UNIVERSITY OF CALIFORNIA, SAN DIEGO

Navigating the Fruit Fly Brain:
Visual Place Learning in *Drosophila Melanogaster*

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Neurosciences

by

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Dedication

This dissertation is dedicated to Anna and to my family.

Table of Contents

List of Figures

List of Tables

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ABSTRACT OF THE DISSERTATION

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How does an animal know where it is, and where it is going? While the impressive navigation abilities of ants, bees, wasps, and other insects clearly demonstrate that insects are capable of visual place learning, little is known about the underlying neural circuits that mediate these behaviors. *Drosophila melanogaster* is a powerful model organism for dissecting the neural circuitry underlying complex behaviors, from sensory perception to learning and memory. Flies can identify and remember visual features such as size, color, and contour orientation. However, the extent to which they use vision to navigate and recall

specific locations remains unclear. In this dissertation, I will: 1) describe the development of a novel place learning assay for investigating spatial memories in *Drosophila*; 2) present evidence that fruit flies are capable of forming and recalling spatial memories; 3) demonstrate that neurons in the central complex are necessary for visual place learning and 4) show that silencing these cells specifically impairs place learning without affecting other sensory or motor systems**.** Together, these studies reveal distinct neuroanatomical substrates for spatial versus non-spatial learning, and substantiate *Drosophila* as a powerful model for the study of spatial memories.

Chapter 1
Background

1.1 Visual Place Learning

How does an animal know where it is, and where it is going? The ability of animals to travel long distances and then return to precise locations, for example a nest or foraging area, has fascinated naturalists for centuries. For most animals, vision provides the most salient cues for directed navigation (just try walking down the block with your eyes closed!). The use of vision and visual memories to direct navigation is not unique to vertebrates. It has long been recognized that many insects, despite their small size and limited neural capacity, possess robust visual place learning capabilities and are able to learn and recall complex routes to specific locations in their familiar environment.

In the late 1920's Niko Tinbergen began his observations of the solitary digger wasp *Philanthus triangulum*. The females of this species dig long deep burrows in the English soil and deposit a single egg in the bottom of the nest. Upon leaving, they carefully conceal the entrance with rocks and dirt and upon taking flight, make several loops of increasing radius (termed "orientation flights") around the nest before striking out for foraging grounds several kilometers away. Following these forging excursions, the successful hunters will return directly to their concealed nest to provision their developing young. How do these insects find their way home and locate the concealed entrance to their nest? In a series of clever experiments, Tinbergen demonstrated that these wasps use visual landmarks surrounding the nest to relocate the entrance. By re-arranging these proximal landmarks (in his first experiments these were pine cones or

pebbles) in a new location, he could induce the wasps to search, and in many cases dig, for the nest entrance in the wrong location (Tinbergen et al. 1938; Tinbergen 1958). While these experiments demonstrated the importance of visual landmarks during the final stage of homing behavior (i.e. locating the next entrance), they did not address the cues these wasps were using to navigate over longer distances (e.g. the several kilometers to and from foraging grounds). In a continuation of his studies, Tinbergen and colleagues displaced conspicuous visual landmarks (potted pine trees) along the wasp's flight path and showed that *Philanthus* uses these large landmarks to orient at a distance (Tinbergen 1958). This series of experiments demonstrated the role of proximal and distal visual landmarks in *Philanthus triangulum* homing behavior.

During the same time period, Tinbergen's compatriot Karl von Frisch was focusing his attention on another hymenopteran insect, the honey bee. Much like Niko's wasps, bees often travel long distances -in some cases several kilometers- to search for food. Amazingly, upon returning to the nest, these social insects are capable of communicating the location and quality of a foraging site to nest mates through the waggle dance (Frisch 1967). While bee navigation has been shown to depend largely on polarized light (i.e. a celestial compass)(Frisch 1967) and optic flow as an odometer (Esch et al. 2001; Labhart et al. 2002), bees also learn and rely on visual landmarks to find their way to and from a nest (Collett 1992; Collett 1996; Fry et al. 2005). When leaving the nest bees, much like wasps, have been observed performing structured orientation flights (Collett et al. 1993; Zeil 1993; Lehrer et al. 1994; Zeil et

al. 1996)[1]. These flights are centered on the nest and are composed of a series of arcs of increasing radius. At the apex of each arc, the bee will often turn to face the nest. It is thought that during these flights bees learn visual landmarks that will later aid in homing.

Much like wasps and bees, ants also display impressive navigating capabilities. Despite their fame as path integrators, ants can use a wide range of sensory modalities for navigation and place learning. This is perhaps not surprising given the extensive radiation of ant species (over 8,800 known species (Hölldobler et al. 1990)) and their ability to adapt to a wide range of environments. When provided with distinct visual landmarks, most ants will use vision to guide navigation (Judd et al. 1998; Nicholson et al. 1999; Fukushi 2001; Durier et al. 2003; Fukushi et al. 2004; Harris et al. 2005; Macquart et al. 2006; Collett et al. 2007; Harris et al. 2007).

Is visual place learning a special adaptation of hymenopteran insects (i.e. ants, wasps, and bees)? While the tendency of these social insects to return repeatedly to the same nest makes these animals convenient models for the study of spatial memories, place learning capabilities are not limited to hymenopteran species. Using an experimental assay where cockroaches (*Periplaneta americana*) must find a hidden cool spot in a warm environment, Mizunami et al. (1998) have shown that roaches are also capable of using visual landmarks to learn and remember locations. In these experiments, they used slivers of aluminum foil to create bilateral lesions in the

[1] These orientation flights are especially prominent in young insects first leaving the nest or in older insects after the nest has been relocated.

mushroom bodies. Following this surgical manipulation, the cockroaches are no longer able to recall the location of the cool spot in the heat maze thus implicating the cockroach mushroom bodies as a necessary substrate for visual place learning. A similar assay was employed to demonstrate place learning capabilities in the cricket (*Gryllus bimaculatus*) (Wessnitzer et al. 2008; Mangan et al. 2009).

How do these insects, with a relatively compact nervous system, perform these navigational feats? With the exception of the mushroom body lesion in the cockroach performed by Mizunami et al. (2008), there is little direct evidence for the neural substrates and coding logic of invertebrate visual place learning. How the senses (particularly vision) are interpreted and integrated to represent a spatial location and how that representation is later read out to guide an insect along a familiar path is currently not know.

1.2 *Drosophila* Sensory Systems

While the focus of this dissertation is on visual place learning, it must be stressed that multiple sensory systems can be used either in isolation or more routinely in combination to guide navigation in a complex environment. Here, I provide a brief overview of *Drosophila* sensory systems that are likely to play a role in navigation.

1.2.1 vision

As the focus of this dissertation is visual place learning, it is perhaps not surprising that this is the first sensor modality to be discussed. In addition to featuring prominently in this work, there is a long and rich history of studies in invertebrate

vision. The first documented investigation of insect eyes comes from Antonie van Leeuwenhoek over 300 years ago. In the intervening centuries, much has been discovered about the molecular, cellular and physiologic aspects of invertebrate vision.

Insect compound eyes differ from those of most vertebrates in that each eye is composed of many facets arranged in a regular hexagonal pattern (~700 per eye, distributed over close to 180 degrees of visual space, (Borst 2009)). This configuration and the early studies of van Leuwenhoek led to the mistaken belief that flies view the world as through the a kaleidoscope (i.e. multiple representations of the same image). We now better understand the optics of insect vision and know this to be not true. Each ommatidium in the dipteran (i.e. fly) eye contains its own photoreceptors and is optically isolated from neighboring ommatidia (termed an apposition eye). Each ommatidium in turn contains 6 peripheral photoreceptors (R1-R6) and two central photoreceptors (R7 & R8, stacked one atop the other). R1-R6 contains a blue sensitive rhodopsin (Rh1, sensitivity peaks in blue/UV spectrum) and are thought to mediate motion detection (Zuker et al. 1985). R7 and R8 are heterogeneous with respect to rhodopsin expression. Along the dorsal rim of the eye, both photoreceptors express Rh3 and are sensitive to short wavelength UV. In many insect species, these dorsal rim photoreceptors are responsible for detecting e-vector orientation of polarized light and contribute to orientation in navigating insects (Wehner et al. 1975; Rossel et al. 1984; Rossel et al. 1986; Wehner 1989; Labhart et al. 1992; Labhart et al. 2002; Heinze et al. 2007; Labhart et al. 2009). Elsewhere in the eye, the ommatidia are

either p-type or y-type. In p-type ommatidia, R7 contains UV sensitive Rh3 while R8 contains blue sensitive Rh5. In y-type ommatidia, R7 contains Rh4 (long wavelength UV), and R8 contains green sensitive Rh6 (Harris et al. 1976; Feiler et al. 1992). Additionally, in some parts of the eye, Rh3 and Rh4 are co-expressed in R7. The different photosensitivity and vertical alignment of the R7/R8 photoreceptors (i.e. they both detect photons from the same point in space) make these cells the primary inputs into the insect color vision pathway (Morante et al. 2008). While there are many similarities between vertebrate and invertebrate photo transduction, there are also marked differences. Most notably is the sign of the response. While vertebrate photoreceptors respond to light by hyperpolarizing, insect photoreceptors respond to light by depolarizing (Zuker 1996). Also different is the speed of this response. In many insects, flickering light up to 300 Hz (the flicker fusion rate) can elicit distinct responses in photoreceptors (Miall 1978). In vertebrates, the flicker fusion rate is no more than 100 Hz. This is an important distinction to keep in mind when designing invertebrate visual experiments.

