IDENTIFICATION, CHARACTERIZATION AND USE OF NOVEL
THERMOGENETIC TOOLS IN DROSOPHILA MELANOGASTER

A Dissertation

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at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

by

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Dr. Troy Zars, Dissertation Supervisor

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The undersigned, appointed by the Associate Vice Chancellor of the Office of
Research

and Graduate Studies, have examined the dissertation entitled

IDENTIFICATION, CHARACTERIZATION AND USE OF NOVEL
THERMOGENETIC TOOLS IN DROSOPHILA MELANOGASTER

presented by Aditi Mishra,

a candidate for the degree of doctor of philosophy of Biology,

and hereby certify that, in their opinion, it is worthy of acceptance.

Professor Troy Zars

Professor David Schulz

Professor Lorin Milescu

Professor Tzyh-Chang Hwang

To Papa and Mama,

ACKNOWLEDGEMENTS

Me: I can't decide if I should set up the avoidance crosses for next week or prep the imaging samples.

Troy: Do them both!

Troy: How do you sit in that chair? It is so low. It feels like I am sitting on the floor. It is for midgets.

Me: Giant...

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ABSTRACT

Extrinsic control of neural activity is necessary to decipher the neural mechanisms underlying behavior. Molecular tools that employ light (optogenetics) or temperature (thermogenetics) are primarily used for extrinsic manipulation of neurons. While the available tools offer precise temporal and spatial resolution, their caveats lie in the limited number of tools that can be used simultaneously to alter neuronal activity. The overlapping spectrum of activation of optogenetic tools prevents their simultaneous use in preparations. Similarly, the lack of thermogenetic tools that can function in the physiological range of organisms has restricted their use. The use of thermogenetic tools is limited to two members from the Transient receptor family of proteins, TrpA1 and TrpM8 to activate neurons, and one protein that reduces synaptic output, Shibire^{ts}. A major drawback to the Trp channels is their response to both temperature and voltage changes. Hence, the discovery of a new temperature sensitive Gustatory Receptor protein provided an opportunity to mine for other temperature sensitive proteins and develop novel thermogenetic tools. In this thesis we report the identification of several thermosensitive proteins, their characterization, and use in studying the learning and memory of freely moving flies.

In the first chapter, we probed several Gustatory receptors for their temperature sensitivity using the heat box. The heat box is a high throughput system that enables us to test and track the behavior of single flies in response to temperature. The top and bottom of heat box chamber has Peltier elements that

allow for control of temperature with a resolution of 1°C. We overexpressed several Gustatory receptors one at a time pan neuronally in *Drosophila melanogaster* and exposed them to various assays. Our initial results imply that at least 2 *Drosophila melanogaster* Gustatory receptors other than Gr28bD are temperature sensitive. To increase the repertoire of thermosensitive proteins, we assayed for temperature response properties of Gr28bD orthologs from 5 other *Drosophila* species that occupy different habitats in the world. We rationalized that flies in different habitats will have Gr28bD orthologs with unique temperature response properties designed to sustain in that habitat. Of the 5 proteins we tested, we found that 4 proteins are temperature sensitive at different temperatures.

While pan-neuronal overexpression is a robust method to determine the temperature responsiveness of a protein, it does not recapitulate the natural environment the protein is present in. In *D. melanogaster*, Gr28bD is present in specialized heat sensing cells in the antenna, called Hot Cells. There are 3 Hot cells on each of the two antennae. There is however no physiological information on where the orthologs are expressed. Since Gr28bD is used for rapid heat avoidance in flies, we rationalized that its orthologs too serve a similar function in their host species and are expressed in the peripheral regions. Hence, in the second chapter, we tested for the avoidance behavior of flies using two choice assays. We made mutant flies that lacked Gr28b proteins, including Gr28bD in the antennae. We then examined the ability of the orthologs to rescue the heat avoidance behavior in these mutants. We found that all the orthologs respond to

temperature differences albeit, at different temperatures. Above a threshold temperature, flies rescued with some orthologs could not differentiate between small temperature differences, suggesting that the activity of the orthologs might saturate beyond certain temperatures. Some homologs responded to temperature only when expressed in Hot Cells, thus leading us to examine the presence of accessory proteins in the hot cells that might be enhancing the thermosensitive properties of these homologs. We found several candidate proteins that can be studied further to determine their role in the temperature sensing in the hot cells.

When used as thermogenetic tools, thermosensitive proteins are in localized environments in small clusters of cells. In the third chapter, we expressed Gr28bD in small clusters of dopaminergic neurons in the fly brain with an aim to understand the role of activation of dopaminergic neurons in operant place learning and memory paradigm. In addition to examining their learning scores at different temperatures, we investigated other behaviors of the flies during the training. Contrary to previous results from our lab that showed that dopaminergic neurons are not important for place learning and memory, we found that activation of a specific subset of dopaminergic neurons does alter place learning and memory. Our findings now laid the groundwork for more experiments to investigate dopaminergic modulation of place learning and memory.

1 Introduction

1.1 Use of genetic and molecular tools in the study of neural circuitry: a brief history.

Over the last 150 years, neuroscientists have designed several molecular and genetic tools to decipher the neuronal architecture in brains, and the functional aspects of neuronal connections. Investigations of structure of neurons gained momentum with Ramón y Cajal's intricate representation of the connectivity of the neurons using the then recently developed Golgi staining. Since then, modifications of Golgi staining, use of viral vectors to trace development of circuits, GFP Reconstitution across Synaptic Partners (GRASP) to label specific synapses, and electron microscopy to identify microstructures have enabled thorough studies of brain anatomies in various organisms [1]. In the meantime, determining the functional connectivity of neural circuits has become feasible with the development of electrophysiology, optogenetics [2,3], chemogenetics (for example, DREADD) [4–6], thermogenetics [7–9], magnetothermogenetics [10,11] and imaging techniques such as FMRI [12].

Of these tools, optogenetic and thermogenetic methods allow for extrinsic control of neuronal activity and are thus widely used in model organisms to tease apart the neuronal circuits underlying specific behaviors. The wealth of genetic tools and tractability, combined with the short lifecycle that enables fast accumulation of data led to the establishment of fruit flies, *Drosophila melanogaster*, as a

popular model system for studying the neural basis of behavior. Studies of neuronal populations and circuitry in fruit flies have contributed to our understanding of different circuits, and the roles of small molecules in establishing functional connections in these circuits. For example, optogenetic excitation of neurons in fruit flies, initiated escape behavior in *Drosophila*. This study identified several lumen-sensitive neurons whose activity helps prevent collision in freely moving animals [13]. Using functional optical imaging, researchers identified discrete clusters of dopaminergic neurons in the fly brain that regulate learning and memory formation in flies [14]. In a recent study, a feedback loop between dopaminergic neurons and Kenyon cells (higher order processing neurons in fly brain) that regulates appetitive memory formation was identified using optogenetics [15]. Similarly, thermogenetic stimulation of serotonergic neurons revealed that serotonin is necessary for place learning and memory formation in fruit flies while octopamine is not [16,17]. In yet another study, Bidaye and colleagues used a thermogenetic screen to identify neurons whose excitation altered the walking direction in flies [18]. They discovered the 'Moonwalker descending neuron' which enables a fly to walk backwards. Optogenetics and thermogenetics have also enabled identification of neural circuits that guide behavior in mammals. Adhikari and colleagues used optogenetic stimulation along with anatomical approaches to identify projections between medial Pre-Frontal Cortex and amygdala that are important for fear and anxiety related behaviors [19]. A recent study combining optogenetic stimulation with pharmacological approaches resolved part of a microcircuit that

regulates motivation in rats. They found that stimulation of the nucleus accumbens in rats leads to downstream effects in the Ventral Tegmental Area which in turn leads to increased motivation [20]. Munshi and colleagues used changes in magnetic field to excite a thermosensitive protein TrpV1 in mice neurons and influence several motor behaviors in freely moving mice [10]. In yet another study, researchers used optical fibers to increase the temperature and excite single cells in several preparations [21].

Optogenetic tools rely on specific wavelengths of light to excite light sensitive molecules in neurons which in turn can alter activity of neurons by depolarizing or hyperpolarizing them [2,22,23]. Light delivery systems to excite optogenetic proteins are spatially and temporally precise with lasers and two-photon microscopes [3,24–26]. However, there are significant drawbacks to these light delivery systems. For example, the wavelengths of light used are not always effective in penetrating the cuticles of insects like *Drosophila* and mammalian skin, thus requiring invasive procedures [10,24,27]. Also, the optically sensitive proteins like Channel-Rhodopsins and Chrimson, have an overlapping spectrum of activation even if their peak emissions are at different wavelengths [28,29]. This limits the use of multiple optogenetic tools in the same system. The limitations of optogenetic tools necessitate the use of tools based on other modalities, such as heat.

Thermogenetic tools use temperature sensitive proteins to modify neuronal activity. While an alternative to optogenetics is thermogenetics, it has even more caveats than optogenetics. Current thermogenetic tools often function outside the

physiological range of organisms and temperatures suitable for cell preparations. In fact, there are very few temperature sensitive proteins used to extrinsically manipulate neuronal activity- TrpA1, TrpV1, TrpM8 and shibire^{ts} [7,30–32] are examples of temperature sensitive proteins used as thermogenetic tools. Moreover, the activity of known thermosensitive proteins changes rapidly with change in temperature, in range of milliseconds to seconds. This makes it difficult to study long lasting changes in neuronal activity upon a brief temperature stimulus. Trps have a disadvantage of being voltage sensitive, thus making it harder to track alterations caused solely by temperature changes [33,34]. Consequently, our ability to control neuronal activity using temperature as a driving factor is limited [24,29]. Hence, there is a need to develop thermogenetic tools with different temporal properties at different temperature scales to complement optogenetic tools in the study of neural circuitry.

1.2 Gustatory receptors in *Drosophila melanogaster*.

The recent discovery of thermosensitive properties of Gustatory receptor (Gr) protein Gr28bD opened new opportunities to mine for and study new thermosensitive proteins [8,35]. Gr28bD is a member of the Gustatory receptor family in *Drosophila melanogaster*. The Gustatory receptors (Grs) were identified using Basic Local Alignment Search Tool with the previously identified Olfactory receptor proteins as template or by algorithms searching for multi-transmembrane proteins in the *Drosophila* genome [36,37]. There are 68 identified taste receptors being coded by 60 genes. The Grs are diverse with 20% or less similarity between their amino acid sequences. There are however

several clusters of genes in the Gr family which share up to 70% sequence similarity within the clusters, possibly due to gene duplications. Many of the genes in a cluster are the products of alternative splicing. Grs which have greater than 30% sequence similarity have been grouped together in subfamilies (Figure 1) [38,39]. As such, the seventh transmembrane domain, i.e, the C terminus is the most conserved domain across all the Grs. Grs are expressed widely, in the labellum, wings, legs and genitalia of the fly [40]. They are expressed in the gustatory neurons in the sensilla and used by flies to detect sweet, bitter, salty, sour (acid) and umami tastes, CO₂, Ca²⁺ and fats, depending on the receptors they express [40–48]. Besides Gr28bD, another gene in the Gr28b cluster, Gr28bE has been suggested to have thermosensitive properties [8]. The Gr family thus encodes a host of receptors whose functions are not limited to taste. *GR28bD* is endogenously expressed in the 6 heat sensing neurons, the so called “Hot Cells”, located at the base of the arista of the fly, 3 on each side [49][50]. These cells are specialized to detect rapid changes in environmental temperature unlike the TrpA1 expressing anterior cells in the brain that allow for thermal preference in response to slow changes in temperature [8,49,51]. Gr28bD is also necessary for the development of microstructure of hot cells [52].

1.3 Goals of the thesis: aims and rationale

The purpose of our study is to develop novel thermogenetic tools that can be used in a variety of preparations and model organisms. We aim to identify thermogenetic tools, characterize their function and use them in understanding the role of dopaminergic neurons in place learning and memory.

The temperature sensing properties of Gr28bD and the diverse functions of the proteins in the Gr family indicated that the Gr family should be mined for other thermosensitive proteins. We tested the hypothesis that several proteins in the Gr family are temperature sensitive. To understand the structure-function relationship of Gr28bD we examined the thermosensitive properties of orthologs of Gr28bD from different species. Comparison across species is beneficial to identifying proteins that have different thermosensitive domains and hence vary in temperature response. The whole genomes of 12 species of *Drosophila* were sequenced as part of an ongoing project, called modENCODE [53]. Of the 12 Species, 5 species of *Drosophila* have orthologs that share 80 to 98% amino acid sequence identity with *Gr28bD*- *D. simulans*, *D. yakuba*, *D. willistoni*, *D. pseudoobscura* and *D. mojavensis* (figure 1-2, Figure 1-3). These species occupy a wide range of habitats, from the rainforests in Africa to the Mojave Desert in America [54] (figure 1-2). We hypothesized that the environmental differences would have led to the development of orthologs of Gr28bD that have unique temperature sensing properties.

To understand the effects that these proteins have in their natural hosts, we examined their temperature response properties in a more natural setting. While the function of Gr28bD, and the temperature sensing neural circuitry downstream of Hot Cells in *Drosophila melanogaster* have been characterized to some extent [8,35,55], there are no published studies on the function of Gr28bD orthologs or the temperature sensing mechanisms in other fruit fly species. However, considering that these proteins share a high percentage of identity with each

other, and that these other species of fruit flies do have temperature preferences [56,57], it is likely that there are similar temperature sensing mechanisms in place in these other species of fruit flies to navigate the fluctuating temperatures that they are exposed to. Hence, we examined the thermosensitivity of the orthologs by overexpressing them in Hot Cells of *Drosophila melanogaster* to uncover the subtle differences in their temperature sensing properties. We tested the hypothesis that the orthologs will have different temperature sensing properties in “Hot cells” of *Drosophila melanogaster*. Our findings in thermosensitivity of previously uncharacterized proteins inspired us to investigate possible accessory proteins in Hot Cells that might be fine tuning the properties of thermosensitive genes. We tested the hypothesis that there are Hot Cell specific accessory proteins that are important for thermosensing in flies.

Following characterization of Gr28bD, we sought to test Gr28bD function in non-native circuits. The limited number of thermogenetic tools had constrained our testing of the role of dopaminergic neurons in an operant place learning and memory paradigm. While the role of dopaminergic neurons has been well explored in several other types of learning and memory such as courtship learning [58,59], olfactory learning [60,61], gustatory learning [41,62] and visual pattern learning [63,64], there have been few studies indicating its role in spatial learning tasks in insects [16,63,65,66]. In fact, previous studies implied that dopamine was not important for place learning and memory [16]. These studies however excluded a cluster of dopaminergic neurons in the protocerebral anterior medial region which have now been shown to be relevant for appetitive learning

in insects [67,68]. We hypothesized that conditional thermogenetic activation PAM cluster of dopaminergic neurons will alter place learning and memory in the heat box paradigm.

In this thesis we have identified several novel *Drosophila* thermosensitive proteins with unique temperature response properties that can be used in non-native systems. We characterized these proteins in several circuits and showed that they can be used in conjunction of existing tools that alter the resting membrane potential of neurons. With the use of one these novel tools we determined that place learning and memory is modulated by activity of dopaminergic neurons. Additionally, we have discussed our findings where we collaborated to quantify the physiological changes in neurons overexpressing these proteins [9] and study their functions in a heterologous system.

Figure 1-1:Cladogram of the Gr subfamilies obtained by neighbor joining.

The red edges indicate candidate thermosensitive GRs [69]. Some families of Grs enable flies to respond to specific compounds. For example, the Gr64 family (purple rectangle) and the Gr43a family (yellow rectangle) is used to detect fatty acids and sugars [42,70,71]. Gr21a helps flies detect Carbon dioxide along with Gr63a [72] (green rectangle) while flies use Grs from the Gr39 [73] family to sense pheromones. The green edges demarcate the Gr28b family of which two members, Gr28bD and Gr28bE were initially found to be thermosensitive [8].

Figure 1-2: Slanted cladogram showing the phylogeny of the *Drosophila* species whose Gr28bD orthologs have been tested for temperature sensitivity in this thesis.

While *D. simulans* and *D. melanogaster* share the maximum identity to each other's genomes, *D. mojavensis* is very distantly related with only 80% identity to *D. melanogaster*. These species of *Drosophila* occupy a wide variety of habitats. We rationalized that different habitats would have resulted in Gr28bD orthologs with unique temperature sensing properties.

