at one end. We entrained the flies to light/dark (LD) cycles for 3–5 d at 25° or 18°C before changing to the next conditions, such as darkness only (16°, 18°, 25°, or 29°C) or thermophase/cryophase (TC) cycles as indicated in the figures.

The TC entrainments in Figures 1 and 7 were performed as follows. The flies were entrained initially using 12 h light/12 h dark cycles for 4 d at 18°C, followed by 7 d of 18 h thermophase (29°C) and 6 h cryophase (18°C) cycles in constant darkness. The thermophase cycles were advanced 8 h relative to the previous LD cycle. After temperature entrainment, the flies were kept at 18°C in the dark (free run).

To perform the TC entrainment experiments shown in Figure 5 (antiphase experiments), the flies were entrained using 12 h LD cycles for 3 d at 25°C followed by 6 d of 12 h thermophase (25°C) and 12 h cryophase (16°C) TC cycles in constant darkness. The warm and cold phases were antiphase to the previous LD cycle, such that the first thermophase coincided with the time when the last night cycle during the LD cycle would have occurred. After the temperature entrainment, the flies were maintained at 25°C for the free running analysis.

Data analyses were performed using ClockLab in conjunction with MatLab, after collecting each file using 30 min bins. To determine the period and Fast Fourier Transfer (FFT) values, we analyzed the data obtained during the free runs (d 2–7) using χ^2 periodograms and batch analysis. If the τ FFT was not 0.042 (which corresponded to a 24 h period), the FFT values for the individual files were calculated manually. If the value of the FFT was >0.05, this indicated that $>5\%$ of the power was at that frequency and considered rhythmic. If the values for the FFT were <0.03, the flies were considered arrhythmic. If the values were between 0.05 and 0.03, the rhythmicities were considered to be borderline.

Results

Loss of *trpA1* altered activity during temperature entrainment of circadian rhythm

The *Drosophila* TRPA1 channel is a candidate for sensing thermal cycles that affect activity rhythms because it participates in thermotaxis (Rosenzweig et al., 2005; Kwon et al., 2008) and is directly activated in a range (>24°C) (Viswanath et al., 2003) that functions in temperature synchronization of circadian rhythms (18°–29°C). This is in contrast to two other thermally-activated *Drosophila* TRP channels, Pyrexia and Painless, which are activated directly by temperatures with much higher thresholds (40°C and 42°C, respectively) (Tracey et al., 2003; Lee et al., 2005). To determine whether loss of the TRPA1 channel affected activity patterns during temperature entrainment, we analyzed *trpA1* mutants using the DAM system (Chiu et al., 2010). Before assaying circadian activity set by TC cycles, we first examined activity entrained by 12 h LD cycles at a constant temperature (25°C). The $trpA1^{+}$ controls (w^{1118}; referred to as wild-type) have activity peaks at "lights-on" (ZT0) and "lights-off" (ZT12; Fig. 1*A*) (Allada and Chung, 2010). Flies increase their activity before the morning (lights on) or evening (lights off). This is called "morning anticipation" and "evening anticipation" and is a hallmark of circadian entrainment (Fig. 1*A*) (Allada and Chung, 2010). The evening anticipation displayed by *trpA1* mutants during the LD cycles was similar to the wild-type control (Fig. 1*A–C*). The *trpA1* flies also displayed morning anticipation, although it appeared to be reduced slightly (Fig. 1*A–C*). After allowing the flies to free run in constant darkness (DD), the morning (dawn) and evening (dusk) peaks remained, and the periodicities were similar to wild-type (Fig. 1*A–C*; Table 1). During the DD cycles, there also appeared to be reduced morning anticipation in *trpA1* mutants (Fig. 1*A–C*). Thus, with the exception of a reduction in morning anticipation, the *trpA1* flies behaved similarly to wild-type during the LD cycle and during the DD free run.

To address whether loss of *trpA1* impaired temperature synchronization of circadian activity, we switched from a LD cycle at 18°C, to constant darkness and used thermophase (29°C; simulates day) and cryophase (18°C, simulates night) cycles (Busza et al., 2007). To clearly resolve the morning and evening peaks, we initiated the first thermophase cycle 4 h into the final night cycle. We used 18 and 6 h TC cycles, which help separate the morning and evening peaks (Busza et al., 2007). Using this paradigm, wild-type flies reestablish morning and evening peaks within ~3 d, including pronounced evening anticipation (Fig. 1*D*,*G*, red and blue arrows on TC day 3, which was day 7 overall; note that except for day 1, each day of