The regular repeating geometry of the compound eye is maintained in the optic lobes, the primary visual neuropils. The optic lobes are composed of the lamina, medulla, lobula and lobula plate. Each of these structures is composed of multiple stereotyped columns (or cartridges). Photoreceptors (R1-R6) from neighboring ommatidia that have parallel visual axes are connected to the same post synaptic target (termed “neural superposition”), leading to an intricate but regular cross-over of photoreceptor axons projecting into the lamina. In the lamina, photoreceptor signals

split into multiple parallel pathways through connections to lamina monopolar cells (L1-L5) and provide the first inputs into the motion vision pathway. It has recently been demonstrated that L1 is the first component of an "On" pathway (detecting contrast changes from dark to light) and L2 provides input into the "Off" pathway (detecting contrast changes from light to dark) (Joesch et al. 2010). Photoreceptors R7 and R8, on the other hand, pass directly through the lamina and make their first synaptic connections in the medulla. Accordingly, the medulla is thought to contain the first inputs into the color vision pathway. In the lobula plate, wide field neurons, termed lobula plate tangential cells (LPTC's) have been identified with dendritic projections spanning large numbers of cartridges (Hausen 1982). All are motion sensitive and can be divided into different classes based on receptive field and direction selectivity. These LPTC's are necessary for normal optomotor responses and are likely to play an important role in flight stabilization and other visually guided behaviors (Heisenberg et al. 1978).

How is information transmitted from the optic lobes to the central brain? Unfortunately, little is known about these connections. There are currently 44 identified pathways from the optic lobes to various locations in the central brain (Otsuna et al. 2006). Many of these neurons terminate in glomerular structures in the ventrolateral protocerebrum termed "optic glomeruli" or "optic foci". The behavioral role of these visual projection neurons and glomerular structures are not currently known; however, they are hypothesized to play a role in the encoding of visual features.

1.2.2 thermosensation

The ability to sense and respond to temperature is critical for fly survival. As a poikilotherm (i.e. and animal who's body temperature closely matches the external environment), the only way for a fly to modulate its internal temperature is by a change in its behavioral state. A fly that is either too hot or too cold needs to quickly navigate to a more suitable location. Given a fly's small size and the speed with which it can desiccate (in warm temperatures) it is not surprising that these animals have developed robust abilities to sense and respond to temperature. The behavioral response to temperature is clearly seen when flies are presented with a choice between a range of temperatures. When placed on a thermal gradient, adult flies will distribute around 24.5 ºC and avoid both warmer and cooler temperatures (Sayeed et al. 1996; Hamada et al. 2008; Gallio et al. 2011). By removing parts of the *Drosophila* antennae, Sayeed et al. (1996) show that these thermal responses are largely mediated by the third antennal segment.

How does a fly sense temperature? In mammals, all known thermo-receptors are TRP channels (Jordt et al. 2003; Patapoutian et al. 2003). This repertoire of thermo TRP's mediates responses to both hot (TRPV1-4) and cold (TRPM8, TRPA1) stimuli (Caterina et al. 1997; Guler et al. 2002; McKemy et al. 2002; Peier et al. 2002a; Peier et al. 2002b; Caterina 2003; Bandell et al. 2004; Chuang et al. 2004; Caterina et al. 2005; Moqrich et al. 2005; Patapoutian 2005; Dhaka et al. 2006; Bandell et al. 2007; Bautista et al. 2007; Caterina 2007; Chung et al. 2007; Dhaka et al. 2007). In the fly, evidence suggests that TRP channels also fill the role of

molecular sensors of temperature. Through a forward genetic screen in *Drosophila* larva, the TRP channel *painless* was identified as being necessary for heat responses (Tracey et al. 2003). This channel, expressed in multidendritic neurons in the larvae body wall, is activated by nociceptive hot temperatures (> 42º C) and also mediates the response to mechanical injury (Tracey et al. 2003; Sokabe et al. 2008; Sokabe et al. 2009). A related TRP channel, *Drosophila* TRPA1 (dTRPA1), is activated at lower temperatures (~29º C) and functions as a warm sensor in both heterologous expression systems (Viswanath et al. 2003) and in the fly brain (Hamada et al. 2008). The fact that dTRPA1 appears to function internally (i.e. in the fly brain) and not at the periphery suggests that there are additional unidentified hot and cold sensors that mediate thermal responses through the antennae. For experiments addressing the identity of these thermo-receptors and the coding logic of *Drosophila* thermosensation, see Chapter 2.2.2 of this dissertation and Gallio et al. (2011).

1.2.3 chemical senses

In the visual place learning studies described in this dissertation, we make significant efforts (both in the design of our behavioral assays and the inclusion of multiple control experiments) to ensure that chemical senses (i.e. olfaction and taste) can NOT be used to solve the place learning task. However, in a natural setting, chemicals do provide salient sensory cues (such as an odor plume) and likely play an important role in fly navigation. Chemosensation can be divided into two systems, gustatory (taste) and olfaction (smell). While the gustatory system and gustatory receptors are capable of guiding a fly along a sugar trail (Dethier 1976), the most

salient chemical cue (with respect to fly navigation) is odor. Anyone who has tried to drink a beer in a fly lab can readily attest to this fact, as flies are quite adept at tracking an odor plume (Frye et al. 2003; Duistermars et al. 2008). The primary olfactory organs of the fly are positioned on the head. Odorant receptor neurons (ORN's) are located in the third antennal segment and to a lesser extent in the maxillary palp. Each ORN expresses one (in a few cases 2 or 3) ligand binding odorant receptors (OR, 62 identified, 30 known to be expressed in the adult) and an obligate co-receptor (OR83b) (Vosshall et al. 2007). The molecular identity of the expressed OR(s) establishes the odor sensitivity of the ORN.

The primary sensory neuropil for the olfactory system is the antennal lobe. Much like the vertebrate olfactory system, there appears to be a 1:1 correspondence between ORN class and targeted glomerulus in the antennal lobe (i.e. all ORN's that express the same OR converge on a common glomerulus in the antennal lobe). Projection neurons from the antennal lobe send axons to the mushroom bodies. In flies, inputs into the mushroom bodies appear to be predominantly olfactory (Stocker 1994; Mizunami et al. 1998), while in other insects the mushroom bodies appear to receive multimodal inputs (Strausfeld et al. 1999). Over the past several decades, there has been much interest in the mushroom bodies as the substrate for learning and memory (Heisenberg 2003). Most of these studies use olfactory conditioning as the assay for learning. In the standard olfactory conditioning experiments, flies are sequentially exposed to two different odors with one of the odors being paired with an aversive stimulus (most often electric shock (Tully et al. 1985)). After conditioning,

flies are then tested for their preference between the two odors. Normal flies will develop an aversion to the odor that was previously paired with electric shock. Depending on the training protocol, this memory can last for hours or days (Tully et al. 1994). Long term memory requires multiple spaced training trials and is dependent on protein synthesis. The memories formed after a single training session do not require protein synthesis and can be divided into short term (lasting minutes), middle term (lasting hours), and anesthesia resistant (consolidated memory developing over first 30 minutes) memory phases (Quinn et al. 1976; Isabel et al. 2004). While these different phases of memory require distinct molecular components, they all depend on the mushroom bodies (de Belle et al. 1994).

