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Thermotaxis, circadian rhythms, and TRP channels in *Drosophila*

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Abbreviations: A1, 1st Antennal Segment; A2, 2nd Antennal Segment; A3, 3rd Antennal Segment; AC, Anterior Cell; AL, Antennal Lobe; AR, Arista; Clk, Clock protein; Cry, Cryptochrome; Cyc, Cycle protein; Dbt, Double Time protein; DN1, DN2, DN3, Dorsal Neuron group 1, 2, 3; GFP, Green Fluorescent Protein; GPCR, G Protein-Coupled Receptor; lLN_v, Ventral Lateral Neuron, large cell body; LN, Lateral Neuron; LN_d, Dorsal Lateral Neuron; LN_v, Ventral Lateral Neuron; LPN, Lateral Posterior Neuron; mdIV, Multidendritic Neuron, class IV; NEL, Nocifensive Escape Locomotion; PAP, Proximal Antennal Protocerebrum; PDF, Pigment Dispersing Factor; Per, Period protein; PKD1, Polycistic Kidney Disease 1; PLC, Phospholipase C; RNAi, RNA interference; SAC, Sacculus; sLN_v, Ventral Lateral Neuron, small cell body; SLPR, Superior Lateral Protocerebrum; SOG, Suboesophageal Ganglion; thermoTRP, thermosensitive TRP channel; Tim, Timeless protein; TRP, Transient Receptor Potential; TRPA, Transient Receptor Potential, group A (ankyrin repeat); TRPC, Transient Receptor Potential, group C (canonical); TRPL, TRP-Like; TRPM, Transient Receptor Potential, group M (melastatin); TRPP, Transient Receptor Potential, group P (polycystic); TRPV, Transient Receptor Potential, group V (vanilloid); VFP, Venus Fluorescent Protein.

The fruit fly *Drosophila melanogaster* is a poikilothermic organism that must detect and respond to both fine and coarse changes in environmental temperature in order maintain optimal body temperature, synchronize behavior to daily temperature fluctuations, and to avoid potentially injurious environmental hazards. Members of the Transient Receptor Potential (TRP) family of cation channels are well known for their activation by changes in temperature and their essential roles in sensory transduction in both invertebrates and vertebrates. The *Drosophila* genome encodes 13 TRP channels, and several of these have key sensory transduction and modulatory functions in allowing larval and adult flies to make fine temperature discriminations to attain optimal body temperature, detect and avoid large environmental temperature fluctuations, and make rapid escape responses to acutely noxious stimuli. *Drosophila* use multiple, redundant signaling pathways and neural circuits to execute these behaviors in response to both increases and decreases in temperature of varying magnitudes and time scales. A plethora of powerful molecular and genetic tools and the fly's simple, well-characterized nervous system have given *Drosophila* neurobiologists a powerful platform to study the cellular and molecular mechanisms of TRP channel function and how these mechanisms are conserved in vertebrates, as well as how these channels function within sensorimotor circuits to generate both simple and complex thermosensory behaviors.

Introduction

As is the case for all animals, the fruit fly *Drosophila melanogaster* must detect and respond to multiple modalities of sensory stimuli in its environment. As a poikilothermic organism, it is especially important that the fly is able to detect and respond appropriately to thermal fluctuations in its environment during both its larval and adult stages in order to maintain an optimal body temperature for growth and development (18°C to 24°C for laboratory wild-type animals). The role of temperature in shaping *Drosophila* biology have been well-characterized in developmental studies of lab-strain animals[1,2] as well as in analyses of latitudinal clines in a variety of morphometric traits observed in wild populations.[3-5] In laboratory studies of behavioral responses to temperature, thermal fluctuations may include relatively modest changes in temperature that the fly navigates to maintain optimal body temperature and to synchronize its behavior to cyclical changes in temperature that accompany the daily cycle of light and dark. Larger temperature fluctuations may be detected to engage behavioral strategies that allow animals to escape non-optimal temperatures that would negatively impact the long-term health of the animal or cause acute tissue damage.

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The Transient Receptor Potential (TRP) family of ion channels consists of a diverse group of cation-selective ion channels that are conserved across the animal kingdom and which serve a variety of physiological functions.[6] TRP channels are particularly known for their essential and diverse functions in multiple modalities of sensory transduction and signaling.[7,8] The canonical TRP channel encoded by the *Drosophila trp* gene was identified for its role in phototransduction in the fly retina.[9-12] The original *trp* mutants were identified based on their visual impairment under intense lighting conditions.[11] These mutants and the *trp* gene itself were later named based on the mutant's transient receptor potential phenotype.[12] Electroretinogram recordings from *trp* mutants show a rapid return to baseline during intense, prolonged light stimulation, unlike wild-type electroretinograms, which display a sustained component during prolonged stimulation.[11] Molecular cloning and characterization of the *trp* gene indicated that it encodes a transmembrane protein, leading to the hypothesis that the TRP protein forms an ion channel.[9,10] Subsequent electrophysiological experiments *in vivo* and in heterologous cells would reveal that TRP forms a Ca^{2+}-permeable ion channel.[13-15]

Other TRP channels have been characterized for roles in chemosensation[16] and the detection of diverse mechanical stimuli, including external mechanical force,[17,18] osmotic deformation,[19] and sound pressure-waves.[20] Several vertebrate and invertebrate members of the TRP family are known for their roles in transducing changes in environmental temperature, and a subset of these channels are directly gated by changes in temperature as part of their sensory function- these channels are known as thermoTRPs.[21,22] Non-thermoTRPs that function in the transduction of thermal stimuli likely function downstream of a primary temperature sensor, either in a primary transduction step or in a modulatory role. During the course of this review, we will consider both thermoTRPs and non-thermoTRPs, their roles in shaping temperature-driven behaviors in *Drosophila*, and their cellular and molecular mechanisms of function.

Temperature-Sensing TRP Channels in *Drosophila Melanogaster*

Channels in the TRP family are divided into groups based upon their amino acid sequences, with members of the same group often sharing similar functions and properties.[23,24] The *Drosophila* genome contains genes encoding 13 TRP channels.[25] In vertebrates, the TRPA, TRPM, and TRPV groups all contain thermoTRP channels that function in sensory transduction of high and low temperatures.[26-31] The *Drosophila* genome contains genes encoding channels that are classified into each of these groups, but to date, only members of TRPA group have been demonstrated to act as thermoTRPs in insects.[32-34] *Drosophila* TRPA channels thus have been the most widely studied for their roles in temperature-sensing behavior in *Drosophila*. However, members of the *Drosophila* TRPV, TRPC, and TRPP groups also play important roles in temperature sensing that may be independent of direct temperature activation (**Table 1**).[35-37]

The Drosophila TRPA1 channel

The vertebrate TRPA1 channel is expressed in nociceptor neurons as well a variety of peripheral tissues and is well-known for its functions in nociception, inflammation, and chemosensation.[26,38-41] The TRPA1 channel is activated by reactive chemicals (such as allyl isothiocyanate),[38] poly-unsaturated fatty acids,[42] and downstream of G protein signaling.[39] While the human TRPA1 channel is not activated directly by changes in temperature, TRPA1 channels from other mammals may be activated by cold stimuli and act in nociceptors to detect noxious cold stimuli.[26] Interestingly, the TRPA1 channels from some reptile species may be activated by heat. These notably include snakes that possess pit organs (e.g. vipers, pythons, and boas), in which the heat-activated TRPA1 functions as part of the transduction mechanism for the detection of infrared and thermal

Table 1. *Drosophila* TRP channels with thermosensory functions

Channel	Group	Activation mechanisms	Thermosensory function
dTRPA1	TRPA	Reactive electrophiles[48]	Detection of temperature fluctuations
		Heat (>26°C for A isoform; within the comfortable range[72,80]	
		>34°C for D isoform)[34,50,51]	Thermotaxis response to elevated temperature[44]
		$G\alpha_q$-PLC signaling[46,49,72]	Noxious heat avoidance[50,150]
			Synchronization of circadian rhythms to temperature cycles[141,142]
Painless	TRPA	Heat[32]	Noxious heat avoidance[18]
Pyrexia	TRPA	Heat[33]	Synchronization of circadian rhythms to temperature cycles[60]
			Noxious heat resistance[33]
TRP	TRPC	$G\alpha_q$-PLC signaling[66]	Thermotaxis responses to cool temperatures[36]
		Polyunsaturated fatty acids[155]	
		Mechanical force[156]	
TRPL	TRPC	$G\alpha_q$-PLC signaling[67]	Thermotaxis responses to cool temperatures[36]
		Polyunsaturated fatty acids[155]	
		Mechanical force[156]	
Inactive	TRPV	Osmotic stimulation[82]	Thermotaxis responses to cool temperatures[35]
Brivido-1	TRPP (non-channel)	—	Thermotaxis responses to cool temperatures[37]
Brivido-2	TRPP (non-channel)	—	Thermotaxis responses to cool temperatures[37]
Brivido-3	TRPP (non-channel)	—	Thermotaxis responses to cool temperatures[37]

stimuli.[43] The *Drosophila* TRPA1 channel (dTRPA1) is 32% identical and 54% similar to its mammalian ortholog by amino acid identity and is activated by elevated temperature as well as in response to reactive chemicals and downstream of intracellular signaling pathways.[44-47] The dTRPA1 channel is expressed in a multiple classes of peripheral sensory neurons[48-50] as well as several groups of central neurons,[44,47] all of which may act as heat sensors for temperature-driven behaviors.