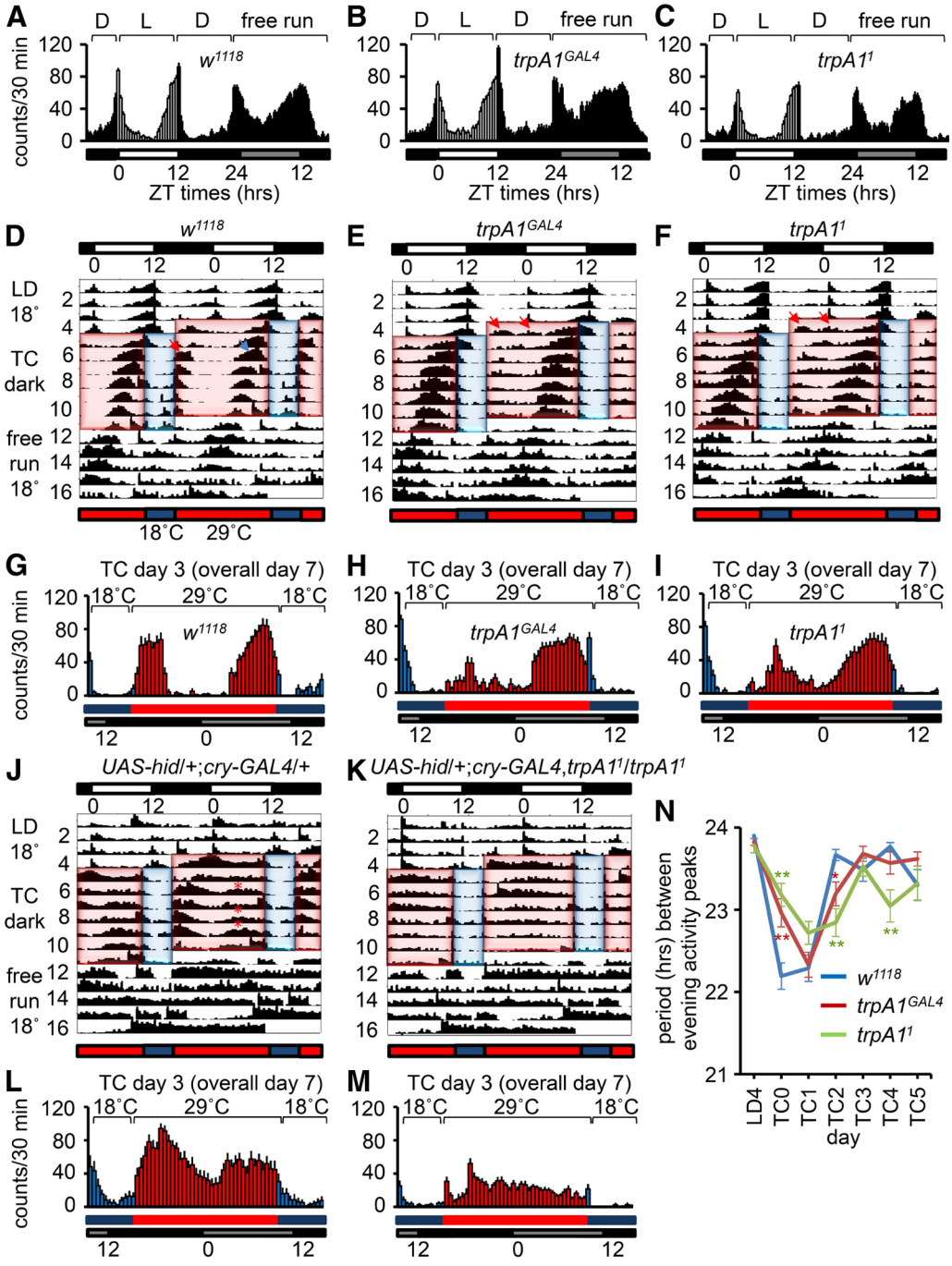

Figure 1. Actograms using LD and TC entrainment. ***A–C***, Average activities of male flies on day 4 under LD conditions (12 h light/dark cycles at 25°C), and day 1 under constant darkness (free run at 25°C). ZT times are indicated. The white, black, and gray bars represent day, night, and subjective day, respectively. ***A***, w^{1118}. ***B***, $trpA1^{GAL4}$. ***C***, $trpA1^{1}$. ***D–F***, ***J***, ***K***, Average normalized activities for the indicated genotypes. Flies were maintained for 4 d at 18°C < 12 h light/dark cycles (LD 18°C) and entrained for 7 d under constant darkness using 18 h (29°C) thermophases/6 h (18°C) cryophases (TC dark). The flies were then kept at a constant temperature (18°C) in the dark (free run 18°C). Except for overall days 1 and 17, the daily activities are presented on two lines. The top white and black bars indicate LD cycles. The bottom red and blue bars indicate TC cycles. The red and blue shading indicates warm and cool phases, respectively. ***D***, w^{1118}. The red and blue arrows on overall day 7 (TC day 3) indicate examples of morning and evening activities, respectively. ***E***, $trpA1^{GAL4}$. ***F***, $trpA1^{1}$. The two arrows on overall day 5 (TC day 1) indicate small double peaks. ***G–I***, Activities from TC day 3 (overall day 7) from ***D–F***, respectively. ***G***, w^{1118}. ***H***, $trpA1^{GAL4}$. ***I***, $trpA1^{1}$. ***J***, *UAS-hid/+;cry-GAL4/+*. Asterisks indicate smaller and broader evening activities. ***K***, *UAS-hid/+;cry-GAL4,*$trpA1^{1}$/$trpA1^{1}$. ***L***, ***M***, Activities from TC day 3 (overall day 7) from ***J*** and ***K***, respectively. ***N***, Periods between evening activity peaks from ***D–F***. The error bars represent SEM. The asterisks indicate significant differences from wild-type (*$p < 0.05$, **$p < 0.01$) based on ANOVA and the Scheffé *post hoc* test.

activity is shown on two lines to facilitate comparisons with the previous and following days).

Both *trpA1* alleles ($trpA1^{GAL4}$ and $trpA1^{1}$) (Kwon et al., 2008; Kim et al., 2010) displayed differences from wild-type during the TC cycles. During the first TC cycle the *trpA1* mutants exhibited double peaks (Fig. 1*E*,*F*, two red arrows on TC day 1, overall day 5). The second peak approximately coincided with the original morning peak established during the LD entrainment, suggesting that this peak persisted. In addition, the morning peaks during the TC cycles were reduced (Fig. 1*D–I*). The evening activity exhibited by the mutant flies initiated slightly earlier than wild-type (advanced activity), and was broader (Fig. 1*D–I*). During

Table 1. Circadian rhythmicity

Genotype	Entrain condition	Entrain temp (°C)	Free run (°C)	*n*	Rhy (%)	Period (h)	FFT
Wild-type	LD	25	25	77	95	23.93 ± 0.03	0.15 ± 0.01
trpA1^{GAL4}	LD	25	25	59	90	23.90 ± 0.04	0.17 ± 0.01
trpA1^{GAL4}/+	LD	25	25	16	100	23.83 ± 0.02	0.21 ± 0.02
trpA1^{1}	LD	25	25	32	94	23.63 ± 0.05	0.15 ± 0.01
Wild-type	LD	29	29	16	100	23.69 ± 0.06	0.15 ± 0.01
trpA1^{GAL4}	LD	29	29	16	100	23.66 ± 0.05	0.14 ± 0.01
trpA1^{GAL4}/+	LD	29	29	15	100	23.68 ± 0.03	0.14 ± 0.01
Wild-type	TC	25/16	25	45	93	23.66 ± 0.08	0.15 ± 0.01
trpA1^{1}	TC	25/16	25	45	96	23.28 ± 0.04*	0.13 ± 0.01
trpA1^{GAL4}	TC	25/16	25	36	83	23.11 ± 0.08*	0.10 ± 0.01
trpA1^{GAL4}/trpA1^{1}	TC	25/16	25	31	100	23.36 ± 0.07*	0.15 ± 0.01
UAS-trpA1-A;trpA1^{GAL4}	TC	25/16	25	28	96	23.64 ± 0.04	0.22 ± 0.01
UAS-trpA1-A;trpA1^{1}	TC	25/16	25	29	97	23.19 ± 0.07*	0.08 ± 0.01
tim-GAL4/+;trpA1^{1}	TC	25/16	25	16	81	23.32 ± 0.12*	0.10 ± 0.01
tim-GAL4/UAS-trpA1-A; trpA1^{1}	TC	25/16	25	16	88	24.12 ± 0.09	0.15 ± 0.02
trpA1^{GAL4}/+	TC	25/16	25	16	100	23.71 ± 0.06	0.20 ± 0.02

The flies were exposed to either LD or TC using a 12/12 h paradigm. Data are shown as the period and FFT values (±SEM) after the LD or TC cycles during days 2–7 of the free runs.

