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Temperature as a universal resetting cue for mammalian circadian oscillators

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Abstract

Environmental temperature cycles are a universal entraining cue for all circadian systems at the organismal level with the exception of homeothermic vertebrates. We report here that resistance to temperature entrainment is a property of the suprachiasmatic nucleus (SCN) network and is not a cell autonomous property of mammalian clocks. This differential sensitivity to temperature allows the SCN to drive circadian rhythms in body temperature which can then act as a universal cue for the entrainment of cell autonomous oscillators throughout the body. Pharmacological experiments show that network interactions in the SCN are required for temperature resistance and that the heat shock pathway is integral to temperature resetting and temperature compensation in mammalian cells. These results suggest that the evolutionarily ancient temperature resetting response can be utilized in homeothermic animals to enhance internal circadian synchronization.

Daily cycles of light and temperature are perhaps the two most reliable environmental timing cues for living systems on Earth. As a consequence, organisms utilize these cues to entrain their endogenous circadian rhythms to the solar day (1). Recent work suggests that most cells in the mammalian body similarly use internal entraining cues to synchronize circadian patterns of gene expression to the rest of the body (2–5). It is now appreciated that most cells in the mammalian body contain cell autonomous circadian oscillators (6–10). These cellular oscillators are synchronized at the organismal level by the suprachiasmatic nucleus (SCN) of the hypothalamus (11). Although a number of factors have been implicated in the resetting of peripheral tissues, no universal entraining mechanism for peripheral oscillators has been defined (2, 4, 12, 13).

Temperature is a primordial entraining agent for circadian rhythms in all organisms with the exception of homeothermic vertebrates (14–18). Although mammals do not normally entrain to external environmental temperature cycles (19), this cue would be ideal as a global *internal* entraining cue in mammals because of the existence of circadian rhythms of body temperature driven by the SCN. Indeed, externally applied temperature cycles can sustain rhythmic clock gene expression in Rat-1 fibroblasts and primary glial cells *in vitro*, as well

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as in peripheral clocks in the whole animal (12, 20). In order for such a system to function *in vivo*, two requirements would be necessary: 1) peripheral oscillators must be sensitive to subtle variations in temperature within the physiologic range (36°C–38.5°C in mice); and, 2) the SCN itself must be resistant to subtle temperature changes or it would be susceptible to feedback which could interfere with entrainment. Consistent with this idea, Brown et al. (12) have shown that altered ambient temperature cycles can shift the phase of rhythms in other brain regions but the SCN remained in phase with the light cycle.

We tested this hypothesis by assessing temperature resetting of the circadian rhythms of tissues from *Per2*^{Luciferase} mice *ex vivo* using real-time analysis of PER2::LUC bioluminescence (10). *Per2*^{Luc} tissues were cultured in Lumicycle (Actimetrics, Wilmette, IL) machines equipped with photomultiplier tubes (PMT). Tissues were maintained at the lower set point of body temperature, 36°C, and then pulsed with temperatures that correspond to the peak set point of 38.5°C (Fig. S1A). All peripheral tissues tested were highly responsive to 38.5°C temperature pulses and exhibited high-amplitude, type 0 resetting. Type 0 phase resetting is characterized by resetting of oscillators to a common new phase following pulses occurring at all phases. Phase transition curves show that both 1-h and 6-h 38.5°C pulses strongly reset the rhythms of peripheral tissues to new phases clustered around CT12–18 and increased the peak to trough amplitude (Fig. 1A and S2). However, at the majority of times across the circadian cycle, the phase of the adult SCN was resistant to identical physiological temperature changes [in contrast to neonatal and juvenile rat SCN tissue which appear more sensitive to temperature cycles (21)]. Tissues of all types that were held at a constant 36°C but removed from the bioluminescence recording device for 6 hours as handling controls showed no phase shifts (Fig. S1B-H).

To test whether the sensitivity of peripheral tissues and the resistance of the SCN also occur under conditions of entrainment to temperature cycles, pituitary and lung cultures from the same animals were exposed to oppositely phased temperature cycles comprised of 12 h of 36°C and 12 h of 38.5°C. Within 3 days, the phase of PER2 bioluminescence of lung and pituitary cultures had assumed the phase of the corresponding temperature cycle (either shifted or unshifted) so that they were ~180° out of phase with each other (Fig. 1B). By contrast, SCN which were cultured in opposing temperature cycles remained unshifted even after 4 days, demonstrating that the SCN is resistant to cyclic temperature changes within the physiologic range (Fig. 1B). The entrainment of peripheral tissues was also observed in response to temperature cycles that mimicked body temperature cycles (22)(Fig. S3). Taken together, these results demonstrate that circadian changes in temperature comparable to that seen with core body temperature rhythms are capable of entraining and enhancing the amplitude of the circadian rhythms of peripheral tissues while the adult SCN remains resistant.