An important feature of the *dTrpA1* gene is its use of multiple transcription start sites and mutually exclusive alternative splicing at the 12th and 13th exons to produce at least 4 dTRPA1 isoforms with differing expression patterns and heat-activation properties (**Fig. 1A**).[46,50,51] The canonical *dTrpA1-A* isoform uses a downstream transcription start site and is spliced to include the 12th exon, while the *dTrpA1-B* isoform uses the same start site, but is spliced to include the 13th exon.[46] GAL4 reporter transgenes that use promoter sequence upstream of the A/B start site drive expression of GFP in a relatively small number of neurons in the brain as well as in peripheral neurons in the antennae and labellum.[44,46-49] The *dTrpA1-C* isoform uses an upstream transcriptional start site and is spliced to include the 13th exon, while the *dTrpA1-D* isoform uses the same start site, but is spliced to include exon 12.[50,51] GAL4 reporter transgenes using promoter sequences upstream of the C/D start site drive expression of GFP in the Class IV multidendritic neurons (mdIVs) that act as larval nociceptors, as well as in the labellum and the neuroendocrine cells of the corpus cardiacum.[50,51]

Studies have demonstrated that the dTRPA1-A channel is a *bona fide* thermoTRP with a threshold of ~25°C,[34] but this is not a property shared by all channel isoforms.[50,51] When expressed in *Drosophila* S2R+ cells along with the genetically encoded calcium sensor GCaMP, the dTRPA1-A isoform mediates calcium transients in response to elevated temperature of around 26°C, while the dTRPA1-D isoform mediated calcium transients with a higher threshold (~36°C).[50] The dTRPA1-B and dTRPA1-C isoforms did not mediate detectable heat-induced calcium transients, but all 4 isoforms produced robust calcium transients in response to allyl isothiocynate (a potent TRPA1 activator).[50] These results are consistent with results obtained from electrophysiological records in *Xenopus* oocytes showing that dTRPA1-A is a more temperature-sensitive channel than dTRPA1-D ($Q_{10} = 130$ vs. 7.5).[51]

These studies of dTRPA1 isoforms expressed in heterologous expression systems indicate that the 37 amino acid residues encoded by exon 12 and the 36 amino acid residues encoded by exon 13 may be essential determinants of temperature activation of the channel, as both splice forms containing exon 12 (i.e. A and D) encode temperature-sensitive channels, while those encoded by splice forms containing exon 13 (i.e., B and C) are not.[50] The amino acids encoded by exons 12 and 13 are contained in the intracellular N-terminus of the protein immediately preceding the first transmembrane domain (**Fig. 1B**), and thus an important role for these residues is generally consistent with studies of chimeric and point-mutant vertebrate TRPA1 channels that indicate an important role for the N-terminal region of the channel in conferring temperature sensitivity.[52,53] A role for the extreme N-terminus of the dTRPA1 protein in determining temperature sensitivity is also suggested by the differing Q_{10} values observed for the dTRPA1-A and dTRPA1-D isoforms in electrophysiological experiments, as well as by mutagenesis studies showing that elimination of basic residues in dTRPA1-D N-terminus can increase the temperature sensitivity of the channel.[51] Interestingly and conversely, recent studies of human TRPA1 have indicated that the N-terminal intracellular region is dispensable for activation of the channel by temperature.[54] These conflicting observations may be reconciled by a recent thermodynamic model of temperature gating that suggests that the temperature sensitivity of temperature-gated channels arises from differences in heat capacity between open and closed conformations and that these heat capacity differences could arise from the solvent exposure of distributed hydrophobic residues, eliminating the need for a dedicated temperature sensor domain.[55] This model has been validated by a recent mutagenesis study in which temperature sensitivity was conferred on normally temperature-insensitive voltage-gated potassium channels by altering the polarity of residues that become solvent-exposed during voltage gating.[56] These developments suggest that the N-terminal intracellular regions of TRPA1 channels might play an allosteric role in controlling

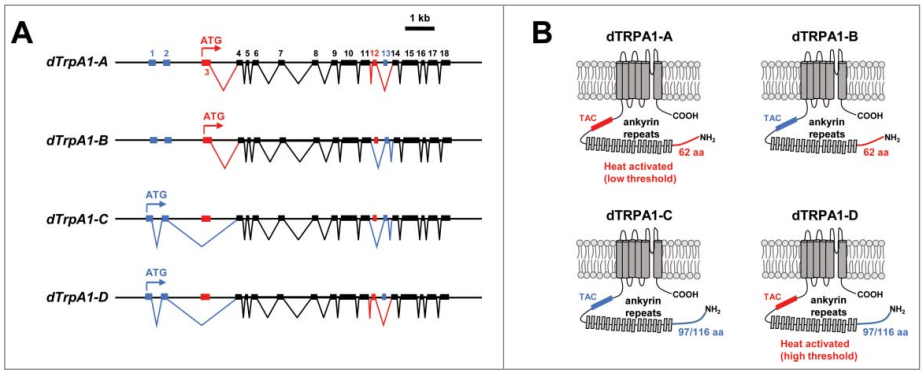

Figure 1. Schematic representation of *dTrpA1* isoforms. (**A**) Gene structures of known *dTrpA1* isoforms. Exons are numbered sequentially starting with the most 5′ exon and irrespective of isoform. The "A" isoform uses a translation start site in exon 3 and is spliced to include exon 12. The "B" isoform uses a translation start site in exon 3 and is spliced to include exon 13. The "C" isoform uses a translation start site in exon 1 and is spliced to include exon 13. The "D" isoform uses a translation start site in exon 1 and is spliced to include exon 12. (**B**) Protein structures of dTRPA1 protein isoforms. N-terminal regions encoded by exons 1 and 2 are drawn in blue, while N-terminal regions encoded by exon 3 are drawn in red. The TAC region between the ankyrin repeats and first transmembrane domain encoded by exon 12 is drawn in red, while the TRP ankyrin cap (TAC) region encoded by exon 13 is drawn in blue.

or modulating temperature sensitivity, but are not themselves a temperature sensor domain.

It is important to note that consensus has not yet been reached on nomenclature for the differing isoforms of the *dTrpA1* mRNA and the dTRPA1 channel subunit. The nomenclature used in this review was introduced upon the discovery that the *dTrpA1-A* and *dTrpA1-B* isoforms are generated by alternative splicing[46] and then extended upon the identification of the *dTrpA1-C* and *dTrpA1-D* mRNA variants.[50] Contemporaneous studies that identified the *dTrpA1-D* mRNA named the subunit encoded by this mRNA as dTRPA1(A), while naming the canonical subunit (i.e. the dTRPA1-A subunit described in this review) dTRPA1 (B), based on the relative positions of the start codons used by these isoforms.[51] It is also important to note that 2 different 5′ ends have been cloned for the *dTrpA1-C/D* mRNAs, one that encodes a 97 amino acid N-terminus[50] and another that encodes a 116 amino acid N-terminus via a slightly upstream start site[51] (**Fig. 1B**). It is unclear whether both start sites cloned in these isoforms are actually used *in vivo*, and thus the 97 amino acid N-terminus may simply be a truncated form of the 116 amino acid N-terminus.