1.3 Content of a Spatial Memory

What are the contents of a spatial memory? The simplest form of visual navigation is orienting towards a beacon. By aiming at a prominent visual landmark in close proximity to a goal, an animal can approach the goal location from a distance without having any special knowledge of the intervening route. Careful reconstruction and analysis of insect paths indicate that beacons are routinely used to navigate (Fourcassie 1991; Fukushi 2001; Fukushi et al. 2004)[2]. However, beacon navigation alone is not sufficient to account for all navigating capabilities in insects. Displacement experiments have long suggested that insects use a much richer representation of visual space (Thorpe 1949; Gould 1986; Menzel et al. 2005). Most

[2] Beacon navigation is used by humans as well. When exploring an unknown city, finding a recommended restaurant is significantly simplified if it lies in close proximity to a prominent and easily identified landmark.

recently, Menzel et al. (2005) have used harmonic radar to reconstruct complete paths for bees arbitrarily displaced from their route to or from a feeder. The authors carefully control the visual landscape at the experiment site to ensure that there are no "beacons" capable of guiding the bees to either the nest for feeder. They find that when a bee is captured and released at an unexpected release site within its familiar area, it will initially continue on the same course it was flying before capture. After a short distance, the bee will slow and make frequent changes to its course. The authors interpret this as the bee "getting its bearings". Following this re-orienting stage, the bee will make a straight and rapid flight either to the hive or to the feeding site. This ability of a bee to choose between two goals (either the nest or the feeder) and then set an arbitrary course to the goal is consistent with a map-like organization of spatial memory. Similar results (i.e. the ability to take direct paths to a goal) are seen in many species of visually guided ants when displaced from a route (Wehner et al. 1996; Fukushi 2001; Durier et al. 2003; Fukushi et al. 2004; Collett et al. 2007; Harris et al. 2007). However, the authors of these studies (in ants and bees) often disagree on the interpretation of their findings. The predominant interpretations can be roughly characterized as either map based or template based navigation.

In a map based scheme, an animal forms a geocentered representation of the spatial relationship between an array of landmarks. If an animal knows where it is in relation to its mental map, then it can directly move between arbitrary locations within the map. The template based theories propose that insects navigate not by a map, but rather by storing a series of image templates. When traveling along a remembered

route, an insect has only to recall the appropriate image template and then move so as to minimize the difference between the remembered template and the current view (Cartwright et al. 1983; Junger 1991; Collett 1992; Collett 1996; Judd et al. 1998; Collett et al. 2002; Durier et al. 2003; Collett et al. 2006a). In this template scheme, visual landmarks provide the animal with procedural, but not positional information (i.e. they tell the animal what to do when, but do not provide a map). While the displacement experiments would seem to favor a map-like representation of space, they do not rule out a template based strategy. Many people argue that the complexity of the natural environments in which most of the displacement experiments are carried out makes it difficult to be sure beacons are not guiding the insects (Collett 1996; Collett et al. 2006a; Wehner et al. 2006). Additionally, they suggest that for some displacements visual templates could still be effective in guiding an animal to (or close to) the goal. Unfortunately, the distinction between these two models has not been successfully resolved experimentally and the existence of an insect cognitive map is still hotly debated.

1.4 Path Integration and Idiothetic Cues

In the absence of visual landmarks, how does an animal find its way? While the focus of this dissertation is visual place learning, it is important to note that path integration is an important component of navigation for most animals. The use of idiothetic cues is likely a necessary part of any spatial representation system and in a

natural setting most insects probably utilize both idiothetic and visual cues.[3] In fact, even insects that are considered predominant path integrators (such as the desert ant *Cataglyphis fortis* which must navigate the often featureless Saharan salt pans) will also learn and use visual cues to aid navigation (Wehner et al. 1996; Akesson et al. 2002). While path integration may be sufficient for navigating short distances, this mode of navigation suffers from the accumulation of errors and at a minimum, external landmarks are likely necessary to reset and re-calibrate the path integrator. Likewise, most models of cognitive maps and visual place memory assume that idiothetic cues and path integration contribute to an underlying coordinate system onto which spatial memories can be superimposed. As such, it is unlikely that we will ever fully understand place learning without also considering the role of idiothetic information.

1.4.1 compass headings

In order to successfully path integrate, an animal must know both the distance and the direction it has traveled. How does an insect determine direction? In the 1940's Karl von Frisch discovered that bees use the position of the sun and the pattern of polarized sky light to set a heading (Frisch 1967). In the intervening years, much has been discovered about the behavioral and physiologic mechanisms for sun compass navigation. In many insects, polarization sensitive photoreceptors have been identified in the dorsal rim of the compound eye (Labhart et al. 1992; Labhart et al.

[3] This is true for most animals. Despite the tendency to divide species into path integrators, visual place learners, etc. most animals, humans included, possess robust and flexible navigation systems capable of using and integrating many different egocentric and geocentric cues to navigate complex environments.

2009). Additionally, in the locust and butterfly, polarization sensitive interneurons have been identified in the optic lobes (Labhart 1996; Homberg et al. 1997; Labhart 1999; Labhart 2000; Labhart et al. 2001) and central brain (Heinze et al. ; Heinze et al. 2007; Heinze et al. 2009a; Heinze et al. 2009b). These cells and circuits are thought to provide the necessary substrate for determining a compass heading.

1.4.2 judging distance

In addition to a compass heading, to successfully path integrate an insect also needs to determine distance traveled. The fact that ants can path integrate is most convincingly demonstrated experimentally through displacement experiments. In the absence of visual landmarks, an ant displaced from its route will continue traveling in the direction it was traveling prior to displacement until it has traveled a distance equal to the distance it would have traveled if not displaced. (Merkle et al. 2006; Narendra 2007). How does an ant judge distance? Clever experiments by Wittlinger et al. (2006) demonstrate that ants do this by counting steps. First, they glued stilts to the ant's legs thereby extending the stride length. If ants were displace from a route following this manipulation, they would travel further than normal. Conversely, if ants legs were clipped (effectively shortening the stride), a displaced ant would travel a shorter distance than normal (Wittlinger et al. 2006). This set of experiments convincingly demonstrates that counting steps provides a robust mechanism for judging distance in a path integrating ant.

Bees also depend on a path integrator. A successful forager upon returning to the nest will perform a "waggle dance". The dancing bee will communicate both

direction and distance to a food sources through the orientation and tempo of the dance (Frisch 1967). While the direction information can set a naive forager on the right path, how does a flying bee measure distance? As opposed to the step counting in ants, bees have been shown to use optic flow for visual odometry (Esch et al. 2001; Hrncir et al. 2003; Tautz et al. 2004; Collett et al. 2006b).

1.4.3 non-visual place learning in the fly

In flies, nearly all evidence for non-visual place learning comes from the labs of Martin Heisenberg and Troy Zars and is based on the "Heat Box" paradigm (for a review, see (Zars 2009)). In the Heat Box, single flies are placed in a small chamber and exposed to hot temperatures when on one side, and cool temperatures when on the other. After training, the box is held at a constant cool temperature and a performance index is calculated for the time spent on the cool associated side vs. time spent in the hot associated side. Normal flies will show a strong preference for the side of the box previously associated with cool temperatures (Wustmann et al. 1996; Wustmann et al. 1997; Putz et al. 2002). This behavior is independent of visual cues and it has been suggested that flies instead rely on idiothetic cues and path integration to solve this task.

1.5 Place, Grid, and Head Direction Cells: Substrates for a Cognitive Map

In mammals, the identification of place, grid, and head direction cells in the medial temporal lobe suggests the existence of a "cognitive map" (Moser et al. 2008). The phenomena of place fields was first reported by O'Keefe and Dostrovsky in 1971 (O'Keefe et al. 1971). While recording from complex spiking cells in the hippocampus

of freely moving rats, O'Keefe realized that the activity of these neurons (i.e. the firing rate) was tightly correlated with the rat's position in space. By recording from many cells, he realized that each cell responds to a specific location in the environment and that the combined effect of many cells is a tiling of familiar space. It is proposed that these place fields provide a map-like representation of local space and encode an animal's position within this space. Much like place cells, grid cells in the entorhinal cortex also show activity that is tightly correlated with an animal's position in space. However, rather than each cell having a receptive field that is correlated with a single position, these cells have multiple receptive fields arranged in a uniform grid which tiles the familiar area (Hafting et al. 2005). Finally, head direction cells (i.e. cells that fire when an animal's head is pointed in a specific direction) have been identified in multiple brain regions (Taube et al. 1990; Chen et al. 1994; Taube 1995).

Identification of these cells suggests they form the neural substrate for a coordinate system (grid cells), a compass (head direction cells), and a map (place cells). However, how these complex receptive fields are established, and how they are later read out to guide an animal remains unclear. Evidence for the role of sensory cues in the establishment of place fields come from experiments where the sensory landscape was manipulated. When distal visual cues are rotated around the arena as a rat navigates, the place fields often show a corresponding shift in location (O'Keefe et al. 1978; Muller et al. 1987). Likewise, enlarging a rectangular arena along one axis results in a corresponding stretching of place fields along the same axis (O'Keefe et al. 1996). However, it is also clear that vision is not the only thing influencing place

fields as olfactory, auditory, and tactile cues can all shape place cell activity (O'Keefe et al. 1978; Tanila et al. 1997; Save et al. 2000). These studies suggest that "place" is coded in the hippocampus independent of a specific sensory modality.