To explore the possible mechanisms of the SCN resistance to temperature pulses, we tested the role of intercellular communication and coupling in the SCN. When communication among neurons in the SCN was impaired by application of the voltage-gated Na^+ channel blocker, tetrodotoxin (TTX), temperature pulses strongly reset the SCN and this effect of TTX was reversible (Fig. 2A and B). To assess the properties of the individual cells within an SCN in its networked state and in the presence of TTX, SCN bioluminescence was imaged with a dual microchannel plate intensified gallium arsenide phosphide cooled CCD camera system. Individual cells within an SCN were typically locked in phase throughout an experiment, but in TTX cells in the same SCN “free run” and eventually became scattered in phase (23) (Fig. 2C). After a 6-h pulse the cells were resynchronized as can be seen as vertical alignment of peak bioluminescence after the pulse in the heatmap in Figure 2C (right panel). The resistance of individual cells was restored after the removal of TTX (Fig. S4D). Thus, TTX treatment rendered the SCN sensitive to temperature resetting.

Because of the presence of voltage-gated calcium channels in the SCN and the role that L-type calcium channels play in the membrane potential of SCN neurons, we tested whether the blockade of L-type calcium channels contributed to the insensitivity of the SCN to temperature pulses (24). Similar to TTX, treatment with an L-type calcium channel blocker, nimodipine, caused a marked decrease in amplitude of *Per2*^{Luc} bioluminescence and reversibly revealed the temperature sensitivity of the SCN to 6-h pulses of 38.5°C (Fig. 2D,E, and S5A). Mibefradil, a T-type calcium channel blocker, did not sensitize the SCN to temperature-induced phase shifts (Fig. 2F and S5B). This suggests that L-type calcium channels play a role in maintaining the SCN's phase in the presence of temperature, possibly through a contribution to intercellular communication.

The ventrolateral and dorsomedial regions of the SCN differ in their expression of the peptides VIP and AVP, their efferent and afferent projections, and in the timing of their development (25, 26). Because of this regional difference, the dorsomedial and ventrolateral regions of the SCN were cultured separately and tested for thermal sensitivity (Fig. 3A and B). Surprisingly, both the ventral and dorsal regions exhibited large phase shifts in response to 6-h 38.5°C pulses very similar to those of SCN slices in the presence of TTX (Fig. 3C). This did not occur if the SCN were split from one another sagittally by a cut from the 3rd ventricle ventrally down through the optic chiasm (Fig. 3D). The sagittally separated SCN were resistant to phase shifts by 6-h 38.5°C pulses (Fig. 3E and F). Pharmacologic blockade of VIP and AVP signaling in the SCN did not reveal strong temperature resetting, however greatly increased the variance of the phase shifts in response to temperature pulses (22) (Fig. S6A and B). The blockade of GABA signaling did not reveal any change in the SCN's resistance to temperature changes (22) (Fig. S6D-F). Thus, the cellular architecture that renders the SCN resistant to temperature resetting is contained within a single SCN and requires communication between the ventrolateral and dorsomedial regions using a GABA-independent signaling pathway that may involve VIP and AVP. Furthermore, cell autonomous circadian oscillators are temperature sensitive in all cell types including the SCN. Logically this argues that the temperature resistance of the SCN must originate as a higher order network property of the mammalian SCN circadian system.

The mechanism of the cell autonomous molecular clock involves a transcription-translation negative feedback loop (27). Recent work shows that Heat Shock Factor 1 (HSF1) and components of the heat shock response pathway are involved in circadian gene expression in mouse liver (13, 28). To test the role of the heat shock response pathway in temperature resetting, we used the benzylidene lactam drug, KNK437, which is a highly potent antagonist of the heat shock signaling pathway (22, 29). One-hour pulses of 100 μM KNK437 alone given to pituitary and lung cultures caused strong Type 0 phase shifts in *Per2*^{Luc} rhythms, however did not affect the phase of SCN cultures (Fig. 4A). To explore the possible mechanisms of phase shifting by KNK437, we compared the phase response curves for resetting by 1-h KNK437 pulses alone with either 1-h 38.5°C 'warm' pulses or 1-h 33.5°C 'cool' pulses. Because warm pulses induce HSF-mediated transcription and KNK437 blocks this transcription, it is reasonable to expect that the pulses induced by KNK437 alone would mimic cool pulses (or a reduction of HSF-mediated transcription). Indeed, when the phase-response curves of 1-h KNK437, 1-h cool (33.5°C), and 1-h warm (38.5°C) pulses were compared, the responses of pituitary and lung to KNK437 and cool pulses were overlapping with breakpoints between delays and advances occurring at ~CT21. In contrast, the phase response curves for warm pulses were distinctly different with breakpoints at ~CT3 (Fig. 4B). Thus, KNK437 pulses mimic a reduction in temperature, which is consistent with a role of HSF1 in temperature resetting to cool pulses.

To test for a role of HSF1 in mediating temperature resetting to warm pulses, we used KNK437 to block HSF1 induction and determined whether temperature induced phase shifts