Other Drosophila TRPA channels

The *Drosophila* genome contains genes encoding 3 additional TRPA channels that do not have mammalian orthologs (Painless, Pyrexia, and Waterwitch).[18,33,57] Electrophysiological studies have demonstrated that the Painless channel is a thermoTRP with an activation threshold of ~42°C when expressed in HEK293 cells.[32] GAL4 reporters for the *Painless* gene drive GFP expression in a large number of central neurons and peripheral sensory neurons, including the multidendritic neurons that tile the larval body wall and in chemosensory neurons of the labellum.[18,45] Like the *dTrpA1* gene, the *Painless* gene makes use of multiple transcription start sites to produce at least 3 different channel isoforms with differing intracellular N-terminal regions (**Fig. 2**).[58] Only the long isoform has been characterized electrophysiologically,[45] but behavioral evidence (described below) suggests that these isoforms may have differing roles in transducing information about elevated temperature.[58]

Like Painless and dTRPA1, the Pyrexia channel is a thermoTRP that is expressed in a wide variety of sensory tissues.[33,59] When expressed in *Xenopus* oocytes or HEK293 cells, Pyrexia mediates heat-activated currents with a threshold of ~40°C.[33] GAL4 reporters that use promoter sequences upstream of the *pyrexia* start codon drive GFP expression in a wide variety of sensory neurons and supporting cells,[33] including an unidentified population of neurons in the second and third antennal segments and in the cap cells that support chordotonal neurons in Johnston's Organ and the femur of adult flies.[59-61] Flies lacking Pyrexia function were initially characterized for their intolerance to thermal stress, as 60% of *pyrexia* mutants were found to become paralyzed following 3 minutes of exposure to a 40°C environment, as compared to less than 10% of wild-type controls.[33] Roles for Pyrexia in temperature sensing and circadian entrainment to temperature cycles have since been discovered and will be described in detail below.[60,61] The final *Drosophila* TRPA channel, Waterwitch, is not known to be activated by changes in temperature or to have any role in behavioral responses to thermal stimuli, instead playing an important role in hygrosensation and behavioral responses to changes in humidity.[57]

Non-TRPA group TRP channels

In vertebrates, the TRPV and TRPM groups also contain characterized thermoTRP channels,[30,31,62,63] but temperature-dependent activation of the *Drosophila* members of these groups have not been observed. Mammalian members of the TRPV subfamily of ion channels have been well-characterized for their activation by elevated temperatures and their roles in the sensory transduction of thermal stimuli. The TRPV1 channel, like TRPA1, is well-known for its central role in nociceptive signaling and is activated by elevated temperatures and by the chemical capsaicin.[30] The mammalian TRPV2, TRPV3, and TRPV4 channels are also heat-activated.[27-29,62] The *Drosophila* TRPV channels, Inactive and Nanchung, are not known to be directly activated by changes in temperature. However, Inactive may play an indirect role in the detection of cool temperatures that will be discussed below.[35] The mammalian TRPM8 channel has a well-described role in detection of cool temperatures and is activated by the ligand menthol.[31,63] However, the *Drosophila* TRPM is not activated by cool temperatures and does not currently have a described sensory function.

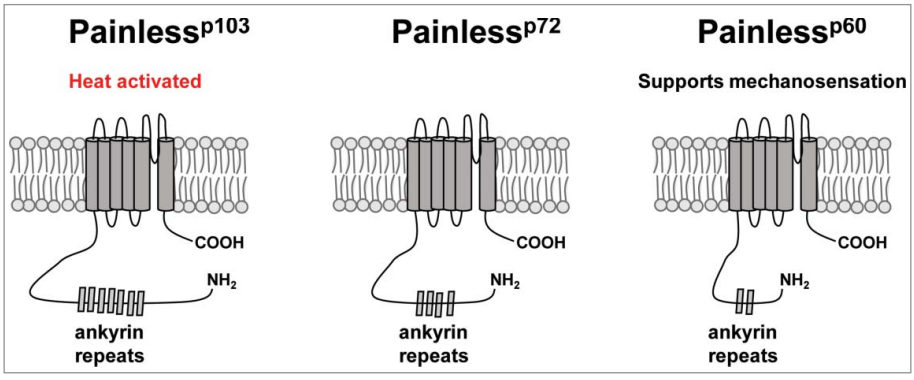

Figure 2. Schematic representation of Painless protein isoforms. The Painless^{p103}, Painless^{p72}, and Painless^{p60} protein isoforms are schematized with varying numbers of ankyrin repeats in their N-terminal intracellular regions.

Drosophila also possess TRP channels from the TRPC and TRPP groups that have established roles in mediating temperature-sensitive behavior,[36,37] although it remains to be determined whether TRP channels from either of these groups are *bona fide* thermoTRPs in *Drosophila*. The TRP and TRPL channels are both *Drosophila* members of the TRPC family who have well-described roles in phototransduction and are activated downstream of

a Rhodopsin-Gα_q-phospholipase Cβ signaling cascade.[9,10,64-68] However, both of these channels also have roles in behavioral responses to cool stimuli that will be described below.[36]

The *Drosophila* Brivido-1, Brivido-2, and Brivido-3 proteins share sequence homology with the TRPP group of mammalian TRP channels, specifically the PKD1 and PKD1-Like proteins.[37] The mammalian PKD1 protein contains 11 transmembrane domains and regulates the function of TRPP2 channel subunits, but is not known to form ion channels on its own.[69,70] Similar to PKD1, the Brivido proteins contain between 8 and 10 transmembrane segments and are not known to form ion channels independently. Despite this, the Brivido proteins function in behavioral responses to cool stimuli and cold-activation of peripheral sensory neurons (as described below).[37] It is possible that the Brivido proteins function similarly to the mammalian PKD1 protein and regulate the function of TRPP ion channels. However, there is no described sensory function for TRPP ion channel subunits in mammals or invertebrates- so the cellular and molecular mechanism for Brivido proteins' functions in cool-detection remains unclear.

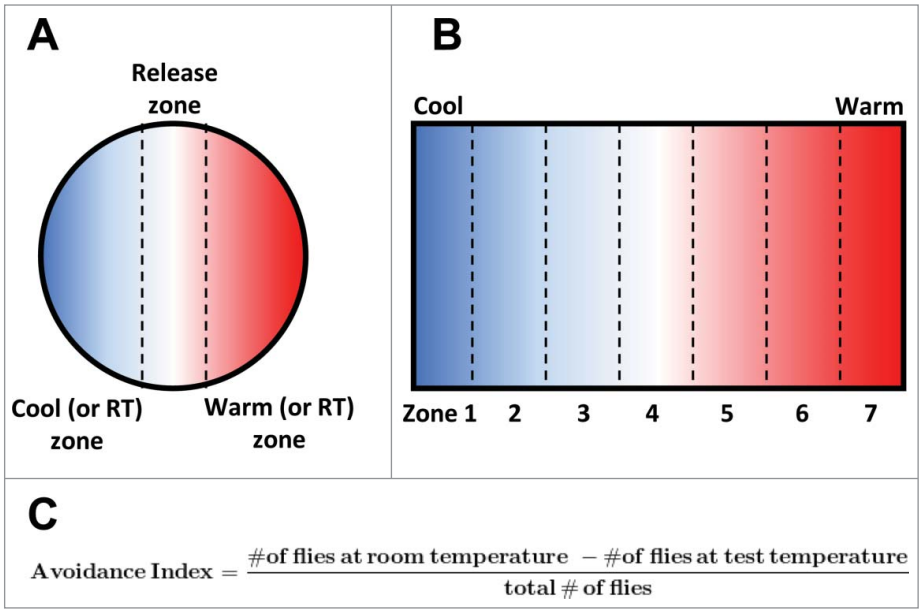

Figure 3. Paradigms for assaying thermosensory behavior in *Drosophila* larvae and adults. (**A**) Schematic of a circular assay chamber in which flies or larvae must choose between warm and cool (or room temperature and test temperature) regions. The distribution of individuals between these regions can then be used to calculate and avoidance (or preference) index. (**B**) Schematic of an assay chamber that can be used to test the distribution of individuals across a temperature gradient. The chamber is heated (or cooled) at one end to produce a temperature gradient and divided into zones. The proportional distribution of flies or larvae across these zones can then be calculated. (**C**) A standard equation used to generate an avoidance index of flies tested in an experimental chamber that requires them to navigate to either room temperature or a test temperature.

Thermotaxis and Behavioral Responses to Changes in Environmental Temperature

In the laboratory, *Drosophila* are commonly reared at temperatures ranging from 15°C to 25°C, with 24°C being the preferred temperature of adult flies[71] and 18°C being the preferred temperature of 3rd instar larvae.[44,72,73] Flies are capable of navigating to optimal temperatures within this "comfortable" range and also capable of avoiding non-optimal temperatures that fall above or below of this optimal range. This temperature-driven navigation is known as thermotaxis, and behavioral studies of thermotaxis behavior in both larvae and adult flies have made significant contributions to understanding the molecular, cellular, and circuit-level mechanisms that underlie temperature sensing. Assays of thermotaxis behavior are generally performed using an arena that can be heated or cooled to produce a temperature gradient that can then be divided into a warm and cool halves or into zones (**Fig. 3A and B**).[44,71,74] Populations of animals are then placed in the arena and allowed to distribute via thermotactic locomotion for a set period of time. The distribution of animals across regions of differing temperature may then be used to calculate an avoidance index to determine the proportion of animals that avoid a warm or cool stimulus (**Fig. 3C**)[73,74] or to create a histogram showing the distribution of animals across a temperature gradient.[71] Multiple molecular and cellular mechanisms contribute to differing aspects of these behaviors, such as sensing small temperature fluctuations within the comfortable range versus large temperature variations above or below the preferred temperature.