MWPIWRQLLSPRDAYGAEQTLFFTYLLGLTPFRLRGH-GTRHFELSRGLYNALLQLTF
 MGKFYKEMFSASDAYGAEQSLLFFTYLLGLTPFRLKGPLGGRHFRLSRLGYLNALIQMSF
 MSGFWRELPHRDAHGSEQTLLCTYILGLTPFRLRGEAGARHYQLSKIGYLNALIQLSF
 MSNTFCLEFKPRDAFGAEQTLFFTYLLGLTPFRLRGQAGGRQFQLSKGLYNALLQLSF
 MSFYFCLEFKPRDAFGAEQTLFFTYLLGLTPFRLRGQAGGRQFHLKIGYLNALQLSF
 MSFLFCDLFKPRDAFGAEQTLFFTYLLGLTPFRLRGQAGGRQFHLKIGYLNALQLSF
 * : : : . **.*.*.*.* : **.*.*.*.* * * * : **.*.*.*.* : * : *

DmojGr
 DwilGr
 DpseGr
 DyakGr
 Gr28bD
 DsimGr

FGYCFLSALVQEQSIVGYFFNSPISQVGDLSQKFIGMTGMFTLFLCCGLRVRLIRHCDM
 FSYCFLSALIEQQSIVNYFFKSEISQVGDLSQKFIGMTGMCILFTCSAIRVRLIKLCDL
 FSYCFLTALIEQQSIVGYFFKSEISQVGDLSQKFIGMTGMSILFLCSTIRVRLIHLNCL
 FSYCFLAALIEQQSIVGYFFKSEISQVGDLSQKFIGMTGMSILFLCSSIRVRLIRIWDNR
 FSYCFLAALIEQQSIVGYFFKSEISQVGDLSQKFIGMTGMSILFLCSSIRVRLIHIWDR
 FSYCFLAALIEQQSIVGYFFKSEISQVGDLSQKFIGMTGMSILFLCSSIRVRLIRIWDNR
 * .***.*.* : .***.*.* : ***.*.* : ***.*.* : ***.*.* : * * : * : *

DmojGr
 DwilGr
 DpseGr
 DyakGr
 Gr28bD
 DsimGr

IACIDNHLDDVGICFNYSRIMSLRHTKLLLIAGVQLAYLASSISMLIYNDVRPSYTVAVA
 VYHIDDKLEIGVCFNYSRIMQLRHVKLLICSVQVAYLASSLCMLLWNNVRPIYTATVA
 ISRIDGHLDDVGIVFNYPAIMRLRHSQFLMSTVQLAYLISSTWMLLRNDVRPSYPAAVA
 ISCVDDRFLNVGVCFNYPAIMRLRHLQILLINGVQLSYLISSNWMLLGNDRPIYTAIVA
 ISYIDDRFLNLGVCFNYPAIMRLRLQLIFLINGVQLGYLISSNWMLLGNDRPIYTAIVA
 ISCIDDRFLNLGVCFNYPAIMRLRLQLIFLINGVQLGYLISSNWMLLGNDRPIYTAIVA
 : : * .***.*.* : ***.*.* : ***.*.* : ***.*.* : ***.*.* : * * : *

DmojGr
 DwilGr
 DpseGr
 DyakGr
 Gr28bD
 DsimGr

FYVPQIFLLSIVVLFGAILHRCWQQFDALNKVLKNLAHQWDRSLKAV-QKQRSLSQCLDS
 FYVPQIFLLSIVVLFGAILHRCWQHKLKVLNEVLKNLAHQWDRSLKAT-QKQRSLSQCLDS
 FYVPLIFLLSTVILFGAFLHRLWQHLEALNKVLKNLSHQWDSHSLKGVVQKQRSLSQCLDS
 FYVPQIFLLSIVVLFHATLHRLWQHFTVLNQVLKNLAHQWDRSLKAVTQKQRSLSQCLDS
 FYVPQIFLLSIVVLFHATLHRLWQHFTVLNQVLKNLAHQWDRSLKAVNQKQRSLSQCLDS
 FYVPQIFLLSIVVLFHATLHRLWQHFTVLNQVLKNLAHQWDRSLKAVTQKQRSLSQCLDS
 ***.*.*.* : ***.*.* : ***.*.* : ***.*.* : ***.*.* : * * : *

DmojGr
 DwilGr
 DpseGr
 DyakGr
 Gr28bD
 DsimGr

FSMYTIVTKDPAEIIQESMEIHQLICEAAATANKYFTYQLLTIISIAFLIIVFDAYYVLE
 FSMYTIVTKDPAEIIQESMEIHQLICEAAATANKYFTYQLLTIISIAFLIIVFDAYYVLE
 FSMYTIVTKDPAEIIQESMEIHQLICEAAATANKYFTYQLLTIISIAFLIIVFDAYYVLE
 FSMYTIVTKDPAEIIQESMEIHQLICEAAATANKYFTYQLLTIISIAFLIIVFDAYYVLE
 FSMYTIVTKDPAEIIQESMEIHQLICEAAATANKYFTYQLLTIISIAFLIIVFDAYYVLE
 FSMYTIVTKDPAEIIQESMEIHQLICEAAATANKYFTYQLLTIISIAFLIIVFDAYYVLE

DmojGr
 DwilGr
 DpseGr
 DyakGr
 Gr28bD
 DsimGr

TLLGKSKRESKFKTFEFVTFSCQMILYLIAIIVSIVEGSNRAIKKSEKTGAIVHSLLNKT
 TLLGKSKRESKFKTFEFVTFSCQMILYLIAIISIVEGSNRAIKKSEKTGGIVHALLNKT
 TLLGKSKRESKFKTFEFVTFSCQMILYLIAIISIVEGSNRAIKKSEKTGGIVHSLMNKT
 TLLGKSKRESKFKTFEFVTFSCQMILYLIAIISIVEGSNRAIKKSEKTGGIVHSLLNKT
 TLLGKSKRESKFKTFEFVTFSCQMILYLIAIISIVEGSNRAIKKSEKTGGIVHSLLNKT
 TLLGKSKRESKFKTFEFVTFSCQMILYLIAIISIVEGSNRAIKKSEKTGGIVHSLLNKT

DmojGr
 DwilGr
 DpseGr
 DyakGr
 Gr28bD
 DsimGr

KSPEVREKLHQFSMQLMHLKINFTAAGLFNIDRTLYFTISGALTTYLIILLQFTSNPSN
 KSVEVKEKLQQFSMQLMHLKINFTAAGLFNIDRTLYFTISGALTTYLIILLQFTSNPSN
 KSPEVKEKLQQFSMQLMHLKINFTAAGLFNIDRTLYFTISGALTTYLIILLQFTSNPHN
 KSAEVKEKLQQFSMQLMHLKINFTAAGLFNIDRTLYFTISGALTTYLIILLQFTSNPNP
 KSAEVKEKLQQFSMQLMHLKINFTAAGLFNIDRTLYFTISGALTTYLIILLQFTSNPNP
 KSAEVKEKLQQFSMQLMHLKINFTAAGLFNIDRTLYFTISGALTTYLIILLQFTSNPNP
 * * * .***.*.* : ***.*.* : ***.*.* : ***.*.* : ***.*.* : * * : *

DmojGr
 DwilGr
 DpseGr
 DyakGr
 Gr28bD
 DsimGr

ESSTGFDCCEAYHNMNT-NHTV
 -NSHLYSCCEALNNITANHTL
 SNGNYYSCCETFNNT-NHTN
 GYNGSSCCETFNNT-NHTL
 GYNGSSCCETFNNT-NHTL
 GYNGSSCCETFNNT-NHTL
 * * * .***.*.* : ***.*.* : ***.*.* : ***.*.* : ***.*.* : * * : *

DmojGr
 DwilGr
 DpseGr
 DyakGr
 Gr28bD
 DsimGr

Figure 1-3: MUSCLE Alignment (EMBL- EBI) of Gr28bD and orthologs.

Red residues indicate small, hydrophobic residues. Blue indicates acidic residues (DE) and magenta indicates basic residues (KR). Residues with hydroxyl, sulphhydryl or amine group are colored green (STYHCNGQ). An * (asterisk) indicates positions where a single residue is conserved across all alignments. A colon indicates positions where amino acids are strongly similar while a period indicates positions where residues are weakly similar [74]. The C terminus of the orthologs is highly conserved. The N terminus hosts the dissimilarities that has the potential to confer unique temperature response properties to the orthologs.

2 Identification of thermosensitive proteins from the Gustatory Receptor family in *Drosophila*.

2.1 Introduction

The ability to extrinsically control neuronal activity is the most powerful way to decipher the neural mechanisms underlying behavior. Ever since their discovery in the 1990s, temperature sensitive molecules have been used to alter neural activity. The most commonly used temperature sensitive tools are Transient Receptor channel proteins (Trps) that activate neurons at different temperatures [7,30,51,75,76], and *Shibire^{ts}* that reduces synaptic output [24,32] at temperatures $>32^{\circ}\text{C}$. Thermosensitive proteins must meet several criteria before being used as tools [24]. At the least, they should function within the physiological range of organisms and preparations and be non-toxic. Ideal tools should function as switches and create lasting changes in neural activity. However, the existing thermogenetic tools have several limitations. They often function outside the physiological range of organisms and temperatures suitable for cell preparations. Although Trp proteins are widely used, their activity changes rapidly with change in temperature, in the range of milliseconds to seconds. This makes it difficult to study long lasting changes in neuronal activity upon a brief temperature stimulus. Additionally, Trp proteins are voltage sensitive [77,78], which makes it difficult to control for temperature-only alteration of neuronal activity. Consequently, our ability to control neuronal activity using temperature as a driving factor is limited.

The recent discovery of temperature response properties of two Gustatory receptors (Gr28bD and Gr28bE) in *Drosophila melanogaster* [8] opened new avenues to study temperature sensitivity of proteins and the effect of this important sensory modality on organisms. Interestingly, the predicted structure of Grs is different from the well-known structure of Trp channels. Trp channels have 6 transmembrane domains and function as tetramers [34]. On the contrary, Grs have a putative inverted GPCR structure with seven predicted transmembrane domains. Recent structural characterizations of a sister family of Grs, the Olfactory receptors (Ors), have revealed that the required cofactor Or83a forms a homo-tetramer with an ion-channel like structure [79]. This suggests that Grs might act via different mechanisms than Trp channels to confer thermosensitivity. The Gustatory receptor family in *Drosophila melanogaster* consists of 68 proteins encoded by 60 genes. The Grs are diverse with 20% or less similarity between their amino acid sequences. There are however, several clusters of genes in the GR family that share up to 70% sequence similarity within the clusters, possibly due to gene duplications. Many of the genes in a cluster are the products of alternative splicing. GRs which have greater than 30% sequence similarity have been grouped together in subfamilies [38,39]. As a first step, we sought to mine the Grs in *D. melanogaster* for thermosensitivity by screening for temperature responsiveness of 31 Grs from different subfamilies. When mis-expressed in muscles, Gr28bD causes an increase in neuronal activity suggesting that it depolarizes neurons [8]. Hence, we investigated the functional properties of Gr28bD by co-expressing it with other ion channels in flies.

We aimed to uncover the structural differences underlying the thermosensitivity of Gr28bD as well as to increase the repertoire of thermogenetic tools available to the scientific community. Hence, our search for temperature sensitive proteins was not limited to Grs from *D. melanogaster*. It encompassed Grs from other *Drosophila* species that live in varied environments. We screened for temperature responsiveness of 5 GR28bD orthologs that have 80-98% identity to Gr28bD, one each from 5 other *Drosophila* species. *D. simulans* is a cosmopolitan species and has a similar distribution to *D. melanogaster*. In contrast *D. mojavensis* is a resident of the Baja California and the Sonoran and Mojave deserts of North America. It breeds in cacti and can survive the harsh arid conditions in temperatures up to 46°C [80]. *D. yakuba* occupies open and semi-open habitats including semiarid areas, savannahs, grasslands and coffee plantations but not rainforests in Africa [81]. *D. willistoni* inhabits the tropical regions of Central and South America [82] and *D. pseudoobscura* is distributed over a wide range of habitats such as forested regions, grasslands and dessert oasis in North America [83]. Different evolutionary histories suggest that genetic variance might have given rise to temperature sensitive orthologs of GR28bD with different temperature response properties in these *Drosophila* species. We sought to use the natural variation among the orthologs to identify temperature sensitive Grs with distinct temperature response properties.

Altogether, we tested the following hypotheses- 1) There are multiple temperature sensitive Grs in *Drosophila*. 2) The behavioral effects of

thermosensitive protein expression can be altered when used in conjunction with hyperpolarizing ion channels.

2.2 Methods

2.2.1 Fly rearing conditions:

Flies were reared on cornmeal-yeast-dextrose medium at 25°C in 12 hour light-day cycle at 60% relative humidity. Adult flies 2-10 days old were collected and transferred to fresh food 2 days before the experiments.

2.2.2 Transgenic fly lines

The *UAS-GR28a*, and *UAS-Gr28bA-E* isoform fly lines were generous contributions of Paul Garrity, Brandeis University. Other *UAS-Grs* in Table 1 were contributed by Anupama Dahanukar, UCSD, and Kristin Scott, USCB. The *nSyb-GAL4*, *OK6-GAL4*, *UAS-Shaw-tr*, *UAS-Shaw-RA*, *UAS-EKO*, *UAS-EKO,222a*, *222b* and *UAS-TrpA1* fly lines were obtained from the Bloomington Stock Center, Indiana. *Canton-S* fly line used for making heterozygous controls is an in-house line. *UAS* lines of orthologs of Gr28bD from 5 other species were made in-house. Coding sequences of the orthologs obtained from Flybase were synthesized and cloned in between Not1 and Xba1 sites (Genscript, USA) in the plasmid pJFRC12[84]. The constructs were sequenced with hsp70 forward primer- “GAGCGCCGGAGTATAAATAGAG” and SV40 reverse primer “CCATTCATCAGTTCCATAGG”[84]. Plasmids with verified constructs were sent to Bestgene, USA, for injection into embryos of fly strain VK05 which has an *attP* site in the third chromosome. ϕ C31-mediated site specific integration was used for insertion of the *UAS* constructs into the *attP* site [85]. Fly lines with successful insertions of the *UAS* constructs were used in experiments. In this manuscript,

the UAS-Gr ortholog fly lines are abbreviated as follows: *UAS-D. simulans* ortholog- DsimGr, *UAS-D. pseudoobscura* ortholog- DpseGr, and *UAS-D. yakuba* ortholog- DyakGr, *UAS-D. willistoni* ortholog- DwilGr, and *UAS-D. mojavensis* ortholog- DmojGr.

For thermotolerance assays, flies were made to overexpress *UAS-Grs* pan-neuronally using *nSyb-GAL4*, or in motor neurons using *OK6-GAL4* [86]. Flies heterozygous for *nSyb-GAL4*, *OK6-GAL4*, or *UAS-genes* were used as negative genetic controls.

2.2.3 Behavioral assays

All behavior experiments were performed in the heat-box paradigm [9,87]. Briefly, activities of flies were observed as they walked the length of a small chamber which is lined on the top and bottom with Peltier elements. The temperature of the chamber was altered with a custom-made software, “Hbox”. An associated software “Heat-Calc” was used for analyzing fly activity.

In the thermotolerance assays, the temperature of the chamber was increased from 24-40°C, in steps of 2°C. At each temperature step, the movements of the flies were recorded for 90s. A fly was considered paralyzed at a specific temperature if no movement was observed for at least 45s in the 90s window and for the rest for the experiment (Figure 2-1).

To determine the time to paralysis at the specific temperatures, flies were subjected to 32, 34, 36, 38, or 40°C following a brief acclimation at 24°C.

Following paralysis, the time to recovery was measured within a 15 min window.

These experiments are referred to as “step-up” experiments in this manuscript (Figure 2-2).

2.2.4 Work flow and statistical methods

Flies were tested for temperature response properties of overexpressed Grs/combinations with behavioral assays in the heat-box. The results were represented with Survival plots that showed the proportion of flies that were paralyzed or recovered at different temperatures with time. Kaplan Meier’s test followed by Gehan’s Wilcoxon post hoc test was used to analyze statistical significance. p values were corrected with Bonferroni correction.

Figure 2-1: Representational activity traces of a Canton-S fly (top panel), and a fly overexpressing a temperature sensitive protein pan-neuronally (bottom panel) in the heat-box.

The temperature of the heat box was increased in 2°C steps from 24-40°C. At each step, activities of the flies were measured for 90s. Blue line indicates fly activity. *Canton-S* fly activity was increased at “high” temperatures of 38-40°C (closely spaced blue lines, top panel). The fly with pan-neuronal overexpression of a temperature sensitive protein was paralyzed at 34°C indicating that the protein is sensitive from 34°C (black arrow, bottom panel). The paralyzed fly did not recover in the duration of the experiment.

Figure 2-2: Example position traces of a non-paralyzed fly (top panel) and of a paralyzed fly that recovered (bottom panel) in a “step up” experiment.

The red area represents the exposure to “high temperature” and the arrows mark the time-to-paralysis (left arrow) and time-to-recovery (right arrow). Flies were exposed to a “high temperature” stimulus for 90s following a 90s acclimation in 24°C. After paralysis their recovery time was measured for 900s.

Measure temperature response
properties if Grs in the heat-box

Fit distribution (non-normal)

Non-parametric tests

Kaplan Meier's Survival
tests with independent
variables

Post hoc Gehan's
Wilcoxon test for multiple
comparisons followed by
BF correction of p values

Figure 2-3: Flow of work and analysis.

Flies overexpressing the Grs were tested for temperature response properties in the heat-box. The data obtained was analyzed with Kaplan-Meier's Survival analysis followed by post-hoc Gehan's Wilcoxon test.