could be blocked by KNK437 treatment. We first validated that 38.6°C temperature pulses induced a heat shock response and that KNK437 could block this (22)(Fig. S7). To test the ability of KNK437 to block temperature-induced phase shifts, 38.5°C temperature pulses were given in the presence of KNK437 in lung and pituitary cultures. At phases at which KNK437 alone produced no shift, but warm pulses alone caused a phase advance, simultaneous application of KNK437 and a warm pulse completely blocked resetting by temperature (Fig. 4C). To confirm the pharmacological blockade by KNK437, we used a second classical HSF1 inhibitor, quercetin (22). Quercetin also completely blocked temperature-induced phase shifts in a manner indistinguishable from KNK437 (Fig. 4C). Thus, two independent inhibitors of HSF1 blocked temperature resetting. To explore the KNK437 blockade at other phases of the circadian cycle, the KNK437 blocking experiment was performed at all phases, and KNK437 completely blocked the phase shifting effects of temperature pulses on peripheral clocks at every phase of the cycle (Fig. 4D and S7D-E). We can also rule out saturation effects in this experiment since non-saturating 1-h temperature pulses were used. By contrast and as observed with temperature pulses, the SCN was also resistant to the phase-shifting effects of KNK437 pulses highlighting another similarity in the differential actions of temperature and KNK437 on central and peripheral oscillators (Fig. 4D). Finally, to circumvent the phase shifting effects of KNK437 pulses, we conducted blocking experiments using chronic application of KNK437. Under these conditions, temperature pulses given to peripheral clocks such as liver, lung, and U2-OS cultures could not induce phase shifts nor induce increases in the amplitude of oscillations (Fig. S8). Taken together, these experiments demonstrate that inhibition of HSF1 mimics the effects of cool pulses and blockade of HSF1 induction is associated with a loss of resetting to warm pulses, implicating a critical role of this pathway in temperature entrainment of mammalian clocks.

In addition to the phase-shifting effects of KNK437, the circadian period increased dramatically with increasing concentration of the drug when administered chronically to SCN cultures, and this effect on period was reversible (Fig. 5A and B). Cultures of pituitary and lung displayed similar period lengthening effects in chronic KNK437 (Fig. 5C). The period lengthening caused by KNK437 inhibition of HSF-mediated transcription is consistent with the long free-running period observed in mice carrying a null allele of *Hsf1* (28). In addition to the entraining effects of temperature, a canonical property of circadian clocks is temperature compensation, which is the ability of the period of a rhythm to remain fairly constant at various physiologically permissive temperatures (1, 18, 22). Intriguingly, treatment with KNK437 greatly impaired the temperature compensation of circadian period of SCN and pituitary. In control media containing DMSO vehicle, circadian rhythms were compensated with temperature coefficient values, Q_{10}, of 1.04 and 0.97 in the SCN and pituitary, respectively. However, in the presence of 100 μM KNK437, the Q_{10} values became 1.34 for the SCN and 1.38 for the pituitary which fall significantly outside the Q_{10} range for temperature compensation (typically between 0.9 – 1.2) (Fig. 5D) (1, 18). In contrast, SCN cultures treated with 1μM TTX displayed a Q_{10} of 1.06 demonstrating that the temperature compensation of the SCN is a cell autonomous phenomenon and does not depend on intercellular coupling (Fig. 5D).

Thus, changes in temperature comparable to the circadian body temperature rhythm are a potent cue for resetting in peripheral tissues. While it has been reported that more than one cue may reset peripheral tissues and that different tissues may use different cues for their entrainment, many of these resetting stimuli appear to involve changes in body temperature or may converge on temperature response pathways (Fig. 5E). For example, while *in vivo* experiments are important for the understanding of entrainment and resetting of rhythms at the organismal level, it is extremely difficult, if not impossible, to separate the effects of activity, sleep, feeding, metabolic and redox state from the effects of temperature.

Paradigms that involve induction of rhythms in the absence circadian cues from the SCN, such as chronic administration of methamphetamine to *Cry1*$^{-/-}$/*Cry2*$^{-/-}$ mice or wild-type mice with SCN lesions, always involve both a change in body temperature as well as activity (30–32). Restricted feeding regimens that alter circadian gene expression in the liver are also associated with deep depressions of body temperature at times when food is unavailable (3). Glucocorticoids, which reset peripheral circadian oscillators (2), also cause an inhibition of the HSF1-mediated transcription, and Hsp90 and Hsp70 act as molecular chaperones for inactive glucocorticoid receptors (33, 34). Redox state, which fluctuates on a circadian basis (35), also regulates HSF1 activation (36). In addition, signaling pathways involving cAMP and calcium, which regulate circadian oscillators (37, 38), also activate the heat shock pathway (39, 40). Thus, changes in body temperature and/or activation of the heat shock pathway may be a final common pathway for resetting circadian clocks in mammals. The experiments presented here show that temperature can act as a universal entraining agent for circadian rhythms throughout the body. This is consistent with the rhythmic binding of HSF1 to HSE *in vivo* and the presence of HSE motifs in the upstream region of the *Per2* gene in many mammalian species (13, 28).

Temperature serves as an important entrainment cue for invertebrates at the organismal level (18). Conidiation rhythms in *Neurospora* can be entrained by either light or temperature cycles, and in some instances temperature cycles can override light cycles (16). The pathways of light and temperature entrainment in *Neurospora* are mechanistically distinct. Temperature changes cause a posttranscriptional, temperature-dependent alternate splicing event in which alternate isoforms of the FRQ protein result from one of two translation initiation sites, whereas light entrainment appears to act through a light-induced increase in *frq* transcription (41–43). In *Drosophila*, temperature pulses act through a similar mechanism to light pulses which are both associated with a CRY-mediated degradation of TIM, and the *cry*^{b} mutation causes reduced circadian resetting to both temperature and light pulses (44, 45). The *Drosophila per* gene also undergoes temperature-dependent splicing, which regulates midday activity (46, 47). Finally, temperature entrainment in *Drosophila* also relies on *nocte* expression (14, 48). Interestingly, this *nocte*-mediated temperature entrainment of clock neurons in *Drosophila* is distinct from that of peripheral oscillators so that the brain clocks require the mechano- and thermo-sensitive chordotonal organs for proper temperature entrainment (48). Thus the temperature entrainment of *Drosophila* requires both the cell autonomous response to temperature changes as well as neuronal communication. It is possible that among mammals a difference in cellular communication within the SCN exists. For example, in rats the expression of clock genes in the ventrolateral and dorsomedial regions of the SCN can be split *in vivo* under 22 hr LD conditions (49). It is possible that this same susceptibility to dissociation within the tissue allows for increased sensitivity to temperature changes in this species (21).

