Ionotropic Receptor 25a C isoform is Responsible for Temperature and Chemical Sensing in *Drosophila. melanogaster*

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ABSTRACT

Ionotropic Receptor 25a C isoform is Responsible for Temperature and Chemical Sensing in *Drosophila melanogaster*

A thesis presented to the Department of Biology

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The ability to sense the environment is essential for animal survival. Insects are easily affected by different environmental changes, so it is critical for them to detect and distinguish various sensory inputs. The *Drosophila melanogaster* Ionotropic Receptor 25a (IR25a), an iGluR-related receptor, is widely expressed. Antennal expression of IR25a is involved in many sensory behaviors, including thermal sensing, hygrosensing and chemical sensing. It is also known that IR25a has four different isoforms, but how they work to distinguish sensory inputs is largely unknown. Here we generated *Ir25a* isoform specific knockout flies by using CRISPR/Cas9. Our immunohistochemistry showed that only the C isoform is expressed in antennae, suggesting that the C isoform is involved in chemical sensing, thermal sensing and hygrosensing. Our
electrophysiology recording from the antennal coeloconic sensilla and from the arista of isoform-specific *Ir25a* mutants further confirmed that the C isoform is involved in chemical sensing and thermal sensing. Our work shows that the same isoform of *Ir25a* mediates chemical sensing, thermal sensing and hygrosensing. These data suggest that the same IR25a protein isoform combines with different co-factors to mediate different sensory modalities and leaves open the functions of the other IR25a isoforms.
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Introduction

Animals rely on sensory systems to perceive information within their environment. They use thermal receptors to achieve optimal temperature for growth and reproduction, humidity sensors to avoid dehydration and over-hydration, and chemical sensors to navigate toward food as well as mating partners. Sensing and responding to changes within their environment is essential for them to avoid noxious damage and unfavorable living conditions. From unicellular organisms to multicellular organisms, optimal temperature ranges usually guarantee best function since either higher or lower temperature will cause dysfunction of proteins, such as enzymes and membrane anchored proteins, which regulate many physiological processes. Therefore, maintaining an optimal temperature is essential for survival of all organisms. Along with temperature, water is also essential for cells. Relative humidity in the ambient environment has great impact on body water evaporation and maintenance. Dehydration can eventually cause death since cells are not able to produce or transport energy for the organism without water. On the other hand, over-hydrated condition can also decrease the viability of organisms, especially for birds or flying insects. In addition, many species need to seek for places that have optimal humidity for laying eggs. Organisms also have to detect and discriminate a vast of number of chemicals in daily life. Our group is interested in studying molecular mechanisms of temperature, humidity and chemical sensing. We use Drosophila melanogaster as our model organism for the following reasons. (1) Fruit flies have small body
size so that they are easily influenced by environmental changes. (2) They have powerful genetics so that it is easy to manipulate the neuronal activity. (3) Their life cycle is about two weeks at 25°C, which is much shorter than mammals. (4) Many important genes in *Drosophila* are conserved across insect species suggesting their evolutionary significance.

There are many of sensory receptors that have been identified in *Drosophila*, such as the *Drosophila melanogaster* Transient Receptor Potential (dTRP) A1 cation channel responding to both noxious chemicals and innocuous warmth[1], the gustatory receptor Gr28B(D) driving rapid avoidance of steep warmth[2], Odorant Receptors (ORs) expressed in the olfactory sensory neurons (OSNs) detecting a large member of volatile odorants[3], and Gustatory Receptors (GRs) detecting a wide range of environmental chemicals.