2.3 Results

2.3.1 Several Gustatory receptors in *Drosophila* are thermosensitive.

We hypothesized that pan-neuronal overexpression (referred to as “overexpression” in this sub-section) of a temperature sensitive protein will paralyze flies at the temperature of activation of the protein due to excessive neuronal activity. TrpA1 has been shown to be temperature sensitive from ~28°C [7,8]. As a positive control, we overexpressed TrpA1 pan-neuronally and measured the paralysis temperature. These flies were paralyzed from 30°C and stayed paralyzed for the duration of the experiment. Our observation confirmed that temperature dependent paralysis was a robust method of determining temperature sensitivity of a protein. There are 68 Gustatory receptors in *D. melanogaster*. We tested 31 of the 68 Grs, and an additional 7 Grs from *Anopheles gambiae* and *D. pseudoobscura* for thermosensitivity (Table 2-1). Our results showed that 3 *D. melanogaster* Grs, Gr28bD, Gr28bB and Gr61a, are temperature sensitive (Figure 2-4 (A), Table 2-1). Flies overexpressing Gr28bD were paralyzed between 34-36°C while those overexpressing Gr28bB and Gr61a were paralyzed at 40°C, indicating that Gr28bB and Gr61a are active at higher temperatures. None of the other tested Grs (Table 2-1) were temperature sensitive within 40°C. To understand the structure-function relationship of Gr28bD, we assayed the temperature response properties of flies expressing Gr28bD orthologs from 5 *Drosophila* species that have 80-98% identity to Gr28bD (Table 2-2). 4 of the 5 orthologs were thermosensitive at different temperatures between 30-38°C (Table 2-2, Figure 2-4 (B)). Flies with

overexpression of DsimGr, DpseGr and DyakGr were paralyzed between 30-32°C. Most flies overexpressing DwilGr were paralyzed at 36°C while those overexpressing DmojGr were not paralyzed within 40°C. Thus, we found 7 thermosensitive proteins that can potentially be used as thermogenetic tools.

An important aspect of using Grs as tools was to ensure that the paralysis was indeed temperature-dependent and temporary, i.e, following paralysis at high temperatures, we expected paralyzed flies to recover when allowed to revive at 24°C. To examine the temperature dependency of the Grs, we assayed the function of temperature on paralysis time and recovery time with “step-up” experiments. Specifically, we measured the time taken by flies overexpressing thermosensitive Grs to incapacitate on exposure to sudden increase in temperature and recover following paralysis (Figure 2-2). Since *D. melanogaster* flies are stressed at temperatures $\geq 40^{\circ}\text{C}$ [88], we chose not to study Gr28bB and Gr61a overexpressing flies further with thermotolerance based paradigms.

The exposure temperatures in “step-up” experiments were chosen from the temperature range that the Grs are thermosensitive in. For example, since Gr28bD was temperature sensitive between 34 and 36°C, we subjected flies overexpressing Gr28bD to 34, 36, 38 or 40°C and measured the time-to-paralysis and time-to-recovery. Interestingly, we found that flies exposed to 36, 38 or 40°C were paralyzed significantly faster than those exposed to 34°C. There appeared to be a threshold temperature between 34 and 36°C beyond which the time-to-paralysis was $\geq 10\text{s}$ (Figure 2-5 (A)). The time taken by 50% of Gr28bD overexpressing flies (P_{50}) is reported in Table 2-3. Few heterozygous effector

(*UAS*) genetic control flies were paralyzed at 40, indicating that there might be some leak expression of *UAS-Gr28bD*. None of the heterozygous driver control (*nSyb-GAL4*) flies were paralyzed at any of the of the tested temperatures. Unlike times-to-paralysis, the-time-recovery of Gr28bD overexpression flies were graded. Flies paralyzed at higher temperatures took longer to recover than those exposed to lower temperatures (Figure 2-5 (B)). The recovery times were significantly different from each other. For instance, 50% of Gr28bD overexpression flies recovered from 40°C induced paralysis in 762s while 50% of flies paralyzed at 38°C recovered within 382s. The times-to-recovery of 50% of flies (R_{50}) is recorded in Table 3. Like Gr28bD overexpressing flies, flies with pan-neuronal overexpression of DsimGr, DpseGr, DyakGr or DwilGr were paralyzed faster when exposed to higher temperature stimulus (Figure 2-6 and Table 2-3). Their recovery was also graded with flies exposed to lower temperature stimulus recovering faster than those exposed to higher temperature stimulus (Figure 2-6 and Table 2-3).

2.3.2 Gr28bD effects can be modulated by co-expressing hyperpolarizing ion channels.

We tested the hypothesis that the paralysis in flies with pan-neuronal Gr28bD overexpression is due to excessive depolarization. In such case, we expected that co-expression of hyperpolarizing ion channels will increase the time to paralysis of flies. The resultant effect would be a right shift in paralysis temperature. Shaw [89] and EKO [90] are ion channels that have been extensively used to hyperpolarize neurons. They are commonly used as tools to

reduce neuronal activity [91]. We co-expressed each of these ion channels with Gr28bD in all motor neurons of flies using *OK6-GAL4* (referred to as “overexpression” in this sub-section). We used *OK6-GAL4* instead of *nSyb-GAL4* as pan-neuronal expression of hyperpolarizing ion channels have been known to be lethal [91]. Similarly to flies with pan-neuronal overexpression of Gr28bD, we found that flies overexpressing Gr28bD in their motor neurons were paralyzed between 34-36°C (Figure 2-7). Most flies overexpressing Shaw-RA+Gr28bD were paralyzed at significantly higher temperature of 38°C. However, flies that co-expressed Gr28bD and Shaw-tr, which is the truncated version of Shaw [92] were paralyzed at the same temperature range as Gr28bD overexpression flies. Flies overexpressing EKO+Gr28bD were also paralyzed at significantly higher temperatures of 38-40°C. Surprisingly, overexpression of Gr28bD+EKO222a, 222b did not affect paralysis temperature significantly (Figure 2-7). Since this line has two insertions of the *UAS-EKO* construct [90], the excessive disruption might be rendering these flies more vulnerable to temperature dependent stress.

We compared the effects of temperature on time-to-paralysis and time-to-recovery of co-expression flies with flies overexpressing either Shaw-RA or EKO or Gr28bD. Very few flies overexpressing Shaw-RA alone or EKO alone were paralyzed at 36 or 38°C (Figure 2-8 (A) and (C)). Gr28bD+Shaw and Gr28bD+EKO flies were not paralyzed at 36°C. In contrast, when exposed to 36°C, at least 50% of Gr28bD overexpressing flies were paralyzed within 20s (Figure 2-8 (A), Table 2-4). When exposed to 38°C, Gr28bD+Shaw-RA and Gr28bD+EKO flies took significantly longer time to incapacitate as compared to

Gr28bD overexpressing flies. Most Gr28bD overexpressing flies were paralyzed within 10s while Gr28bD+EKO and Gr28bD+Shaw-RA flies took at least 10s longer to be paralyzed (Figure 2-8 (A) and (C), and Table 2-4). The times-to-recovery of flies was graded. Following paralysis, Gr28bD+Shaw-RA and Gr28bD+EKO flies recovered significantly faster as compared to Gr28bD overexpression flies. At least 50% of Gr28bD+EKO flies recovered within 130s and 50% of Gr28bD+Shaw-RA flies recovered within 10s after paralysis at 38°C. In contrast it was 287s before 50% of flies Gr28bD overexpression flies recovered (Figure 2-8 (B) and (D), and Table 2-4). Thus, the shift in paralysis and recovery times indicate that Gr28bD activity can be modulated with co-expression of hyperpolarizing ion channels.

Figure 2-4: Several Gustatory receptors are thermosensitive between 24-40°C.

Flies overexpressing Gr genes had their locomotor activity recorded for 90 seconds at 24–40°C in two-degree steps. The plots show the cumulative proportion of flies not incapacitated at each temperature. **A)** Most flies with pan-neuronal overexpression of TrpA1 were paralyzed by 30°C while those that overexpressed GR28bD were temporarily incapacitated at 34 or 36°C. Gr28bB and Gr61a overexpression had paralyzing effects at the very high end of the tested temperature range. Flies overexpressing other tested Grs (Table 1) were not paralyzed within 40°C indicating that those Grs are not temperature sensitive. 52-80 flies were tested per genotype. Statistical comparisons done by Kaplan-Meier's analysis shows that $p < 0.00001$. Post hoc analysis was done with Gehan's Wilcoxon test for pairwise comparisons of non-paralyzed vs paralyzed flies with BF correction. $p < 0.0125$ is considered significant. **B)** Flies with overexpression of DsimGr, DpseGr and DyakGr orthologs were paralyzed

between 30 and 32°C. Most flies overexpressing DwilGr ortholog were incapacitated at 36°C while those overexpressing the DmojGr ortholog were not paralyzed within 40°C. 54-62 flies were tested per genotype. In statistical analysis performed by Kaplan-Meier test, $p < 0.000001$. In post hoc Gehan's Wilcoxon tests for pairwise comparisons of every ortholog with *D. melanogaster* GR28bD, $p < 0.01$ is significant with BF correction. For all comparisons, $p < 0.00001 = ****$.

Table 2-1: Paralysis temperatures of flies with pan-neuronal Gr overexpression in <i>D. melanogaster</i> . Driver: <i>nSyb-GAL4</i> . NP: Not paralyzed							
Sl. No.	<i>UAS-Gr</i>	Paralysis temperature (°C)	N	Sl. no	<i>UAS-Gr</i>	Paralysis temperature (°C)	N
1	28a	NP	65	21	64f	NP	73
2	28bA	NP	65	22	98c	NP	65
3	28bB	40	66	23	5a	NP	73
4	28bC	NP	65	24	22c	NP	63
5	28bD	34-36	65	25	22e	NP	55
6	28bE	NP	65	26	59c	NP	64
7	61a	40	64	27	93a	NP	63
8	66a	NP	103	28	94a	NP	54
9	33a	NP	101	29	93c	NP	70
10	21a	NP	67	30	93d	NP	63
11	32a	NP	72	31	2a	NP	64
12	39aa	NP	64	32	AgGr15	NP	66
13	43a	NP	77	33	AgGr16	NP	62
14	63a	NP	66	34	AgGr17	NP	65
15	23a	NP	43	35	AgGr25	NP	63
16	64a	NP	71	36	DpGr64a	NP	56
17	64b	NP	70	37	DpGr64d	NP	60
18	64c	NP	70	38	DpGr64f	NP	59
19	64d	NP	70				
20	64e	NP	74				

Table 2-2: Summary of paralysis temperatures of flies with pan-neuronal overexpression of Gr28bD orthologs.					
	Orthologs	NCBI Accession numbers	% identity to Gr28bD	Paralysis temperatures (°C)	N
	Gr28bD	NT_033779	100	34-36	65
1	DsimGr	XP_016023952.1	98	30-32	54
2	DyakGr	XP_015053535.1	96	30-32	60
3	DpseGr	XP_015035526.1	85	30-32	54
4	DwilGr	XP_002066897.2	81	34-36	62
5	DmojGr	XP_017857911.1	80	No paralysis < 40	60

Figure 2-5: Time-to-incapacitation and time-to-recovery of Gr28bD overexpression flies are temperature dependent.

Flies were exposed to sudden increase in temperature for 90s and allowed to recover at 24°C for 900s. Their times-to-paralysis and times-to-recovery were measured. **A)** Time-to-paralysis at different temperatures: flies overexpressing Gr28bD and flies heterozygous for the *nSyb-GAL4* driver and *UAS-Gr28bD* construct were tested. Most flies with pan-neuronal Gr28bD overexpression were incapacitated within 10 seconds at 36, 38, and 40°C, but it took much longer at 34°C (Table 3). Few flies from the heterozygous genotypes were incapacitated at the temperatures tested. Statistical difference between control and experimental genotypes was determined with a Kaplan-Meier's test where $p < 0.000001$. Gehan's Wilcoxon test with BF correction was used for post hoc analysis. Pairwise comparisons of times-to incapacitation of overexpression genotypes with heterozygous genetic controls revealed that $p < 0.00001$ at all tested

temperatures. **B)** Time-to-recovery from incapacitation: Following paralysis, the recovery times of flies were noted at 24°C for 900s. Gr28bD overexpression flies that were subjected to lower paralysis temperatures recovered faster. For example, flies exposed to 34°C had significantly lower R_{50} than those exposed to 36°C (Table 3). Statistical difference between recovery curves at different temperatures was determined with a Kaplan-Meier's test where $p < 0.000001$. Gehan's Wilcoxon test with BF correction was used for post hoc analysis. $p < 0.00001$ for pairwise comparisons of times-to-recovery. In each experiment, 60–80 flies of each genotype were tested. $p < 0.00001 = ****$

Figure 2-6:Gr28bD orthologs show similar trends as Gr28bD in time-to-paralysis and time-to-recovery following paralysis.

Flies with overexpression of GR28bD orthologs and those heterozygous for *nSyb-GAL4* or *UAS-Gr-ortholog* were exposed to sudden increase in temperature for 90s and allowed to recover at 24°C for 900s. Their times-to-paralysis and times-to-recovery were measured. **A, C, E, G) Time-to-paralysis:** Majority of flies with overexpression of the orthologs were paralyzed within 20s (Table 3). In contrast genetic controls were not paralyzed. Statistical comparisons with Kaplan-Meier analysis, and post hoc Gehan's Wilcoxon test for pairwise comparisons show significant differences in time-to-incapacitation between experimental groups and controls for each genotype at every tested temperature. $p < 0.00001 = ****$. **B, D, F, H) Time-to-recovery following paralysis:** In every genotype, flies that were paralyzed at higher temperatures took longer to recover. The recovery of flies is graded with significant differences between recovery times of flies subjected to higher temperatures vs those subjected to lower temperatures (Table 3). Statistical comparisons with Kaplan Meier's analysis and post hoc Gehan's Wilcoxon test show that the time to recoveries are significantly different from each other. 50-60 flies were tested in C, D, E, F and G, and 30-35 flies were tested in A and B. $p < 0.000001 = ****$.

Table 2-3: Summary of P50 and R50 of flies exposed to different paralyzing temperatures.					
	<i>nSyb-GAL4 > UAS-Gr</i>	Paralysis temperatures (°C)	P₅₀ (s)	R₅₀ (s)	N
1	Gr28bD	34	20	20	96
		36	10	160	77
		38	10	382	66
		40	10	762	60
2	DsimGr	32	10	310	39
		34	10	429	33
3	DyakGr	32	20	170	67
		34	10	260	65
4	DpseGr	32	10	120	51
		34	10	330	45
5	DwilGr	36	10	120	59
		38	10	310	60

Figure 2-7: Co-expression of Gr28bD with hyperpolarizing ion channels alters temperature dependent incapacitation.

Flies overexpressing *UAS-Gr28bD* + *UAS-Shaw-RA*, *UAS-Gr28bD* + *UAS-EKO*, *UAS-Gr28bD* + *UAS-Shaw-tr*, *UAS-Gr28bD* + *UAS-EKO* or *UAS-Gr28bD* + *UAS-EKO 222a, 222b* in their motor neurons using *OK6-GAL4* driver were exposed to temperatures of 24-40°C in steps of two-degrees with 90s at each step. The plot shows the cumulative proportion of flies that were not paralyzed with increase in temperature. Flies with overexpression of *UAS-Gr28bD* were paralyzed at 36°C. Most flies co-expressing *UAS-Shaw-RA* or *UAS-EKO* with *UAS-Gr28bD* were

paralyzed at 38°C. Flies co-expressing *UAS-Shaw-tr* or *UAS-EKO 222a, 222b* were paralyzed between 36-38°C. Statistical analysis performed with Kaplan Meier's tests, and post hoc Gehan's wilcoxon tests show that paralysis temperatures of flies co-expressing *UAS-Gr28bD* with *UAS-EKO* or *UAS-Shaw-RA* are right shifted, and significantly different from those overexpressing *UAS-Gr28bD* alone. 49-60 flies were tested per genotype. $p < 0.00001 = ****$.

Figure 2-8: Modulation of time-to-paralysis and time-to-recovery of Gr28bD overexpression flies by Shaw-RA and EKO ion channels.

Flies overexpressing Gr28bD+EKO, Gr28bD+Shaw-Ra, Shaw-RA, EKO, or Gr28bD in motor neurons were exposed to 36 or 38°C for 90s, following which they were allowed to recover for 900s. The times-to-paralysis and times-to-recovery were measured. **A and C) Time-to-paralysis:** Statistical analyses using Kaplan Meier's tests showed that co-expression flies took significantly longer to be paralyzed at both temperatures as compared to Gr28bD overexpression flies. Most flies overexpressing Shaw-RA or EKO alone were not paralyzed. Post hoc Gehan's Wilcoxon test was used for pairwise comparisons of flies which were paralyzed vs those that were not paralyzed. **B and D) Time-to-recovery:** There was a significant difference in the recovery times of flies. Flies overexpressing Gr28bD+EKO or Gr28bD+Shaw-RA recovered significantly faster than those expressing Gr28bD alone at both 36 and 38°C. 50-60 flies were tested per genotype. Statistical analyses were performed with Kaplan-Meier's tests followed by post hoc Gehan's Wilcoxon test for pairwise comparisons. $p < 0.00001 = ****$ in all comparisons.

Table 2-4: Summary of P ₅₀ and R ₅₀ of co-expression flies and control flies exposed to different paralyzing temperatures.					
	<i>OK6-GAL4 > UAS-genotypes</i>	Paralysis temperature (°C)	P ₅₀ (s)	R ₅₀ (s)	N
1	Gr28BD heterozygous	36	20	40	59
		38	10	287	57
2	Shaw-RA heterozygous	36	NP	0	58
		38	NP	0	57
3	Gr28bD+Shaw-RA	36	NP	0	44
		38	20	91	50
4	EKO heterozygous	36	NP	0	50
		38	NP	0	49
5	Gr28bD+EKO	36	NP	0	59
		38	20	130	44

2.4 Discussion

We discovered several temperature sensitive proteins in the Gustatory receptor family of proteins in *Drosophila*. These temperature sensitive proteins have different temperature response properties than other known thermosensitive proteins like TrpA1. 3 of the 31 tested *D. melanogaster* Grs were temperature sensitive from varying temperatures between 24-40°C. Our experiments showed that on pan-neuronal overexpression of Gr28bD, flies were paralyzed in between 34-36°C. Thus, Gr28bD activates at temperatures at least 4 degrees higher than Trpa1. Two other proteins, Gr28bB and Gr61a displayed temperature response properties at 40°C. Notably, flies overexpressing Gr28bE were not paralyzed in our experiments, suggesting Gr28bE is not temperature sensitive. This contrasts with previous findings that when expressed in specialized heat sensing cells, the so called “Hot Cells” Gr28bE could rescue the loss of function mutation in mutant flies [8]. Thus, the temperature sensitivity of Gr28bE might be limited to certain cell types.