Warmth sensors in the central nervous system

Both larvae and adult flies engage in thermotaxis behavior to escape temperatures higher than 25°C.[44,71] These behaviors require the dTRPA1 channel and likely use the innate temperature sensitivity of the channel as a direct temperature sensor, as the temperature threshold for channel activation in heterologous cells is equivalent to the temperature threshold for activation of temperature-sensing neurons and for the behavior *in vivo*.[34,44,47]

Drosophila larvae lacking dTRPA1 function show a significantly reduced preference for cooler temperatures when tested in an assay that forces them to choose between the warmer (i.e., less comfortable) and cooler (i.e. more comfortable) portions of a temperature gradient.[44] It should be noted that in this experiment, the entire thermal gradient (27°C–41°C) was above the threshold for dTRPA1 activation, and thus these results are consistent with dTRPA1 acting as a direct temperature sensor. The site of action of dTRPA1 for these experiments has not been positively identified, but it is likely that dTRPA1 functions in a small number of central neurons, as opposed to peripheral neurons. Expression of

tetanus toxin under the control of a *dTrpA1-A/B-Gal4* driver that drives expression in a small number of dTRPA1-expressing neurons in the brain and in the neuroendocrine cells of the corpus cardiacum was sufficient to reduce heat avoidance similar to the effect observed in a *dTrpA1* mutant.[44]

Similar to the effect observed in *Drosophila* larvae, adult flies lacking dTRPA1 function also show defective avoidance of warmer temperatures.[47] When allowed to passively distribute on a temperature gradient ranging from 18°C to 32°C, flies lacking dTRPA1 function displayed increased accumulation within the 28°C to 32°C range as compared to wild-type controls.[47] As in larvae, dTRPA1 is expressed in a small number of central neurons in the adult fly brain (**Fig. 4**).[47] These dTRPA1-positive neurons (as detected by anti-dTRPA1 antisera) have been grouped into 3 groups based on position in the brain: the lateral cell, ventral cell, and anterior cell (AC) clusters. The AC neurons are likely to be the principle central thermosensors for adult warmth avoidance. First, restoration of dTRPA1 expression to the AC neurons (but not the lateral cell or ventral cell neurons) of a *dTrpA1* mutant is sufficient to restore wild-type heat-avoidance behavior.[47] Second, tissue-specific knockdown of *dTrpA1* transcript in the AC neurons using *dTrpA1*-specific RNAi and an AC-expressed *dTrpA1-Gal4* driver caused loss of heat avoidance similar to that observed in a *dTrpA1* mutant.[47] Thus, dTRPA1 function in the AC neurons is both necessary and sufficient for wild-type thermal preference in adult flies. Finally, measurements of heat-induced calcium transients in the AC neurons using GCaMP show that the cells are activated by heat with a threshold of ~27°C.[47] This threshold is similar to the threshold for activation of heterologously expressed dTRPA1,[34] and indeed, the heat-induced transients are absent from the AC neurons in *dTrpA1* mutants.

While the AC neurons are highly likely to be central temperature sensors for thermotaxis behavior, the complete neural circuit that underlies their behavioral function is unknown. Projections from the AC neurons innervate the olfactory lobe (**Fig. 4**), the site of first-order synapses between olfactory sensory neurons and olfactory projection neurons.[47] These results suggest the possibility of multimodal integration of thermal stimuli with chemosensory signals within the olfactory lobe. The AC additionally project to the suboesophageal ganglion,[47] the superior lateral protocerebrum,[47] and the proximal antennal protocerebrum (PAP).[37] The possible significance of these projections is unknown, but projections from the AC neurons to the PAP overlap with projections from warmth-sensing neurons in the aristae (as described below) to the PAP,[37] suggesting that the PAP may function to integrate information from multiple warmth sensors (**Fig. 4**). Additionally, the AC neurons receive inputs from peripheral warmth-sensing neurons in the second antennal segment (as described below), suggesting that the AC neurons themselves may be a site of integration for cell-intrinsic and cell-extrinsic temperature signals.[61]

Warmth sensors in the antennae

The antennae of many insect species are multimodal sensory structures.[75,76] As such, the antennae of *Drosophila* have long been considered potential sites of action for thermosensory neurons that initiate thermotaxis behavior. Flies in which the third antennal segment and aristae have been bilaterally ablated have been shown to distribute broadly across a 18°C to 31.5°C temperature gradient, as compared to un-ablated controls or flies in which only the aristae had been removed, which accumulated with a peak at ~24°C.[71] Furthermore, mutations in the *spineless* gene, which produce severe defects in antennal development, produce similar defects in thermotaxis behavior.[71,74] Together these results suggested a role for the third antennal segment, better known for its role in olfaction, in responses to both hot and cool stimuli. The role of the third antennal segment in behavioral responses to heat has been rendered unclear, however, by subsequent studies that indicate a more specific role for the third antennal segment in thermotaxis responses to uncomfortably cool temperatures, but not uncomfortably warm temperatures.[47]

Despite the unclear role for antennal temperature sensors in the generation of behavioral responses to elevated temperature, it is clear that the antennae contain

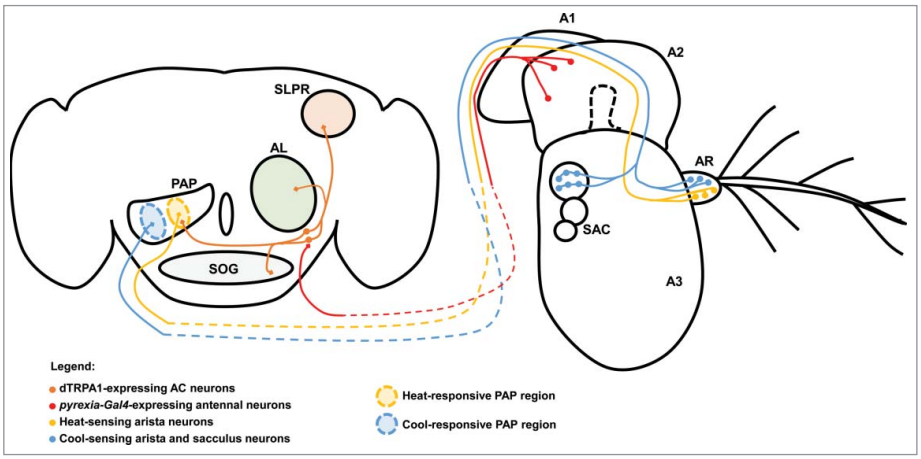

Figure 4. Central and peripheral neural mechanisms for detecting changes in temperature. The adult *Drosophila* brain and antenna are schematized here, with colored circles representing neuronal cell bodies, colored lines representing axonal connections, and colored diamonds representing presynaptiC-terminals. Heat-responsive and cool-responsive regions of the proximal antennal protocerebrum (PAP) are schematized as filled ovals with dashed outlines. Heat-sensing neurons of the aristae (AR) connected to the third antennal segment (A3) send projections to the brain that synapse in the heat-sensitive region of the PAP. Cool-sensing neurons of the aristae and sacculus (SAC) send projections to the cool-responsive region of the PAP. Putative *pyrexia-Gal4*-expressing neurons in the second antennal segment (A2) project to the brain and synapse on the dTRPA1-expressing AC neurons (it is important to note that the exact location and identity of these neurons are unknown). The AC neurons project to the heat-responsive region of the PAP, as well as to the suboesophageal ganglion (SOG), the superior lateral protocerebrum (SLPR), and the antennal lobe (AL). Also depicted is the first antennal segment (A1).

at least 2 independent heat sensors. The first of these temperature sensors is thought to be located in the second antennal segment and is dependent on the function of the Pyrexia TRPA channel, suggesting that the sensor is comprised of as-yet unidentified *pyrexia*-expressing neurons in that structure (**Fig. 4**).[61] Within the second antennal segment, the *pyrexia-Gal4* reporter is most obviously expressed by the cap cells that support the chordotonal neurons in Johnston's Organ,[59] a structure that is well-known for its role in detecting and generating behavioral responses to gravitational forces and auditory stimuli.[77,78] However, unidentified *pyrexia-Gal4*-expressing neurons send projections to the AC neurons, which act as central temperature sensors, and appear to influence their electrical activity.[61] Wild-type AC neurons expressing GCaMP display a 2-peaked calcium response during a temperature-ramp protocol, with the first peak occurring at ~25°C and the second at ~27°C.[61] The first peak occurs near the threshold for dTRPA1 activation and is significantly decreased in *dTrpA1* mutants. The second peak is absent in animals in which the second antennal segments have been removed and in *pyrexia* mutants, consistent with a model in which *pyrexia*-expressing neurons in the second antennal segment send information about environmental temperature to the AC neurons.[61] The functional significance of temperature sensing in *pyrexia*-expressing antennal neurons is unclear, as *pyrexia* mutants have no defect in thermotaxis,[61] but these results do demonstrate that multiple heat sensors function in the *Drosophila* nervous system and may be integrated, in this case, in the AC neurons (**Fig. 4**).