1.6 The Power of *Drosophila* Genetics

Following the above discussion of the place learning capabilities of rodents, ants, bees, and other animals, the reader may be left wondering why we would choose to study this complex behavior in the "simple" fruit fly. While strong arguments can be mounted against the perceived "simplicity" of the fly, the most compelling argument focuses on the power of *Drosophila* genetics. Flies have a long and celebrated history as genetic models. Additionally, their small size and short generation time have made flies a standard for large scale genetic screens (initially through the use of chemical mutagens and radiation, later through the use of p-elements). In the 1960's Seymour Benzer adapted many of these genetic techniques for the study of behavior (a field now termed "neurogenetics"). During the past 50 years, flies have become standard models for studying basic (visual, olfactory, locomotor, auditory, gustatory, etc.) and complex (memory, aggression, attention, etc.) behaviors. In many cases, striking similarities have been identified in the underlying neural mechanisms between vertebrates and invertebrates.

The primary "power" of the fruit fly comes from the development of the GAL4/UAS system (Brand et al. 1993). GAL4 is a yeast transcriptional activator that can drive expression of genes downstream of its DNA binding site (UAS). By placing the GAL4 transgene downstream of endogenous genetic enhancers, GAL4 can be

expressed in restricted and reproducible subsets of cells. Large collections of enhancer-GAL4 lines (tens of thousands) allow either large classes or small subsets of neurons to be precisely and reliably targeted (Pfeiffer et al. 2008; Jenett et al. 2009). The expression patterns from these driver lines can be further refined through the use of various intersectional strategies. Two enhancers can be combined, one driving GAL4, the other driving GAL80 (a repressor of GAL4) (Luan et al. 2007). In this way, GAL4 activity can be blocked in cells where expression from the two enhancers overlaps (negative intersection). Conversely, two enhancers can be used to express each half of split GAL4 (Luan et al. 2006), resulting in GAL4 activity being reconstituted only in cells where expression of the two enhancers overlap (positive intersection). These highly specific GAL4 lines can then be used to drive effectors to:

*determine neuroanatomy or target electrophysiologic recordings (GFP)

*kill cells (diphtheria toxin, grim)

*silence neurons (tetanus toxin, shibire^{ts}, Kir2.1, halorhodopsin)

*activate neurons (NaChBac, channelrhodopsin, dTrpA1)

*optically monitor calcium currents (GCamP, TN-XXL)

Temporal specificity can be achieved through the use of an effector that is thermally (shibire^{ts}, dTrpA1) or optically (halorhodopsin, channelrhodopsin) gated, or through the use of GAL80^{ts}. GAL80^{ts} is a thermally sensitive version of GAL80 that is active (i.e. blocks GAL4) at 18ºC, but is inactive at 30ºC (McGuire et al. 2004). By using GAL80^{ts} in combination with GAL4, flies can be reared at 18ºC and expression of the effector is only induced after flies are temperature shifted to 30ºC.

Recent additions to the *Drosophila* tool kit include two more exogeneous transcriptional activators, the LexA/LexAop system (Lai et al. 2006) and Q system (Potter et al. 2010). By using combinations of these three systems (GAL4, LexA, and Q), multiple subsets of neurons can be simultaneously probed. For example, hypothesis can be generated about connectivity using GRASP (**G**FP **R**econstituted **A**cross **S**ynaptic **P**artners) (Feinberg et al. 2008). With GRASP, one half of GFP can be expressed in a pre-synaptic neuron while the other half is driven in potential post-synaptic targets. GFP fluorescence is only reconstituted at sites of likely synaptic contact. Functional connectivity between neurons can be later confirmed by expressing ChR2 in presynaptic cells while optically (or electrophysiologically) monitoring post-synaptic activity using GCamP. The modularity of these systems (i.e. the ability to mix and match various drivers and effectors) and the rapid life cycle of *Drosophila melanogaster* allows for the rapid and systematic probing of the fruit fly brain.

1.7 Experimental Questions and Overview of the Dissertation

In studying place learning in the fruit fly, we do not wish to provide just another "me too" example of an insect capable of forming visual place memories. Rather, we hope to leverage the genetic tools and relative simplicity of the fruit fly brain to make a significant contribution towards an understanding of the neural underpinnings of visual place learning. As this behavior has not been previously described in the fruit fly, the first challenge was to create a visual place learning assay

for testing *Drosophila* place memory[4]. In Chapter 2 of this dissertation, I will describe the development of a thermal-visual arena inspired by the Morris Water Maze (Morris 1984) that allows us to directly address the role of a visual landscape in *Drosophila* place memory. Using this novel *Drosophila* place learning assay, I show (in Chapter 3) that *Drosophila* are capable of using peripheral visual cues to remember and find distinct locations in space. What are the neural substrates for place learning in the fly? In Chapter 4, I will describe the behavioral screen that has allowed us to identify subsets of cells in the central complex as critically required for visual place learning. Furthermore, I will show results from control experiments that lead us to believe these cells play a specific role in place learning, rather than a more general role in sensory or motor processing. Finally, in Chapter 5, I will discuss the broader significance of these findings and present some preliminary work in developing a "virtual" place learning assay with a head fixed fly. Additionally, I will present some ideas on future directions and discuss some of the obvious next steps for these studies (such as recording from central complex neurons while a fly navigates in a "virtual" thermal-visual landscape).

[4] At the beginning of these experiments, it was not at all clear that fruit flies were even capable of forming visual place memories. While the size of *Drosophila* optic lobes would suggest vision plays a prominent role in fly survival, the fly brain is much smaller (~1 order of magnitude fewer neurons) than the brains of most other visual place learners (i.e. bees and wasps).

Chapter 2
Development of a *Drosophila* Place Learning Assay

In this chapter, I will discuss the technologic developments that contributed to the success of our *Drosophila* place learning experiments. In addition to describing the mechanics of our assay, I will also present behavioral data that while not specific to place learning, was critical to achieving reliable and reproducible fly behavior. Finally, I will present experiments that address the mechanisms and logic of temperature coding in the *Drosophila* brain. Additional details and data from these thermosensation studies, performed in close collaboration with Dr. Marco Gallio, can be found in our paper "The Coding of Temperature in the *Drosophila* Brain" (Gallio et al. 2011).

2.1 Development of the Thermal-Visual Arena

To explicitly test for visual place learning in *Drosophila*, we developed a thermal-visual arena inspired by the Morris Water Maze (Morris 1981; Morris 1984). In the Morris Water Maze, animals (traditionally rats or mice) are placed in a large tank filled with an opaque liquid (Figure 2-1). A small platform is placed just beneath the surface of the water so that it cannot be seen by the animal. However, the animal is able to see visual landmarks positioned either around the perimeter of the tank or the room. During the first couple trials, animals will explore the Water Maze randomly until they locate the hidden platform. During successive trials, normal animals will improve in the speed and efficiency with which they locate the hidden platform, and after multiple rounds of training, will swim directly to the hidden platform using peripheral visual cues to guide navigation.

In adapting the logic of the Morris Water Maze for use with flies, we use heat, rather than water as an aversive stimulus (Mizunami et al. 1998; Wessnitzer et al. 2008). In this "Heat Maze", flies must find a hidden cool spot (as opposed to a submerged platform) in an aversive warm environment. Panoramic visual landmarks are presented around the outer perimeter of the arena and, in principle, can be used by the fly learn and later recall the location of the cool spot.

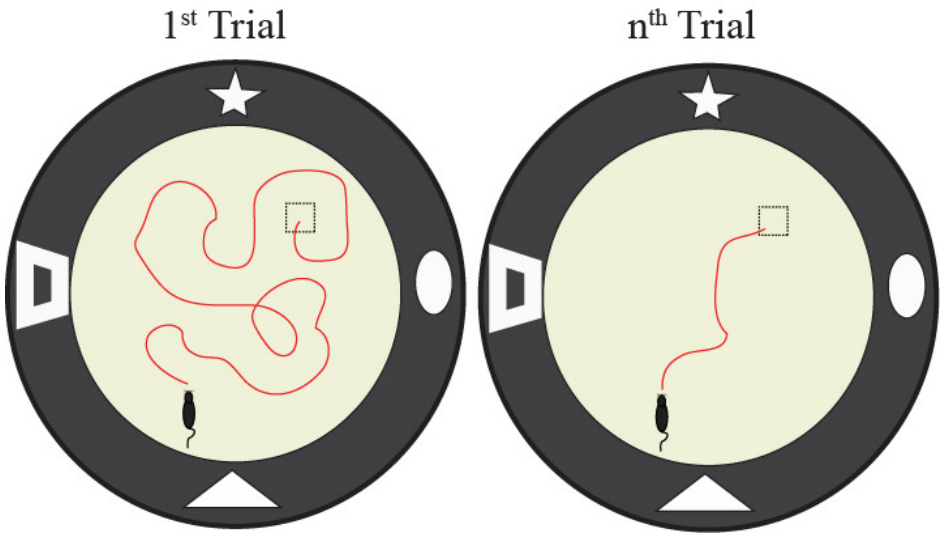

Figure 2-1: Diagrammatic illustration of the Morris Water Maze.
Shown is a diagram of the Morris Water Maze. In the first trial (left), a rat is placed in a tank filled with a milky liquid and swims randomly (red line) until it locates a submerged platform. After multiple trials (right), the animal will learn to us peripheral visual cues decorating the edge of the tank to swim directly to the hidden platform.