In summary, we have shown that cellular communication within the SCN and between the ventrolateral and dorsomedial SCN confers resistance to temperature resetting. This observation is consistent with the ability of an animal's behavioral rhythms to "free-run" through environmental temperature cycles (18, 19) and suggests that resistance to temperature entrainment in vivo is conferred by the SCN. When communication between cells within the SCN is blocked, the tissue exhibits temperature sensitivity equal in magnitude to that of peripheral tissue, revealing that temperature-sensitive resetting is a cell autonomous property. Finally, the sensitivity of peripheral clocks to small temperature changes is abolished in the presence of KNK437 or quercetin, thus revealing a critical role of the heat shock response pathway in resetting of circadian clocks to thermal stimuli and in temperature compensation of circadian period.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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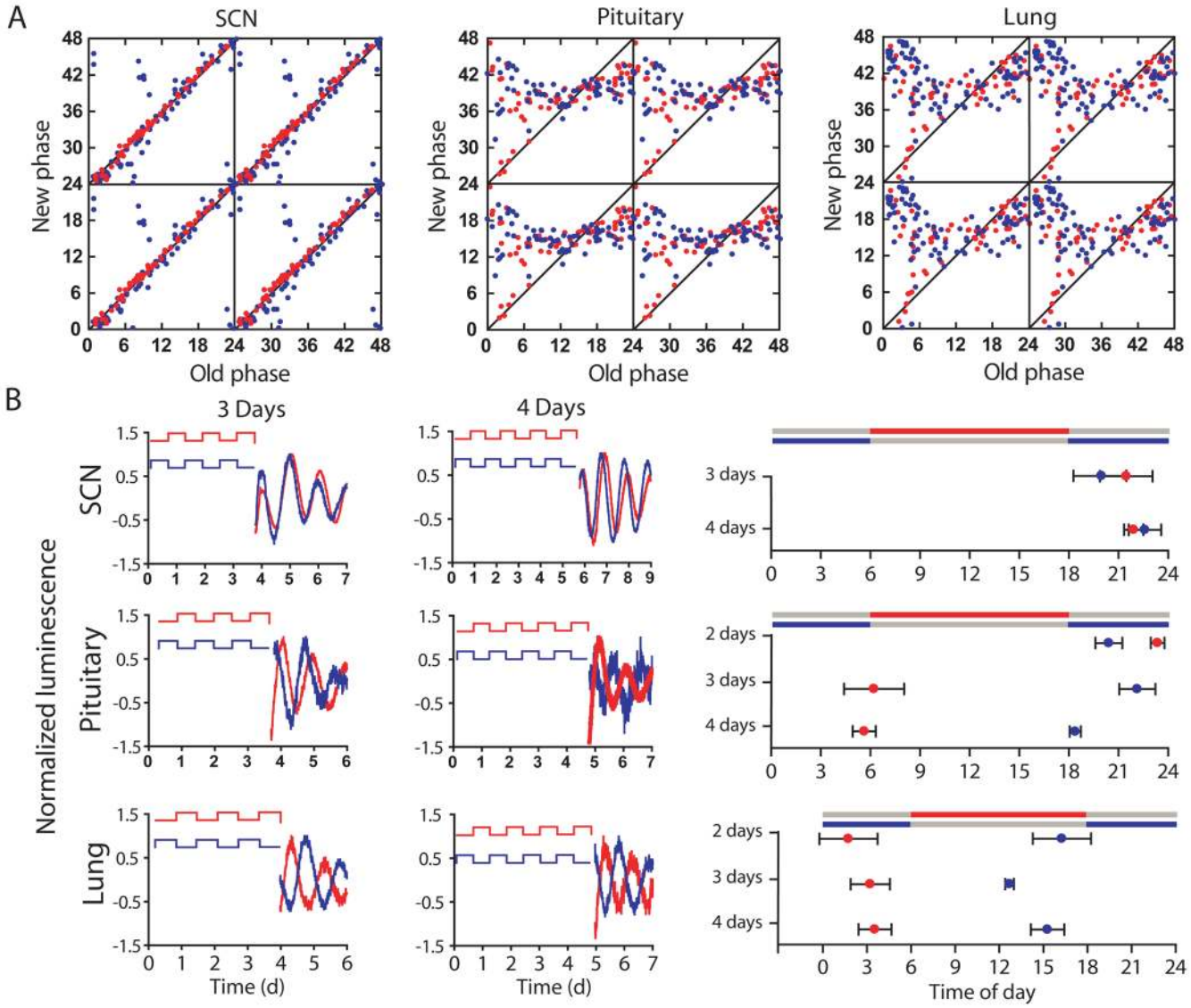