*$p < 0.05$, significant differences from wild-type using ANOVA and the Scheffé *post hoc* tests. Rhy, Rhythmicity.

Table 2. Circadian rhythmicity

Genotype	Entrain temp (°C)	Free run (°C)	*n*	Rhy (%)	Period (h)	FFT
Wild-type	29/18	18	29	86	23.52 ± 0.07	0.11 ± 0.01
trpA1^{1}	29/18	18	30	90	23.32 ± 0.07	0.15 ± 0.01
trpA1^{GAL4}	29/18	18	30	80	23.48 ± 0.14	0.13 ± 0.01
UAS-hid/cry-GAL4	29/18	18	32	41	27.12 ± 1.15	0.19 ± 0.01
UAS-hid/cry-GAL4;trpA1^{1}	29/18	18	32	6	23.00 ± 0.90	0.08 ± 0.02
per^{01}	29/18	18	31	23	26.50 ± 1.64	0.05 ± 0.01
UAS-trpA1-A /cry-GAL4;trpA1^{1}	29/18	18	16	94	22.03 ± 0.13	0.09 ± 0.01
UAS-trpA1-B/+;trpA1^{1}	29/18	18	32	97	23.73 ± 0.09	0.20 ± 0.01
tim-GAL4/+;trpA1^{1}	29/18	18	32	88	24.14 ± 0.38	0.20 ± 0.01
UAS-trpA1-B /tim-GAL4;trpA1^{1}	29/18	18	17	100	23.90 ± 0.10	0.19 ± 0.01
Wild-type	29/18	29	15	73	23.33 ± 0.25	0.09 ± 0.01
trpA1^{1}	29/18	29	16	100	22.68 ± 0.11*	0.10 ± 0.01
trpA1^{GAL4}	29/18	29	15	67	22.72 ± 0.09*	0.08 ± 0.01
per^{01}	29/18	29	16	0		
UAS-hid/cry-GAL4	29/18	29	16	38	22.94 ± 0.19	0.08 ± 0.01
UAS-hid/cry-GAL4;trpA1^{1}	29/18	29	16	6	22.9[1]	0.122[1]
Wild-type	25/16	16	16	75	23.16 ± 0.20	0.07 ± 0.01
trpA1^{1}	25/16	16	16	100	21.88 ± 0.26*	0.07 ± 0.01
UAS-trpA1-A;trpA1^{1}	25/16	16	16	81	21.78 ± 0.54*	0.07 ± 0.01
UAS-trpA1-A /tim-GAL4;trpA1^{1}	25/16	16	15	93	22.56 ± 0.21	0.08 ± 0.01
UAS-trpA1-A /cry-GAL4;trpA1^{1}	25/16	16	16	69	22.31 ± 0.14*	0.05 ± 0.01
per^{01}	25/16	16	16	0		

The flies were entrained under 18 h thermophase/6 h cryophase conditions and either 29°/18°C or 25°/16°C TC cycles. The free runs were performed at either 18° or 29°C. The period and FFT values (±SEM) are based on the free runs (days 2–7).

*$p < 0.05$, significant differences from wild-type, based on ANOVA and the Scheffé *post hoc* tests. [1]No error bars are presented because only one *UAS-hid/cry-GAL4;trpA1^{1}* fly showed rhythmicity.

the TC cycles the *trpA1* mutants required an additional day compared with wild-type to establish a constant period between the evening activity peaks (Fig. 1*N*). By TC day 2, wild-type displayed a period comparable to that during LD4 (Fig. 1*N*; TC2, 23.67 ± 0.07; LD4, 23.90 ± 0.00). However, the *trpA1* mutants did not exhibit a similar period until TC day 3 (Fig. 1*N*; *trpA1^{GAL4}*, 23.68 ± 0.09; *trpA1^{1}*, 23.53 ± 0.17).

Because *trpA1* mutants still have rhythmic activity induced by temperature entrainment, we tested whether the effect of removing *trpA1* would be enhanced by genetically ablating a subset of pacemaker neurons. In the *Drosophila* brain there are ~150 pacemaker neurons. In addition to six lateral posterior neurons (LPNs), the other pacemaker neurons sort into two major groups (Allada and Chung, 2010). These include the lateral neurons (LNs), which are located in the anterior cortex between the central brain and optic lobe, and the dorsal neurons (DNs) situated in the cortex associated with the dorsal protocerebrum. The LNs are divided into five small and four large ventrolateral neurons (s-LNvs and l-LNvs, respectively) and six dorsolateral neurons (LNds) in each hemisphere. There are also three groups of DNs (DN1, DN2, and DN3). Several pacemaker neurons have been suggested to participate in temperature entrainment of circadian rhythms. These include LNvs (Busza et al., 2007), DN1s (Zhang et al., 2010), DN2s (Busza et al., 2007; Picot et al., 2009), and LPNs (Busza et al., 2007; Yoshii et al., 2009). In addition, the concentration of Per in LPNs changes strongly in response to TC cycles but not to LD cycles (Busza et al., 2007). With the exception of the LPNs and DN2s, most pacemaker neurons including the LNvs, LNds, and some DN1s and DN3s are removed by expressing the proapoptotic gene *UAS-hid* under the control of the *cry-GAL4* (Busza et al., 2007). Therefore, we compared temperature entrainment of circadian activity in *UAS-hid*;*cry-GAL4* flies in the presence or absence of the *trpA1^{1}* mutation. As previously