2.1.1 mechanical design

To precisely control the thermal landscape, we developed an array of sixty-four 1 inch-square individually addressable thermoelectric modules (TEM, peltier tiles) arranged in an 8x8 grid (Figure 2-2, developed in collaboration with Oven

Industries, Mechanicsburg, PA). The arrays are built in 4x4 subunits (i.e. 16 TEMs each), and 4 of these subunits are combined and mounted on a water cooled heat sink to form the floor of our *Drosophila* place learning arena. This TEM floor is covered with black masking tape to create a uniform, featureless surface that can be easily replaced between experiments. Electronic control of the TEM array occurs via RS-232 serial port communication with the controlling computer and custom Matlab code (see Appendix 6.1).

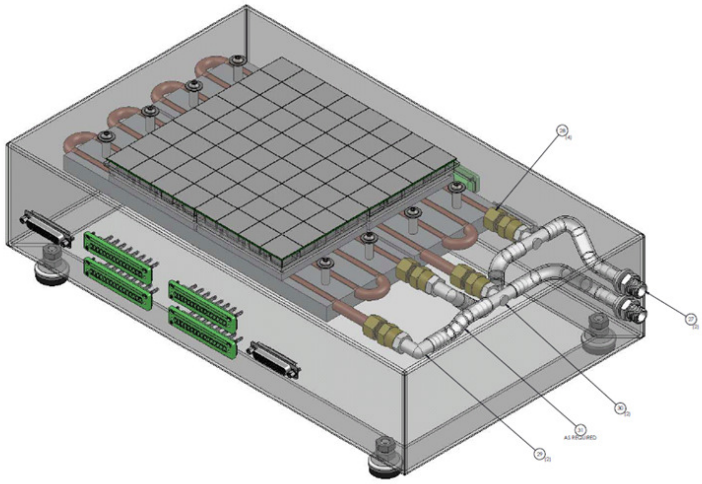

Figure 2-2: Diagram of a 64 peltier tile array.
Shown is a diagram of the 64 tile TEM array. The array is composed of 4 array subunits with each array subunit containing 16 TEM tiles arranged in a 4x4 grid. The 4 array subunits are all mounted on a water cooled heat sink and constitute a flat square surface 8 inches wide and composed of 64 individually addressable TEM tiles.

Following assembly, each TEM array is calibrated. Initial efforts to calibrate using a single thermocouple proved impractical with the 64 tile array due to complex inter-tile interactions. To address these calibration challenges, a thermal imaging

camera (Micro Infrared Microscope, Optotherm) and custom Matlab code (see Appendix 6.1) was used to perform automated iterative calibration of the 64 tile array. Following calibration, each tile of the array will hold within ±0.1º C of the set temperature (measured for temperatures between 20-40º C), stabilizes to a new set temperature within 10 seconds, and displays no significant temperature gradient across the array surface with all tiles set to a uniform temperature (Figure 2-3). To confine flies to this surface, a 3mm high, 8 inch diameter aluminum ring was placed around the outer perimeter of the arena and covered with a glass disk coated with a slippery silicon film (Sigmacote, Sigma-Aldrich). To keep flies from walking on the walls, the aluminum ring was heated to >50º C using insulated resistance wire (29 AWG Nichrome 60 w/Kapton, Pelican Wire).

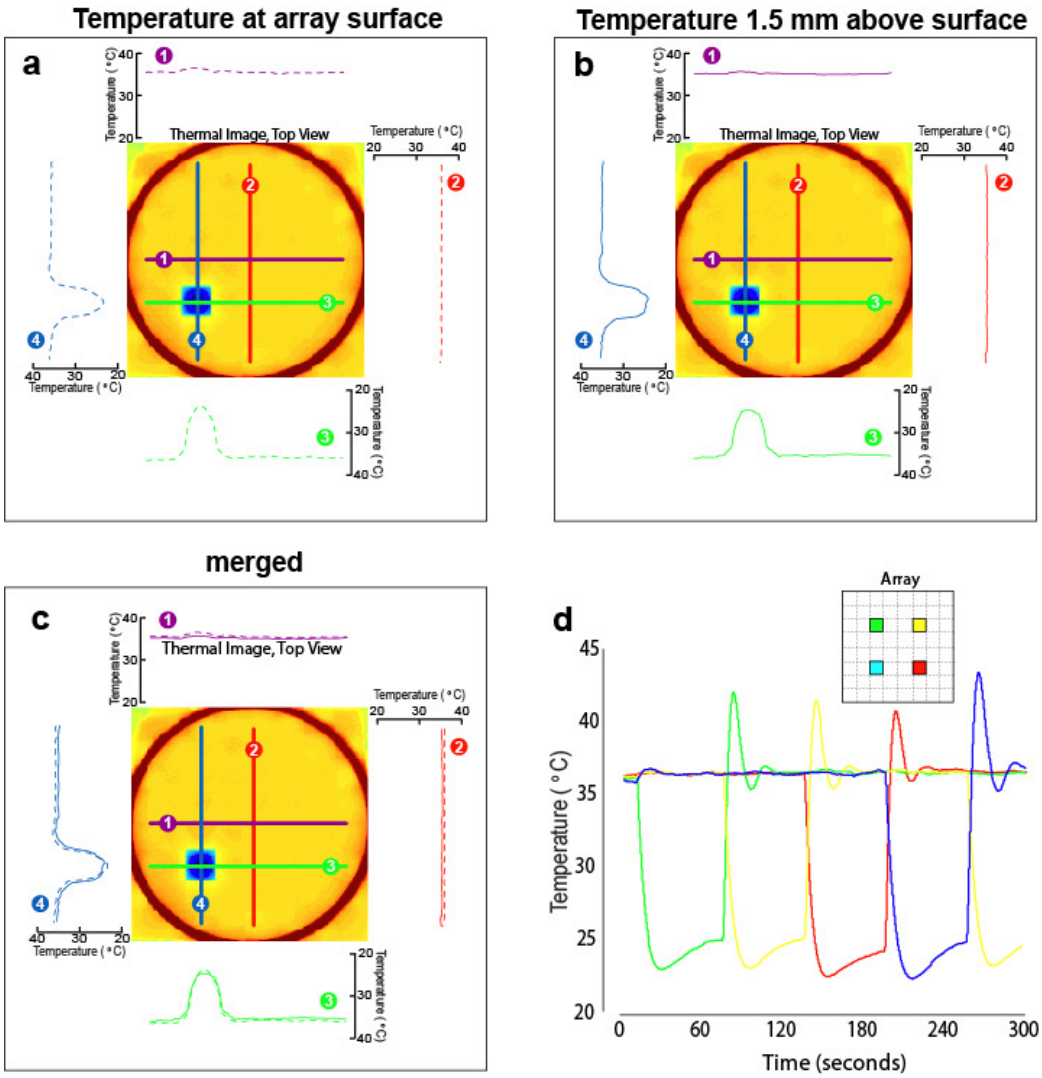

Figure 2-3: Temperature measurements in the thermal arena
Temperature readings (± 0.1 degree) were made across the TEM array at locations indicated by lines 1 to 4 superimposed on a thermal image of the array. (**a**) Temperature readings at the array surface (dashed line, data from thermal imaging camera) or (**b**) a thermocouple 1.5 mm above the array surface (solid line). (**c**) Temperature readings from panels **a** and **b** plotted on the same axis; note a slight temperature difference in the vertical direction. No gradients are detected significantly beyond the boundaries of the test cool tile. (**d**) Temperature readings over a period of 5 minutes were made at 4 TEM tiles as diagramed (top right of panel). The 4 TEM tiles were sequentially cooled for 60s and then reheated (starting with green, yellow, red, then blue). Note the rapid change in tile temperature after sending a new temperature setting, as well as the brief overshoot when a tile is re-heated.

Peripheral visual cues were provided using an electronically controlled LED display positioned around the outer perimeter of the arena (Reiser et al. 2008). The use of an LED display (refreshed at >372 Hz), rather than a more conventional CRT or LCD monitor was necessary because the refresh rates of CRT and LCD displays are typically slower than the flicker-fusion rate of many laboratory insects (Miall 1978).

To record video and to track fly activity in the arena under changing light conditions, the entire arena was illuminated with infrared light (850nm, IR) (Smart Vision Lights, Muskegon, MI) and fly activity was recorded with a Basler (Ahrensburg, Germany) 622f CMOS camera fitted with an IR passing, visible blocking filter. Since flies are unable to see in the infrared spectrum(Harris et al. 1976; Menne et al. 1977; Desalomon et al. 1983), we were able to uncouple the illumination necessary for monitoring fly activity (850nm peak, IR light) from the LED display visual cues (565nm peak, green light).