While the AC neurons and thermosensory neurons of the second antennal segment appear to generate physiological and behavioral responses to temperatures warmer than those preferred by wild-type *Drosophila*, the antennae also contain neurons that generate responses to small temperature fluctuations within the comfortable range (18°C–24°C). Two-photon calcium imaging of the *Drosophila* antennae has revealed 6 ciliated sensory neurons in the aristae that are temperature sensitive.[37] These neurons can be divided into 2 populations: 3 neurons that respond specifically to cooling stimuli and are inhibited by heat (to be discussed below) and 3 neurons that are stimulated by heat and inhibited by cooling stimuli. Remarkably, the calcium responses in both of these populations of neurons are activated by temperature fluctuations as low as ~0.5°C.[37] The projections of the warmth-sensing and cool-sensing neurons of the aristae terminate in distinct regions of the PAP, and the projections of the warmth-sensing neurons overlap in the PAP with those of the AC neurons, suggesting that the PAP may be thermotopically organized and act as an integration region for thermosensory information (**Fig. 4**).[37]

Further studies of the warmth-sensing neurons in the *Drosophila* aristae have identified novel molecular mechanisms for the detection of thermal stimuli as well as provided important insights into the organization of *Drosophila* thermosensory behavior. Blockade of synaptic transmission in the warmth-sensing neurons of the aristae by cell-specific expression of the tetanus toxin light chain produces a significant defect in rapid avoidance of warm temperatures.[37,79] This behavior is distinct from thermotaxis responses that occur over a longer time scale, as neither dTRPA1 nor AC neuron function is required for this rapid avoidance.[79] These results suggest that *Drosophila* thermotaxis and thermosensory behaviors may be broken down into simpler component behaviors that require differing sensory neurons, neural circuits in the brain, and molecular temperature sensors.

While the central circuits that allow different types of thermosensory neurons to contribute to different aspects of thermosensory behavior remain to be elucidated, it is certainly clear that these circuits make use of differing molecular mechanisms. While thermotaxis by adult flies over long time scales along a shallow temperature gradient requires dTRPA1 function in the AC neurons (as described above),[47] more rapid thermotaxis behaviors mediated by the warmth-sensing neurons of the aristae require the gustatory receptor, GR28B(D).[79] Loss of GR28B(D) function results in a loss of rapid thermal avoidance behavior, and expression of GR28B(D) is sufficient to confer thermal sensitivity upon a wide variety of tissues.[79] These results suggest that GR28B(D) is a *bona fide* thermosensor, although the cellular and molecular mechanisms for its function remain unknown.

Warmth sensors in other tissues

When tested for temperature preference along a thermal gradient (see **Fig. 3**) *Drosophila* larvae are capable not only of avoiding uncomfortable low (<18°C) and high (>24°C) temperatures, but also of navigating to an optimal temperature (18°C) within a comfortable range (18°C–24°C).[72,80] The heat-activated dTRPA1 channel is required for this thermal preference behavior, but the ability of larvae to make fine thermal discriminations below the described temperature threshold for dTRPA1 activation (~25°C) suggests that TRPA1 is not functioning as direct temperature sensor for this behavior.[72] However, the simultaneous discovery that, in addition to a requirement for dTRPA1, the $G\alpha_q$ subunit, Gαq, and the phospholipase C enzyme, NorpA, are also required for thermal discrimination suggested that dTRPA1 could function downstream of a G protein-coupled receptor (GPCR) signaling cascade.[72]

This hypothesis was confirmed by the identification of the Rh1 rhodopsin, NinaE, as essential for larval thermal preference within the comfortable range.[80] The role of NinaE was found not to be light-dependent, and the thermal preference function of NinaE occurs not in Bolwig's Organ cells that function as larval photoreceptors, but in some other unidentified cells that express both TRPA1 and NinaE.[80] Epistasis analysis supported the existence of a NinaE-Gαq-NorpA-dTRPA1 signaling cascade, defining a novel signaling mechanism for temperature discrimation.[80] Questions remain to be elucidated about this thermosensory mechanism. For instance, how does NinaE function as a thermosensor? The instrinsic thermal sensitivity is far too low and too slow to mediate the behaviors described above, suggesting the possibility that an accessory factor is required.[80,81] Additionally, the site of action of the signaling cascade is unknown. dTRPA1 is not known to be expressed in the Bolwig's Organs, the traditional site of NinaE action. Single-cell RT-PCR analysis of cultured larval cells reveals that cells co-expressing

NinaE and dTRPA1 do exist, but their identities are currently unknown.[80]

Cool sensors in the peripheral nervous system

In order to avoid tissue damage and maintain an optimal growth rate, *Drosophila* larvae and adult flies must also avoid sub-optimally cool temperatures. When released in the middle of a cool temperature gradient (~15°C–21°C), *Drosophila* larvae show strong preference for movement toward the warmer portion of the gradient and away from the cooler portion.[36] This cool-avoidance behavior requires the function of the TRP and TRPL channels,[36] which have been historically characterized for their roles in phototransduction and which are not known to be directly temperature sensitive.[10,36,64] A similar cool-avoidance paradigm likewise reveals a role for the TRPV channel, Inactive, in avoiding uncomfortably cool temperatures.[35] Like TRP and TRPL, Inactive is not known to be a temperature-activated channel, having more well-described roles in auditory transduction.[82]

Despite the well-described roles for TRP and TRPL in phototransduction in the larval photoreceptor neurons, their roles in cool temperature avoidance are distinct from their roles in phototransduction at a cellular and molecular level. Elimination of the larval photoreceptor neurons of the Bolwig Organ by cell-specific overexpression of the pro-apoptotic protein Hid or mutation of the transcription factor GLASS eliminates phototaxis responses without altering responses to uncomfortably cool stimuli.[36] Thus TRP and TRPL must function in different sets of neurons to fulfill their roles in thermotaxis and phototaxis. While the site of action of TRP and TRPL has not been formally demonstrated, the neurons of the terminal organ are an appealing candidate site. The terminal organ neurons show robust responses to cold stimuli, as measured with the genetically encoded calcium sensor, Cameleon, and extracellular electrophysiological recording.[73] Furthermore, elimination of synaptic vesicle release in these neurons via cell-specific expression of the tetanus toxin light chain produces cool-avoidance defects similar to those observed in *trp* and *trpl* mutants.[73] The molecular mechanism for TRP and TRPL functions in phototransduction and thermotransduction are also distinct from each other. Larvae that are mutant for NorpA, the phospholipase C enzyme essential for activating TRP and TRPL in phototransduction, show significant phototaxis defects, but no defects in avoiding the cool (<18°C) half of a thermotaxis arena.[36] These findings, which show a TRP-/TRPL-dependent, NorpA-independent mechanism for cool avoidance, stand in contrast to experiments discussed above, which demonstrate that NorpA, TRPA1, and NinaE function are required for larvae to avoid mild elevated temperatures (19°C–24°C).[72,80] Clearly, distinct molecular mechanisms must exist for larval behavioral responses to warm and cool temperatures.

Adult flies also avoid uncomfortably cool stimuli using a mechanism that is cellularly and molecularly distinct from that used for warmth avoidance. A group of 3 ciliated sensory neurons in the aristae of the *Drosophila* antenna, as well as a cluster of neurons in the sacculus of the third antennal segment, have been shown to respond to cooling stimuli with calcium transients, as measured with GCaMP.[37] The cool sensors for these neurons require the TRPP family subunits Brivido-1, Brivido-2, and Brivido-3. A GAL4 reporter for *brivido-1* drives GFP expression in these cool-responsive neurons, as well as a small population of non-temperature-sensitive neurons in the third antennal segment.[37] RNAi-knockdown of either *brivido-1* or *brivido-2* results in defective cooling-sensitive calcium responses, while knockdown of any of the 3 channels results in defective behavioral avoidance of cool temperatures.[37] As described above, the Brivido subunits most closely resemble human PKD1 proteins, which do not form ion channels, but instead regulate the function of TRPP2 channels.[69,70] Thus it is unlikely that Brivido homomers or heteromers form cooling-activated ion channels on their own. However, it is possible that Brivido subunits instead support the function of some as-yet unidentified cooling-activated ion channel.

Regulation of Drosophila Circadian Rhythms by Temperature

Drososphila adults and larvae use thermotaxis to avoid non-optimal temperatures in their environment. There is also substantial adaptive value in coordinating daily patterns of behavior to avoid being active during times of non-optimal temperature, such as hot middays or cold nights. Circadian clocks are biological oscillators that allow organisms to synchronize their physiology and behavior to the 24-hour cycles of light and dark and concomitant temperature variation that result from the rotation of the Earth on its axis. The central components of a cellular circadian clock are the molecular oscillations of clock genes and their products, which are altered in their abundance, subcellular localization, and phosphorylation state over a 24-hour cycle.[83,84] These molecular oscillations occur in peripheral tissues as well as in so-called "pacemaker neurons" in the central nervous system that integrate environmental information in order to synchronize the circadian clock to those stimuli.[85-87]

Molecular and cellular components of the Drosophila circadian clock

The cyclical accumulation and subcellular translocation of the Period (Per) and Timeless (Tim) proteins are perhaps the most fully understood molecular oscillations in *Drosophila* pacemaker cells and are often considered to be the centerpiece of the circadian clock.[83,84,88] Briefly, the helix-loop-helix transcription factors, clock (Clk) and cycle (Cyc), form a heterodimer that binds the *per* and *tim* promoter regions to activate transcription, leading to accumulation of *per* and *tim* mRNAs.[89-91] Tim interacts with Per to form a heterodimer, a configuration that blocks the phosphorylation and proteasomal degradation of Per.[92-94] However, Tim is quickly degraded in the presence of light, preventing either protein from accumulating during the light phase of the light-dark cycle.[95-98] During the dark phase of the light-dark cycle, however, Per and Tim accumulate in the cytoplasm and eventually translocate into the nucleus in complex with the Double-time (Dbt) kinase.[99,100] In the nucleus, Per, Tim, and Dbt bind to the Clk-Cyc dimer and allows for Dbt-dependent

phosphorylation of Clk.[100,101] Phosphorylation inhibits binding of the Clk-Cyc dimer to DNA, thus preventing further *per* and *tim* transcription (and presumably transcription of other cycling genes).[100] This negative feedback loop is the molecular basis for the cyclic accumulation of *per* and *tim* gene products.