Figure 1. Peripheral tissues, but not SCN are sensitive to temperature changes within the physiological temperature range

(**A**) Phase transition curves for SCN, pituitary, and lung in response to 6-h (blue) or 1-h (red) 38.5°C temperature pulses from 36°C. Pulse times are plotted as the time in circadian hours of the end of the pulse from the previous trough of bioluminescence. (**B**) SCN, pituitary, and lung cultures exposed to opposing square-wave 12hr:12hr 36°C:38.5°C temperature cycles. Phase graphs show time of peak $PER2^{LUC}$ bioluminescence the day after the temperature cycle. Colored bars above represent the times of warm temperature for the points of corresponding color below. Data are mean+/− SEM. n=4 for each SCN point and n=5 for each pituitary and lung point.

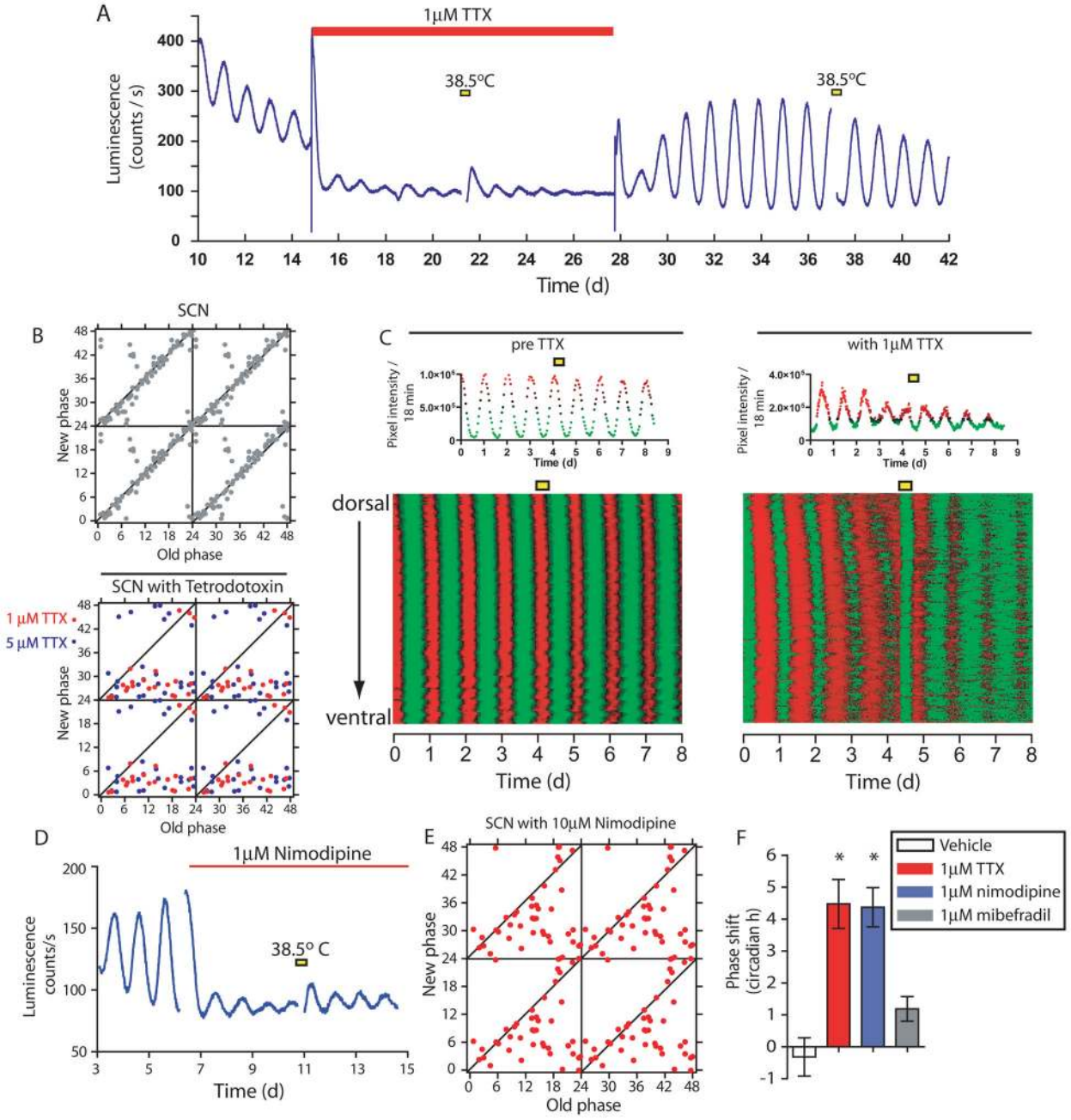

Figure 2. Tetrodotoxin reveals temperature sensitivity of SCN cultures

(**A**) Bioluminescence record from a *Per2*^{Luc} SCN treated with 1 μM TTX as indicated. Six-hour 38.5°C pulses noted with yellow bars. (**B**) Phase transition curve of individual SCN cultures containing 5 μM (blue) or 1 μM (red) TTX or without drug (gray). (**C**) A *Per2*^{Luc} SCN was imaged using an intensified CCD camera. Identical regions of interest equal to or smaller than the size of a single cell were measured. Heat maps display voxels measured from dorsal (top) to ventral (bottom) where red corresponds to peak bioluminescence and green to trough with and without TTX for the same SCN. (**D**) 1μM nimodipine reduces amplitude of bioluminescence rhythms and reveals temperature sensitivity. (**E**) Phase transition curve of individual SCN cultures receiving a 6-h temperature pulse of 38.5°C in 10 μM nimodipine. (**F**) Phase shifts in response to 6-h 38.5°C pulses which ended 22 hours