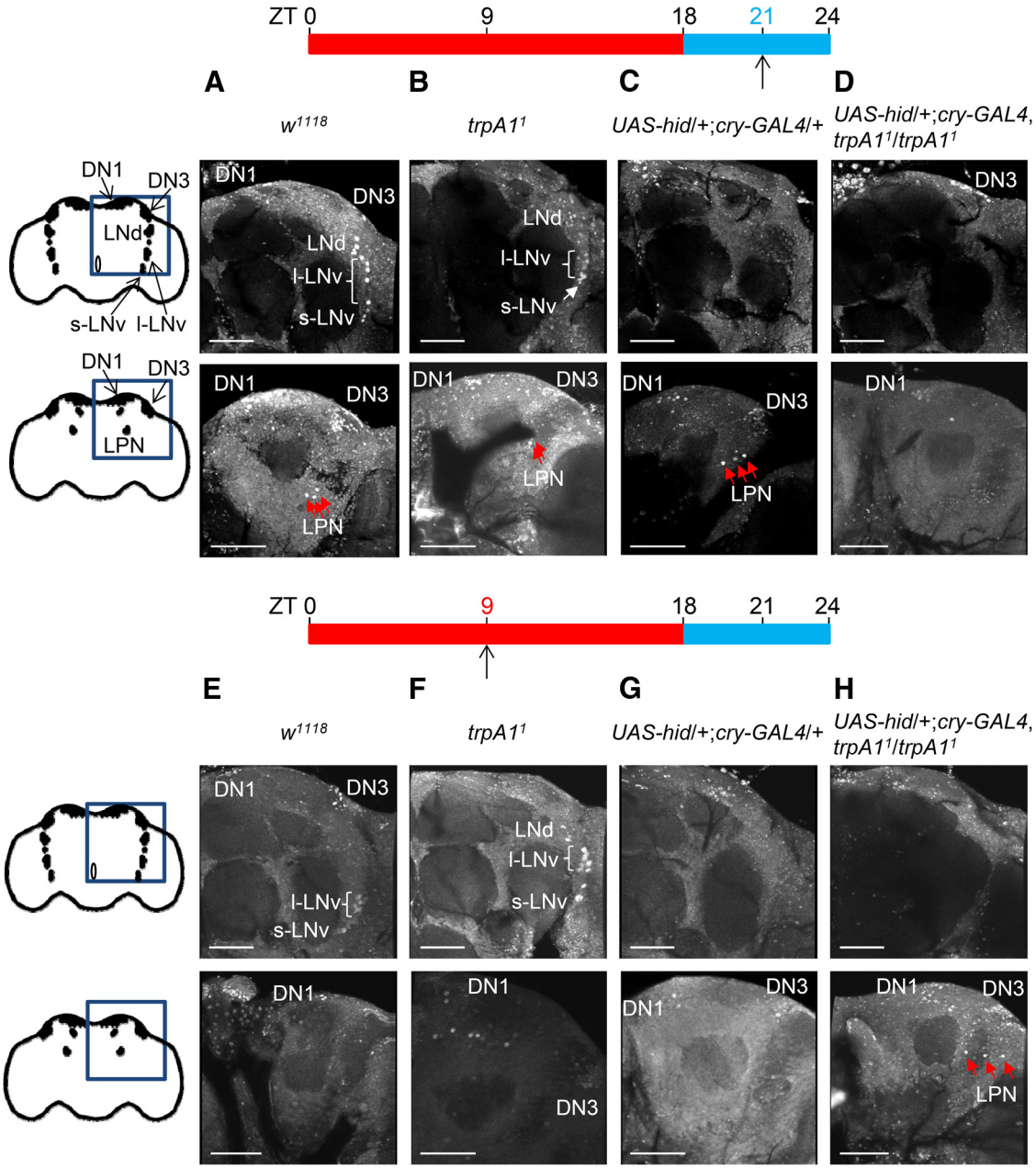

Figure 2. Relative Per levels during a TC cycle at ZT21 and ZT9. Three- to 7-d-old flies were entrained under a TC cycle (29°/18°C) for 6 d and dissected either at ZT21 or ZT9, and stained with anti-Per. The genotypes are indicated. More than 10 hemispheres were examined per genotype. The upper and bottom are anterior and posterior views, respectively. The diagrams represent adult brains. The boxes indicate the brain regions shown. The positions of the clusters of pacemaker neurons are presented. ***A–D***, ZT21. ***E–H***, ZT9. Scale bars, 50 μm.

described, during a LD cycle, the *UAS-hid*;*cry-GAL4* flies were missing the usual morning and evening peaks of activity, except for brief startle responses when the lights were turned on and off (Stoleru et al., 2004; Busza et al., 2007) (Fig. 1*J*). When these flies were switched to a TC cycle, they displayed two broad peaks (Fig. 1*J*,*L*) (Busza et al., 2007). During the free run at 18°C, 41% of the flies displayed rhythmicity (Table 2).

Of significance here, when we combined the *trpA1*^{1} mutation with the *UAS-hid*;*cry-GAL4* transgenes, the activity peaks during the TC cycles were virtually eliminated, and the flies were nearly arrhythmic during the free runs (6% rhythmicity) (Fig. 1*K*,*M*; Table 2). We obtained similar results when switched from the TC cycles to a free run using a constant thermophase (29°C) instead of cryophase (Table 2). Because introduction of the *trpA1* mutation into the sensitized *UAS-hid*;*cry-GAL4* background virtually eliminated the activity peaks during the TC cycles, and caused the flies to lose nearly all rhythmicity during the free runs, TRPA1 appears to contribute to TC entrainment of circadian rhythm. Even without the sensitized background (*UAS-hid*;*cry-GAL4*), loss of *trpA1* alone shortened the periodicity during the 29°C free run following 29°/18° TC cycles (18/6 h phases) (Table 2), and during the 16°C free run following 25°/16°C cycles (18/6 h phases) (Table 2).

Increased variation in Per expression in *trpA1*^{1}

In wild-type pacemaker neurons, the concentration of the clock protein Period (Per) (Konopka and Benzer, 1971; Citri et al., 1987) oscillates during the circadian cycle (Siwicki et al., 1988; Yoshii et al., 2005; Allada and Chung, 2010). Per is high at ZT21 (middle of the night) and ZT3 (early morning), and low at ZT9