Ionotropic Receptors (IRs) are a family of ionotropic glutamate receptor (iGluR) relatives first characterized as chemosensory receptors in *Drosophila*[4]. The iGluRs mediate synaptic transmission by interacting with the neurotransmitter glutamate throughout vertebrate and invertebrate nervous system. IRs have a similar modular organization to iGluRs but variable ligand-binding domains, which lack of glutamate-interacting residues[4]. Another difference is that IRs usually concentrate in sensory dendrites but not synapses. In *Drosophila*, the third segment of the antenna contains a large number of sensory neurons, where IRs have been detected in the olfactory neurons that do not express ORs or GRs[5]. In the IR family, there are two widely expressed co-receptors, IR25a and IR8a, detected in partially overlapping populations of neurons throughout antenna. They function by forming heterotetramers with other odor-specific IRs[6]. The main difference between co-receptor IRs and odor-specific IRs is that co-receptor IRs have the amino-terminal domain, which is assumed to interact with other
IRs to form heterotetrameric complexes[4]. Antennal coeloconic (ac) sensilla are sensory sensilla that can be distinguished into four groups according to sensory specificities. Both IR25a and IR8a are expressed in ac1, ac2 and ac4 sensilla, but only IR8a is detected in ac3 sensilla. IR25a is also expressed in the arista but IR8a is not. In the sacculus, a pouch located inside the antenna which also contains sensory neurons, both IR25a and IR8a are expressed [5]. Studies have shown that IR25a is responsible for chemosensing[6], thermal sensing[7] as well as hygrosensing [Zachary Knecht and Paul Garrity, unpublished]. There are four different transcription start sites for the IR25a gene, resulting in three different protein isoforms that are slightly different from each other at their amino-terminus. However, the function(s) of each protein isoform of IR25a have not been explored.

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) 9 system has been widely applied for genetic modification across many species. It was discovered in bacteria and archaea as a RNA-mediated adaptive immune system that protect organisms from invasion of exogenous DNA or plasmids[8]. Among three types of CRISPR/Cas systems, the type II system is most commonly used. Both of the type I and type II systems require precursor CRISPR RNA (pre-crRNA) modification by specialized Cas endonuclease and multi-Cas protein complex assembly to cleave targeting DNA sequences[9]. In the type II system, however, Cas9 is the only protein involved and the pre-crRNA can be processed by RNase III at the presence of Cas9[9]. The versatile guide RNA (gRNA) used for recognizing DNA sequence and recruiting Cas9 protein consists of a 5’ target recognition sequence and a hairpin structure retaining the base-pairing interactions, which is derived from the dual-RNA model formed by crRNA and trans-activationg crRNA[10]. When the gRNA is
binding to a DNA strand, it will recruit Cas9 protein to cleave at a position three to eight base pairs prior to protospacer adjacent motif (PAM) sequence, NGG, which is a targeting component for determining the invading virus or plasmid[11]. The efficiency of success in generating mutant flies was highly increased by using U6:3 promoter for gRNA expression, actin promoter for Cas9 protein, and the gene targeting during oogenesis with lethality inhibitor and CRISPR/Cas (Golic+) tool kit to integrate our arbitrary DNA fragments into target genomic loci[12, 13].

We generated several Ir25a isoform-specific knockout fly lines by using CRISPR/Cas9 tool[13] and used them to assess the expression patterns of different IR25a isoforms as well as their isoform-specific function. Immuno-staining results showed no IR25a protein expression in the antennal section of IR25a(C) knockout mutant, suggesting that IR25a(C) is the only isoform expressed there. Recordings of neuronal activity showed that IR25a(C) knockout fly lost responses to both cold temperature and aversive chemicals. Therefore, we concluded that IR25a(C) is involved in thermal sensing and chemical sensing in Drosophila and further hypothesize that, as it appears to be the only isoform expressed in the sacculus, that it will also be required for hygrosensing.
Materials and Methods

Molecular biology

Ir25a isoform-specific knock out strains were made by CRISPR-Cas9 technology as described[12, 13].. The gRNAs for making Ir25a(B), Ir25a(C) and Ir25a(D) alleles were designed by Belinda Barbagallo in Paul Garrity’s lab. Ir25a(B) gRNA was constructed in a vector with wild-type white-eye (w+) gene marker (a gift from Tzumin Lee’s lab) and Ir25a(C) and Ir25a(D) gRNAs were constructed in pCFD3 vector with a wild-type vermilion (v+) gene marker. Plasmids were injected into fly germ line cells by Rainbow Transgenic Flies Inc.

Ir25a(B) gRNA: 5’ – GTGGTTGCTGATGGCGTTG – 3’

Ir25a(C) gRNA: 5’ – GTTATTAAGCAGCTTTAGTT – 3’

Ir25a(D) gRNA: 5’ – TTGGCCAATGCACCTTTAACC – 3’

To test the genotype of each Ir25a isoform-specific mutant fly, genomic DNA from single flies were extracted and amplified by using Phusion® High-Fidelity DNA polymerase (from New England Biolabs® Inc.) Sequencing for mutant flies was done by Eton Bioscience Inc.