after the trough of bioluminescence displayed as mean +/− SEM. Vehicle (n=14), 1 μM TTX (n=11), 1 μM nimodipine (n=13), 1 μ mibefradil (n=11). * indicates $p < 0.05$, ANOVA, Tukey post-hoc analysis.

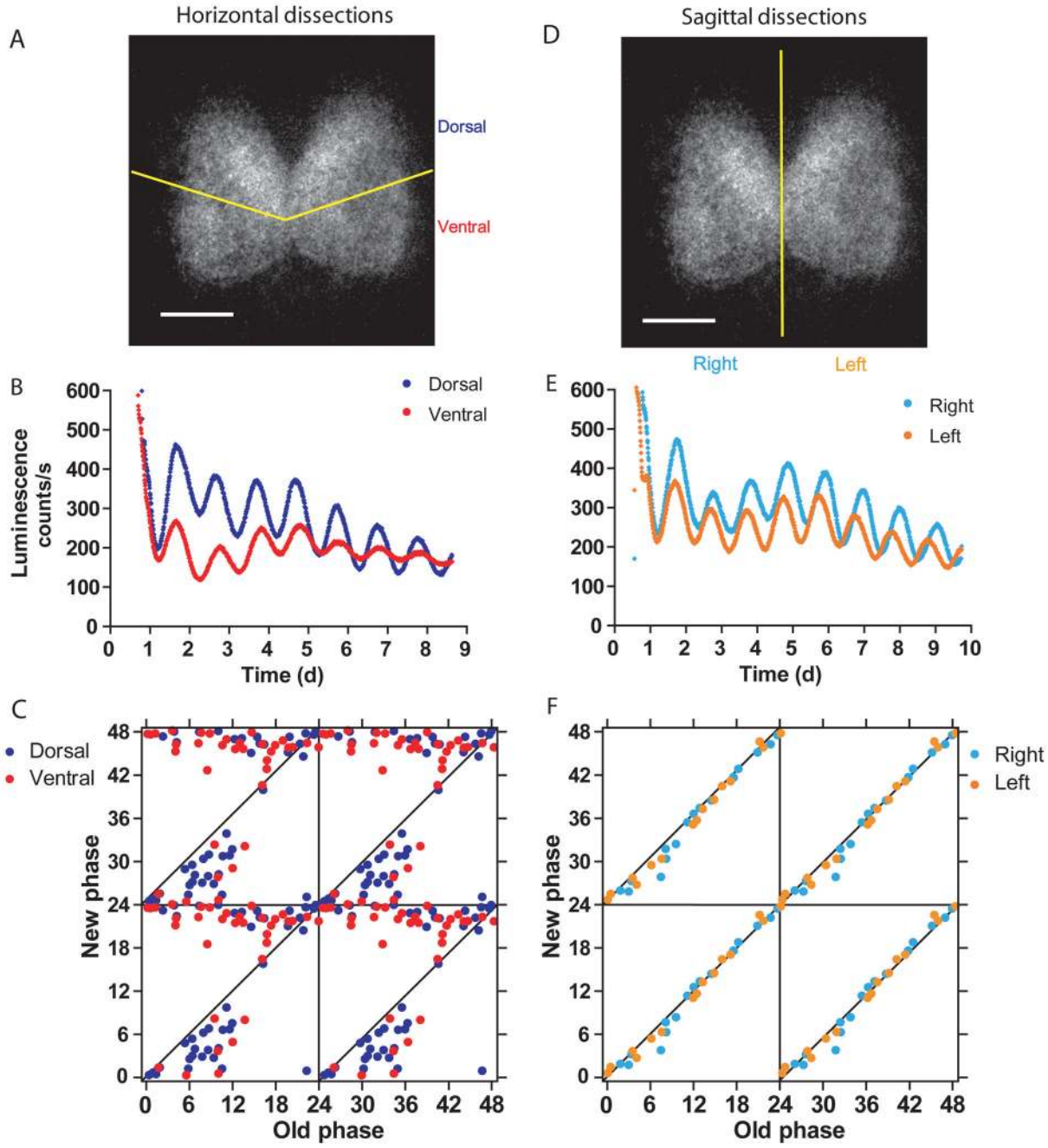

Figure 3. SCN resistance to temperature pulses relies on the integrity of the ventral and dorsal regions

(**A**) Yellow line shows approximate dissection of the dorsomedial and ventrolateral regions of a coronal SCN slice. White bar represents 500 μm. (**B**) Dorsal (blue) and ventral (red) sections from the same SCN cultured in separate dishes. (**C**) Phase transition curves of dorsal and ventral SCN sections in response to 6-h pulses of 38.5°C (**D**) Coronal sections of the SCN were also dissected sagittally so that the left and right SCN were cultured separately. (**E**) Bioluminescence of right and left SCN from the same animal. (**F**) Phase transition curves of right (blue) and left (orange) SCN sections in response to 6-h pulses of 38.5°C.