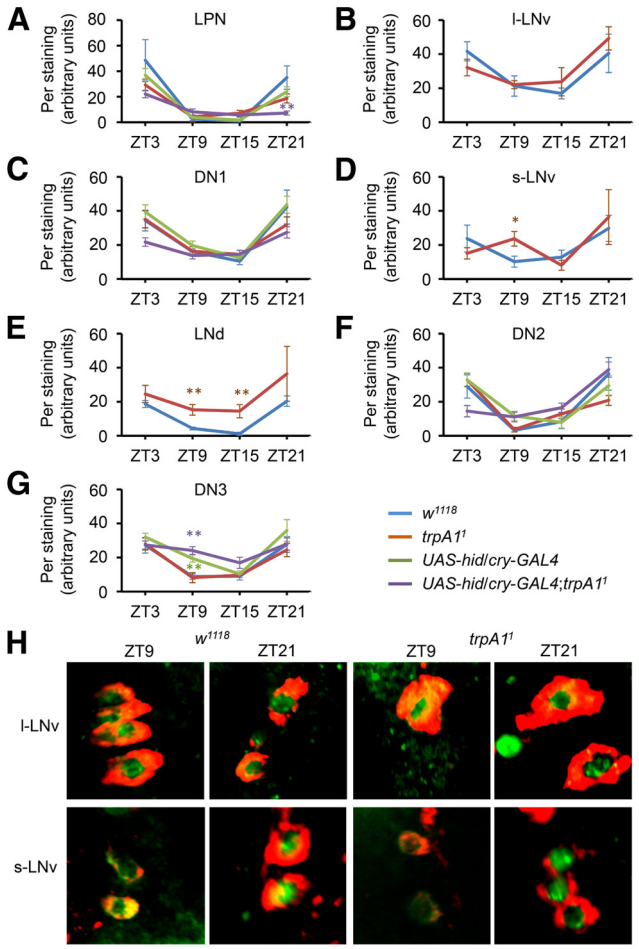

Figure 3. Quantification of Per oscillations in clock neurons. Three- to 7-d-old flies were entrained under a TC cycle (29°/18°C) for 5 d. The brains were then dissected at ZT3, ZT9, ZT15, or ZT21 and stained with anti-Per. ***A–G***, The genotypes and the types of pacemaker neurons are indicated. Data are not indicated for Per levels in l-LNvs, s-LNvs, and LNds since these pacemaker neurons are eliminated in *UAS-hid/cry-GAL4;trpA1*^{1} (Busza et al., 2007). We confirmed this finding by immunostaining with anti-Per and anti-PDF (data not shown), the latter of which labels only l-LNvs and s-LNvs in wild-type (Renn et al., 1999). The fluorescence intensities were quantified using MacBiophotonics ImageJ software using 3D reconstruction images (10–27 brain hemispheres per sample). The formula for the relative intensity (*I*) is $I = 100(S - B)/B$, where *S* is the fluorescence intensity and *B* is the background intensity of the region adjacent to the pacemaker neuron (Picot et al., 2009). The error bars indicate SEM. The asterisks indicate significant differences from wild-type ($*p < 0.05$, $**p < 0.01$) based on ANOVA and the Scheffé *post hoc* tests. ***H***, Colabeling with anti-Per (green) and anti-PDF (red) from *w*^{1118} and *trpA1*^{1} l-LNvs, and s-LNvs at ZT9 and ZT21.

(middle of the day) and ZT15 (late afternoon (Figs. 2*A*,*E*, 3*A*–*G*). The pacemaker neurons in the *trpA1* mutants displayed oscillations in Per, but the patterns of the oscillations and concentrations of Per among pacemaker neurons were more variable in *trpA1*^{1}, especially in combination with *UAS-hid*;*cry-GAL4*. The Per intensities and oscillations in *trpA1*^{1} brains were similar to wild-type in some pacemaker neurons (e.g., l-LNvs, Figs. 2*A*,*B*,*E*,*F*, 3*B*). At ZT9, when Per expression was relatively low in wild-type, Per was elevated in some *trpA1*^{1} and *UAS-hid*;*cry-GAL4*,*trpA1*^{1} pacemaker neurons (e.g., s-LNvs and LNds, Figs. 2*E*,*F*, 3*D*,*E*; or DN3, Figs. 2*H*, 3*G*). Per expression also tended to be lower at ZT21 in *trpA1*^{1} LPNs, DN1s, and DN2s, although the differences were not statistically significant using ANOVA (Figs. 2*A*,*B*, 3*A*,*C*,*F*). However, in a *UAS-hid*;*cry-GAL4*;*trpA1*^{1} background, Per was virtually undetectable in LPNs at ZT21 (Figs. 2*D*, 3*A*), and showed only mild oscillations in Per expression in multiple pacemaker neurons (e.g., LPNs, DN1s, and especially DN3s, Fig. 3*A*,*C*,*G*).

We also examined the subcellular localization of Per. In wild-type pacemaker neurons, with the exception of the l-LNvs, Per was primarily in the cytosol at ZT9, and in the nucleus at ZT21 (Figs. 2*A*,*E*, 3*H*). However, we occasionally detected Per nuclear localization in l-LNvs earlier than s-LNvs (Fig. 3*H*). At ZT9, Per was distributed in both the cytosol and nucleus of l-LNvs, and primarily in the cytosol of s-LNvs (Figs. 2*E*, 3*H*). The subcellular distribution of Per was similar in the mutant pacemaker neurons; however, Per expression in *trpA1*^{1} tended to be higher and more variable than in wild-type at ZT9 (Figs. 2*E*,*F*, 3*H*). This suggests that the oscillation of Per in *trpA1*^{1} was slightly advanced, consistent with the behavioral results (Table 2).

Expression of *trpA1* in pacemaker neurons contributes to temperature synchronization of locomotor activity

Peripheral neurons participate in temperature entrainment of circadian rhythm (Sehadova et al., 2009). Thus, TRPA1 could promote TC control of circadian activity either by functioning in peripheral neurons that send axons to pacemaker neurons, and/or by acting in pacemaker neurons. To address this question, we first examined the presumptive expression pattern of *trpA1* using a *GAL4* reporter that we inserted previously at the position of the endogenous *trpA1* translation initiation site (Kim et al., 2010) in combination with a *UAS-nuclear*::*lacZ*::*GFP*. The *trpA1* reporter was expressed in many pacemaker neurons, which also expressed Per (Fig. 4). These included all three LPNs (Fig. 4*A*), and 1–3 neurons in other groups of pacemaker neurons (Fig. 4*B*–*E*).

To address whether *trpA1* has a role in pacemaker neurons for temperature synchronization of activity rhythms (29°/18°C TC cycles), we set out to perform rescue experiments with a transgene that expressed TRPA1-A (Kwon et al., 2010). However, this greatly reduced viability due to activation of the TRPA1 channel at 29°C (Viswanath et al., 2003). Therefore, we used lower temperatures for the TC cycles (25° and 16°C), which had no adverse effects on viability. We entrained the flies with a LD cycle, and initiated the first cryophase (simulates night) when the light cycle would have initiated, as previously described (Glaser and Stanewsky, 2005). This "antiphase paradigm" causes wild-type flies to display a robust single peak in the middle of the thermophase (Glaser and Stanewsky, 2005) (Fig. 5*A*). During the subsequent free running period at 25°C, the single peak during the thermophase persisted for many days (Fig. 5*A*).