All instrument control and integration was performed using custom Matlab code. To facilitate high experiment throughput, as well as reliable experiment data and meta-data collection and organization, a Matlab graphical user interface (GUI) was created to run the standard experiment protocols (Figure 2-4). In addition to coordinating and executing the experiment protocol, metadata (such as fly genotype, fly age, relative circadian cycle, experiment date, experiment time, room temperature, room humidity, rearing temperature, etc.) was collected for all experiments.

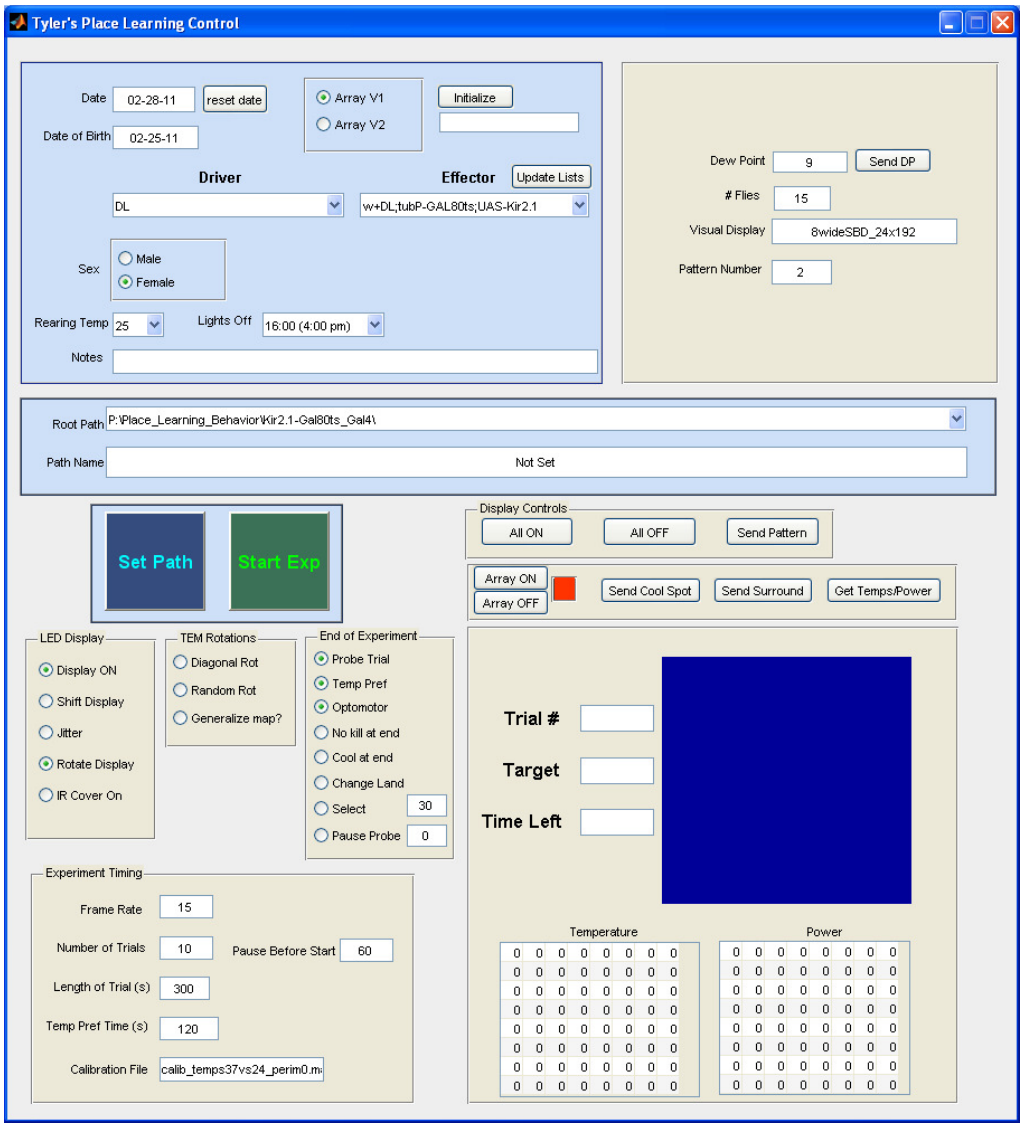

Figure 2-4: Place learning experiment control GUI
Shown is a screen shot of the custom graphic user interface (GUI) for running standard visual place learning protocols. In addition to controlling the arena, this interface allows efficient collection and storage of a meta-data record attached to each experiment. For standard place learning experiments, the user needs only to set the parameters highlighted in the blue box. Standard fields such as *Date* are automatically populated. Other fields, such as *Driver*, *Effector* and *Rearing Temp* are selected from a drop-down menu prior to starting each experiment. Clicking *Set Path* creates a unique file path for the experiment and if all required fields have been entered *Start Exp* will begin the experiment. Options for changing the experiment protocol or manually controlling the TEM array and LED display are located in gray boxes.

2.1.2 data processing pipeline

High resolution (1024 x 1024 pixel) AVI videos of fly activity were recorded at 15 fps using custom Matlab code. The large amount of raw video collected (up to 1.25 terabytes a day) necessitated a stream-lined data processing and analysis pipeline capable of matching our rate of data generation. Following the end of an experiment, all raw video is transferred from a local hard drive to a network directory and processed on the Janelia 4000 core computing cluster. As long term storage of raw AVI video would be prohibitive, videos are first compressed using a custom background subtraction algorithm (Static Background Fly Movie Format, SBFMF). These SBFMF files are then used to track flies using Ctrax fly tracking software (Branson et al. 2009). Using this tracking software, populations of flies can be tracked and individual fly identity maintained over the course of an experiment (Figure 2-5). Behavioral metrics are derived from the fly centroid and body axis orientation data using custom Matlab code. Derived behavior metrics are then stored in a custom database and accessed via Matlab code or through a "data browser" (also created in Matlab) for easy visualization of experiment data and rapid data mining of experiment results (Figure 2-6).

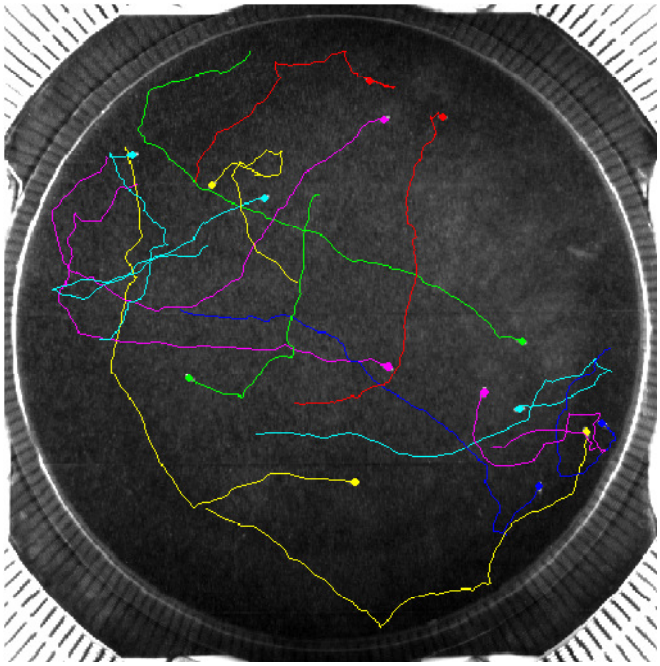

Figure 2-5: Fly tracking using Ctrax
Shown is a sample frame from an overhead video of 15 flies in the thermal-visual arena after flies have been tracked with Ctrax. Colored dots indicate the current fly position and colored lines indicate positions during the preceding 15 seconds.