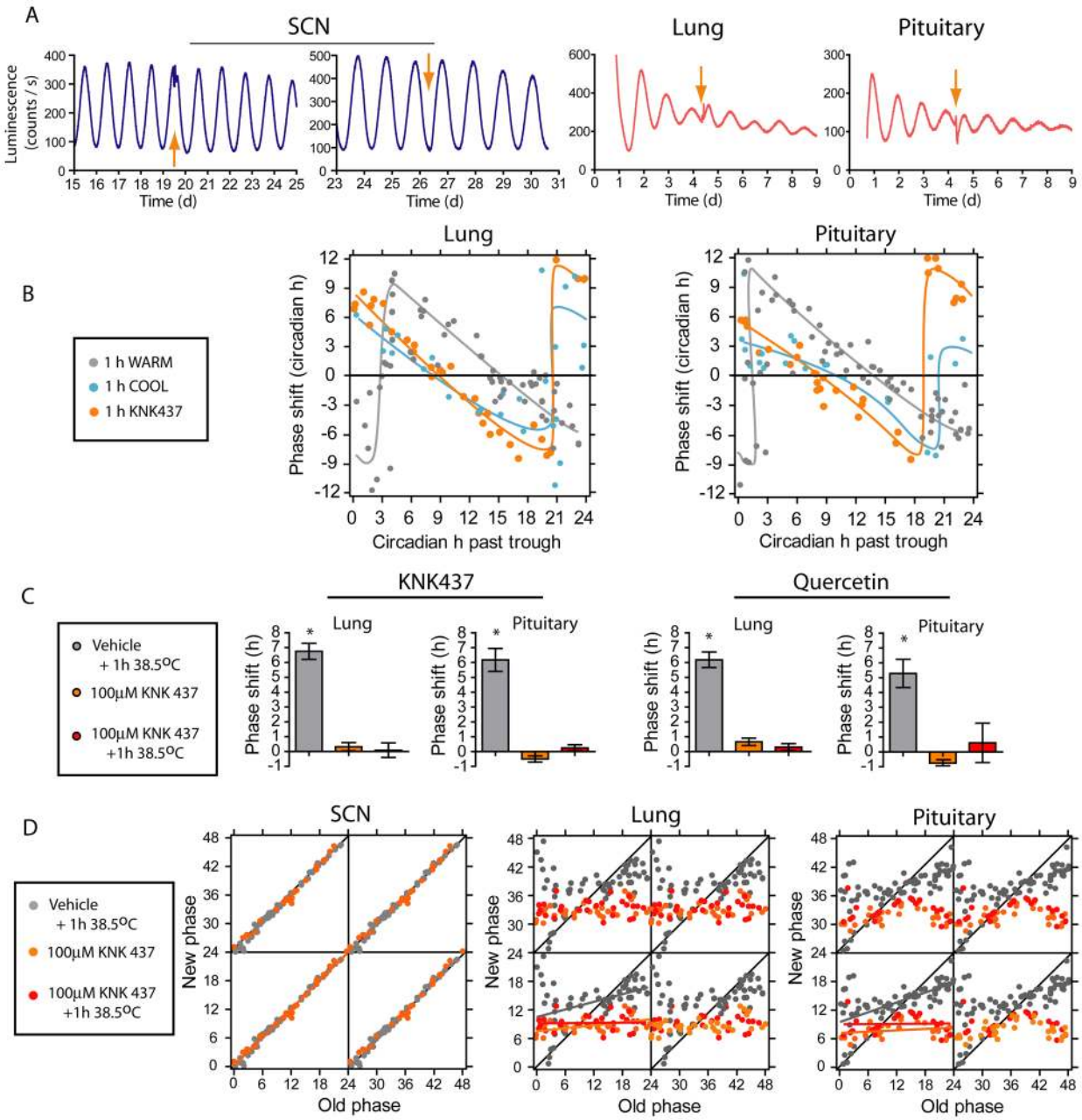

Figure 4. KNK437 phase shifts the clock and blocks temperature-induced phase changes

(**A**) SCN, lung, or pituitary cultures receiving a 1-h pulse of 100 μM KNK437 indicated by an orange arrow. (**B**) Phase response curves of lung and pituitary receiving a 1-h 38.5°C "warm" pulse, 33.5°C "cool" pulse, or 100 μM KNK437 pulse. (**C**) Average phase shifts (mean +/− SEM) from lung or pituitary cultures receiving a 1-h 38.5°C pulse in DMSO (n=6), 1-h 38.5°C pulse in 100 μM KNK437 or 100 μM Quercetin (n=5), or a 1-h pulse of 100 μM KNK437 or 100 μM Quercetin alone (n=4) given 9–11 h (lung) or 4–8 h (pituitary) past peak luminescence. * indicates $p < 0.01$, ANOVA, Tukey post-hoc analysis. (**D**) Phase transition curves of SCN, lung, and pituitary receiving 1-h pulses of indicated treatments.