Using the lower TC temperatures (25°/16°C), the *trpA1* mutant phenotype was relatively subtle, indicating that TRPA1 was more important for temperature controlled circadian activity using higher thermophase conditions (29°C). Nevertheless, there were two differences from wild-type, which we used to test for rescue. First, during the first 4 d of the TC cycles, *trpA1*^{1}, or *UAS-trpA1-A*;*trpA1*^{1} flies exhibited additional activity near the end of the cryophase cycle, which corresponded approximately with the former evening period under the LD cycles (Fig. 5*B*,*C*, red asterisks). Second, during the free run, the circadian cycle was slightly shorter in *trpA1*^{1} and *trpA1*^{GAL4} (23.28 ± 0.04 and 23.11 ± 0.08, respectively) (Table 1) than in *w*^{1118} (23.66 ± 0.08; Table 1). To test for rescue, we expressed *UAS-trpA1-A* under the control of the *tim-GAL4*, which was expressed in pacemaker neurons (Dubruille and Emery, 2008). The extra peaks in the *UAS-trpA1-A*;*trpA1*^{1} control flies during days 2–4 of the TC cycles (Fig. 5*C*, asterisks) were diminished or eliminated (Fig. 5*D*), and the length of the circadian cycle during the free run was similar to

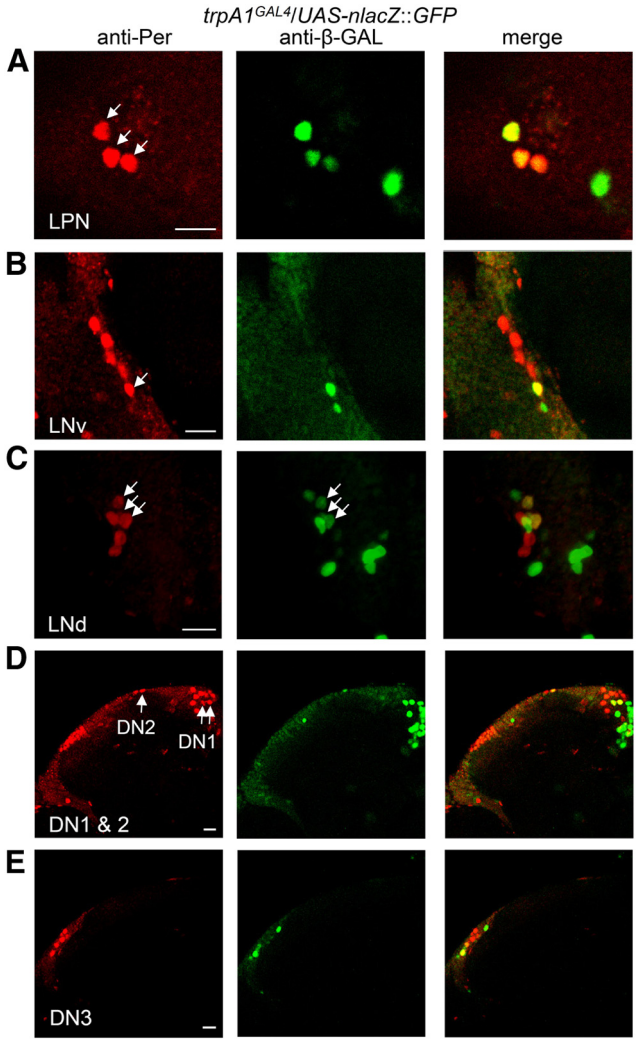

Figure 4. Coexpression of the *trpA1*-reporter and Per in pacemaker neurons. Brains from 3- to 7-d-old *UAS-nlacZ::GFP/+;trpA1^{GAL4}/+* flies immunostained with anti-β-GAL and anti-Per at ZT23 during LD cycles. The right panels are the merged images. ***A***, LPN. ***B***, LNv. ***C***, LNd. These 3D images were reconstituted from confocal stacks. ***D***, DN1 and 2. ***E***, DN3. The arrowheads indicate colabeling of Per and the *trpA1* reporter. Scale bar, 10 μm.

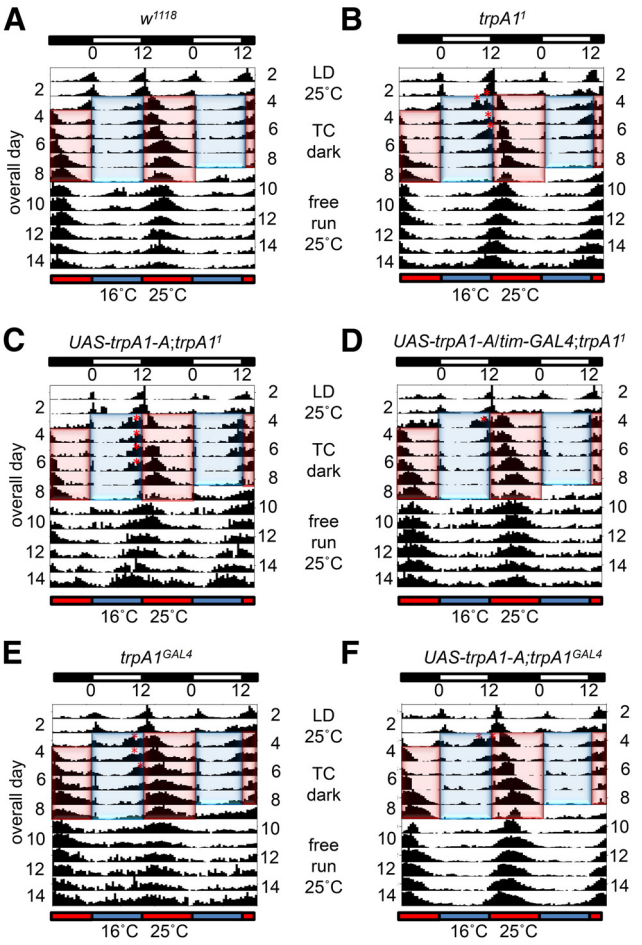

Figure 5. Suppression of the *trpA1* phenotype by expression of the wild-type *trpA1* transgene in pacemaker neurons. Average actograms using flies that were entrained to 12 h light/dark cycles for 3 d at 25°C followed by 6 d of 12 h thermophase (25°C) and 12 h cryophase (16°C) cycles in constant darkness. The warm and cool cycles, which simulate day and night, were the reverse phase from the night and day during the LD cycles (antiphase paradigm). After the temperature entrainment, the flies were kept at 25°C (free run). The white and black bars at the top indicate the LD cycles and the red and blue bars at the bottom indicate the TC cycles. The thermophases and cryophases are highlighted on the actograms with red and blue boxes, respectively. Asterisks indicate sustained peaks that were not detected in the *w^{1118}* actogram. ***A***, *w^{1118}*. ***B***, *trpA1^{1}*. ***C***, *UAS-trpA1-A;trpA1^{1}*. ***D***, *UAS-trpA1-A /tim-GAL4;trpA1^{1}*. ***E***, *trpA1^{GAL4}*. ***F***, *UAS-trpA1-A;trpA1^{GAL4}*.

