Title: *Drosophila* Ionotropic Receptor 25a mediates circadian clock resetting by temperature

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Summary:

Circadian clocks are endogenous timers adjusting behaviour and physiology with the solar day[1]. Synchronized circadian clocks improve fitness[2] and are crucial for human physical as well as mental wellbeing[3]. Visual and non-visual photoreceptors are responsible for synchronizing circadian clocks to light[4,5], but clock-resetting is also achieved by alternating warmer ('day') and colder ('night') temperatures with 2°-4°C difference only[6-8]. This temperature sensitivity is even more remarkable considering that the period of circadian clocks (~24 h) is largely independent of the surrounding ambient temperature[1,8]. Here we show that the *Drosophila* Ionotropic Receptor 25a (IR25a) is required for behavioural synchronization to low-amplitude temperature cycles. We found that this channel is expressed within sensory neurons of internal stretch receptors in the fly body, which have previously been implicated in temperature synchronization of the circadian clock[9]. IR25a is required for temperature-synchronized clock protein oscillations in specific subsets of central clock neurons, defining the neural substrates for temperature sensitivity within the circadian clock circuit. Extracellular leg nerve recordings reveal temperature-and IR25a-dependent sensory responses and misexpression of IR25a confers temperature-dependent firing of action potentials in heterologous neurons. We propose that IR25a is part of a temperature input pathway to the circadian clock that is responsible detecting small temperature differences. This pathway is operating in the absence of the known temperate-preference regulating 'hot' and 'cold' sensors in the fly antenna[10,11], and hence revealing the existence of novel periphery-to-brain temperature signalling routes involving IR25a function in peripheral sensory organs.

Main Text:

In *Drosophila*, daily activity rhythms are controlled by a network of ~150 clock neurons expressing the clock genes *period* (*per*) and *timeless* (*tim*), which encode repressor proteins that negatively feedback on their own promoters resulting in 24 h oscillations of clock molecules. Temperature cycles (TC) synchronize molecular clocks present in external body parts and the PNS in a tissue autonomous manner[9,12], while synchronization of clock neurons in the brain largely depends on temperature input from peripheral temperature receptors located in the chordotonal organs (ChO) and on the gene *nocte*, which is also expressed in ChO[9,12,13].

To identify novel proteins involved in temperature entrainment we expressed tagged NOCTE versions in flies followed by purification of interacting partners and their identification by mass-spectrometric analysis (see Methods and[14]). *nocte* mutants show defects in ChO morphology, pointing to a structural role of NOCTE in ChO cilia[9]. Consequently, the majority of the identified proteins (10/16) likely regulate function and dynamics of the ChO neuron cilia (Extended Data Tab. 1). Since we were mainly interested in identifying potential temperature receptors, we focused on other NOCTE-interacting proteins, particularly on Ionotropic Receptor 25a (IR25a) (Extended Data Tab. 1). We verified the interaction by co-immunoprecipitation after overexpressing IR25a and NOCTE in all clock cells using *tim-gal4* (Extended Data Fig. 1a). IR25a is a member of a divergent subfamily of ionotropic glutamate receptors, which function in chemosensory detection rather than synaptic transmission, consistent with IR25a expression in many different populations of sensory neurons in the antenna and labellum[15-17]. The function of IR25a has only been analysed in olfactory neurons of the third antennal segment, where it acts as a co-receptor with different odour-sensing IRs[15].