Primers for IR25a isoforms

Ir25a (B) sense: 5’ – ATGGGTTCACGCTTGGATTG – 3’

Ir25a (C) sense: 5’ – TTCGAAAATCCTGTGGCTGC – 3’

Ir25a (B) & IR25a(C) anti-sense: 5’ – TAGCTTCCAGCAGCACCTTGG – 3’

Ir25a (D) sense: 5’ – GGGCAAACAAAAAACGC – 3’
**Ir25a (D) anti-sense**: 5’ – CTCGGAAATAATCCGC – 3’

**Electrophysiology**

Extracellular recordings of ac4 sensilla from 2-5 days old individual flies were performed as described[5]. Odorants ordered from Sigma-Aldrich in the highest purity were diluted in either water or glycerol to make 1% (v/v in solvent) solutions. 40 μl of 1% odorants were then added onto filter paper strips and placed inside 1 ml glass pipettes, which could be linked to a syringe. Six ml of odorant stimuli were added into a tube towards the fly and delivered by a constant wet air stream. Response quantification was calculated by the equation: Response (Δ spikes/s) = (Number of spikes per second during stimulus) – (Number of spikes per second before the stimulus). We compared each isoform knockout mutant with wild-type flies for a given stimulus by t-test.

**Immunohistochemistry**

Immunofluorescence on antennal sections was performed as described[14]. The primary monoclonal antibodies against IR25a and secondary antibodies with Cy3 Fluorescence System were previously described[5].
Results

Generation of isoform-specific knockout flies

To determine the function of each \textit{Ir25a} isoform, we utilized CRISPR-Cas9 to generate isoform-specific knockout flies. The genetic crossing scheme used to of generate mutant flies is diagrammed in Figure 1 and Figure 2.

We obtained five \textit{Ir25a(B)} alleles that had mutations or deletions in the exon that is specific for the B isoform. The \textit{Ir25a(B)}-5, with a one base-pair deletion, and \textit{Ir25a(B)}-8, with an eight base-pair deletion, both created translational frameshifts and should cause early termination of translation. \textit{Ir25a(B)}-9, with an 18 bp deletion, \textit{Ir25a(B)}-10, with a 27 bp deletion, and \textit{Ir25a(B)}-13, with an eight bp deletion and a five bp insertion, caused in-frame changes (Figure 3). We chose \textit{Ir25a(B)}-8, later referred as \textit{Ir25a(B)}, as the representative for a B isoform-specific knockout mutant as it generated both a deletion and a translational frameshift.

The three alleles obtained that disrupted the exon specific for the C isoform were \textit{Ir25a(C)}-3, with a one bp deletion, \textit{Ir25a(C)}-4/57, with a five base-pair deletion, and \textit{Ir25a(C)}-5/27, with a different one bp deletion different than \textit{Ir25a(C)}-3 (Figure 3). We chose \textit{Ir25a(C)}-3, as the representative for a C isoform-specific knockout mutant, and it will be referred as \textit{Ir25a(C)} below.

We obtained six alleles that disrupted an exon only present in the D isoform. Both \textit{Ir25a(D)}-5 and \textit{Ir25a(D)}-1 had 14 bp deletions; both \textit{Ir25a(D)}-6 and \textit{Ir25a(D)}-8 had 7bp
deletions; \textit{Ir25a(D)}-4 had a two bp deletion; and \textit{Ir25a(D)}-10 had a three bp insertion (Figure 3).

We chose \textit{Ir25a(D)}-5 to represent IR25a(D) isoform specific knockout mutants, later referred as IR25a(D).