wild-type (Table 1). We confirmed that the *tim-GAL4* was expressed in LPNs, as the *tim-GAL4/UAS-nlacZ::GFP* was coexpressed with the Per protein (Fig. 6*A–C*). We also rescued the *trpA1^{GAL4}* mutant phenotype using the *UAS-trpA1-A;trpA1^{GAL4}* transgenes (Fig. 5*E*,*F*; Table 1). Moreover, we performed the lower TC temperatures (25°/16°C) using the 18/6 h paradigm that we used with the higher temperature TC cycles (29°/18°C). Under these latter conditions, the main difference from wild-type was that the periodicity was shorter (wild-type, 23.16 ± 0.20; *trpA1^{1}*, 21.88 ± 0.26) (Table 2). The rescue of this phenotype by expression of *UAS-trpA1-A* under control of the *tim-GAL4* was statistically significant, whereas the suppression resulting from expression of *UAS-trpA1-A* under control of the *cry-GAL4* was not (Table 2).

Recently it was reported that TRPA1 is expressed as at least four isoforms, two of which (TRPA1-B and TRPA1-C) are not activated directly by elevated temperatures (Kang et al., 2012; Zhong et al., 2012). Therefore, to address whether or not TRPA1 might be a direct thermosensor in pacemaker neurons, we expressed *UAS-TRPA1-B* in *trpA1^{1}* mutant animals pacemaker neurons under the control of the *tim-GAL4*, and used the 16 h 29° and 8 h 18°C TC paradigm described above. We found that introduction of TRPA1-B in pacemaker neurons (*UAS-trpA1-B/tim-GAL4;trpA1^{1}*) restored the morning peak (Fig. 7*C*), which was reduced in *trpA1* mutant control flies (Fig. 7*A*,*B*). In addition, expression of TRPA1-B in pacemaker neurons reduced the advanced evening activity, and decreased the mid-thermophase activity displayed by the *trpA1^{1}* mutant animals (Fig. 7*A–C*). Using this TC paradigm, wild-type flies were capable of establishing the same periodicity between the evening peaks on TC2, as was displayed on LD4 (Figs. 1*N*, 7*D*, LD4, 23.49±0.09; TC2, 23.41 ± 0.09). The *trpA1* mutant flies required an additional day, and did not show a periodicity similar to LD4 until TC3 (Figs. 1*N*, 7*D*, *UAS-trpA1-B/+;trpA1^{1}* and *tim-GAL4/+;trpA1^{1}*: LD4, 23.72 ± 0.05 and 23.73 ± 0.04, respectively; TC2, 23.01 ± 0.16 and 22.76 ± 0.14, respectively; TC3, 23.53 ± 0.11 and 23.45 ± 0.09, respectively). However, the rescue flies (*UAS-trpA1-B/tim-GAL4;trpA1^{1}*) showed a periodicity comparable to LD4 on TC2 (Fig. 7*D*, LD4, 23.75 ± 0.07; TC2, 23.90 ± 0.00).

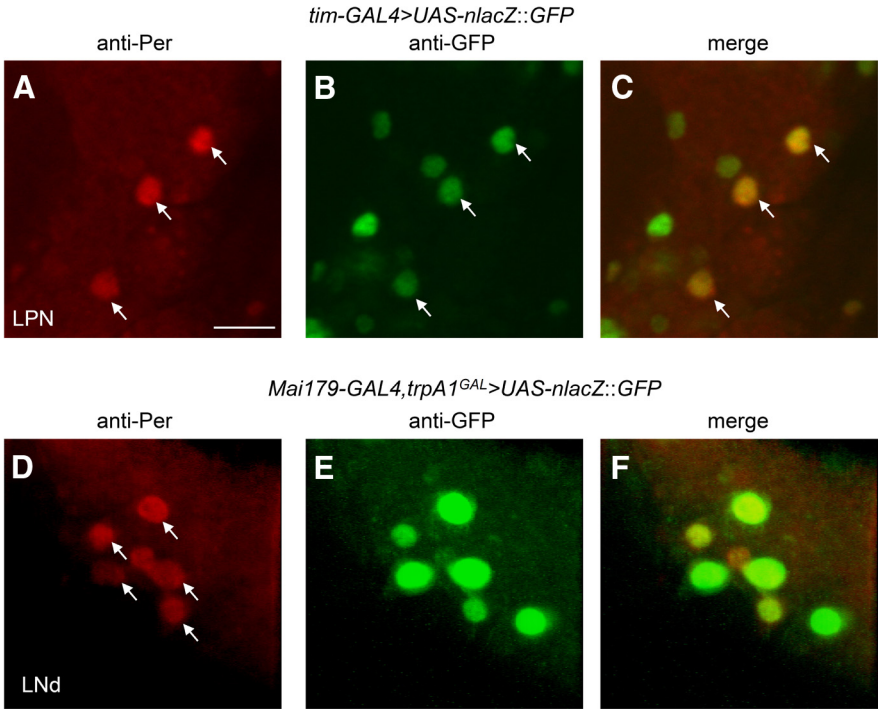

Figure 6. Testing for coexpression of anti-Per with reporters in LPNs and LNds. ***A–C***, The *tim-GAL4* reporter was expressed in LPNs. A brain was dissected from *UAS-nlacZ::GFP/tim-GAL4* flies at ZT21, after the flies were exposed to 18 h thermophase (29°C)/6 h cryophase (18°C) cycles in the dark for 6 d. The brain was stained with anti-Per and anti-GFP. ***A***, anti-Per (red). The arrows point to LPN neurons that were Per positive. ***B***, anti-GFP (green). ***C***, The merged image from ***A*** and ***B***. ***D–F***, Colabeling with anti-Per (red) and anti-GFP (green) from *UAS-mCD8::GFP/Mai179-GAL4;trpA1^{GAL4}/+* flies at ZT21 after entraining flies under TC cycles for 6 d in the dark. ***D***, anti-Per (red). A total of six LNds are labeled. The arrows point to LNds that were Per and GFP positive in merged image in ***F***. ***E***, Anti-GFP (green). ***F***, The merged image from ***D*** and ***E***. Scale bar, 10 μm.