To investigate if *IR25a* is co-expressed with *nocte* in ChO we first analysed *IR25a* expression in femur and antennal ChO using an *IR25a-gal4* line, previously shown to at least partially reflect the IR25a expression pattern in the third antennal segment[15] (Extended Data Fig. 2a). *IR25a-gal4* driven *mCD8-GFP* prominently labelled subsets of ChO neurons in the femur, which showed substantial overlap with *nompC-QF* driven *QUAS-Tomato* signals (Fig. 1 a-c). *nompC-QF* is expressed in larval ChO[18] as well as in the adult femur ChO (Fig. 1d, e) Comparison of *IR25a*-driven *mCD8-GFP* and nuclear *Ds-Red* signals with those of other ChO neuron drivers (*F-gal4* and *nocte-gal4* [9]), suggests that *IR25a* is transcribed in a small subset of femur ChO neurons and Johnston's Organ (JO) neurons (Fig. 1c, Extended Data Fig. 1b-g). We also detect endogenous *IR25a* mRNA in the femur and leg (Extended Data Fig. 2b, e). To determine if *IR25a-gal4* ChO signals reflect endogenous IR25a expression, we performed IR25a antibody staining on femur ChO (Fig. 1f, g). Double labelling with a neuronal marker revealed IR25a signals within ChO neuron cell bodies and ciliated dendrites, similar as in coeloconic neurons of the antenna[16]. This subcellular distribution of IR25a was confirmed after co-expression of an mCherry-IR25a fusion protein with the dendritic cap marker NOMPA-GFP (Fig. 1h), which showed expression along the ChO cilia, clearly distinct from the dentritic cap. Together, these results show that IR25a is expressed in subsets of antennal and femur ChO neurons and the *IR25a-gal4* driver reflects this pattern.

Since *nocte*^{1} mutants do not synchronize to 16°C : 25°C TC in constant light (LL)[9,12] (Extended Data Fig. 3a) we analysed *IR25a*$^{-/-}$ mutants[16] under these conditions. Unlike *nocte*^{1}, the *IR25a*$^{-/-}$ flies synchronized well to this regime and we obtained similar results at warmer TC (Extended Data Fig. 3a). To test the possibility that *IR25a* is specifically required for synchronization to

small temperature intervals[7,13], we subjected *IR25*$^{-/-}$ flies to a series of TC with an amplitude of 2°C only. Surprisingly, and in contrast to wild type, *IR25a*$^{-/-}$ mutants did not synchronize to any of the shallow TC in LL or constant darkness (DD) (Fig. 2a-e, Extended Data Fig. 3b, 4c). While in LL wild type and *IR25a* rescue flies showed a clear activity peak in the 2nd part of the warm period before and after the 6 h shift of the TC, *IR25a*$^{-/-}$ mutants were constantly active throughout the TC, apart from a short period of reduced activity at the beginning of the warm phase of TC1 (Fig. 2a, Extended Data Fig. 3b). In DD, control flies slowly advanced (or delayed) their evening activity peak during phase-advanced (or delayed) TC (Fig. 2b, Extended Data Fig. 4c). The phase of this activity peak was maintained in the subsequent free running conditions (DD, const. 25°C) indicating stable re-entrainment of the circadian clock (Fig. 2b, Extended Data Fig. 4). In contrast, *IR25a* mutants did not shift their evening peak during the TC; instead it remained at its original phase throughout the experiment (Fig. 2b; see also Extended Data Fig. 4c and Fig. 2d for phase quantification).

To quantify entrainment in LL, we determined the 'Entrainment Index' (EI) for each genotype and condition, whereas for most DD experiments we calculated the phase difference of the main activity peak upon release into constant conditions between *IR25a* mutants and controls (see Methods). In all 2°C-amplitude TC tested the EI of *IR25a*$^{-/-}$ flies was significantly lower (LL) and phase calculation indicated no, or a significantly reduced phase shift compared to controls (Fig. 2c-e). The same non-synchronization phenotype was observed in *IR25a*$^{-}$*/Df(IR25a)* flies, and temperature synchronization was fully restored in *IR25a*$^{-/-}$ flies expressing a genomic rescue construct (*rescue*) (Fig. 2a-d, Extended Data Fig. 3b). *IR25a*$^{-/-}$ mutants synchronize to light and have normal free-running and temperature compensated periods (Fig. 2b, Extended

Data Fig. 4d, Extended Data Tab. 2). Combined, the results suggest that rather than mediating synchronization to a specific temperature range, IR25a enables the circadian clock to sense subtle temperature changes across the entire physiological range. Indeed increasing the TC amplitude to 4°C restored temperature entrainment in *IR25a*$^{-/-}$ flies (Extended Data Fig. 4a, b).

Although temperature receptors are located in fly antennae and arista, they are not required for temperature-synchronized behaviour[9,11,19]. We therefore tested if temperature entrainment of *IR25a*$^{-/-}$ flies can be rescued in the complete or segment-specific absence of antenna. Although *IR25a* is expressed in the outer segments of the antennae (Extended Data Fig. 1c, 2a)[16], we found that antennal *IR25a* function is not required for temperature entrainment (Extended Data Fig. 5).