Figure 1 Crosses set-up for generating \textit{IR25a(B)} knockout flies. The vector of \textit{IR25a(B)} gRNA containing a \textit{w+} gene as a biomarker for sorting flies were inserted into the third chromosome. Red eye male flies carrying the gRNA were crossed with \textit{w-}; Sp:Sb/S:T virgin female flies. Then, red eye (gRNA positive) male flies with balancer (S:T) were crossed with virgin female flies with homozygous \textit{w+ act5-Cas9} on X chromosome. Next, male flies without balancer (S:T) were crossed with \textit{w-}; Sp:Sb/S:T virgin females. In the next generation, single white eye (without gRNA or \textit{act5-Cas9}) males with balancer were crossed with \textit{w-}; Sp:Sb/S:T virgin female flies. After self-crosses within the progeny carrying \textit{Ir25a(B)}/S:T, homozygous flies with potential \textit{Ir25a(B)} mutations were sorted and sequenced.
Figure 2 Crosses set-up for generating IR25a(C) and IR25a(D) knockout flies. The vectors of IR25a(C) and IR25a(D) gRNAs containing a vermilion (v+) genes as a biomarker for sorting flies were inserted into the third chromosome. The male flies carrying gRNA were crossed with v-; Sb/TM3 Ser virgin female flies. Then, male flies (gRNA positive) with balancer (TM3 Ser) from were crossed with virgin female flies with homozygous v+ act5-Cas9 on X chromosome. Then, virgin females without balancer (TM3 Ser) were crossed with v-/y; Sco/CyO males. In the next generation, single males (v-) without gRNA or act5-Cas9 were crossed with white-eye v+; Sco/CyO virgin female flies. Another cross between v+ males and white-eye v+; Sco/CyO virgin females were done on order to turn the background into v+. After self-crosses within the progeny carrying Ir25a(C) or Ir25a(D)/S:T, homozygous flies with potential Ir25a(C) or Ir25a(D) mutations were sorted and sequenced.
Figure 3 Genotypes of IR25a isoform-specific knockout flies. The dash lines within gene sequences represent deletions and red characters mean insertions.
Only IR25a(C) isoform is expressed in antennae

Antennal expression of IR25a is involved in chemical sensing, thermal sensing and hygrosensing. To understand the function of each isoform, we first examined their expression pattern in antennae by immunohistochemistry. IR25a is widely expressed in the third segment of antenna, including the arista (a feather-like projection), the sacculus (a pocket-like organ buried in the antenna) and in coeloconic sensilla (hair-like structures distributed over the surface the antenna), which mediate thermal sensing, hygrosensing and chemical sensing, separately (Figure 6). To assess the expression pattern of each isoform, we did IR25a staining on the third segment of antenna using our isoform-specific mutants. The IR25a antibody is able to detect all IR25a isoforms, as it is directed against the C-terminus of IR25a, and the IR25a isoforms only differ from each other at the N-terminus.

In wild-type flies, IR25a antibody labeled a large number of IR25a expressing cells in arista, sacculus and coeloconic sensilla. We did not detect IR25a in Ir25a^2 mutants, which are null for all isoforms, suggesting that the antibody specifically labels IR25a. Among three mutants, the staining results for Ir25a(B) and Ir25a(D) mutants were similar to the wild-type, but Ir25a(C) mutant flies had no signal for IR25a protein, similar to the Ir25a^2 null mutant, suggesting that IR25a(C) is the only isoform expressed in antenna (Figure 6). As the antennal expression of IR25a is involved in chemical sensing, thermal sensing and hygrosensing, we supposed that IR25a(C) isoform might be responsible for all these sensory modalities.
Figure 4 Immuno-staining for IR25a on antennal section of WT, Ir25a<sup>2</sup>, Ir25a(B), Ir25a(C) and Ir25a(D) flies. The first figure shows a diagram of sensory organs on the third segment of antenna[4]. In WT, Ir25a(B) and Ir25a(D) mutants, IR25a proteins are detected in arista, sacculus and coeloconic sensilla. However, there is no IR25a detected in either Ir25a<sup>2</sup> or Ir25a(C) mutant flies.