Several of the orthologs showed distinct temperature response properties. On overexpression of DsimGr, DpseGr or DyakGr, flies were paralyzed from 30-32 °C. Their activation temperature is 2°C higher than TrpA1. In contrast, majority of flies with overexpression of DwilGr were paralyzed in between 36-38°C, suggesting that the activation temperature of DwilGr is 6°C higher than the activation temperature of TrpA1. Thus, the temperature sensitive proteins can be broadly grouped into three categories; the low threshold group that includes DsimGr, DpseGr and DyakGr, and the intermediate threshold groups that include

DwilGr and Gr28bD, and finally, the high temperature threshold groups that include Gr28bB and Gr61a. Since the activation temperature of the high threshold proteins is outside the physiological range of *D. melanogaster*, and most model organisms and invitro preparations, we chose not to study them further at this time.

Interestingly, DmojGrD, which is 80% identical to Gr28bD did not display any temperature response properties within 40°C. *Drosophila mojavensis* is a desert fly that is endemic to the Mojave Desert. It has higher thermotolerance than most other species of *Drosophila* and can tolerate temperatures well above 40°C for at least 20 min in laboratory conditions [80]. Incidentally, it is high temperature avoidance, not water balance that enhances survival of *D. mojavensis* flies in the dessert. [57,93]. Hence, DmojGr might have evolved to detect temperatures higher than 40°C. In our behavior experiments, we tested for temperature responsiveness within 40°C to avoid temperature dependent stress on our model system. Thus, our experimental constraints might have prevented us from detecting the thermosensitivity of DmojGr. Nonetheless, the temperature response properties of DmojGr can be deciphered with different model systems that tolerate higher temperatures.

The times to paralysis and recovery from paralysis of flies overexpressing thermosensitive proteins are temperature dependent. Transgenic flies that overexpressed Gr28bD or its temperature sensitive orthologs were subjected to the upper and lower limits of their activation temperatures. Flies exposed to the lower limits of the activation temperatures were paralyzed slower as evidenced

by the tails of the distributions (Figure 2-5, A, C, E, G) and recovered faster (Figure 2-5, B, D, F, H) than those exposed to the upper limits. This could be due to differences in sensitivity of the thermosensitive proteins at different temperatures, or due to differences in time taken by fly neural systems to re-establish homeostasis following paralysis at different temperatures.

To measure the physiological changes underlying fly behavior, we co-expressed GR28bD or its orthologs with Gcamp6f in *Drosophila* motor neurons [9]. There were profound changes in Gr28bD induced neuronal activity with increase in temperature that could be measured as changes in Gcamp6f fluorescence. Essentially, the activity of neurons expressing thermosensitive proteins increased with increase in temperature indicating that fly paralysis is a result of excessive neuronal activity due to activation of thermosensitive proteins (Figure 2-9). Flies co-expressing DmojGr and GCamp6f emulated the lack of change in fluorescence with increase in temperature in heterozygous UAS-Gcmap6f control flies. This confirmed that DmojGr is not active between 24-40°C (in prep). Furthermore, the threshold temperatures of peak neuronal activities of different thermosensitive proteins were comparable to the observed paralysis temperatures, thus providing a direct association between the physiological changes in fly behavior and fly paralysis (in prep).

When expressed in a heterologous system, such as *Xenopus* oocytes, Gr28bD or its thermosensitive orthologs function as non-selective cation channels that depolarize cells on activation ([9], [in prep](#)). In addition to physiological studies, this observation also corroborates our hypothesis that hyperexcitability of

neurons paralyzes flies with thermosensitive Gr overexpression. Interestingly, the current densities rapidly follow the changes in temperature ([9], in prep). It also indicates that unlike voltage gated cation channels, the thermosensitive proteins do not inactivate after a period. These observations clarified that the differences in the times-to recovery of flies subjected to different temperatures in “step-up” experiments are not due to the sensitivity of the thermosensitive protein products but a function of neural systems recovering from extrinsic activation. It accounted for our finding that co-expression of hyperpolarizing ion channels such as Shaw-RA and EKO with Gr28bD increased the time-to-paralysis and decreased the times-to-recovery. Perhaps these ion channels decreased the Resting Membrane Potential (RMP) [91] to an extent that more number of Gr28bD channels needed to function longer to depolarize the neurons sufficiently to induce paralysis at a temperature, thus increasing the time-to-paralysis. Similarly, faster hyperpolarization due to the overexpressed Shaw-RA and EKO channels could help reestablish RMP faster, thus reducing the recovery times. Consistent with previous observations, DmojGr did not display significant depolarizing current (in prep). Thus, biophysical studies support our observations of fly behavior with thermosensitive protein overexpression (Figure 2-9).

The thermosensitive Grs have several important biophysical properties that set them apart from other thermosensitive proteins. For example, the temperature response properties of the Grs in oocytes indicate that the Grs do not need any explicit co-factors to be active, thus distinguishing them from the well characterized ORs that require a co-factor, Or83a to function [79,94]. Moreover,

current clamp experiments proved that unlike Trp proteins that are voltage dependent ([7,30,78]), at constant temperature, activity of thermosensitive Grs is independent of changes in voltage ([9], in prep). Grs are specific to Arthropods, and there are no identified proteins in mammals similar to Grs. A cursory BLAST of Gr28bD revealed that the first 20000 proteins that share some identity to Gr28bD exist exclusively in Arthropods. Thus, they can potentially be used in mammalian preparations without risk of interactions with endogenous proteins.

Altogether our experiments verify our hypotheses. We demonstrated that the thermosensitive Grs not only alter fly behavior but are also functional in other cell systems. When expressed in conjunction with ion channels, they significantly alter fly behavior. Co-expression of hyperpolarizing ion channels shifts the times and temperatures of paralysis to the right and hastens recovery from paralysis. In future, comparisons of their protein sequences will shed light on the bases of their thermosensitivity. Thus, our findings suggest that the thermosensitive Grs can be used as thermogenetic tools in different systems.

Figure 2-9:Paralysis temperatures in flies, physiological changes in fly motor neurons and current density in oocytes confirm that several Gr orthologs are temperature sensitive.

Gr28bD, DsimGr, DyakGr, DpseGr, and DwilGr have significant changes in fluorescence compared to control when co-expressed with GCaMP6f in fly motor neurons. Flies overexpressing these orthologs pan-neuronally are paralyzed at different temperatures ranging from 34-36°C. On heterologous expression in oocytes, these orthologs display inward currents with increase in temperature. Flies and oocytes expressing DmojGr are deficient in all 3 tests suggesting that DmojGr is not temperature sensitive within 40°C. Our observations for these thermosensitive proteins are consistent across our experiments in different systems.

3 Characterization of thermogenetic tools in a sensory system in *Drosophila melanogaster*

3.1 Introduction

Insects survive extreme temperature conditions through a host of mechanisms. Their strategies involve behavioral avoidance, accumulation of genetic variations, physiological mechanisms like cold hardiness to tolerate low temperatures, shivering in the cold and basking in the heat to raise body temperatures, and evaporative cooling to reduce body temperatures [95]. As with most insects, because of their small size, the body temperature of fruit flies is synonymous with the ambient temperature in few seconds. Hence it is necessary for the flies' survival to avoid extremely hot or cold regions. Proteins from the Transient Receptor family, TrpA1, TrpM8 and Pyx enable flies to determine a comfortable temperature in a thermal gradient [51,96,97] and protect flies from high temperature stress [98,99]. Early evidence with experiments on antenna-less flies suggested that the antenna of fruit flies is necessary to avoid rapid changes in temperature [87]. Recent studies show that the 5th antennal segment (arista) of fruit flies houses specialized cells, called "Hot Cells". These cells endogenously express the thermosensitive protein Gr28bD and detect fast temperature changes which enable the flies to avoid hot regions [8,100]. However, mutant flies that lack Gr28b proteins did not display any preference when given a choice between 24 and 36°C [8]. These mutants could be rescued by restoring Gr28bD

expression in the antenna [8], suggesting that the Gr28bD is required for heat avoidance.

Previously, we identified several thermosensitive orthologs of Gr28bD through mis-expression studies. Flies that endogenously express these orthologs have diverse evolutionary histories (figure 1-2). If these orthologs were to be arranged in descending order of the temperatures their hosts they inhabit, being from warmer climates, orthologs from *D. mojavensis* and *D. willistoni* would probably occupy the top positions, followed by *D. pseudoobscura*, *D. yakuba* and *D. simulans*. While mis-expression induced paralysis is a robust first step to study the orthologs, it is not an accurate representation of the endogenous function of these proteins. To understand their function in a more natural context, we sought to study the thermosensitivities of these proteins by expressing them in the Hot Cells. We hypothesized that the proteins will have different sensitivities when expressed in the Hot Cells. Essentially, we tested for the ability of thermosensitive proteins to rescue *GR28b^{mi}* null mutants by assaying for behavioral avoidance of hot regions. We also investigated if the orthologs were sensitive to small temperature differences or absolute temperatures. We hypothesized that orthologs obtained from flies that inhabit warmer regions will be more sensitive at higher temperatures as compared to those that inhabit lower temperatures. Our findings reveal differences in the thermosensitivities of the orthologs. These differences are reminiscent of the thermal aspects of the environment of the species' from which the orthologs are derived. We found that several orthologs which did not display temperature sensitivity in our

thermotolerance assays were sensitive when expressed in the Hot Cells. A possible cause is the presence of accessory proteins of Gr28bD in Hot Cells that might be enhancing temperature sensing abilities of these proteins. We hypothesized that the Hot Cells have specific accessory proteins that are important for thermosensing. We discuss our findings and reveal several genes that could be potentially crucial to thermosensing in flies.

Altogether, we tested the following hypothesis: 1) Gr28bD orthologs will have unique temperature response properties when expressed in the Hot Cells, 2) orthologs obtained from flies that inhabit warmer regions will have altered sensitivity at higher temperatures as compared to those that inhabit lower temperatures, and 3) Hot Cells have specific accessory proteins that are important for thermosensing.

3.2 Methods

3.2.1 Flies

Flies were made to express Gr28b paralogs or orthologs in Hot Cells of *Drosophila* using *Hot-Cell-GAL4* (*HC-GAL4*) in a *Gr28b^{mi}* or heterozygous *Gr28b^{mi}* background. The *HC-GAL4* fly line was a generous contribution of Paul Garritty. The *Gr28b^{mi}* fly line was obtained from BDSC. Flies were maintained on standard cornmeal and yeast medium (2.2.1). 2-10 days old flies were used for experiments.

3.2.2 Behavioral assays

In the thermosensitivity assay, the temperature of one half of the chamber was increased from 24 to 39°C in steps of 3°C while maintaining the temperature of the other half at 24°C. The hot side of the chamber alternated between the two sides at every temperature step during the experiment. At each step, the preference of flies to either side of the chamber was observed for 1 min (Figure 3-1). We tested 80-100 flies per experimental genotype, spread over 12-14 trials with 7 flies per trial. The Avoidance Indices of the flies were measured as follows- Equation 3-1:

$$\text{Avoidance index (AI)} = \frac{\text{Time spent in cool side} - \text{time spent in hot side}}{\text{Total time spent in the chamber}}$$

A positive AI indicates a larger proportion of time spent in the cool side, while a negative AI indicates that the flies spent majority of time in the hot side. Wild-type (CS) flies avoid the warm side in the heat box [87]. On the other hand, “antennae-less” display reduced avoidance [87], presumably due to the disrupted

activation of Hot Cells. We tested if *Gr28b^{mi}* flies rescued with candidate Gr28b genes or orthologs can avoid the hot side in the thermosensitivity assay.

To examine if *Gr28b^{mi}* flies rescued with Gr28bD or the orthologs in the hot cells respond to small temperature differences, we modified the thermosensitivity assay above to have two degrees difference in every step from 28 to 38°C with a break of 24°C after every step (Figure 3-2). Since Gr28bD excites cells after a threshold temperature [9], we introduced the 24°C break to reduce any residual effect of neuronal excitation in a step on the next one.

3.2.3 RNAi knockdown of candidate genes

Even though some of the orthologs were not thermosensitive in our misexpression assay (for example, Gr28bE), when expressed in Hot Cells in *Gr28b^{mi}* flies, the heat sensing abilities of the transgenic flies could be rescued to large extent. Since Gr28bD formed a fully functional channel upon expression in a heterologous system, one possible explanation for Gr28bE having thermosensitive properties in the hot cells is that the Hot Cells have accessory proteins that are important but not necessary for thermosensing. To test this possibility, we overexpressed several RNAi genes in Hot Cells of flies with heterozygous *Gr28b^{mi}* background. Given that Gr28bD is predicted to function in the membrane, an accessory protein important for its function is likely to be membrane-associated. Neely and colleagues identified several genes which when knocked down, altered thermotolerance of flies [101]. We tested if flies with reduced expression of a subset of these genes that are membrane associated. To confirm our positive results, we tested additional RNAi lines from VDRC for

candidate BDSC RNAi lines whose knockdown reduced avoidance scores in heterozygous *Gr28b^{mi}* mutant flies. During screening, we used an *empty-GAL4* line as a control. For verification experiments, we used *CS* to make heterozygous controls.

3.2.4 Work flow and statistical methods

To analyze our data, we followed a specific route (Figure 3-3). Since our data was non-parametric, we used a Kruskal-Wallis test followed by Dunn's post hoc test for comparisons of rescues or mutants with genetic controls. For analysis of values, for example, Avoidance Indices, for the same genotype across an experiment, we used a Friedman's test for dependent variables followed by post hoc Sign test for multiple comparisons. The p values were corrected per the number of comparisons with Bonferroni correction.

Figure 3-1: Schematic of Avoidance assay to study thermosensitivity.

Flies were introduced in a heat box chamber one half of which was maintained at 24°C. The temperature of the other half across a hypothetical midline increase in steps of 3°C from 24 to 39°C with every step lasting for 60s. Thus, the flies had 60 second to choose between the low and high temperature sides at every step. The hot side of the heat box alternated at every step to eliminate any bias the flies might have for a side. The Avoidance Index was calculated for every temperature step.

Figure 3-2: Schematic of Avoidance sensitivity assay to study thermosensitivity.

Flies were introduced in a heat box chamber in which a 2°C difference was maintained at every alternate step. Following a brief acclimation period of 30s, one half of the chamber was increased to 28°C and the other half to 30°C. After a 60s interval at 24°C, the temperature was again increased to provide a choice between 30 and 32°C for 60s. This trend was continued until the chambers reflected 36 and 38°C in the final step. The hot side of the heat box alternated at every step to eliminate any bias the flies might have for a side. The Avoidance Index was calculated for every temperature step.

Figure 3-3:Schematic of flow of statistical analyses.

Non-parametric tests were used to analyze the Avoidance Indices obtained from various experiments.

3.3 Results

3.3.1 Hot Cell specific overexpression of several Gr genes rescued thermosensitivity in mutant flies.

We tested if Hot Cell specific overexpression of orthologs rescued ability of *GR28b^{mi}* mutants to sense and avoid heat. Flies were made to overexpress *UAS-Gr* orthologs in hot cells using *HC-GAL4* in a *GR28b^{mi}* mutant background. Flies heterozygous for respective *UAS-Grs* in *GR28b^{mi}* mutant background were used as effector controls. Flies heterozygous for *HC-GAL4* in *GR28b^{mi}* mutant background were used as driver controls.

Flies rescued with Gr28bD, Gr28bE and Gr28bB in Hot Cells had significantly higher positive avoidance indices compared to their controls from temperature difference $\geq 6^{\circ}\text{C}$ (Figure 3-4). In contrast overexpression of Gr28bC did not rescue avoidance behavior in flies (Figure 3-4). Since the hot side of the heat box is alternated at every temperature step, flies have to cross over to the opposite side to avoid the hot side. The time they spend crossing over prevents the Avoidance Index (AI) from reaching 1. Hence, we consider an $\text{AI} \geq 0.8$ as “maximal avoidance”. Flies that were rescued with Gr28bD, DsimGr, DyakGr or DpseGr had median AI of ~ 0.8 from at $24\text{--}30^{\circ}\text{C}$ temperature choice (Figure 3-5). In contrast, flies that were rescued with DwilGr had a median AI of 0.8 at temperature difference $\geq 9^{\circ}\text{C}$ (Figure 3-5). Flies rescued with D. mojGr had an equivalent AI at an even higher difference of 15°C (Figure 3-5).