To reveal the importance of IR25a expression within ChO neurons, we performed *gal4* mediated *IR25a* knock-down using RNAi. Pan-neuronal *elav-gal4* knock down decreased *IR25a* mRNA >75%, or >90%, using one or two different RNAi lines combined, respectively (Extended Data Fig. 2d). This reduction was sufficient to interfere with behavioural synchronization to TC (Extended Data Fig. 6c). Spatial restriction of *IR25a-RNAi* using *IR25a-gal4* also resulted in a lack of synchronization to TC (Extended Data Fig. 6a, c) confirming that IR25a is important for temperature entrainment. Next, we used various drivers with known activity in all or subsets of ChO neurons (Fig. 1, Extended Data Fig. 1), resulting in a lack of entrainment to temperature cycles (Extended Data Fig. 2e, 6b, c). In contrast, *IR25a* knock-down in multidentritic, TRPA1, or clock neurons did not impair temperature entrainment (Extended Data Fig. 6c). These findings are consistent with the absence of *IR25a* expression in clock neurons and the brain (Extended

Data Fig 2e-g). Together, these results show that *IR25a* expression within ChO neurons is required for temperature entrainment to 25˚C : 27˚ TC in LL.

To identify the neural substrates underlying the lack of behavioural synchronization, we investigated clock protein levels in *IR25a*$^{-/-}$, wild type, and *IR25a*$^{-/-}$ *rescue* flies exposed to shallow TC in LL. While TIM expression was robustly rhythmic and synchronized in all clock neuronal groups in controls, TIM levels in the Dorsal Neuron 1 (DN1) and DN2 clock neurons of *IR25a*$^{-/-}$ flies were barely detectable (Fig. 3a, Extended Data Figure 7a, b). Moreover, in the small and large ventral Lateral Neurons (s-LNv and l-LNv), TIM expression exhibited an additional peak during the warm phase (Fig. 3a, Extended Data Figure 7a, b). TIM oscillations in the DN3 showed an earlier decline compared to controls and were normal in the dorsal Lateral Neurons (LNd). In TC and DD TIM levels in DN1 were also blunted, but oscillations in the DN2 and DN3 were similar to controls. In contrast to LL TIM was not oscillating in the s-LNv and l-LNv and at constantly low levels (Fig 3b), consistent with the behavioural results obtained under these conditions (Fig 2b, d). The alterations of TIM expression are temperature specific, as we observed normal oscillations in LD cycles at 25°C (Extended Data Fig. 7c). An increase of the TC amplitude to 4°C also restored normal TIM expression in *IR25a*$^{-/-}$ flies, in agreement with the behavioural rescue (Extended Data Figs. 7d, 4a, b). In summary, in low amplitude TC IR25a is required for normally synchronized TIM oscillations in DN1-3 and LNv in LL and in DN1 and LNv clock neurons in DD.

To test if the clock neurons affected by the lack of IR25a are indeed involved in regulating behavioural synchronization to shallow TC, we blocked synaptic transmission by expression of tetanus-toxin (TNT). Indeed, expression of active TNT in the DN1 and DN2 blocked

synchronization to shallow TC in LL, whereas in DD only DN1 blockage interfered with temperature entrainment (Fig. 3c, d)[20]. Consistent with the differential effect on TIM oscillations in LL and DD (Fig. 3a, b) these results strongly suggest that IR25a is required for the synchronized output of the DN1 (LL and DD) and DN2 (LL) to control temperature-entrained behaviour.