**IR25a(C) isoform is responsible for chemical sensing as well as thermal sensing**

IR25a is responsible for the ability of sensory neurons in the coeloconic sensilla to detect various chemical cues, including acids and amines. Loss of IR25a results in loss of IR25a-dependent responses to many odorants[4]. In order to determine the functions of different isoforms in chemosensing, we used single sensillum recording to detect neuronal activity in coeloconic sensilla. There are four distinct classes of coeloconic sensilla that contain IR-expressing neurons named ac1-ac4. The ac1, ac2 and ac4 sensilla contain an IR8a expressing neuron as well as two IR25a expressing neurons[5]. Due to the expression of different odor-
specific IRs in different neurons, these coeloconic sensilla have distinct but also partially overlapping sensory specificities [15]. In ac4 sensilla, IR8a expressing neurons and IR25a expressing neurons sense phenylacetic acid (PAA) and phenylethylamine (PEA), respectively, and these sensitivities are specific to the ac4 sensilla’s neurons [4]. We applied the odorant of PAA first to identify an ac4 sensillum, and then tested the chemosensing ability of IR25a-expressing neurons by applying the odorant of PEA. In wild-type flies, neuronal activities increased while applying PAA as well as while applying PEA (Figure 5, 6). However, in Ir25a2 null flies, the sensilla that responded to PAA did not respond to PEA, suggesting no functional IR25a was present. The Ir25a(B) and Ir25a(D) mutants had the similar ability to sense PAA and PEA with wild-type flies. The Ir25a(C) mutant, however, responded to PAA, but completely failed to respond to PEA (Figure 5, 6). These data suggest that Ir25a(C) is the key IR25a isoform for chemical sensing.

The arista is a feather-like projection on the third segment of antenna, which is an important temperature sensory organ in Drosophila. There are three cold cells and three hot cells at the base of arista, responding to cold and warm temperatures, respectively. IR25a is expressed in the temperature-responsive neurons, and the loss of IR25a leads to a defect in the cold response of cold cells [L. Ni, M. Klein, A. Samuel and P. Garrity, unpub]. In order to determine which isoform is responsible for cold sensing, Gonzalo Budelli (a post-doctoral fellow in the Garrity lab) performed extracellular recordings from the arista. These experiments indicate that the C isoform, but not B and D isoforms, is responsible for cold sensing in the arista (data not shown).
Figure 5 Representative traces for the responses of ac4 sensilla to 1%(v/v) phenylacetic acid or 1%(v/v) phenylethylamine. The black bars above the traces indicate the stimulus time. The left panel represents neuronal responses to phenylacetic acid, which is IR8a dependent. And the right panel represents the responses to phenylethylamine, which is IR25a dependent.
Figure 6 Quantification of mean responses (± s.e.m) to PAA and PEA from ac4 sensilla. Red bars represent the frequency changes of IR8a expressing neurons in ac4 sensilla after applying PAA. Blue bars represent the frequency changes of IR25a expressing neurons in ac4 sensilla after applying PEA. *** Significant difference from wild-type flies (t-test, p<0.001).
Using the CRISPR-Cas9 system, I generated \textit{Ir25a} isoform-specific knockout flies, providing the genetic tool for the studies of each isoform. Using these mutants I found that only the IR25a(C) isoform is expressed in antennae, indicating its function of the IR25a-dependent sensory processing in antennae. Electrophysiological recordings further confirmed that the IR25a(C) isoform is responsible for both cold temperature and chemical sensing.

In addition to the functions of IR25a(C) in thermal and chemical sensing, we also propose that this isoform will also be involved in humidity sensing. The sacculus, the pocket-like organ buried in the third segment of antenna, is known to contain hygroreceptor neurons[16] and the loss of IR25a causes a defect in avoiding aversively moist environment [Zachary Knecht, unpublished]. At present, it was difficult to confirm that IR25a(C) is the isoform responsible for respond to humidity using electrophysiology, due to the difficulty in penetrating the electrode to record neuronal activities inside the sacculus. Our lab is looking for a solution by using a genetically encoded calcium indicator to monitor changes of neuronal activity of the terminals of the sacculus neuron axons in the brain while applying humidity stimuli to flies.

In order to complete the functional analysis of each IR25a isoform, it is necessary to rescue the defects with each of isoforms and also to test them in behavioral assays. Preliminary data have shown that IR25a(C) isoform is sufficient to rescue the thermal and chemical sensing defects in \textit{Ir25a}\textsuperscript{2} mutants (Z. Knecht, L. Ni, and P. Garrity, unpublished observations). In addition,
the IR25a(B) isoform is unable to rescue the thermosensory defect in Ir25a$^2$ mutants, suggesting that different isoforms form distinct receptors (L. Ni and P. Garrity, unpublished observations). Functions of IR25a(B) and IR25a(D) isoforms have not been characterized so far. IR25a has been shown to be expressed in other body parts such as appendages and proboscis, as well as in larval stages[17, 18]. IR25a(B) and IR25a(D) isoforms might play a role in different tissues or different developmental stages. Our isoform specific knockout flies provide the genetic tools to investigate these hypotheses.
References