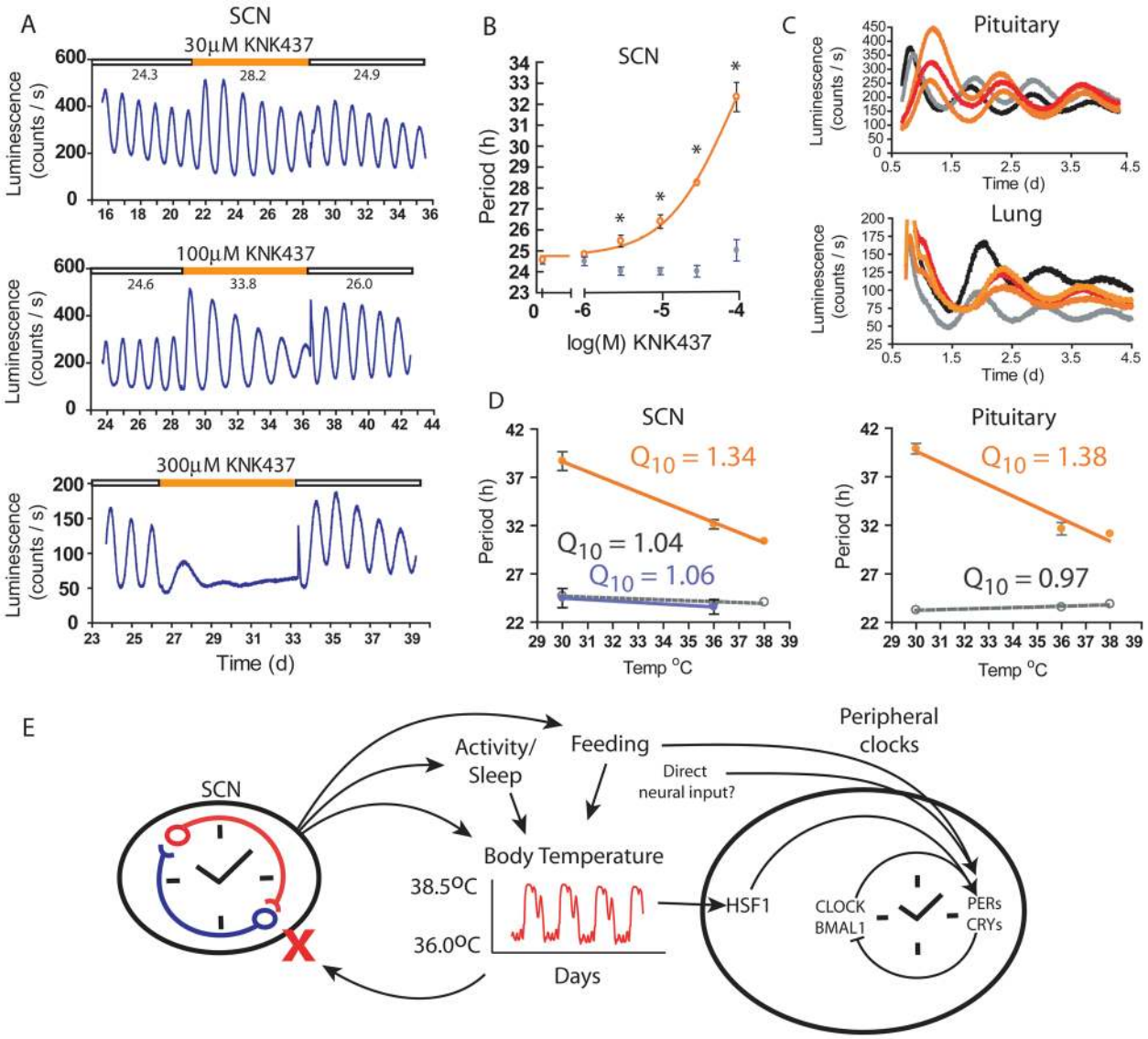

Figure 5. Inhibiting HSF-mediated transcription lengthens circadian period and impairs temperature compensation

(**A**) SCN in KNK437 where marked by an orange line. Numbers indicate the period of the rhythm underneath the line. (**B**) Periods of SCN in KNK437 (orange) at 100 μM (n=5), 30 μM (n=5), 10 μM (n=5), 3 μM (n=5), and 1 μM (n=5) displayed as mean +/− SEM. Gray points indicate the periods of the same SCN cultures after KNK437 was removed. * indicates $p < 0.05$ from paired t-tests corrected for multiple measures. (**C**) Traces of bioluminescence from pituitary or lung cultured with DMSO (black/gray) or 100 μM KNK437 (orange/red). (**D**) Periods (mean +/− SEM) of SCN or pituitary in 100 μM KNK437 or DMSO. SCN: 30°C DMSO n=8, KNK n=5, and TTX n=; 36°C DMSO n=9, KNK n=8, and TTX n=8; 38°C DMSO n=8 and KNK n=8. Pituitary: 30°C DMSO n=6 and KNK n=6; 36°C DMSO n=9 and KNK n=7; 38°C DMSO n=7 and KNK n=8. $p < 0.05$ comparing drug treatment or temperature within KNK groups (ANOVA); N.S. for temperature in vehicle groups (ANOVA). (**E**) Model representing the communication between dorsal and ventral SCN which confers phase resistance to body temperature

changes which are regulated by the SCN. Body temperature then entrains peripheral oscillators by acting through HSF mediated transcription.