Next, we tested if ChO may directly sense temperature in an IR25a-dependent manner. We recorded leg nerve activity in restrained preparations and identified ChO units in the compound signal (Fig. 4a). As expected, in both wild type and *IR25a*$^{-/-}$ flies spontaneous leg movement changed as a function of temperature along with motor and sensory activity. In addition, presumed ChO activity of wild type flies also increased during periods without movement (see 3rd insert in Fig. 4b). This temperature-induced, but movement-independent ChO activity unrelated to movement was absent in *IR25a*$^{-/-}$ flies showing that temperature is sensed in the legs in an IR25a-dependent manner (Fig. 4c). In order to test if IR25a may contribute to direct sensing of temperature changes and because it is not expressed in clock neurons (Extended Data Fig. 2f), we decided to ectopically express this channel in the physiologically well-characterized l-LNv clock neurons[ref]. Isolated brains were exposed to a temperature ramp and spike frequency of individual l-LNv was recorded continuously. Control l-LNv did not show a significant temperature-dependent change in neural activity (Fig. 4d). This was in contrast to the linear and reversible temperature-dependent increase in action potential firing frequency (Q10 >4, Fig. 4i, $p<0.01$, Fig 4e) seen with *IR25a* expression. Other cellular parameters like membrane potential (Fig. 4f, $p=0.25$), input resistance (Fig. 4g, $p=0.78$), and spontaneous firing rate (Fig. 4h, $p=0.29$) showed no difference. Increasing the temperature by only 2-3°C also lead

to a reversible increase in firing frequency of 1.03±0.20 Hz (Fig. 4j). As a positive control, the temperature sensitive *Drosophila* TRPA1 channel[21] was expressed in the l-LNv. As expected, firing rate of TRPA1 expressing neurons drastically increased linearly with temperature, as did other cellular parameters (Extended Data Fig. 8). To test for IR25a specificity, we ectopically expressed another ionotropic receptor, *IR8a*. Here neurons showed a generally increased firing rate, independent of changes in temperature (Extended Data Figure 8a, b, f). Therefore ectopic expression of *IR25a* results in significant temperature dependent changes of neuronal firing, not observed in naïve l-LNv.

IRs evolved the capacity to respond to a variety of external stimuli like odours and tastants, and our data suggest the functions of this family extend also to temperature sensing as described for the gustatory receptor family member Gr28b[22]. IR25a is the most conserved IR[23], and although it functions as co-receptor in the olfactory system[15], our data suggest that IR25a contributes to temperature sensing within ChO. We cannot rule that IR25a has a similar role in other sensory neurons of the PNS or the brain, but we show that IR25a expressing sensory neurons in the leg are capable of sensing temperature and mediating temperature entrainment. IR25a responds to small temperature changes and we predict that the fly continuously integrates temperature signals received from multiple ChO across the whole body for synchronization of the clock. This potential reliance on weakly responding temperature receptors may also explain why the *Drosophila* circadian clock is quasi inert to brief temperature pulses[24], which may help to maintain synchronized clock function in natural conditions of rapid and large temperature fluctuations with fast responding hot and cold sensors mediating temperature preference behaviour[10,11,19,21]. Similar to the complex light

entrainment pathways, our results suggest that multiple thermosensors and mechanisms contribute to temperature entrainment of the clock[9,12,13], with IR25a specifically required for sensing small, but regular temperature changes.

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Author Contributions C.C., E.B., R.S., J.H., R.B. and K.S.L. conceived, designed, and supervised the project. C.C., E.B., M.X., V.C., and J.R. performed experiments. C.C., E.B., and J.R. analysed data, and R.S. wrote the paper.

Fig. 1. IR25a is expressed in ChO neurons. **a,** Overview of the femur ChO adapted from[13]. **b, d,** Double labelling of the femur ChO by *IR25a-gal4* (**b**) and *F-gal4* (**d**) driven mCD8-GFP and *nompC-QF* driven *QUAS-Tomato*. **c, e**, higher magnification of circled areas in (**b**). **f,** IR25a immunolabeling of femoral ChO cryosections of *IR25a-gal4/UAS-mCD8-GFP* flies. From left to right, GFP, anti-IR25a, 22C10, and merged images are shown. **g,** anti-IR25a and 22C10 labelling of femur ChO sections of *IR25a*$^{-/-}$ flies. **h,** Subcellular distribution of an mCherry-IR25a fusion protein co-labelled with the dendritic cap marker nompA-GFP in the femur ChO. Scale bar = 20µm