In order to test if flies rescued with orthologs were sensitive to temperature differences or to absolute temperatures, we subjected flies to a modified

thermosensitivity assay (Figure 3-6). Flies had to choose between a high and a low temperature sides that were two degrees apart. The temperature range 28-38°C encompassed the activation temperatures of Gr28bD, DsimGr, DyakGr, DpseGr and DwilGr. We observed that flies rescued with the orthologs were not sensitive to all temperature differences. For example, flies rescued with Gr28bD did not have significantly different AIs from the genetic controls at any tested temperature difference. At 28-30°C, and 32-34°C choices, all other rescues had significantly higher AIs than their respective genetic controls. At 30-32°C choice, the AI of DwilGr rescue was not significantly different from the driver control. Similarly, at 34-36°C choice, flies rescued with DsimGr and DpseGr did not have significantly different AIs than the driver control. At 36-38°C choice, the AIs of flies rescued with DsimGr and DyakGr were not significantly different from their effector controls. Thus DsimGr, DpseGr and DyakGr are probably not as sensitive to 2°C differences at higher temperatures as DwilGr.

To determine whether the AIs are affected by the intensity of the temperature, we performed a thorough analysis of differences in AIs of flies at lower temperatures and compared it with AIs at higher temperatures in the Avoidance sensitivity assay. As compared to 28-30°C choice, the median AIs of flies rescued with DsimGr, DpseGr and DyakGr was significantly reduced at 36-38°C choice. In contrast flies rescued with DwilGr had significantly higher median AI at 34-36°C as compared to 28-30°C choice. Flies rescued with Gr28bD had AIs that were indistinguishable across the experiment. Thus, our observations that certain orthologs are more sensitive at lower temperatures were corroborated by

comparisons within the rescues. Flies rescued with DmojGr had high AI at higher temperature differences (Figure 3-5). Since *D. melanogaster* flies are stressed at temperatures >40°C, we chose not to pursue this sensitivity test with flies rescued with DmojGr.

3.3.2 Several proteins are potentially important for hot cell mediated thermosensing in flies.

In our mis-expression studies, pan-neuronal Gr28bE overexpression did not paralyze flies within 24-40°C. Similarly, lack of inward current on Gr28bE expression in oocytes [9] bolstered the conclusion that Gr28bE is not thermosensitive. However, when overexpressed in hot cells, Gr28bE could rescue *Gr28b^{mi}* mutants' heat sensing ability. Ni and colleagues [8] also reported a similar finding showing that Gr28bE could substitute for lack of Gr28bD in Hot Cells. We sought to understand if Gr28bE activity is a Hot Cell specific phenomenon. We investigated the presence of accessory proteins of Gr28bD in Hot Cells that could potentially fine-tune the functions of certain proteins to enable them to function as thermosensors. To identify such proteins, we knocked down several genes that code for membrane or membrane-associated proteins in Hot Cells in heterozygous *GR28b^{mi}* background. The genes were a subset of a larger set of genes that are potentially involved in thermosensing (Neely et al, 2010). Following RNAi knockdown, we screened for changes in avoidance behavior (Figure 3-1). During screening, of the 26 of genes we tested, knockdown of 5 had significantly reduced avoidance behavior at different

temperature differences (**Error! Reference source not found.**) as compared to the driver control. Of these,

knockdown of 4 genes (*fz*, *CG5890*, *CG5160* and *mld*) created deficits in avoidance as compared to both the driver and effector controls (Figure 3-7). We confirmed our observations by knocking down these genes with RNAi lines from different source (VDRC) (Figure 3-8, **Error! Reference source not found.**).

Figure 3-4: Hot cell specific overexpression of several *Gr28b* genes rescued Avoidance behavior in *Gr28b^{mi}* mutants.

Flies overexpressing *UAS-GR28bB*, *UAS-GR28bE* or *UAS-GR28bC* genes in Hot cells were tested for avoidance behavior in the thermosensitivity assay (solid lines). Flies heterozygous for *HC-GAL4* driver in *Gr28b^{mi}* mutant background were used as driver control. Flies heterozygous for *UAS-Gr* genes in *Gr28b^{mi}* mutant background were used as effector controls (dotted lines). The plot shows the Avoidance Index of flies at different temperature choices. Colored vertical

lines indicate quartiles and the boxes depict the median AIs. Flies rescued with *UAS-Gr28bB*, *UAS-Gr28bD* and *UAS-Gr28bE* had significantly higher avoidance scores than their respective effector controls, and the driver control from temperature difference $\geq 6^{\circ}\text{C}$ with respect to 24°C . Kruskal-Wallis test followed by Dunn's post hoc test for multiple comparisons was used to determine statistical significance of rescues with respect to controls. After Bonferroni correction, $p < 0.025$ was considered to be statistically significant ($p < 0.00002 = ****$).

Figure 3-5: Hot Cell specific overexpression of Gr28bD or several orthologous genes rescued avoidance behavior in *Gr28b^{mi}* mutants.

Flies overexpressing *UAS-Gr28bD*, *UAS-DsimGr*, *UAS-DyakGr*, *UAS-DpseGr*, *UAS-DwilGr* or *UAS-DmojGr* genes in Hot Cells were tested for avoidance behavior in the thermosensitivity assay (solid lines). Flies heterozygous for *HC-GAL4* driver in *Gr28b^{mi}* mutant background were used as driver control. Flies heterozygous for *UAS-Gr* genes in *Gr28b^{mi}* mutant background were used as

effector controls (dotted lines). In the plot the median AIs (colored boxes) of the flies with the quartiles (vertical lines) are shown at different temperature choices. Flies rescued with all *UAS-Gr* genes had significantly higher avoidance scores than their respective effector controls and the driver control from temperature difference $\geq 6^{\circ}\text{C}$ with respect to 24°C . *UAS-Gr28bD*, *UAS-DsimGr*, *UAS-DyakGr* and *UAS-Dpse* rescues reached their maximal avoidance score within 6°C difference. Flies rescued with *UAS-DwilGr* reached their maximal avoidance at 9°C difference with respect to 24°C while those rescued with *UAS-DmojGr* had maximal avoidance scores at 15°C difference. The avoidance score of *DwilGr* rescue at $24\text{-}30^{\circ}\text{C}$ was significantly lower than the AIs of *DsimGr*, *DpseGr* and *DyakGr* rescues (black stars). Similarly, flies rescued with *DmojGr* had significantly reduced avoidance scores than other rescues at 6 , 9 , and 12°C differences (pink stars). Kruskal-Wallis test followed by multiple comparisons with Dunn's post hoc test was used for statistical comparisons. After Bonferroni correction, $p < 0.025$ was considered significant ($p < 0.00002 = ****$).

Figure 3-6: Flies rescued with Hot Cell specific overexpression of several Gr28bD or specific orthologous genes are sensitive to two degrees temperature differences.

Flies overexpressing *UAS-Gr28bD*, *UAS-DsimGr*, *UAS-DyakGr*, *UAS-DpseGr* or *UAS-DwilGr* in Hot Cells in *Gr28b^{mi}* mutant background were tested for their sensitivity to 2°C temperature differences in the range of their activation temperatures. *HC-GAL4, Gr28b^{mi}/Gr28b^{mi}* mutant flies were used as driver control. *UAS-Gr, Gr28b^{mi}/Gr28b^{mi}* flies were used as effector controls. The median AIs of the flies (colored boxes) along with the quartiles (vertical lines) are

shown for each genotype. Kruskal-Wallis test followed by Dunn's post hoc test was used for statistical comparisons of rescues with their genetic controls. While, flies rescued with Gr28bD did not have significantly different avoidance scores from the genetic controls at any tested temperature difference, flies rescued with DsimGR, DpseGr and DyakGr had significantly different avoidance scores from controls at 28-30, 30-32 and 32-34°C choices only. In contrast flies rescued with DwilGr had significantly higher avoidance scores than controls at 28-30, 32-34, 34-36 and 36-38°C choices ($p < 0.025$). Friedman's test followed by post hoc Sign test was used for statistical comparisons of AIs within the rescues. The median AIs of flies rescued with *UAS-DsimGr*, *UAS-DpseGr* and *UAS-DyakGr* was significantly reduced at 36-38°C choice as compared to 28-30°C choice ($p < 0.002$). In contrast flies rescued with *UAS-DwilGr* had significantly higher AI at 34-36°C as compared to 28-30°C choice ($p < 0.004$). The AIs of flies rescued with Gr28bD did not vary significantly across the experiment. Since there were two comparisons for every condition, after Bonferroni correction, $p < 0.025$ was considered statistically significant both categories of tests mentioned above.

Figure 3-7: Targeted knockdown of RNAi genes in Hot Cells alters thermosensitivity of flies.

Flies were made with specific candidate genes knocked down in hot cells in a heterozygous $Gr28b^{mi}$ mutant background. Heterozygous $Gr28b^{mi}$; UAS-empty RNAi flies were used as effector controls. Heterozygous HC-GAL4, $Gr28b^{mi}$ flies were used as driver control. In the plot, the dots depict the median AIs and the

vertical bars represent the quartiles of data for each genotype. Of all the knockdown mutants tested, flies with knockdown of *CG5890*, *CG5160*, *stan*, *fz*, *ninaE* or *CpN* had significantly reduced thermosensitivity than genetic control flies (circled). Kruskal-Wallis test followed by Dunn's post hoc test was used to determine statistical significance. $p < 0.025$ is considered significant after Bonferroni correction.

Figure 3-8: Knockdown of candidate RNAi genes in hot cells of heterozygous *Gr28b^{mi}* mutant flies alters thermosensitivity.

Flies heterozygous for *UAS-fz*, *UAS-CG5890*, *UAS-CG5160* or *UAS-mld* in heterozygous *Gr28b^{mi}* mutant background were used as effector controls. Flies heterozygous for *HC-GAL4* in heterozygous *Gr28b^{mi}* mutant background were

used as driver controls. In the plot, the dots depict the median AIs and the vertical bars represent the quartiles of data for each genotype. In contrast to the control flies, knockdown of *fz*, *CG6890*, *CG5160*, and *mld* significantly reduced thermosensitivity in flies when they had to choose between 24 and 33°C. Kruskal-Wallis test followed by Dunn's post hoc test was used to determine statistical significance. $p < 0.025$ is considered significant after Bonferroni correction.

3.4 Discussion

Fruit flies are tiny creatures whose body temperatures reflect the ambient temperature in few seconds. Thus, it is essential for flies to develop strategies to avoid high temperature areas [95]. The presence of Hot Cells which house the thermosensitive Gr28bD protein is one such adaptation. We rationalized that different the thermosensitive properties of Gr28bD are not limited to *D. melanogaster* alone. Flies indigenous to different climatic conditions will have Gr28bD orthologs that have adapted to their environment. We tested the hypothesis that different evolutionary backgrounds will have Gr28bD orthologs that have different temperature sensing properties.

Our tests revealed that orthologs from different *Drosophila* species have different temperature sensing properties. Mutant flies that were rescued with DyakGr, DpseGr, and DsimGr had significantly high avoidance scores from 24-33°C. Of these, *D. yakuba* flies reside in cool rainforests, and *D. pseudoobscura* flies occupy grasslands and semi-arid regions. *D. simulans* flies are cosmopolitan. In contrast *D. melanogaster* flies that were rescued with orthologs from flies that inhabit warmer regions like the tropics in Mexico (*D. willistoni*) or the Mojave Desert (*D. mojavensis*) had high avoidance scores at larger temperature differences (24-36°C, 24-40°C).

We had hypothesized that orthologs from flies from warmer regions will be more sensitive at higher temperatures compared to orthologs from cooler regions. Our observations proved our hypothesis. We found that flies overexpressing DwilGr were less sensitive at the lower temperatures and temperature differences

(Figure 3-5, Figure 3-6) in contrast to *DsimGr*, *DyakGr*, and *DpseGr* overexpressing flies that were more sensitive. Interestingly flies rescued with *Gr28bD* had indistinguishable avoidance scores in Avoidance sensitivity experiment. It might be that *Gr28bD* is not evolved to detect such low temperature differences. Unlike the other fly lines, the *Gr28bD* line is not an in-house line. There is a small chance that the genetic background might be affecting the thermosensing ability of flies despite overexpression of *Gr28bD* in the Hot Cells.

Interestingly, *Gr28bE*, a paralog of *Gr28bD* which was not temperature sensitive in our previous assays with pan-neuronal overexpression could also rescue the mutant flies' heat sensing ability when expressed in the Hot Cells. This observation inspired us to hypothesize that accessory proteins specific to hot cells that are important for thermosensing. The presence of accessory protein(s) that provides some redundancy to heat sensing or fine tune the function of thermosensitive proteins might be of evolutionary significance. In our bid to identify possible accessory proteins, we knocked down several genes one at a time in hot cells in heterozygous *Gr28b^{mi}* background. Targeted knockdown of membrane associated genes revealed *CG5890*, *CG5160*, *fz*, *mld* genes to be important for thermosensing in flies. KD of these genes reduced the flies' ability to sense heat. *frizzled (fz)* is an extensively studied gene that encodes a family of receptors to wingless (wnt) proteins. Along with several other proteins, *fz* helps establish planar cell polarity during development [102]. While the function of *CG5890* gene products have not been studied, their roles might lie in regulating

release of calcium from ER to cytoplasm in neurons during activation or downstream processing [103]. *molting-defective (mld)* expresses a nuclear zinc finger protein that is involved in biosynthesis of ecdysone; steroid hormones that regulate various events including molting [104], growth [105], sleep [106], immunity [107] and long term courtship memory [108] in insects. Unfortunately, there is barely any information on the role of *CG5160* gene products.

How could these proteins influence thermosensing in flies? Since signaling through *fz* is necessary for establishing cell polarity in sensory organ precursors that form hot cells [109,110], it is possible that altered development of Hot Cells in *fz* KD flies affected their thermosensing ability. While molting is a temperature sensitive process [106], there is no evidence for *mld* involvement in temperature sensing. Similarly, ecdysones have not been directly implicated in thermosensing. However, reduced expression of *cyp41*, a cytochrome involved in ecdysone biosynthesis decreased flies' ability to select a preferred temperature in a gradient assay [111], suggesting that ecdysone biosynthesis might be important for temperature sensing. Reduction in *mld* levels might be altering ecdysone biosynthesis thus leading to low thermosensitivity. Due to lack of information on the *CG5890* and *CG5160* gene products, it is difficult to establish any link between their involvement in temperature sensing. Further experiments that test temperature sensing in null mutants of the genes discussed above and trace the molecular mechanisms at different developmental stages are necessary to shed light on their specific roles in thermosensing in Hot Cells.

ur observations give us an opportunity to discuss the potential effect of the thermosensors on the neural circuitry of thermosensing in flies. Appropriate alteration of the neural circuit connected to the Hot Cells is as important as the thermosensor expressed in the Hot cells. In *D. melanogaster*, the Hot cells project their axons to the posterior antennal lobe (PAL) where they synapse on the dendrites of thermosensory Projection Neurons (tPNs). The tPNs project their axons to three higher order brain structures- the calyx of the Mushroom Body (MB), the Posterior Lateral Protocerebrum (PLP) and the Lateral Horn (LH) [55]. Typically, a thermosensor will alter the Resting Membrane Potential of the Hot Cells leading to excitation at raised temperatures which in turn would potentially excite warmth-specific PN's [112]. Even though Gr28bE and Gr28bD can rescue the *Gr28b^{mi}* mutants' heat sensing ability, there is no evidence for Gr28bE exciting neurons. Gr28bE was not functional in *Xenopus oocytes*, a heterologous system [9]. Additionally, Gr28bD is important to establish the microstructure in Hot cells during development [52]. It would be interesting to examine whether substitution of Gr28bD with Gr28bE in mutants results in a similar rescue in Hot Cell structure and the alteration in Hot Cell excitability responsible for thermosensing.

Table 3-1: RNAi KD of several genes in heterozygous *Gr28b^{mi}* background did not alter Avoidance behavior in flies.