One major feature of cells that contain circadian clocks is their ability to sustain molecular oscillations in the absence of outside input from the animal's environment or from other cells. As such, when flies are housed in constant darkness, the rhythmic accumulation of Per and Tim proteins described above will continue to occur in cells containing a circadian oscillator with a period of around 24 hours.[102,103] Likewise, flies housed under these conditions will continue to exhibit circadian behavior patterns with a similar period. However, a second essential feature of cells that contain circadian clocks is their ability to synchronize their molecular oscillations between each other and entrain them to cyclical environmental stimuli or *Zeitgebers* ('time givers') such as light and temperature.[84,104] Synchronization of the circadian clock to light stimuli is largely dependent on the blue-light photoreceptor, Cryptochrome (Cry).[105-107] When Cry is activated by light, it binds to Tim and causes its light-dependent degradation (as part of the negative feedback loop described in the preceding paragraph).[108,109]

The central circadian clocks in adult *Drosophila* are the so-called "pacemaker neurons" in the central nervous system.[85,86] There are roughly 150 pacemaker neurons in the adult *Drosophila* brain, and these can be divided into 3 major groups based on their anatomical positions (**Fig. 5**).[87] These are the dorsal neurons (DNs), the lateral neurons (LNs), and the lateral posterior neurons (LPNs). The DN and LN groups can be further subdivided based on neuronal position and morphology. Thus the DNs are divided into the DN1, DN2, and DN3 subgroups based on their positions within the brain, while the LNs are divided into a dorsal LN_d group along with small and large ventral LN_v groups (sLN_vs and lLN_vs) (**Fig. 5**).

While pacemaker neurons of all subgroups are capable of sustaining clock gene oscillations, they also differ within and between subgroups in their connectivity, gene expression profiles, and function. For example, only the LN_v neurons express pigment dispersing factor (PDF), a major neuropeptide output of circadian system that functions to synchronize the individual circadian clocks.[110,111] Pacemaker neurons can also be categorized by their roles in generating or supporting the morning or evening peaks in *Drosophila* locomotor behavior, which anticipate the daily onset of light and dark respectively.[112] Four of the sLN_vs are responsible for the morning peak and are considered to be the master oscillators of the *Drosophila* circadian system, as they alone are sufficient to sustain circadian locomotor rhythms under constant darkness (**Fig. 5**).[113-116] The fifth sLN_v and the LN_ds are responsible for supporting the evening activity peak (**Fig. 5**).[113,114] The DN1s may actually support either the morning or evening peak of activity in an ambient temperature dependent manner.[117] Additionally, not all pacemaker neurons share intrinsic light sensitivity, as only subsets of the the LN_vs, LN_ds, and DN1s express Cry, while the photoreceptor is not present at all in the LPNs, DN2s, or DN3s (**Fig. 5**).[105,118,119]

Circadian entrainment and phase shifting in response to thermal stimuli

Light is the most thoroughly characterized *Zeitgeber* in *Drosophila*, producing robust entrainment of molecular and behavioral rhythms. However, cyclical changes in temperature have also long been known for their powerful ability to shift and entrain behavioral rhythms in *Drosophila*.[104,120,121] It is likely that in a poikilothermic organism such *Drosophila*, one of the major selective advantages of entraining behavior to a light-dark

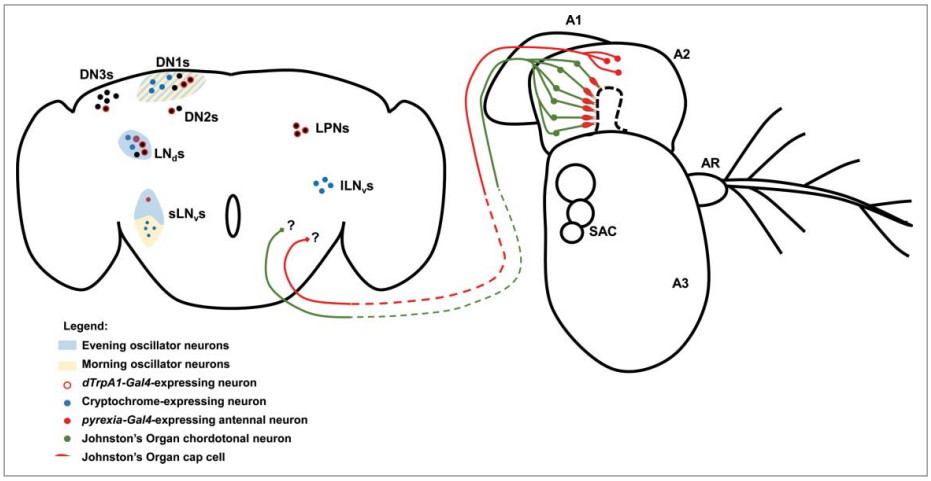

Figure 5. Central and peripheral neural mechanisms for circadian entrainment to temperature. The adult *Drosophila* brain and antenna are schematized here, with colored circles representing neuronal cell bodies, colored lines representing axonal connections, and colored diamonds representing presynaptiC-terminals. Shaded areas are used to represent morning and evening oscillators in the circadian circuit. Unidentified *pyrexia-Gal4*-expressing neurons of the second antennal segment (A2) project to the brain and potentially provide input to central circadian pacemakers via unknown synapses. Chordotonal neurons in Johnston's Organ of the second antennal segment and also in the body (not shown) are associated with Pyrexia-expressing cap cells and provide input to the central circadian pacemakers via unknown synapses. The morning oscillator (cream shading) is comprised of 4 of the small ventral lateral neurons (sLN_vs) and a subset of the first group of dorsal neurons (DN1s). The evening oscillator is comprised of the fifth sLN_v neuron, the dorsal lateral neurons (LN_ds), and a subset of the DN1s. Cryptochrome functions as the central nervous system photoreceptor of the circadian system and is expressed in the sLN_vs, the large ventral lateral neurons (lLN_vs), a subset of the LN_ds, and a subset of the DN1s. dTRPA1 functions as a component of a central nervous system temperature sensor for the circadian system. *dTrpA1-Gal4* is expressed in the lateral posterior neurons (LPNs) as well as subsets of the sLN_vs, the LN_ds, the DN1s, the second group of dorsal neurons (DN2s), and the third group of dorsal neurons (DN3s).

cycle is that behavior will also be synchronized with concomitant cycles of environmental temperature, allowing the fly to avoid cool nights and hot middays. Given the importance of temperature as an environmental factor influencing *Drosophila* development and fitness, it is not surprising that environmental temperature may impinge upon the circadian system in multiple different ways. In addition to the *Zeitgeber* function mentioned above, ambient temperature may play a modulatory role in pacemaker neuron function to alter the precise relationship between light-dark cycles and behavior. Exposure to non-physiological or noxious temperatures may cause phase shifts in the circadian rhythm, likely due to disruption of the molecular interactions between clock components.

An essential feature of the circadian clock is temperature compensation. Although the clock may be entrained or modulated by temperatures within a physiological range, the period length of the molecular and behavioral rhythms themselves are relatively temperature insensitive in wild-type animals.[122] For example, in constant darkness, behavioral rhythms are neither sped up by high temperatures nor slowed down by cool temperatures. However, non-physiological temperatures may produce phase shifts or disrupt clock function by acting directly on the clock's molecular components and their interactions. Brief, noxious heat pulses (~37°C) have been demonstrated to cause dramatic decreases in Per and Tim protein abundance and phase shifts in locomotor activity rhythms.[123,124] This phenomenon is not entirely understood, but likely involves disruption of the Per-Tim complex via a Cry-dependent mechanism.[125] It is important to note that this mechanism by which non-physiological temperatures impinge on the circadian clock is likely independent from the mechanisms by which temperatures in a physiological range can entrain behavior or modulate entrainment. Temperatures within a physiological range depend, at least in part, upon cellular temperature sensors similar to those used for thermotaxis behavior.