Fig. 2. *IR25a* is required for temperature synchronization to low-amplitude temperature cycles. **a,** Upper part shows double plotted average actograms depicting the daily activity levels and environmental conditions during the entire experiment. White areas: LL and 25°C; orange areas: LL and 27°C. Histograms show daily average activity levels during the initial LL treatment and the last 3 days of each TC. Light orange: 25°C, dark orange: 27°C, white bars: activity levels in LL. Error bars indicate SEM, numbers in the upper right corner 'n', x-axis: Zeitgeber time (h) and y-axis total activity (beam crossings/30 min). **b,** As in (**a**) but flies were initially kept in LD 25°C, before being exposed to a 7 h phase advanced TC in DD (dark histogram bars) and free-running conditions (DD and 25°C). Actogram shading as in (**a**) but grey areas indicate darkness. Green and red arrows indicate the position (phase) of the main activity peak during the final free run for control and mutant flies, respectively. **c-e,** Quantification of entrainment in LL and DD. **c, e,** EI values (mean ± s.e.m.) during (**c**) 25°C : 27°C TC in LL (delay as in (**a**)), and as indicated in (**e**) (all delay, except 25°C : 27°C: advance) (see Extended Data Fig. 3b for actograms

and daily average plots. In (**c**) *per*^{01} and *nocte*^{1} flies were used as negative controls. ***p<0.001, **p<0.01, n.s.: not significant, One way ANOVA followed by Bonferroni correction (**d**) Phase difference during DD and constant temperature after TC between *IR25a*$^{-/-}$ (n > 10 for each TC) and *y w* control (n > 9) and *IR25a*$^{-/-}$ rescue flies (n > 11). ****p<0.0001, ***p<0.001, **p<0.01; F-statistic (Watson-Williams-Stevens test).

Fig.3. IR25a is required for clock protein oscillations in central clock neurons **a, b,** TIM levels in clock neurons during LL (**a**) and DD (**b**) 25°C : 27°C TC at the indicated time points (ZT). At least 8 brain hemispheres per time point were analysed for each genotype. Error bars indicate s.e.m. See Extended Data Fig 7a, b for neuronal images and rescue experiments, and Extended Data Fig. 7c, d for normal TIM oscillations during LD 25°C and high amplitude TC in LL in *IR25a*$^{-/-}$ brains. **c,** Progeny of *UAS-IMP-TNT* and *UAS-TNT* females crossed to *Clk4.1M-gal4* (DN1>, upper panel) or *Clk9M-gal4;Pdf-gal80* (DN2>, lower panel) males, were exposed to 2, 6h-delayed TC (12h 25°C: 12h 27°C in LL). Left: actograms, shading as in Fig 2a. Right: EI calculations, numbers in bars indicate n. ** p<0.01; One way ANOVA followed by Bonferroni correction. **d**, Same genotypes as in (**c**) were exposed to an 8h-delayed 25°C: 12h 27°C TC in DD. Left: actograms plotted as in Fig 2b, Right: phase difference of activity peaks during final constant conditions between controls (DN1/DN2 > *UAS-IMP-TNT,* n=9/12, respectively) and the indicated genotypes (DN1/DN2 > TNTE, n=16/10). ****p<0.0001, n.s. not significant, F-statistic (Watson-Williams-Stevens test).

Fig.4. IR25a is required for temperature-induced leg nerve responses and confers temperature sensitivity to l-LNv. **a,** Schematic of the setup **b,** Recording of a control fly leg nerve including motor and sensory axons. The first extended insert shows a discharge of presumed ChO sensory units in response to manual extension of the tibia (green bars). Heating the preparation from 20°C to 30°C (middle, red trace) lead to both spontaneous leg movement and concurrent motor and sensory activity (2nd insert) but also to increased sensory firing in the absence of leg or motor activity (3rd insert), which was reversible (4th insert). **c,** Same recording protocol as in (**b**) in an *IR25a*$^{-/-}$ fly shows similar responses to tibia extension and temperature-dependent leg movement, but no sensory activity in response to elevated temperature. **d,** Whole cell current clamp recordings of control and Pdf > IR25a brains exposed to the indicated temperature ramp. **e,** Quantification of the temperature response from multiple recordings (mean, s.e.m.). (**f**) Vm, membrane potential; (**g**) Rin, input resistance; (**h**) F, spontaneous firing rate at 18°C. (**i),** Q10, temperature coefficient. Error bars indicate s.e.m., number in bars = n, ** p<0.01; t-test. **j,** *IR25a* expressing l-LNv respond to small (2-3°C) temperature changes.

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