Fly lines in this screen were obtained from the Bloomington Drosophila Stock Center

		24-24			24-27			24-30		
Genotype	N	Median	Lower quartile	Upper quartile	Median	Lower quartile	Upper quartile	Median	Lower quartile	Upper quartile
<i>HC-GAL4, Gr28b^{mi} > UAS-CadN-RNAi</i>	130	-0.15	-0.32	0.07	0.22	-0.07	0.56	0.21	-0.06	0.51
<i>HC-GAL4, Gr28b^{mi} > UAS-caps-RNAi</i>	128	-0.18	-0.29	-0.03	0.10	-0.11	0.33	0.13	-0.09	0.37
<i>HC-GAL4, Gr28b^{mi} > UAS-Rab2-RNAi</i>	123	-0.09	-0.25	0.05	0.18	-0.01	0.38	0.19	-0.02	0.56
<i>HC-GAL4, Gr28b^{mi} > UAS-Eps-15-RNAi</i>	118	-0.23	-0.37	-0.13	0.14	-0.08	0.39	0.22	-0.08	0.46
<i>HC-GAL4, Gr28b^{mi} > UAS-stan-RNAi</i>	119	-0.20	-0.28	-0.07	0.15	-0.01	0.39	0.29	0.07	0.62
<i>HC-GAL4, Gr28b^{mi} > UAS-fz-RNAi</i>	124	-0.16	-0.30	-0.04	0.20	-0.03	0.51	0.19	-0.05	0.52
<i>HC-GAL4, Gr28b^{mi} > UAS-Cpn-RNAi</i>	107	-0.19	-0.33	-0.04	0.04	-0.21	0.29	0.05	-0.21	0.34
<i>HC-GAL4, Gr28b^{mi} > UAS-fz-RNAi</i>	125	-0.13	-0.30	0.00	0.05	-0.08	0.31	0.07	-0.09	0.28
<i>HC-GAL4, Gr28b^{mi} > UAS-ninaE-RNAi</i>	109	-0.23	-0.38	-0.10	0.06	-0.19	0.33	0.13	-0.08	0.35
<i>HC-GAL4, Gr28b^{mi} > UAS-unc-13-4A-RNAi</i>	127	-0.15	-0.33	0.03	0.28	0.00	0.58	0.36	0.04	0.75
<i>HC-GAL4, Gr28b^{mi} > UAS-ctbp-RNAi</i>	133	-0.17	-0.34	-0.02	0.20	-0.06	0.50	0.29	-0.07	0.69
<i>HC-GAL4, Gr28b^{mi} > UAS-Eps-15-RNAi</i>	112	-0.12	-0.36	0.11	0.25	-0.04	0.59	0.33	0.08	0.62
<i>HC-GAL4, Gr28b^{mi} > UAS-fz-RNAi</i>	121	-0.15	-0.27	0.03	0.18	-0.08	0.55	0.31	0.06	0.60
<i>HC-GAL4, Gr28b^{mi} > UAS-Rab2-RNAi</i>	116	-0.15	-0.26	-0.03	0.14	-0.08	0.38	0.27	0.02	0.57
<i>HC-GAL4, Gr28b^{mi} > UAS-CadN-RNAi</i>	109	-0.20	-0.39	-0.03	0.18	-0.16	0.56	0.21	-0.12	0.58
<i>HC-GAL4, Gr28b^{mi} > UAS-AdoR-RNAi</i>	125	-0.10	-0.29	0.02	0.46	0.16	0.72	0.36	0.09	0.66
<i>HC-GAL4, Gr28b^{mi} > UAS-CG31183-RNAi</i>	119	-0.24	-0.39	-0.02	0.10	-0.17	0.58	0.31	-0.02	0.68
<i>HC-GAL4, Gr28b^{mi} > UAS-CG11638-RNAi</i>	109	-0.13	-0.27	0.01	0.17	-0.08	0.54	0.11	-0.16	0.40
<i>HC-GAL4, Gr28b^{mi} > UAS-emptyRNAi (attp2 control)</i>	119	-0.11	-0.21	-0.01	0.08	-0.08	0.34	0.08	-0.09	0.29

			24-33			24-36			24-39	
Genotype	N	Median	Lower quartile	Upper quartile	Median	Lower quartile	Upper quartile	Median	Lower quartile	Upper quartile
<i>HC-GAL4, Gr28b^{mi} > UAS-CadN-RNAi</i>	130	0.31	-0.01	0.58	0.53	0.20	0.78	0.74	0.43	0.88
<i>HC-GAL4, Gr28b^{mi} > UAS-caps-RNAi</i>	128	0.15	-0.08	0.37	0.31	0.11	0.53	0.54	0.34	0.79
<i>HC-GAL4, Gr28b^{mi} > UAS-Rab2-RNAi</i>	123	0.24	0.02	0.54	0.48	0.26	0.81	0.71	0.47	0.87
<i>HC-GAL4, Gr28b^{mi} > UAS-Eps-15-RNAi</i>	118	0.23	0.01	0.45	0.32	0.12	0.65	0.62	0.39	0.84
<i>HC-GAL4, Gr28b^{mi} > UAS-stan-RNAi</i>	119	0.44	0.15	0.72	0.77	0.44	0.92	0.83	0.64	0.91
<i>HC-GAL4, Gr28b^{mi} > UAS-fz-RNAi</i>	124	0.25	-0.03	0.53	0.43	0.13	0.68	0.56	0.38	0.78
<i>HC-GAL4, Gr28b^{mi} > UAS-Cpn-RNAi</i>	107	0.13	-0.18	0.39	0.18	0.00	0.49	0.47	0.27	0.80
<i>HC-GAL4, Gr28b^{mi} > UAS-fz-RNAi</i>	125	0.11	-0.01	0.32	0.22	0.06	0.49	0.44	0.25	0.71
<i>HC-GAL4, Gr28b^{mi} > UAS-ninaE-RNAi</i>	109	0.12	-0.12	0.36	0.19	0.04	0.42	0.31	0.17	0.59
<i>HC-GAL4, Gr28b^{mi} > UAS-unc-13-4A-RNAi</i>	127	0.46	0.17	0.75	0.61	0.32	0.89	0.82	0.66	0.91
<i>HC-GAL4, Gr28b^{mi} > UAS-ctbp-RNAi</i>	133	0.32	0.05	0.62	0.52	0.11	0.83	0.76	0.56	0.89
<i>HC-GAL4, Gr28b^{mi} > UAS-Eps-15-RNAi</i>	112	0.43	0.06	0.76	0.42	0.21	0.72	0.66	0.39	0.85
<i>HC-GAL4, Gr28b^{mi} > UAS-fz-RNAi</i>	121	0.25	0.01	0.58	0.40	0.22	0.76	0.70	0.44	0.84
<i>HC-GAL4, Gr28b^{mi} > UAS-Rab2-RNAi</i>	116	0.24	0.01	0.55	0.44	0.22	0.71	0.54	0.33	0.81
<i>HC-GAL4, Gr28b^{mi} > UAS-CadN-RNAi</i>	109	0.34	0.06	0.65	0.43	0.18	0.79	0.68	0.43	0.82
<i>HC-GAL4, Gr28b^{mi} > UAS-AdoR-RNAi</i>	125	0.37	0.11	0.70	0.41	0.21	0.66	0.63	0.45	0.83
<i>HC-GAL4, Gr28b^{mi} > UAS-CG31183-RNAi</i>	119	0.27	-0.14	0.69	0.44	0.11	0.79	0.69	0.39	0.92
<i>HC-GAL4, Gr28b^{mi} > UAS-CG11638-RNAi</i>	109	0.30	0.00	0.56	0.41	0.12	0.72	0.67	0.41	0.85

Table 3-2: RNAi KD of several genes decreased flies' AIs (pink shade).

These lines were obtained from VDRC.

Genotype	N	24-24			24-27			24-30		
		Median	Lower Quartile	Upper Quartile	Median	Lower Quartile	Upper Quartile	Median	Lower Quartile	Upper Quartile
Column1	Column2	Column3	Column4	Column5	Column6	Column7	Column8	Column9	Column10	Column11
UAS-CpN-RNAi/+ (KK 101962)	121	-0.11	-0.27	0.10	0.22	0.01	0.52	0.21	0.04	0.50
HC-GAL4,G/28b TM >UAS-CpN-RNAi (KK 101962)	113	-0.07	-0.19	0.03	0.13	-0.04	0.31	0.20	0.03	0.40
UAS-Iz-RNAi/+ (KK 105493)	118	-0.10	-0.25	0.04	0.26	-0.05	0.56	0.26	0.01	0.53
HC-GAL4,G/28b TM >UAS-Iz-RNAi (KK 105493)	119	-0.11	-0.21	0.00	0.16	0.01	0.36	0.21	0.04	0.42
UAS-CpN-RNAi/+ (GD 13086)	110	-0.19	-0.44	-0.02	0.15	-0.07	0.56	0.50	0.13	0.73
HC-GAL4,G/28b TM >UAS-CpN-RNAi (GD 13086)	105	-0.20	-0.35	-0.02	0.15	-0.13	0.56	0.21	-0.05	0.62
UAS-CG5160/+ (GD 22019)	111	-0.18	-0.30	-0.02	0.17	-0.04	0.43	0.21	-0.02	0.56
HC-GAL4,G/28b TM >UAS-CG5160-RNAi (GD 22019)	109	-0.22	-0.36	-0.04	0.13	-0.10	0.36	0.17	-0.07	0.53
UAS-Iz-RNAi/+ (GD 43077)	107	-0.14	-0.41	0.06	0.15	-0.12	0.63	0.42	0.08	0.71
HC-GAL4,G/28b TM >UAS-Iz-RNAi (GD 43077)	105	-0.23	-0.36	-0.01	0.20	-0.07	0.59	0.30	0.00	0.63
UAS-ninaE-RNAi/+ (GD 44178)	107	-0.22	-0.39	-0.06	0.15	-0.07	0.44	0.27	0.07	0.55
HC-GAL4,G/28b TM >UAS-ninaE-RNAi (GD 44178)	110	-0.23	-0.35	-0.12	0.34	0.07	0.75	0.27	0.05	0.55
UAS-mld/+ (GD 17329)	87	-0.16	-0.32	-0.03	0.18	0.04	0.57	0.32	0.07	0.66
HC-GAL4,G/28b TM >UAS-mld-RNAi (GD 17329)	92	-0.24	-0.36	-0.11	0.19	-0.11	0.42	0.13	-0.05	0.35
UAS-ctbp-RNAi/+ (GD 37609)	83	-0.25	-0.41	-0.11	0.19	0.01	0.50	0.14	-0.05	0.49
HC-GAL4,G/28b TM >UAS-ctbp-RNAi (GD 37609)	76	-0.35	-0.45	-0.16	0.17	-0.11	0.58	0.28	0.01	0.66
UAS-stan-RNAi/+ (GD 51379)	55	-0.31	-0.49	-0.08	0.05	-0.30	0.48	0.05	-0.40	0.40
HC-GAL4,G/28b TM >UAS-stan-RNAi (GD 51379)	74	-0.36	-0.58	-0.16	0.12	-0.17	0.60	0.21	-0.25	0.57
UAS-stan-RNAi/+ (GD 51382)	90	-0.26	-0.40	-0.15	0.15	-0.19	0.62	0.15	-0.10	0.40
HC-GAL4,G/28b TM >UAS-stan-RNAi (GD 51382)	88	-0.31	-0.47	-0.14	0.25	-0.14	0.48	0.25	-0.12	0.60
UAS-ctbp-RNAi/+ (KK 107313)	87	-0.08	-0.21	0.01	0.09	-0.02	0.27	0.09	-0.04	0.25
HC-GAL4,G/28b TM >UAS-ctbp-RNAi (KK 107313)	94	-0.13	-0.22	-0.03	0.16	-0.02	0.38	0.08	-0.09	0.22
UAS-stan-RNAi/+ (KK 107993)	97	-0.20	-0.30	-0.08	0.10	-0.10	0.29	0.13	-0.07	0.30
HC-GAL4,G/28b TM >UAS-stan-RNAi (KK 107993)	90	-0.14	-0.27	-0.07	0.09	-0.05	0.24	0.06	-0.11	0.22
UAS-unc-13-4A-RNAi/+ (KK 109304)	89	-0.17	-0.29	-0.01	0.08	-0.06	0.32	0.19	0.06	0.47
HC-GAL4,G/28b TM >UAS-unc-13-4A-RNAi (KK 109304)	92	-0.10	-0.26	0.09	0.22	-0.01	0.52	0.14	-0.03	0.39
UAS-CG5890/+ (KK 105766)	86	-0.13	-0.33	-0.06	0.09	-0.04	0.35	0.15	-0.02	0.28
HC-GAL4,G/28b TM >UAS-CG5890-RNAi (kk 105766)	107	-0.12	-0.25	-0.03	0.12	-0.12	0.30	0.11	-0.13	0.28
HC-GAL4,G/28b TM / ⁺	125	-0.16	-0.38	0.00	0.15	-0.11	0.45	0.26	0.02	0.58

Genotype	N	24-33			24-36			24-39		
		Median	Lower Quartile	Upper Quartile	Median	Lower Quartile	Upper Quartile	Median	Lower Quartile	Upper Quartile
UAS-CpN-RNAi/+ (KK 101962)	121	-0.11	-0.27	0.10	0.22	0.01	0.52	0.21	0.04	0.50
HC-GAL4,G/28b TM >UAS-CpN-RNAi (KK 101962)	113	-0.07	-0.19	0.03	0.13	-0.04	0.31	0.20	0.03	0.40
UAS-Iz-RNAi/+ (KK 105493)	118	0.31	0.14	0.49	0.45	0.25	0.72	0.65	0.37	0.85
HC-GAL4,G/28b TM >UAS-Iz-RNAi (KK 105493)	119	0.16	0.07	0.37	0.27	0.11	0.42	0.38	0.22	0.58
UAS-CpN-RNAi/+ (GD 13086)	110	0.45	0.18	0.77	0.73	0.40	0.88	0.79	0.63	0.88
HC-GAL4,G/28b TM >UAS-CpN-RNAi (GD 13086)	105	0.32	0.06	0.61	0.33	0.14	0.66	0.56	0.29	0.88
UAS-CG5160/+ (GD 22019)	111	0.27	0.08	0.59	0.38	0.21	0.72	0.72	0.49	0.87
HC-GAL4,G/28b TM >UAS-CG5160-RNAi (GD 22019)	109	0.14	-0.02	0.37	0.31	0.09	0.45	0.40	0.26	0.67
UAS-Iz-RNAi/+ (GD 43077)	107	0.38	0.16	0.70	0.46	0.23	0.80	0.74	0.41	0.87
HC-GAL4,G/28b TM >UAS-Iz-RNAi (GD 43077)	105	0.28	0.06	0.62	0.39	0.13	0.63	0.56	0.25	0.84
UAS-ninaE-RNAi/+ (GD 44178)	107	0.34	0.11	0.64	0.57	0.15	0.80	0.79	0.53	0.90
HC-GAL4,G/28b TM >UAS-ninaE-RNAi (GD 44178)	110	0.42	0.05	0.72	0.35	0.15	0.69	0.71	0.40	0.89
UAS-mld/+ (GD 17329)	87	0.46	0.15	0.81	0.65	0.36	0.82	0.78	0.51	0.90
HC-GAL4,G/28b TM >UAS-mld-RNAi (GD 17329)	92	0.12	-0.07	0.43	0.30	0.09	0.70	0.56	0.22	0.82
UAS-ctbp-RNAi/+ (GD 37609)	83	0.34	0.03	0.64	0.31	0.09	0.62	0.62	0.38	0.85
HC-GAL4,G/28b TM >UAS-ctbp-RNAi (GD 37609)	76	0.20	0.01	0.55	0.36	0.12	0.61	0.37	0.16	0.58
UAS-stan-RNAi/+ (GD 51379)	55	0.33	-0.01	0.63	0.37	-0.04	0.63	0.66	0.18	0.85
HC-GAL4,G/28b TM >UAS-stan-RNAi (GD 51379)	74	0.25	-0.11	0.59	0.19	-0.14	0.65	0.43	0.17	0.78
UAS-stan-RNAi/+ (GD 51382)	90	0.24	-0.05	0.48	0.31	0.06	0.65	0.55	0.22	0.81
HC-GAL4,G/28b TM >UAS-stan-RNAi (GD 51382)	88	0.22	-0.05	0.52	0.29	0.04	0.69	0.50	0.26	0.72
UAS-ctbp-RNAi/+ (KK 107313)	87	0.10	-0.02	0.33	0.17	0.06	0.38	0.38	0.19	0.57
HC-GAL4,G/28b TM >UAS-ctbp-RNAi (KK 107313)	94	0.09	-0.05	0.30	0.16	0.00	0.30	0.24	0.10	0.42
UAS-stan-RNAi/+ (KK 107993)	97	0.12	-0.06	0.28	0.22	0.09	0.50	0.37	0.18	0.65
HC-GAL4,G/28b TM >UAS-stan-RNAi (KK 107993)	90	0.12	-0.04	0.28	0.19	0.00	0.35	0.37	0.21	0.52
UAS-unc-13-4A-RNAi/+ (KK 109304)	89	0.23	0.06	0.40	0.28	0.13	0.61	0.44	0.25	0.76
HC-GAL4,G/28b TM >UAS-unc-13-4A-RNAi (KK 109304)	92	0.21	0.04	0.44	0.27	-0.01	0.55	0.40	0.18	0.62
UAS-CG5890/+ (KK 105766)	86	0.20	0.07	0.38	0.25	0.09	0.45	0.40	0.25	0.64
HC-GAL4,G/28b TM >UAS-CG5890-RNAi (kk 105766)	107	0.10	-0.04	0.31	0.16	0.00	0.35	0.30	0.15	0.50

4 Exploring the role of dopaminergic neurons in place learning and memory in flies using a thermogenetic tool.

4.1 Introduction

The ability to learn, i.e., alter one's behavior based on experience while a stimulus is being provided, and the ability to remember, i.e., retrieve learned information in absence of said stimulus, is crucial for an organism's survival. Biogenic amines like dopamine and serotonin form the functional connections that mediate learning and memory. By far, dopamine is the most well studied biogenic amine and neurotransmitter. Dopaminergic signalling has been implicated in learning and memory in both vertebrates and invertebrates. In vertebrates, dopamine regulates working memory formation [113], reward seeking behaviour [114], determines the value of a stimulus [115–117] and sets the motivation to work [118]. Dopamine is also involved in forming associative memories [119–121] in both classical and operant conditioning.

Experiments in invertebrates, specifically in fruit flies, have provided valuable insights into the mechanistic aspects of dopaminergic signalling that regulate learning and memory formation. With an arsenal of transgenic flies, molecular tools, behavioral paradigms, and knowledge of brain circuitry, fruit flies make an

excellent system for determining the role of dopamine in learning and memory. Dopaminergic neurons in fruit flies can be grouped into 8 distinct clusters [14]. The processes from these clusters project into different areas of the fly brain. For example, dopaminergic neurons in the anterior medial protocerebrum (PAM) and in the posterior lateral protocerebrum (PPL1) clusters synapse primarily with the Mushroom body, the seat of associative memory formation [122,123] in *Drosophila melanogaster* brain, while those in the PPM3 cluster project to the central complex [14]. Even though the Mushroom body is known to mediate olfactory and gustatory learning and memory formation [41,124], it was found to be unimportant for place learning [125]. Instead the central complex that regulates locomotor behavior [126], orientation [127–129] and sleep-wake behaviour (Liu, Liu, Kodama, Driscoll, & Wu, 2012) was found to be required for visual pattern learning [131], spatial orientation memory [65,132] and has been suggested to be involved in place learning.