While daily temperature oscillations of relatively low magnitude (<5°C) within a physiological range have long been known to be sufficient to synchronize circadian locomotor rhythms,[120] the neural and molecular substrates of this entrainment have remained slightly more elusive. Temperature cycles are sufficient to entrain circadian behavior under constant light or constant dark conditions (i.e., in the absence of light *Zeitgebers*), and this behavioral entrainment is reflected in the cycling of clock genes in all or almost all pacemaker neurons in the brain.[126,127] However, experiments in which flies are exposed to simultaneous light and temperature cycles that are out of phase with each other demonstrate that the that clock gene cycling in the DN and LPN neuron groups tends to remain in phase with temperature cycles, while clock gene cycling in the LNs tends to remain in phase with light cycles.[128] These results and those described below suggest the presence of independent temperature- and light-entrainable oscillators in the brain. The hypothesis that the DN and LPN pacemaker neuron groups function as the temperature cycle-sensitive oscillators of the circadian system is strengthened by ablation studies that target the PDF-expressing pacemaker neurons, a manipulation that eliminates the LN_v groups, but spares the DN and LPN groups. When the PDF neurons are ablated, animals retain a weak ability to entrain to temperature cycles, and clock gene expression continues to cycle in the LPN and DN groups, indicating that these groups are sufficient to synchronize circadian rhythms of behavior to temperature cycles.[126,129] Interestingly, *Drosophila* adults also display a circadian rhythm in temperature preference, showing a stronger preference for warmer temperatures during the late portion of the light phase than during the dark phase.[130] This circadian rhythm is dependent on the DN2 neurons, which also control entrainment to environmental temperature rhythms in larvae.[130,131]

The potential presence of separate light- and temperature-entrainable oscillators in the fly brain raises interesting questions about how light and temperature information are integrated in the circadian system. For instance, how are light-entrained rhythms modulated by temperature and vice versa? In studies that use a paradigm in which flies are exposed to simultaneous light and temperature cycles that are 12 hours out of phase with each other, clock gene oscillation largely depended on Cry expression. Cyclic expression of Tim in Cry-expressing neurons tended to remain in phase with the light-dark cycle, while expression of Tim in Cry-negative cells stayed in phase with the temperature cycles.[132] Interestingly, when this experiment was performed in mutants lacking Cry, all pacemaker neurons showed cyclical Tim expression in phase with temperature cycles.[132] Consistent results were obtained in behavioral experiments in which behavior largely entrained to the light-dark cycle when light and temperature cycles were out of phase, but readily entrained to the temperature cycle when the Cry-expressing neurons were ablated.[132] Subsequent experiments have shown that the DN and LN_d groups of pacemaker neurons are capable of supporting behavioral entrainment to temperature cycles under constant darkness conditions, but not under constant light conditions.[133] However, in a *cry* mutant background, the DN and LN_d were sufficient to support behavioral entrainment to temperature cycles during constant light conditions.[133] Together these experiments support a model in which most pacemaker neurons can entrain to temperature cycles, but Cry antagonizes this entrainment in a light-dependent manner in a subset of them. As such, flies will generally entrain to light-dark cycles instead of temperature cycles when the 2 are out of phase, but will readily entrain to temperature cycles in the absence of Cry function.

Ambient temperature within a physiological range may also modulate entrainment of the circadian clock in a non-*Zeitgeber* fashion. For instance, flies entrained to a light-dark cycle and housed at low temperatures in the physiological range (18°C) display a phase-advanced evening peak of activity, while those housed at a high physiological temperature (29°C) display a phase-delayed activity peak in the evening.[134] The molecular basis for this shift depends at least in part on regulated splicing of the 3′ end of the *per* mRNA.[134] The splicing event is not restricted at low temperatures and short day length, but inhibited synergistically by high temperature and long day length.[135,136] Thus Per can accumulate more quickly and advance the circadian clock under cool and short-day conditions. Interestingly, this splicing event is also highly dependent on NorpA function outside of the visual system.[135,136] Another example of this

temperature modulation of circadian entrainment has been observed specifically in the DN1 neurons.[117] The DN1s are sufficient to support morning and evening peaks of behavior when entrained to a light dark cycle, but these peaks are differentially sensitive to ambient temperature- the morning peak is suppressed at low ambient temperatures, while the evening peak is suppressed by high temperature.[117] These types of adaptations, in which ambient temperature has a modulatory effect on entrainment, may allow flies to adapt to seasonal changes in day length, advancing their evening peak of activity to avoid cold winter nights and delaying their evening peak to avoid hot summer afternoons.[104,134,135]

Peripheral temperature sensors and the circadian system

Drosophila are capable of entraining to relatively low-amplitude temperature rhythms that exist well within the temperature compensation range of the molecular clock and which do not appear to impinge directly on its cycling.[120] Thus identifying the temperature sensor or sensors that provide input to the circadian system is an important goal for understanding temperature entrainment. In some tissues, the molecular clock appears to detect temperature oscillations using a cell-autonomous mechanism.[137] Molecular oscillations of clock gene activity can be assayed by measuring luciferase activity in tissue cultured from transgenic flies expressing *luciferase* under the control of *per* regulatory sequences. In these assays, cycles of luciferase activity in cultured peripheral tissues can be entrained to environmental temperature cycles, suggesting the presence of a tissue-intrinsic temperature sensor in the circadian clock of many types of circadian tissues.[137] However, the pacemaker neurons in the central nervous system do not appear capable of entraining to temperature oscillations in this *ex vivo* system, suggesting that peripheral input is required.[104,138]

The role of the peripheral nervous system, specifically the chordotonal organs, in providing information about temperature cycles to the central circadian oscillator was clarified by the discovery of mutants for the *nocte* gene, which display strong defects in their ability to synchronize locomotor rhythms to cyclical changes in temperature and in their ability to phase shift appropriately in response to temperature pulses.[137] The *nocte* locus encodes a large glutamine-rich protein that is broadly expressed in the adult fly, notably in the peripheral nervous system, as determined by GAL4 reporter-driven GFP expression. Expression of RNAi targeting *nocte* in the chordotonal organ neurons, external sensory organ neurons, and a small population of brain neurons produced the same defect in temperature entrainment as that observed in the *nocte* mutant.[137] The chordotonal organs of *nocte* mutants were further shown to have structural defects and mislocalization of structural proteins, and mutants for genes encoding additional structural components also show defects in circadian temperature entrainment.[137] These pieces of evidence combined point to the chordotonal organs as the peripheral temperature sensors that provide information to the pacemaker neurons for temperature entrainment (**Fig. 5**). This is consistent with behavioral experiments demonstrating a role for the larval chordotonal neurons in behavioral avoidance of cool temperatures and also with calcium imaging experiments demonstrating that the chordotonal organ neurons show calcium transients in response to relatively small temperature deflections within the comfortable range.[35,73]

The TRPA channel, Pyrexia, is a candidate component of the temperature sensor of the chordotonal organs for their role in circadian entrainment to temperature cycles. GAL4 reporters using *pyrexia* promoter sequences are expressed in the cap cells of the adult chordotonal organs and in a putative population of second antennal segment neurons that synapse on the AC neurons in the brain.[33,59,61] Mutants lacking Pyrexia function show defects in synchronizing their behavior to temperature cycles, and these defects are specific to low temperatures, as *pyrexia* mutants are unable to entrain to 16°C–20°C temperature cycles, but are unaffected in their ability to entrain to cycles that include higher temperatures.[60] This result is consistent with a model in which multiple peripheral and central temperature sensors provide input to the pacemaker neurons of the brain to allow accurate circadian entrainment over a broad range of temperatures (**Fig. 5**).

The central targets of the chordotonal organ neurons and *pyrexia*-expressing neurons in the circadian system of the brain are unknown, and thus the circuit-level mechanism for the function of multiple circadian temperature sensors remains to be elucidated. It is also important to note that Pyrexia's function in circadian entrainment seems to occur at temperatures that are much lower than those that have been demonstrated to activate Pyrexia in heterologous cells (<20°C vs. >40°C),[33] suggesting that Pyrexia alone may not sense temperature directly, but instead may be a component of a heat-sensitive complex or signaling pathway. Additionally, if Pyrexia functions in the cap cells of the chordotonal organs to support entrainment to temperature cycles, it is unclear how this non-neural site of action would produce signals that are then transmitted to central circadian oscillators. The cap cells of the chordotonal organs are tightly coupled to chordotonal neurons via extracellular matrix and play an essential role in neural morphogenesis.[139,140] Thus it is possible that Pyrexia function in the cap cells impinges on chordotonal neuron function or development.