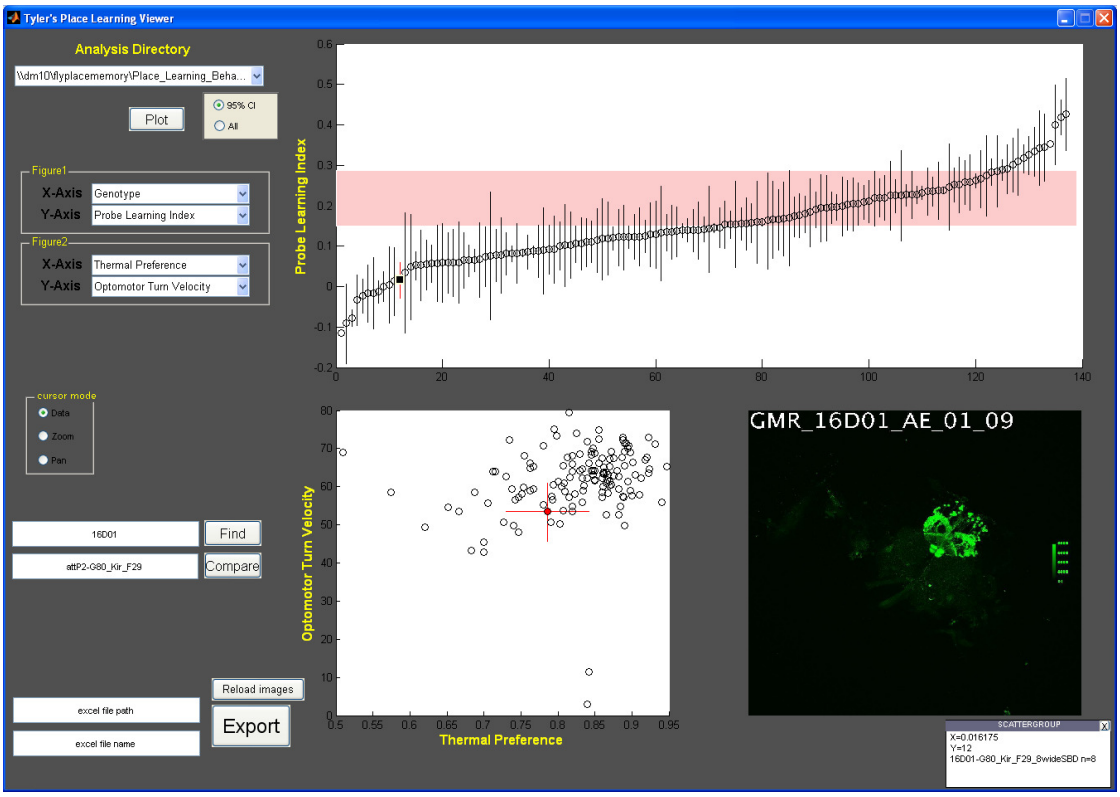

Figure 2-6: Screen shot of the Place Learning Viewer
Shown is a screen shot of the custom data browser for efficiently viewing place learning data. Following the completion of an experiment, a database is automatically populated with derived behavior metrics. Place learning and/or control behavior metrics can be plotted in the top or lower left axes and can be sorted according to various experiment parameters such as genotype, date, room humidity, etc. To find a specific experiment, a search string can be entered and upon clicking *find*, matching data points are highlighted in red. Finally, by clicking on a data point in the browser, a representative GFP image of the expression pattern for the selected line is displayed in the lower right panel. This browser provides a convenient interface for accessing behavioral data and correlating behavioral phenotypes with GAL4 expression patterns. All representative GFP images were kindly provided by the Janelia Fly Light Project.

2.2 Behavioral Optimization

To fully appreciate the power of quantitative behavioral analysis and to successfully employ a large scale behavioral screen, it is essential that we are able to

compare experiments performed over days, weeks, months, and even years. While reproducibility can be partially achieved via the careful design, calibration, and repeated validation of the mechanical aspects of our assays (i.e. minimize the technologic variability), it is equally important to understand and minimize the biologic variability. To this extent, I will describe some of the behavioral parameters that we have explored and found critical to generating reliable and reproducible data.

2.2.1 circadian effects on behavior

It has long been recognized that *Drosophila*, like most organisms, undergo biochemical, physiologic, and behavioral cycles that are intimately linked to a ~24 hour circadian clock (Konopka et al. 1971; Ashburner 1987; Zeng et al. 1996; Krishnan et al. 1999; Helfrich-Forster et al. 2002; Levine et al. 2002; Hendricks et al. 2004; Hardin 2005; Rosato et al. 2006). In order to characterize the effects of this circadian cycle on fly activity in our thermal-visual arena, we developed a protocol to maintain flies for multiple days in the arena while recording and tracking fly activity. The primary challenge was finding a way to feed flies in our assay. Initial efforts to place food in the arena proved unsuccessful. The algorithm for tracking fly behavior assumes a static background. Flies are distinguished from the background by subtracting a time-averaged background image from each frame of an experiment video. Normal food placed in the arena will change size and shape as it is consumed and dehydrated, leading to significant artifact in the background subtracted images and failure of the fly tracking algorithm. In order to perform multi day experiments, we developed a novel "fly feeder" to deliver food and water to flies enclosed in the

thermal-visual arena (Figure 2-7 a and b). The fly feeder is composed of a hollow cylinder 10mm in diameter and 3 mm high. Short glass capillary tubes are evenly spaced around the outer perimeter of this cylinder. The cylinder can then be filled with a solution of 5% (wt/vol) sugar and 5% (wt/vol) yeast extract. Flies can freely feed through the capillary tubes and have been maintained and tracked for >5 days with this setup (without the feeder most flies die within 24 hours).

To analyze the effect of circadian cycle on fly activity in the thermal-visual arena, flies were entrained for 3 days on a 16 hour light/8 hour dark cycle and then placed in the thermal-visual arena and tracked for 36 hours. Flies show a sharp peak in activity when the lights turn on in the subjective morning and a broader, more sustained peak in activity during 4 hours in the subjective afternoon leading up to the expected lights turning off (Figure 2-7). In order to minimize variability between tests, we perform all of our other behavioral experiments, unless otherwise noted, during this afternoon peak in activity (hours 11-16, where hour 0 corresponds to the "morning" transition from dark to light).

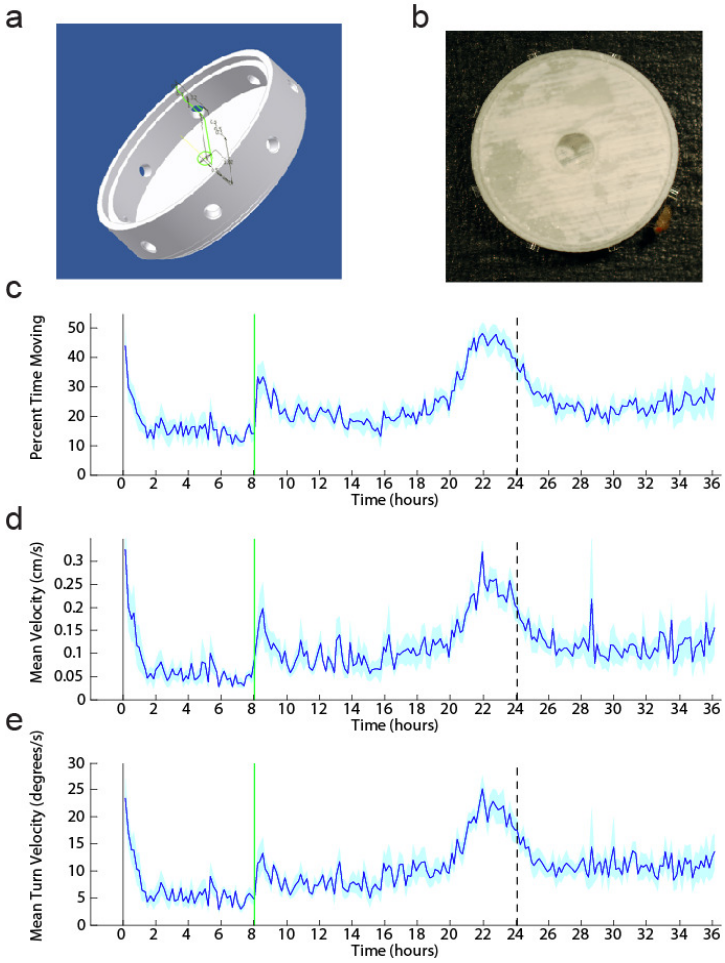

Figure 2-7: Circadian activity in the thermal-visual arena

(**a**) Shown is a CAD drawing used to print (via a 3D printer) prototype fly feeders. The fly feeder is 3mm tall and 10 mm in diameter. (**b**) Small glass capillary tubes are inserted around the outer circumference of the feeder. After filling with a solution of 5% (wt/vol) sugar and 5% (wt/vol) yeast extract, flies can freely feed from these capillary tubes. Importantly, flies crawling on or around this feeder are not hidden from the camera view. (**c-e**) Flies entrained for 3 days on a 16 hr light/8 hr dark circadian cycle are placed in the arena. Time 0 (vertical black line) indicates the time at which the lights turn off and data logging begins in the evening. Hour 8 (green line) indicates the time when lights turn back on in the morning. Hour 24 (dotted black line) indicates the time where the light would normally turn back off. However, in this experiment the light does not turn off but rather stays on until the completion of the experiment. Flies show a peak in (**c**) percent time moving, (**d**) mean walking velocity and (**e**) mean turn velocity in both the subjective morning and evening. n=6 experiment, 20 flies per experiment. Light blue shading is ± SEM.