In fruit flies, dopamine has been shown to be important for courtship learning (Berry, 2012a), forgetting [133], olfactory learning and memory formation [134,135], retrieval and reconsolidation (Felsenberg et al., 2018; Felsenberg, Barnstedt, Cognigni, Lin, & Waddell, 2017) and gustatory learning and memory [62,137–139]. Early studies have implicated that serotonin is necessary for place learning and memory formation in the heat-box paradigm (Putz & Heisenberg, 2002; Sitaraman et al., 2008; Wustmann & Heisenberg, 1997; Zars, Fischer, Schulz, & Heisenberg, 2000) while dopamine is not [16,143]. These studies targeted a subset of dopaminergic neurons covered under the *TH-GAL4* driver

which targets dopaminergic neurons that have been shown to regulate aversive learning [144,145]. However, the *TH-GAL4* driver sparsely encompasses the PAM cluster of dopaminergic neurons (Liu et al., 2012) that typically enable appetitive learning and memory formation [61,139,147,148]. So, while initial conclusions were that dopamine is not involved in place learning and memory in flies [16], the recent realization that a cluster of dopaminergic neurons was not tested in these studies leaves open the question. That is, whether the PAM cluster of dopaminergic neurons modulates place learning and memory is unknown.

Recent studies have implicated the PAM cluster of dopaminergic neurons in modulating spatial learning and memory [66] and have shown that visual and olfactory memory share common circuitry [63]. We sought to examine whether the PAM dopaminergic neurons modulate place learning and memory. We hypothesized that conditional activation of PAM dopaminergic neurons that modulate appetitive learning and memory will alter aversive place learning and memory in the heat-box paradigm. In this paradigm, the flies must choose between a high temperature associated side (punishment side) and a room temperature associated side in a small chamber devoid of other stimuli. Learning is associated with increased preference for the room temperature associated side as opposed to the punishment side and memory is continued preference of the room temperature associated side in the absence of the punishment stimulus. We used one of our new thermogenetic tools, Gr28bD [8,9] to use the high temperature that flies experience when they choose the punishment side to

activate subsets of dopaminergic neurons. In this case, activation of the PAM dopaminergic neurons by high temperature would be potentially rewarding to the flies. The aversive high temperature stimulus will thus be associated with the rewarding properties of PAM dopaminergic neurons' activation, and therefore interfere with appropriate place learning. We also investigated whether conditional thermogenetic activation of *TH-GAL4* neurons that encompass the non-PAM clusters of dopaminergic neurons influences place learning and memory. In this case, both the high temperatures and activation of non-PAM neurons are potentially "aversive". Through our analyses, we sought to understand whether the finer components of fly behaviour, such as approach to the punishment associated side and total time spent there were altered during the place learning paradigm. We determined that appetitive and aversive dopaminergic neurons contribute differently to place learning and memory when dopamine release from these neurons interacts with an aversive high temperature stimulus. In doing so, we provide the first evidence that dopamine, released from PAM neurons, is able to influence place learning and memory.

4.2 Methods

4.2.1 Flies and maintenance

The *UAS-Gr28bD* line was a generous contribution of Paul Garritty. The *0273-GAL4* line was a gift from Scott Waddell. The *TH-GAL4* line was obtained from BDSC. Flies were made to overexpress *UAS-Gr28bD* in PAM dopaminergic neurons with *0273-GAL4* and *R58E02-GAL4*. *TH-GAL4* was used to overexpress *UAS-Gr28bD* in non-PAM dopaminergic neurons. An in-house *Canton-S* (CS)

line was used to make controls. Flies heterozygous for *UAS-Gr28bD*, *TH-GAL4* and *R58E02-GAL4* were used as genetic controls. Flies were maintained on cornmeal-yeast medium at 25°C in a 12 hours light-dark cycle at 60% relative humidity. Adult flies used in experiments were 2–10 days old.

4.2.2 Behavioral assays

4.2.2.1 Place learning and memory paradigm

The heat box paradigm has been described in detail previously (Putz & Heisenberg, 2002; Zars, Wolf, Davis, & Heisenberg, 2000). Briefly, flies acclimate at 24°C for 30s (pre-training) following which they undergo a training phase for 10 mins. During the training phase, one side of the heat-box is associated with a “punishment” temperature. When flies cross a hypothetical mid-line to the punishment associated side, the whole chamber heats up to the punishment temperature (Figure 1). The chamber cools down when the flies move to the non-punishment associated side. Typically, in few minutes, flies learn to avoid the “place” associated with punishment and prefer to stay in the non-punishment associated side. The final phase tests the memory of the flies by measuring their “place” preference at 24°C for 3 mins (Figure 4-1). The Performance Index (PI) is calculated as follows: (time spent by the flies in the cool side-time spent by flies in the warm side)/total time spent in the chamber. A positive PI suggests that the flies preferred the cool side while a PI of negative PI indicates the flies’ preference to the punishment side.

4.2.2.2 Activity test

Flies were allowed to acclimate at 24°C for 30s. They were exposed to a high temperature stimulus (36, 38 or 40°C) for 2 minutes followed by a recovery period for 3 minutes. Their activity was measured every 0.1s at 126 different positions in the heat-box and categorized into 30s intervals for analysis (Figure 4-2).

4.2.3 Work flow and statistical methods

Flies were tested for the learning and memory paradigm and the activity paradigm. Different aspects of behaviour during learning were measured. The data were analysed as per the flowchart in Figure 4-3 with R, Statistica 8.0 (StatSoft, USA), and XLSTAT 2019.

4.3 Results

4.3.1 PAM dopaminergic neurons modulate place learning and memory in a temperature dependent manner.

Previous experiments showed that dopamine is not involved in modulating place learning and memory in the heat-box paradigm [16]. However, those experiments assumed that the commonly used *TH-GAL4* was effective in all dopaminergic neurons. Since then, there is updated information that in flies, a cluster of dopaminergic neurons in the PAM cluster is sparsely covered by *TH-GAL4* (Liu et al., 2012). In this study we tested if this group of neurons influences place learning and memory.

Flies that overexpressed Gr28bD in PAM dopaminergic neurons (*0273-GAL4>UAS-Gr28bD* and *R58E02-GAL4>UAS-Gr28bD* flies) had abnormal conditioned behaviour on being trained at 36 or 38°C (Figures 4A and 4B). The training scores of *0273-GAL4>UAS-Gr28bD* flies were significantly lower than heterozygous controls. Despite the differences in their training scores (learning phase), test scores (memory phase) at 36 and 38°C were not significantly different from controls (Figure 4A). *R58E02-GAL4>UAS-Gr28bD* flies had reduced learning at 36°C but their memory was intact. While their training PI was significantly lower than both the controls at the 0.05 level, their test PIs were not significantly different (Figure 4-4B).

The alteration in conditioned behaviour was temperature dependent. At 40°C, the learning and memory scores of *0273-GAL4>UAS-Gr28bD* and *R58E02-*

GAL4>UAS-Gr28bD flies were normal and not significantly different from their heterozygous controls (Figures 4A and 4B). Incidentally, learning and memory of *R58E02-GAL4>UAS-Gr28bD* flies was unaltered at 38°C (Figure 4B). *0273-GAL4* spans several other neurons in addition to the PAM cluster [66,150] such as cholinergic neurons. Since *R58E02-GAL4* spans smaller number of neurons than *0273-GAL4*, and is specific to dopaminergic neurons, the lack of change in behavior could be attributed to activation of lesser number of neurons (Burke et al., 2012; Liu et al., 2012). Activation of PAM dopaminergic neurons might not be sufficient to influence place learning when flies are exposed to more aversive temperatures. In contrast to observations with activation of PAM dopaminergic neurons, conditioned behaviour of flies overexpressing *UAS-Gr28bD* in non-PAM neurons was unaltered (Figure 4A). There were no significant differences in learning or memory scores at any of the conditioning temperatures. The pre-training scores in all experiments did not vary suggesting that the flies were not biased towards a side. As expected, flies had significantly lower learning scores during training at lower temperatures as opposed to higher temperatures (Zars & Zars, 2006).

4.3.2 Activity at high temperatures was altered for specific genotypes at certain temperatures.

In the heat-box, flies orient themselves in the dark to navigate the paradigm successfully [140]. A locomotor deficit would prevent them from performing and give false results. To ensure that the locomotor activity of flies was unaltered, we

exposed the flies to an “activity assay” (Figure 4-2), and recorded their median activity scores every 30s (Table 1). We observed that the activity of *R58E02-GAL4>UAS-Gr28bD* flies were comparable to their respective heterozygous genetic controls during high temperature exposures. Similarly, activities of the *TH-GAL4>UAS-Gr28bD* flies were largely unaltered compared to the heterozygous controls. They moved slower than their heterozygous controls during the first minute of exposure to 38°C but that difference in activity ameliorated in the second minute of the exposure. However, *0273-GAL4>UAS-Gr28bD* flies had significantly reduced activity after 30s of high temperature exposure to 36 or 38°C as compared to the genetic controls. The reduction in activity scores was not severe as the scores were >150. In fact, several of the tested fly lines had reduced median activities (<100) during 40°C exposure (Table 1). The activities of any tested line were however, not different from their heterozygous genetic controls. Despite their reduced movement, a one-way Wilcoxon Signed Ranks test showed that the median activities of the flies were >0. There was a general increase in locomotor activity of all flies with increase in temperature and decrease in activity following the sudden decrease in temperature to 24°C. These phenomena have been observed previously and are well documented [152].

4.3.3 Approach to hot side is altered in the place learning and memory paradigm at specific temperatures.

A decrease in learning score could either be a consequence of the flies

approaching the high temperature associated side more frequently or due to the flies spend spending longer time in the high temperature associated side, or both. To investigate these components of fly behavior, we measured the number of approaches to the heat-associated side and the total time spent by flies in the heat-associated side during the training phase. When conditioned at 36°C, flies overexpressing *UAS-Gr28bD* in their PAM dopaminergic neurons, i.e., *0273-GAL4>UAS-Gr28bD* and *R58E02-GAL4>UAS-Gr28bD* flies spent significantly more time in the heat-associated side as compared to their respective genetic controls (Figure 4-5A). However, the total number of approaches did not differ significantly (Figure 4-5B). *0273-GAL4>UAS-Gr28bD* flies spent explored the heat-associated side for significantly longer duration as compared to control flies during training at 38°C (Figure 4-5C). Similarly to their behaviour in 36°C, their number of approaches to the heat-associated side however did not vary significantly at 38°C (Figure 4-5D). *R28E02-GAL4>UAS-Gr28bD* flies that were trained at 40°C approaches the heat-associated side significantly lesser number of times as compared to control flies (Figure 4-5F). In contrast, the total time spent in the heat-associated side did not vary significantly (Figure 4-5E). Additionally, the median number of approaches and hot side dwells of *R58E02-GAL4>UAS-Gr28bD* flies that were trained at 38 or 40°C were significantly lower than those trained at 36°C (Mann-Whitney U test, $p < 0.019$). Similarly, *0273-GAL4>UAS-Gr28bD* flies that were trained at 36 or 38°C approached and dwelled in the heat-associated side significantly more than those trained at 40°C (Mann-Whitney U tests, $p < 0.001$). Our observations suggest that the low learning

scores might be due to the flies spending longer time in the heat-associated side rather than a variation in the number of approaches.

4.4 Discussion

While the role of serotonin and octopamine in place learning and memory has been well elucidated [16,17,143], the role of dopamine is unclear. Previous experiments showed that dopamine does not influence place learning and memory. However, those experiments excluded the now well studied PAM dopaminergic neurons that help reinforce appetitive memory (Burke et al., 2012; Liu et al., 2012). Here, we show that thermogenetic activation of PAM dopaminergic neurons alters place learning in a temperature dependent manner. Place memory, however, is not altered to statistical significance.

We tested the role of PAM and non-PAM dopaminergic neurons in aversive place learning and memory. In this operant paradigm, flies are exposed to an aversive high temperature stimulus when they move to one side of a chamber. Returning to the other side cools the chamber down. Flies learn that high-temperature associated side is aversive while the other side is rewarding [149]. In our experiments, every time a *0273-GAL4>UAS-Gr28bD* fly is in the high-temperature associated side, the PAM neurons presumably are activated, thus releasing dopamine [153,154]. Hence, a potentially rewarding neural stimulus (dopamine release) is combined with the aversive environmental stimulus (heat). In *TH-GAL4>UAS-Gr28bD* flies, activation of non-PAM neurons that are known to reinforce aversive memories are combined with an aversive heat stimulus. The heterozygous controls experience only the aversive heat stimulus in the high-temperature associated side. We observed that activation of PAM dopaminergic

neurons alters the learning scores of flies at 36 and 38°C. The low learning scores of flies upon activation of PAM neurons at 36 (*0273-GAL4>UAS-Gr28bD*, *R58E02-GAL4>UAS-Gr28bD*) and 38°C (*0273-GAL4>UAS-Gr28bD*) could be attributed to the release of dopamine that makes the aversive stimulus seem appetitive. On the other hand, activation of *TH-GAL4>UAS-Gr28bD* neurons did not significantly increase aversion to heat. Combined with previous knowledge that inactivation of *TH-GAL4* neurons does not alter place learning and memory [16], perhaps, the non-PAM dopaminergic neurons indeed do not play any role in place learning and memory.

The alteration in conditioned behavior of flies depended on the strength of the aversive stimulus. Flies experience a temperature difference of 12 or 14 degrees when they are trained at 36 and 38°C respectively. Even though flies are uncomfortable, these temperatures and temperature differences might not be as noxious as being exposed to 16 degrees difference at 40°C. This was reflected in our observations. Flies that were trained at lower temperatures approached the heat-associated side more. At uncomfortable temperatures such as 36 °C, the thermogenetic stimulation of appetitive PAM dopaminergic neurons was sufficient to override flies' innate aversion to heat (Figures 4A and 4B). We hypothesized that the flies would spend more time in the high-temperature associated side without serious side-effects, thus decreasing the learning scores. Indeed, the flies that had significantly higher dwelling times also had significantly lower learning scores. The higher learning scores at 40°C might more potently activate the flies' innate aversion to noxious stimuli which in turn would override the

“desire” to approach and stay in the high-temperature associated side induced by activation of PAM dopaminergic neurons, thus resulting in a higher learning score. During training at 40°C, *R58E02-GAL4>UAS-Gr28bD* and *0273-GAL4>UAS-Gr28bD* flies not only approached the heat-associated side less, but also remained on that side for less time (Table 1). Their learning scores were not significantly different from controls but were higher than those trained at lower temperatures.

We ensured that the locomotor activities of flies were not differentially affected by the high temperature stimuli during training phases. In our activity tests, flies were exposed to high temperature for 2 minutes continuously. Unlike the place learning paradigm, during the activity test the flies do not have a choice to avoid the high temperature stress. We specifically analyzed their activities during high temperature exposure. The activities of *R58E02>UAS-Gr28bD* and *TH-GAL4>UAS-Gr28bD* flies were not significantly different from their controls at 36 and 40°C. Even though activity of *0273-GAL4>UAS-Gr28bD* flies were significantly lower than genetic controls during exposure to 36 and 38°C, the locomotor deficit was not severe as the median activity scores were significantly >0. At 36 and 38°C, the high temperature stress did not affect their locomotor activities in the heat box severely, thus confirming that the difference in learning scores in flies overexpressing *UAS-Gr28bD* in PAM dopaminergic neurons was a result of temperature dependent hyperactivation of dopaminergic neurons. At 40°C, the activity scores were lower in general. Thus, in the place learning paradigm, it is possible that the flies avoided the high temperature associated

side as it was too noxious for them.

Taken together, our data provide the first demonstration that dopamine, released from PAM neuron clusters, can influence place learning and memory in fruit flies. These observations serve as a platform to reassess the role of dopamine in operant conditioning procedures where stimuli with opposing valence are presented to flies. Organisms encounter different combinations of appetitive and aversive stimuli in nature. Generally, animals consider the interaction between the stimuli [155,156] to develop an optimal response [157–159]. While the neural mechanisms underlying appetitiveness and aversiveness alone are well studied [16,17,160–163], few studies have examined the neural basis of response to the interaction of appetitive and aversive stimuli. In a classical conditioning paradigm, when sugar laced with quinine is provided to flies along with an olfactory cue, hungry flies prefer the sugar reward that contains a below threshold levels of quinine [62]. Similarly, crabs that are subjected to a looming visual stimulus associated with a food reward developed aversive memories that are dependent on the strength of the stimulus [164]. Dopamine was found to modulate the formation of these appetitive and aversive memories in both cases.

Although classical conditioning procedures have shed light on how organisms process simultaneous presentation of risk and reward, these procedures do not address the animals' preference during training. Studies using operant conditioning procedures on mammals found that dopamine mediates reward learning and approach [165]. Few studies have examined the role of biogenic amines in operant paradigms using insects. In honey bees, both dopamine and

octopamine regulate avoidance learning in a place preference test [161], while dopamine regulates aversive memory formation and octopamine mediates appetitive memory formation in crickets [166]. In our experiments, an aversive heat stimulus was combined with either appetitive stimulus (activation of PAM dopaminergic neurons) or more aversive stimulus (activation of non-PAM dopaminergic neurons). Comparable to findings in the classical conditioning experiments, flies were attracted to the aversive stimulus if it was below a threshold. Flies preferred the warm side during training when the temperature was $<40^{\circ}\text{C}$ (Figure 4-4). Thus, we show that in the heat-box paradigm, flies consider both the valences of the two components, and the consequence of their interaction to determine an optimal response. In future, it will be interesting to address brain regions involved in this novel context of dopaminergic modulation.