Central temperature sensors and the circadian system

In addition to an extrinsic temperature sensor located in peripheral sensory neurons, some clock neurons also use a cell-intrinsic temperature sensor to synchronize their circadian clock with environmental temperature cycles. A subset of pacemaker neurons express a GAL4 reporter for *dTrpA1* (**Fig. 5**).[141,142] These *dTrpA1-Gal4*-expressing cells include the LPNs, the non-cryptochrome-expressing LN_ds, and small subset of DN neurons (**Fig. 5**). *dTrpA1* mutants display a mild, but statistically significant, defect in behavioral entrainment to warm-cool temperature cycles as well as defects in rhythmic Per protein accumulation under these conditions.[141] Interestingly, when the *cry*-expressing pacemaker neurons are ablated in a *dTrpA1* mutant background, entrainment of locomotor behavior to temperature cycles is lost entirely.[141] This suggests a potential model in which 2 independent temperature sensors provide partially redundant temperature information to different populations of clock neurons for

entrainment of behavior to temperature cycles. One temperature sensor appears to be contained within the *dTrpA1*-positive, *cry*-negative clock neurons (i.e. the LPNs and LN_ds), while a second temperature sensor appears to act through the *cry*-positive pacemaker neurons. It is possible that these neurons are the targets of temperature information from the chordotonal organs or other extrinsic temperature sensors.

While dTRPA1-expressing clock cells appear to comprise a temperature sensor for circadian entrainment, the temperature-activation properties of dTRPA1 do not appear to be required for this feature. First, *dTrpA1* mutants are defective entraining to temperature cycles that are below the characterized temperature activation threshold of the dTRPA1 channel (16°C–25°C), suggesting that the channel is not directly sensing the environmental temperature change.[141] Second, the temperature entrainment defect of the *dTrpA1* mutant can be rescued by expression of the dTRPA1-B isoform,[141] which does not respond to elevated temperature when expressed in S2R+ cells.[50] This information suggests that dTRPA1 may actually function downstream of some other primary temperature sensor. While the identity of this temperature sensor and the mechanism of dTRPA1 activation in the transmission of temperature information is unknown, it is reasonable to hypothesize the presence of a $G\alpha_q$-PLC signaling cascade that acts upstream of dTRPA1, as such a mechanism has been identified for the transmission of thermal information for thermotaxis[80] and mutants for the *Drosophila* PLC, *NorpA*, also show defects in entrainment to temperature cycles.[137,143]

Escape Responses to Noxious Temperatures

In addition to navigating toward optimal body temperatures and avoiding those higher or lower than the optimal range, *Drosophila* larvae and adults also execute rapid escape responses to temperatures that have the potential to cause acute tissue damage. The best understood example of this behavior is the nocifensive escape locomotion (NEL) performed by *Drosophila* larvae in response to noxious high temperature,[18] as well as in response to noxious mechanical stimulation[144] and short-wavelength light.[145] During NEL, larvae halt their normal peristaltic locomotion and execute a series of lateral rolls around their long body axis. The principle sensory neurons that detect noxious stimuli to evoke NEL are the Class IV multidendritic neurons (mdIV), which extend elaborate dendritic arborizations that tile the larval body wall.[146] While the ethological relevance of this behavior is not fully understood, it is suggested that NEL may help *Drosophila* larvae avoid parasitization following ovipositor penetration by parasitoid wasps.[146,147]

Painless mediates larval responses to noxious heat

The first identified nociception-defective mutants were those in the *Painless* gene, which encodes a TRPA channel that is not directly orthologous to the vertebrate TRPA1 channel (**Table 1**), but does have orthologs in other insect species. Mutant larvae lacking functional Painless channels show significant defects in NEL responses to thermal stimuli above 39°C as well as to harsh mechanical stimuli.[18] When expressed in HEK293 cells, the Painless channel mediates a calcium-dependent, heat-activated current with a threshold of ~42°C in the presence of intracellular calcium.[32] This temperature threshold is similar to the threshold observed for the stimulation of NEL as well as for multidendritic neuron action potential firing (~39°C), as observed by extracellular recording.[18] These results raise the intriguing possibility that Painless is the primary noxious heat sensor in the mdIV neurons for nociception behavior, although this hypothesis has not yet been directly tested.

As with the *Drosophila TrpA1* gene, the *Painless* gene produces multiple transcription units via alternative splicing and the use of alternative transcription start sites.[58] The longest isoform (Painless^{p103}) possesses a 468 amino acid intracellular N-terminal domain that contains 8 ankyrin repeats, while the shorter Painless^{p72} and Painless^{p60} isoforms possess 183 amino acid and 83 amino acid N-terminal domains respectively (**Fig. 2**).[58] These multiple isoforms appear to have important consequences for nociceptor function, as the Painless^{p103} and Painless^{p60} isoforms show distinct subcellular localization patterns and roles in thermal and mechanical nociception. When tagged with a C-terminal Venus Fluorescent Protein (VFP), the Painless^{p103} isoform localizes to the cell bodies, axons, and dendrites of larval multidendritic neurons and is sufficient to rescue the thermal nociception defect, but not the mechanical nociception defect, of a *painless* mutant. Conversely, the VFP-tagged Painless^{p60} isoform localizes exclusively to the cell bodies of multidendritic neurons and rescues the mechanical nociception defects, but not the thermal nociception defects, of a *painless* mutant.[58] These results support an important role for mRNA processing of ion channel genes in shaping sensory neuron sensitivity, but important questions remain. For instance, the expression patterns and relative expression levels of each Painless isoform are not known. Additionally, while Painless^{p103} is a *bona fide* temperature-activated channel,[32] the intrinsic properties of the other isoforms are unknown. Finally, the contributions of differences in channel subcellular localization to function remain to be elucidated.

Specific isoforms of dTPRA1 determine the sensitivity of larval nociceptors

In vertebrates, the TRPA1 channel plays a central role in nociceptive signaling, with imputed roles in chemical, mechanical, and cold nociception.[39,148] It is also an essential mediator of chronic pain following tissue damage and inflammation.[41,149] Like its mammalian ortholog, dTRPA1 plays a similarly central role in nociceptive signaling. Mutant larvae lacking dTRPA1 function show defects in NEL responses to both noxious thermal and noxious mechanical stimuli.[50,150] Expression of *dTrpA1-C/D-Gal4* is restricted almost exclusively to the mdIV neurons in the peripheral nervous system, and expression of the dTRPA1-C cDNA in the mdIV neurons of *dTrpA1* mutant larvae is sufficient to rescue the nociception defective phenotype, indicating that dTRPA1 normally functions in the nociceptor neurons.[50]

While a role for dTRPA1 in thermal nociception is obvious based on analysis of mutant phenotypes, the exact role of the dTRPA1 channel in transducing/transmitting thermal stimuli is

less clear. Of the 4 dTRPA1 isoforms described above, only the –C and –D isoforms appear to be expressed in the mdIV neurons, based on analysis of GAL4 reporters.[50] dTRPA1-C is able to restore heat-responsive NEL to a *dTrpA1* mutant when expressed in the mdIV neurons, but does not mediate heat-activated calcium influx when expressed heterologously in S2R+ cells.[50] The fact that the non-heat-activated dTRPA1-C isoform is sufficient for behavioral responses to noxious heat suggests that the role of dTRPA1 in thermal nociception is not that of a primary sensory transducer. Instead, dTRPA1 may act as an amplifier for thermal stimuli transduced by other heat-activated receptors such as Painless, Gr28B(D), or NinaE. This downstream signaling role of dTRPA1 would be consistent with activation of the vertebrate TRPA1 channel downstream of calcium influx and GPCR signaling and the role of dTRPA1 in larval thermotaxis behavior at 18°C.[39,72,151]

The role of RNA processing in regulating thermal nociception also remains to be elucidated. Based on the analysis of GAL4 reporter expression, both dTRPA1-C and dTRPA1-D are potentially expressed in the mdIV neurons, but have differing thermal-activation properties.[50] In theory, the alternative splicing decision that chooses between dTRPA1-C and –D expression could have a large effect on the thermal sensitivity of the mdIV neurons. The temperature threshold for activation of dTRPA1-D is lower than the temperature threshold for NEL. Thus it is tempting to hypothesize that an increase in dTRPA1-D expression via regulated alternative splicing underlies the decrease in NEL temperature threshold observed following UV-induced damage to the larval epidermis.[152,153]

Painless and dTRPA1 are required for behavioral responses to noxious heat in adult flies

Adult *Drosophila* must also avoid noxious thermal stimuli. This behavior is measured by assaying flies' ability to avoid entering and being paralyzed by the noxious (46°C) portion of a heated chamber.[150,154] A genome-wide RNAi screen for genes required for this behavioral avoidance identified both *Painless* and *dTrpA1*, as RNAi knockdown of either gene results in a reduced ability to avoid the noxious portion of the chamber (a result that was subsequently confirmed in genetic mutants).[150] The sensory neurons and neuronal circuitry underlying the avoidance of noxious heat in adult flies remains incompletely understood. Inhibition of synaptic transmission in *Painless*-expressing neurons results in complete loss of heat avoidance, confirming the importance of *Painless*-expressing cells.[150] Refinement of the inhibition of synaptic transmission reveals that the mushroom bodies do not appear to have a role in the behavior, while lesion experiments suggest that the antennae and labella may play minor roles in noxious heat avoidance.[150] A possible role for the adult multidendritic neurons has not been described.

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