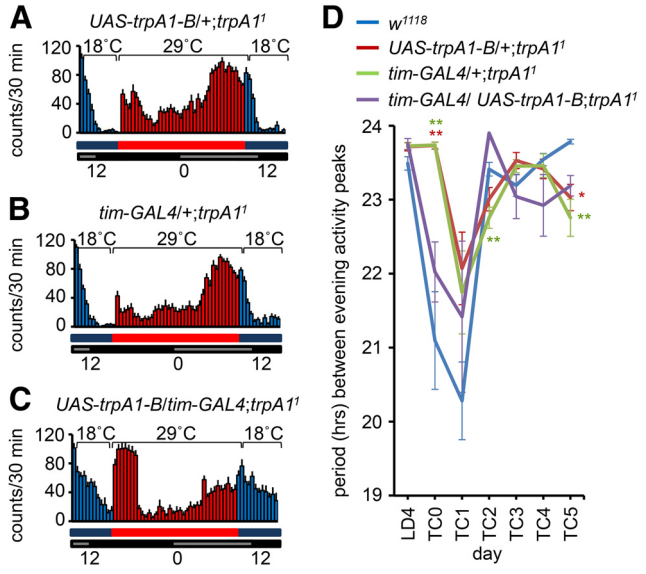

Figure 7. Suppression of the *trpA1* phenotype by expression of the *trpA1-B* isoform in pacemaker neurons. Flies were maintained and entrained using the same paradigm as in Figure 1*D–K*. ***A–C***, Average activities of male flies on TC day 3 (overall day 7) for the indicated genotypes. ***D***, Periods between evening activity peaks from LD4 to TC5. The error bars represent SEMs. The asterisks indicate significant differences from wild-type (*$p < 0.05$, **$p < 0.01$) based on ANOVA and the Scheffé *post hoc* test.

Discussion

Temperature entrainment of the central clock depends at least in part on peripheral sensory neurons, and on the phospholipase C (PLC) encoded by *norpA*, and a novel glutamate-rich protein referred to Nocte (Glaser and Stanewsky, 2005; Sehadova et al., 2009). However, it was unclear whether TC synchronization of the circadian clock depends exclusively on temperature sensation in peripheral neurons or whether there exist one or more sensors in the pacemaker neurons that promotes temperature-controlled activity patterns. Here, we conclude that a subset of central pacemaker neurons functions cell-autonomously in temperature synchronization of circadian rhythm, and that TRPA1 contributes to this behavior.

TRPA1 may not function in all pacemaker neurons since it appeared to be expressed in just a subset of these cells. The LPNs would appear to constitute one class of pacemaker neurons in which *trpA1* is functionally important since *trpA1* was expressed in all thee LPNs, and flies with intact LPNs but not other pacemaker neurons (*UAS-hid;cry-GAL4*) were still capable of TC entrainment of circadian rhythms. Moreover, entrainment was eliminated if the *UAS-hid;cry-GAL4* transgenes were combined with a *trpA1* mutation. These data are consistent with previous studies suggesting that the LPNs contribute to temperature control of circadian rhythm (Busza et al., 2007; Yoshii et al., 2009). The TRPA1 channel may also function in other pacemaker neurons since the *trpA1* reporter was expressed in at least one neuron in most clusters of pacemaker neurons, including LNvs, DN1s, and DN2s, which have also been proposed to contribute to TC entrainment (Busza et al., 2007; Picot et al., 2009; Zhang et al., 2010). We suggest that the *trpA1*-expressing neuron in each cluster might be the central core sensory neuron that responds to temperature changes, and mediates TC sensitivity in other neurons within the clusters.

In addition to affecting TC-induced activity patterns, mutation of *trpA1* also impacted on TC-dependent cycling of Per expression in some pacemaker neurons. Thus, although central brain neurons are not capable of undergoing TC entrainment in the absence of peripheral input (Sehadova et al., 2009), it appears that a temperature sensor in pacemaker neurons promotes normal fluctuations in Per expression. Because we observed alterations in Per cycling in just a subset of pacemaker neurons, it appears that the central neurons are not uniformly sensitive to responding to temperature in a cell autonomous manner.

It has been proposed that separate sets of central neurons are the most critical for light and temperature entrainment, and *cry*-negative neurons may be the more important temperature sensors (Busza et al., 2007; Miyasako et al., 2007; Picot et al., 2009; Sehadova et al., 2009). Such specialization may limit temperature interference with light entrainment (Sehadova et al., 2009). In this context, it is worth noting that some *trpA1*-positive central neurons were *cry* negative. For example, the *trpA1* reporter was expressed in three LNds (Fig. 4*C*), and that *cry* was expressed in only three of six LNds. The *Mai179-GAL4* is expressed in the three Cry-positive LNd cells (Grima et al., 2004; Picot et al., 2007), and five of six LNd cells were labeled with both reporters (Fig. 6*D–F*), indicating that at least two *trpA1*-positive cells did not overlap with the Cry-positive LNd cells.

TRPA1 contributes to temperature control of circadian rhythm, but does not appear to be a direct temperature sensor in pacemaker neurons for two reasons. First, loss of TRPA1 impairs 25°/16°C TC cycles, and these temperatures are below the thermal threshold for activation of TRPA1, which is ~27°C. Second, introduction of a temperature-insensitive TRPA1 isoform (TRPA1-B) (Kang et al., 2012; Zhong et al., 2012) in *trpA1* mutant flies restored the morning peaks during 29°/18°C TC cycles, reduced the advanced evening activity, decreased the mid thermophase activity of the mutants and eliminated the need for one additional day to establish a constant period between the evening activity peaks. These data suggest that TRPA1 might function downstream of another temperature sensor. Although the identity of the thermosensor remains to be identified, the activity of TRPA1 might be coupled to the PLC that is encoded by *norpA*. This is plausible because NORPA contributes to this temperature entrainment pathway (Glaser and Stanewsky, 2005), and TRPA1 acts downstream of NORPA to promote temperature sensation in larvae (Kwon et al., 2008).

TRPA1 cannot define the only pathway that contributes to temperature synchronization of circadian rhythm because mutation of *trpA1* alone had a modest effect, although when combined with the *UAS-hid*;*cry-GAL4* transgenes, temperature entrainment was eliminated. If a second cation channel promotes TC synchronization, it may not belong to the TRP superfamily, because no other *Drosophila* TRP appears to be activated by environmental temperatures in the range that functions in control of circadian rhythms. Given that input from peripheral tissues, such as chordotonal organs, impacts on temperature entrainment (Sehadova et al., 2009), we suggest that thermal sensors exist in both central pacemakers and in peripheral neurons to synchronize activity by TC cycles. It is also plausible that additional thermosensory input is mediated through central brain cells that are not pacemaker neurons. Nevertheless, because TRPA1 is one channel that promotes temperature control of circadian rhythm, our findings demonstrate that the roles of a thermoTRP in a poikilothermic organism are not limited to the avoidance of undesirable temperatures.

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