Figure 4-1: Schematic of place learning and memory paradigm in the heat-box.

Pre-training: Flies were allowed to acclimate at 24°C for 30s in a small chamber.

Training: One side of the chamber (A) is associated with a conditioning temperature of 36°C for 10 minutes. When the fly moves to side A, the whole chamber heats up to 36°C. The chamber cools down to 24°C when the fly moves to side B. Test: The chamber temperature is maintained at 24°C. At each phase in the conditioning paradigm the PI of the fly is calculated to measure the fly's preference to a side of the chamber. Adapted from the Mishra et al. article in this issue.

Figure 4-2: Schematic of activity assay in the heat-box.

Acclimation: Flies were allowed to acclimate at 24°C for 30s. High-temperature

exposure: Flies were exposed to a high temperature stimulus, for example, 36°C,

for 2 minutes. Recovery: Activity of flies were recorded for an additional 3

minutes at 24°C.

Figure 4-3:Schematic of flow of experiments and data analysis.

The learning and memory of flies were tested in the heat box. The PIs were obtained from “heat-calc”, an associated software. The number of approaches and the total time spent by flies on the heat-associated side were analysed separately using R. Since the data were non-normal, non-parametric tests were performed to compare PIs, activities and number of approaches of different genotypes with their heterozygous controls.

Figure 4-4: Thermogenetic activation of PAM dopaminergic neurons alters place learning.

Flies were made to express *UAS-Gr28bD* in PAM dopaminergic neurons using *0273-GAL4* (A) or *R58E02-GAL4* (B) and in non-PAM dopaminergic neurons using *TH-GAL4* (C). Flies heterozygous for *0273-GAL4*, *R58E02-GAL4* and *UAS-Gr28bD* lines were used as controls. Flies were trained at 36 (1), 38 (2) or 40°C (3) in the heat-box. Their Performance Indices (PIs) were measured at every step of the experiment. The median PIs are shown as horizontal bars with the vertical lines representing the quartiles of data. **A) Conditioning with 36 and 38°C (1 and 2):** *0273-GAL4>UAS-Gr28bD* flies had significantly lower training PIs than heterozygous controls. PIs in pre-training and test phases did not vary significantly from controls. **Conditioning with 40°C (3):** There was no significant difference in pre-training and training PIs of *0273-GAL4>UAS-Gr28bD* flies with respect to controls. The test phase PI of overexpression flies was significantly higher than the driver control and lower than the effector control, making deductions inconclusive. **B) Conditioning with 36°C (1):** *R58E02-GAL4>UAS-Gr28bD* flies had significantly lower training scores than the driver control. Their median PI was significantly lower than the effector controls at the 0.05 but not at 0.025 level. The pre-training and test PIs were not significantly different from controls. **Conditioning with 38 and 40°C (2 and 3):** There were no significant differences in pre-training, training and test PIs of *0273-GAL4>UAS-Gr28bD* flies with respect to controls. **C) *TH-GAL4>UASGr28bD*** flies did not have significantly different PIs from heterozygous controls in the pre-training, training and test

phases in all the tested temperatures. Kruskal-Wallis H test followed by Dunn's post hoc test with BF correction was used for statistical comparisons (**= $p < 0.0025$ and ***= $p < 0.0001$).

Figure 4-5: Thermogenetic activation of PAM dopaminergic neurons alters number of approaches and duration of hot side dwells during training.

The horizontal bars depict the median scores, colored boxes represent the quartiles of data, and the whiskers represent the non-outlier range (coefficient=1). During training at 36°C (yellow): **A)** *R58E02-GAL4>UAS-Gr28bD* and *0273-GAL4>UAS-Gr28bD* flies spent significantly longer time in the heat-associated side. **B)** *R58E02-GAL4>UAS-Gr28bD* flies also had significantly higher number of approaches as compared to heterozygous genetic controls. The number of approaches of *TH-GAL4>UAS-Gr28bD* and *0273-GAL4>UAS-Gr28bD* flies to the heat-associated side did not vary significantly from the respective genetic controls. During training at 38°C (orange): **C)** *0273-GAL4>UAS-Gr28bD* flies spent significantly longer time in the heat-associated side compared to genetic controls. **D)** However, they approached the heat-associated sides as many times as heterozygous controls. **C and D)** *R58E02-GAL4>UAS-Gr28bD* flies approached the heat-associated side fewer times as compared to heterozygous genetic controls but there was no difference in the time the flies spent on the heat-associated side from the genetic controls. During training at 40°C (red): **E and F)** The total number of approaches of all the experimental genotypes and the time they spent on the heat-associated side were not significantly different from their respective genetic controls. Mann-Whitney U tests with Bonferroni correction were used to compare different groups (*= $p < 0.025$, **= $p < 0.001$ and ***= $p < 0.00001$).

	<i>0273-GAL4/+</i>	<i>0273-GAL4>UAS-Gr28b^D</i>	<i>R58E0 2-GAL4/+</i>	<i>R58E0 2-GAL4>UAS-Gr28b^D</i>	<i>TH-GAL4/+</i>	<i>TH-GAL4>UAS-Gr28b^D</i>	<i>UAS-Gr28b^{D/+}</i>	
Low temp (36°C)	220.0	202.0	192.5	166.0	159.0	187.0	215.5	Acclima tion at 24°C
	234.0	207.5	229.0	209.0	200.0	192.5	227.0	36°C exposur e
	233.5	162.5	225.0	186.5	187.0	189.0	209.5	
	216.5	166.5	208.5	186.0	199.0	185.5	197.0	
	213.5	151.0	202.5	183.0	186.0	189.5	192.0	
	171.5	169.0	143.5	142.5	158.0	159.5	176.5	Recove ry at 24°C
	184.5	176.0	147.5	125.5	144.0	129.5	168.5	
	180.5	176.5	144.0	122.0	142.0	148.0	167.0	
	185.0	177.0	154.5	130.0	140.0	151.0	173.5	
	166.5	173.0	155.0	131.0	120.0	142.5	167.5	
	179.5	177.5	168.0	122.5	132.0	143.5	166.0	
Medium temp (38°C)	222.0	196.5	180.0	180.0	181.0	180.0	211.5	Acclima tion at 24°C
	232.0	197.0	236.0	207.0	212.0	180.0	214.5	38°C exposur e
	212.0	179.0	219.0	204.0	200.0	177.0	210.5	
	205.0	149.0	205.0	150.0	169.0	179.0	185.5	
	205.0	171.0	230.0	212.0	192.0	172.0	203.5	
	167.0	180.0	130.0	122.0	153.0	151.0	161.5	Recove ry at 24°C
	165.0	174.5	116.0	109.0	118.0	148.0	160.5	
	172.0	170.0	124.0	113.0	144.0	129.0	163.5	
	167.0	173.5	130.0	117.0	127.0	158.0	151.5	
	174.0	173.5	140.0	120.0	131.0	150.0	152.0	
	167.0	165.5	113.0	113.0	128.0	135.0	142.5	
High temp (40°C)	206.5	196.0	189.0	191.5	178.5	165.0	212.0	Acclima tion at 24°C
	217.0	165.0	210.0	187.0	194.5	169.0	161.5	40°C exposur e
	212.5	115.0	186.0	144.5	188.5	164.0	129.0	
	207.0	124.0	193.0	148.0	179.0	158.0	144.5	
	203.0	143.0	182.0	173.0	167.0	149.0	147.5	
	113.5	158.0	55.0	71.5	120.5	106.0	60.0	Recove ry at 24°C
	90.0	153.0	42.0	65.0	93.5	110.0	57.5	
	94.5	170.0	41.0	74.5	104.5	133.0	94.0	
	122.5	175.0	61.0	106.5	99.0	120.0	115.0	
	119.5	179.0	44.0	127.0	73.0	142.0	136.5	
	124.5	178.0	50.0	121.0	82.5	130.0	96.0	

Table 4-1: Temperature exposure altered activity of specific genotypes.

0273-GAL4>UAS-Gr28bD, *R58E02-GAL4>UAS-Gr28bD*, *TH-GAL4>UAS-Gr28bD* flies and their respective heterozygous controls were allowed to acclimate in the heat-box for 30s at 24°C. They were exposed to a high temperature stimulus of 36, 38 or 40°C for 2 minutes and then allowed to recover for 3 minutes at 24°C. Each row in the table shows the median activity scores that were measured every 30s during the experiment. The activities of *TH-GAL4>UAS-Gr28bD* flies were indistinguishable from the heterozygous controls during exposure to 36°C and in the recovery period (Kruskal-Wallis test with Dunn's post hoc test, $p < 0.025$). During exposure to 36°C, and 38°C, the median activities of *0273-GAL4>UAS-Gr28bD* flies were significantly lower than heterozygous controls ($p < 0.025$) but significantly greater than zero (One-sample Wilcoxon Sign rank test, $p < 0.0001$). *TH-GAL4>UAS-Gr28bD* flies had significantly lower activity than their heterozygous controls during the first minute of exposure to 38°C. The activities during the last minute were comparable to that of controls. Similarly, the activities of *R58E02-GAL4>UAS-Gr28bD* flies were not different from both the heterozygous controls during, or after exposure to 38°C. The activities of *0273-GAL4>UAS-Gr28bD* flies were significantly higher than controls during the recovery phase post exposure to 40°C. There were no significant differences in their activities while being exposed to 40°C. Similarly, activities of *R58E02-GAL4>UAS-Gr28bD* and *TH-GAL4>UAS-Gr28bD* flies during exposure to 40°C or in the recovery period were not significantly different from their respective controls. Even though *R58E02-GAL4>UAS-Gr28bD* flies

moved significantly less than controls during the last 150s of recovery ($p < 0.025$), their activities were significantly greater than zero (One-sample Wilcoxon Sign rank test, $p < 0.0001$).

5 Conclusion and Summary

Extrinsic control of neural activity is necessary for deciphering the mechanisms through which neural circuits regulate behavior. The current tools rely heavily on a single modality, light. While optogenetic tools have proven to be powerful and precise, they have significant drawbacks related to the mode of delivery of light and the invasive procedures necessary to target light to specific neurons [24,27,167]. The overlapping spectrum of activation of light sensitive proteins has limited the efficient simultaneous use of more than one optogenetic tool in a system [28,29]. Optogenetic tools continue to be developed today [22,168] but their caveats call for the need to explore tools that rely on other modalities, such as, temperature. Thermogenetic tools are limited both in number and function. Some of the temperature sensitive proteins, especially those belonging to the Transient receptor potential family of proteins that are used in studies today are sensitive to changes in both temperature and voltage [7,21,24]. They often activate outside the feasible range for various preparations. We need thermogenetic tools that can be used in various preparations to complement the use of existing tools in dissecting complex neural circuitry. Here, we have identified several thermosensitive proteins from the Gustatory receptor (Gr) family of proteins with unique characteristics that can be used as thermogenetic tools in heterologous systems and preparations, and in organisms to understand how neural circuits regulate behavior. We have characterized the thermosensitive proteins in various subsets of neurons in *D. melanogaster* with several behavioral studies. With the use of one of these thermogenetic tools, we

have clarified a controversy, and showed that dopaminergic neurons modulate place learning and memory.

To characterize the thermosensitive proteins behaviorally, we expressed them in several circuits in the fly and used different behavioral paradigms. First, we identified their temperature response properties by overexpressing them pan-neuronally in *D. melanogaster*. Second, we examined the effects on fly behavior when a thermosensitive protein was co-expressed with hyperpolarizing ion channels that are also used as tools to inhibit neuronal activity. Third, we tested for changes in avoidance behavior and the subtle differences in their temperature sensing properties. Fourth, we investigated the presence of accessory proteins that might be important for thermosensing in hot cell. Fifth, we used one of the proteins as a thermogenetic tool to investigate the role of activation of subsets of dopaminergic neurons in place learning and memory.

Our approaches for studying the thermosensitive proteins were not limited to behavioral experiments. We followed a three-part system: behavioral studies, physiological measurements in fly motor neurons, and electrophysiological studies in *Xenopus* oocytes. We investigated the physiological basis of behavior by measuring the calcium transient induced changes in fluorescence in *Drosophila* motor neurons [9], unpublished). Our observations from physiological studies corroborate our results from behavior experiments. Moreover, expression in *Xenopus* oocytes, a heterologous system showed that several Gr orthologs are functional in a heterologous system, thus bolstering their potential as thermogenetic tools. Additionally, unlike the Trp channels, the thermosensitive

proteins were independent of changes in voltage [9], unpublished). These characteristics make the thermosensitive Gr proteins excellent candidates for use as thermogenetic tools in other model systems.

Several proteins isolated from protozoans, viruses and other animals have been used successfully in mammals for altering genes, as reporters of cellular activity and for visualizing proteins. For instance, Green Fluorescent protein that was isolated from jelly fish has been used for decades to visualize gene expression and protein function [169–172]. Other engineered versions of GFP are being used as calcium reporters (GCaMP) and for deciphering protein-protein interactions [3,173–175]. Cas9, that was isolated from a bacterium, *Staphylococcus*, has proven extremely useful in gene editing with the CRISPR-Cas9 system [176,177]. Similarly, two other proteins Cre recombinase [178–180], and loxP repressor [84,181,182] that were isolated from bacteria and viruses respectively, are used extensively in making transgenic mammals. Yet another example is that of the bioluminescent protein, luciferase obtained from a firefly that is used as a reporter for gene expression [183–186]. Despite their diverse functions, one property these proteins share is that they have no known homologs in mammalian cells. Similarly, a BLAST of Gr28bD with all databases failed to identify any homologs in mammals. The presence of these Grs appears to be limited to Arthropods (figure?). Thus, in the future, it would be interesting to test the function of these thermosensitive Grs in mammals. While there have been developments to delivering proteins into targeted neurons in mammals using viruses [11,179], there are several caveats yet to be addressed. A major drawback to using

thermosensitive proteins in mammals is that the body temperatures of mammals that are typically around 37°C will keep some of the Grs constantly active. This would make conditional activation difficult. However as Munshi and colleagues have shown [10,11], coupling thermosensitive proteins with magnetic materials can be used to conditionally activate these proteins by heating the proteins locally.

The thermosensitive Grs activate at particular temperatures and remain active until the temperature is decreased. Our experiments show that they follow the temperature changes precisely (Mishra 2018, unpublished). We are attempting to engineer chimeric proteins that can activate as well as deactivate at specific temperatures allowing us to alter neuronal activity with short stimuli. Another potential is to use temperature as a driving factor to activate these proteins and induce cellular changes while simultaneously altering the expression of target genes [187–190]. A first step towards designing chimeric proteins is to determine the biophysical properties of these proteins and understand their structure function relationship. Preliminary experiments mutation for sequences in a putative loop region eliminated response to temperatures within 40°C (Figure 5-1). This experiment has laid the foundation for future experiments to mutate single or multiple amino acids in strategic positions to decipher the contributions of different domains of the protein to temperature sensitivity. Even though the Grs have a putative inverted GPCR structure [36,37], numerous studies have shown that they function as ion channels [8,9,71,191]. Being closely related to the Olfactory receptors, it is highly likely that the Grs will also function as

tetramers [79]. In future we will work towards identifying the thermosensitive domain(s) in the Grs through mutational studies and determine the structure of the functional unit formed by these proteins through cryo-EM. A correlation between the structure and function of these proteins will aid our interests in designing proteins with desired temperature response properties.

Figure 5-1: Substitution of 4 amino acids in a putative loop region eliminates thermosensitivity of Gr28bD.

Flies were made to express Gr28bD or Gr28bDmt (IEQQ → AAAA) pan-neuronally using *nSyb-GAL4*. While *nSyb-GAL4>UAS-Gr28bD* flies were paralyzed as in Figure 2-4, *nSyb-GAL4>UAS-Gr28bDmt (IEQQ → AAAA)* were not paralyzed within 40°C. Gehan's Wilcoxon test, $p < 0.0000$.

6 Bibliography

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VITA

My journey in researching started when I was at high school with a project on determining the economic value of pearl culture in my hometown. I knew then that I wanted to be a scientist. After my high school I earned a Bachelors in Science (Research) from the Indian Institute of Science where I majored in Biology, and minored in Chemistry. I had several opportunities to learn and work independently during my undergraduate career. Of all the model systems I worked with, the versatility of fruit flies and the wonderful genetics that accompanies them captured my interest. It was astonishing to see the flies behave and be able to alter their appearance and behavior genetically.

I took my passion from my undergraduate career and moved to the US to work with Dr. Troy Zars on the development of novel thermogenetic tools. My graduate work is inundated with various behavioral assays, genetics, and a newer concept, neuroeconomics. Our collaborations extended my skills to biophysics, and molecular biology. In graduate school, I also acquired a different set of skills to communicate science to the public. Through teaching, and public speaking, I discovered a new passion in talking about science to scientists and non-career scientists.

In future, I want to pursue a career in academia in neuroeconomics, a field that will allow me to explore the application of psychology and economics in neuroscience. My immediate plans involve moving to England for post-doctoral research studying learning and memory using flies.