2.2.2 Drosophila thermosensation

As thermosensation (or more appropriately, the ability to sense and avoid warm temperatures) plays a prominent role in our place learning paradigm, we put significant effort into investigating and understanding the fly thermosensory system. As discussed in Chapter 1.2.2 flies posses central and peripheral thermosensors that transmit thermal information to the brain and are thought to direct thermosensory behaviors (e.g. an aversion to hot or cold stimuli). How and where this information is encoded in the fly brain has been unclear. To test fly thermosensory behavior using our TEM array, we developed a two choice thermal aversion assay. For two choice assays 15 flies are placed in a thermal arena consisting of four 1" square, individually addressable peltier tiles (TEM array, described above). Since each arena covers 4 tiles on the 64 tile TEM array, we can test flies in 16 arenas in parallel. Initial efforts using 3mm high acrylic for the walls of the arena proved problematic as flies would spend a significant amount of time walking on this smooth vertical surface and as a result experience a different thermal profile than flies walking directly on the array surface. In the place learning experiments, we discourage contact with the wall by heating an aluminum ring encircling the arena to 50ºC. While this works well for other experiments it is not feasible for these studies since we want to explicitly investigate both hot and cold thermal responses. To solve this difficulty, we developed a novel "jagged" arena. The edge of this jagged arena is composed of spikes 6.0 mm long spaced 1.4 mm apart and was cut from a sheet of 3mm thick acrylic (Figure 2-8). The width between spikes is narrow enough to prevent fly entry. Flies largely avoid

walking on this jagged wall, possibly because the spikes provide little surface area for the fly to stand on, and remain upright on the TEM array surface.

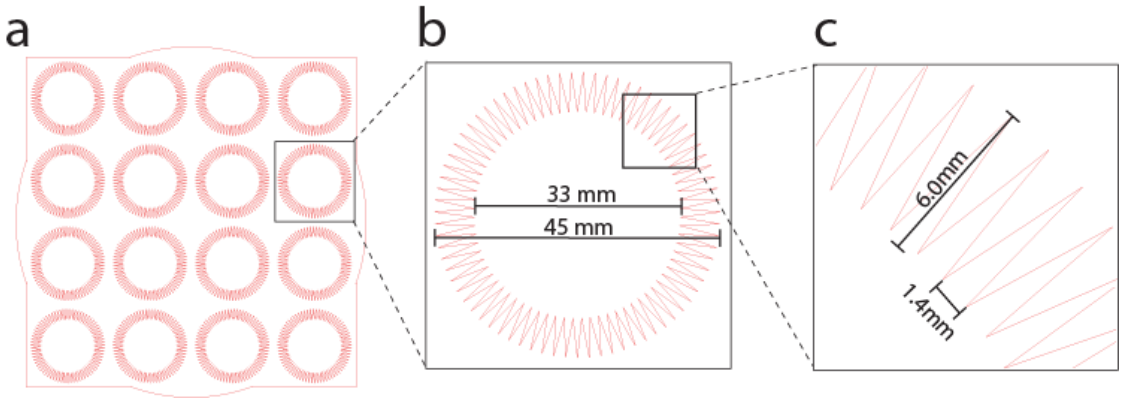

Figure 2-8: Diagram of the thermal aversion arena.
Shown is a diagram of the "jagged" arena used in thermal aversion experiments. (**a**) 16 arenas (each covering 4 TEM tiles) were cut out of a 3mm thick sheet of acrylic. (**b**) Each arena has an inner diameter of 33 mm and outer diameter of 45 mm. (**c**) The walls of each arena are arranged as inward facing spikes. Each spike is 6.0 mm long and the spacing between the inner points of neighboring spikes is 1.4mm.

To assay thermal aversion, flies are tested in a series of trials where they are presented with a choice between two temperatures. At the beginning of each trial, flies are dispersed by raising the temperature of all four tiles to 33°C for 20 seconds. Next, two tiles are set to 25°C and two at a test temperature between 11 and 39°C (temperatures are tested at 2°C intervals for a total of 15 comparisons). Fly location is recorded continuously for 3 minutes using a CMOS camera (BASLER A622f). Next, the temperatures of the 4 tiles are reversed for a 3 min re-test. Following the re-test, a new trial is initiated until all 15 temperature comparisons have been performed. The videos are analyzed off-line using a custom Matlab script to calculate an avoidance

index for each test temperature. The avoidance index (AI) is defined as (#flies at 25°C - #flies at test temp) / total # flies and is reported as the mean avoidance index in the period from 60 to 180 seconds of each temperature comparison. Wildtype flies tested with this paradigm show a preference for 25°C and an aversion to warmer and cooler temperatures (Figure 2-9).

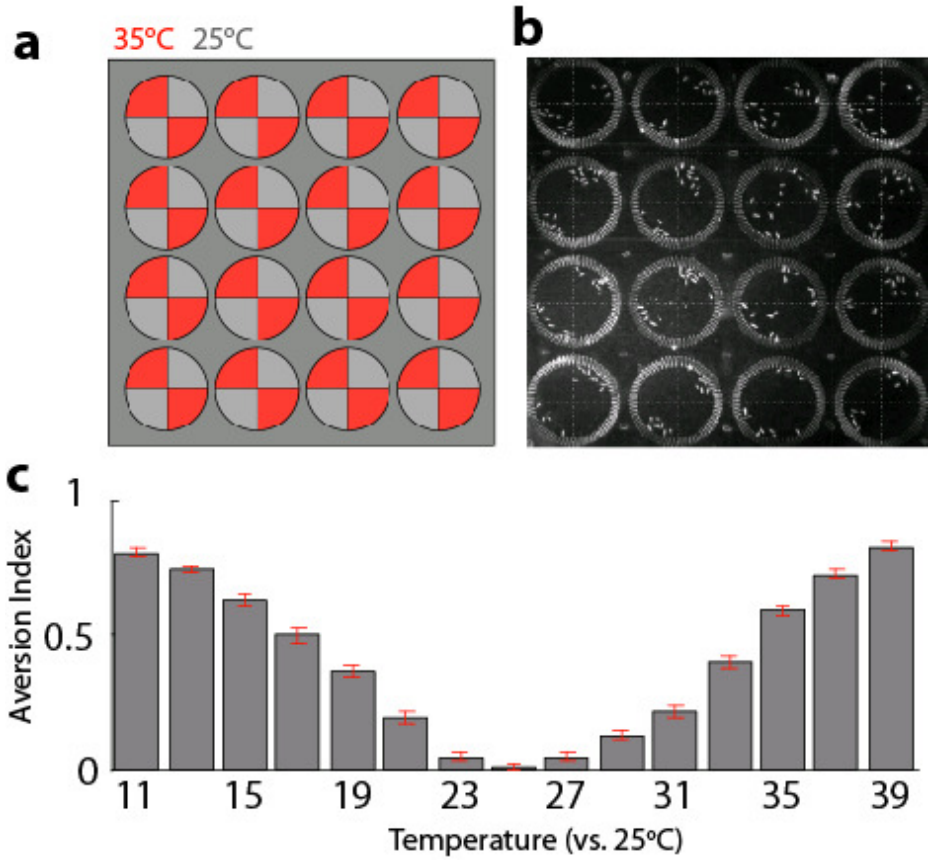

Figure 2-9: *Drosophila* thermal aversion
(**a**) Schematic view of the 16 chamber assay. Each chamber consists of four temperature controlled tiles (1"x1"). (**b**) Shown is an image of the arena with flies loaded in each chamber. The temperature settings correspond to the diagram in (a). The preference for 25°C can be seen clearly as flies avoid the 35°C tiles. (**c**) By serially comparing a range of temperatures (from 11 to 39°C) with the preferred temperature of 25°C, a complete preference curve showing the Aversion Index can be generated within 2 hours.

In developing the two-choice thermal aversion assay, we noticed that flies show a stronger behavioral aversion to cool temperatures when tested with the lights on as compared to when tested in the dark (Figure 2-10 panels **a** and **b**). How is visual input affecting thermosensory behavior? The most likely explanation is that vision does not directly affect the perception of temperature[5] (i.e. the thermo-sensory input), but rather affects the ability of the fly to successfully navigate the thermal landscape. For a fly to display a thermal aversion in our assay, it must both find and then remain at the preferred temperature. It is likely that when a fly leaves its preferred temperature (25ºC) and enters a cold location, it uses visual cues to return to the preferred location. In the dark, flies may have a difficult time re-locating the preferred location leading to a reduced thermal aversion score. The observation that cool aversion is more strongly affected than warm aversion may be related to the stronger reinforcing properties of warm temperatures (Zars et al. 2006). We see this trend when performing thermal aversion assays with references temperatures warmer or cooler than 25ºC (Figure 2-10 panel c). When presented with a choice between an aversive warm and an aversive cold temperature, flies usually choose the cold temperature. This stronger aversion to warm temperatures may mean that flies are less likely to leave the preferred tile when confronted with a hot comparison and as a result, are less dependent on visual cues to relocate the preferred location.

[5] While I believe this to be a less likely explanation, there is precedence for the behavioral state of an animal affecting the tuning of sensory inputs. In a series of recent papers (Borst et al. 2010, Chiappe et al. 2010, Maimon et al. 2010) it has been shown that the behavioral state of an animal affects the gain of wide field motion detectors in the fly optic lobe (lobula plate tangential cells, LPTC's). This is one example of how feedback from other neural centers may affect sensory perception.

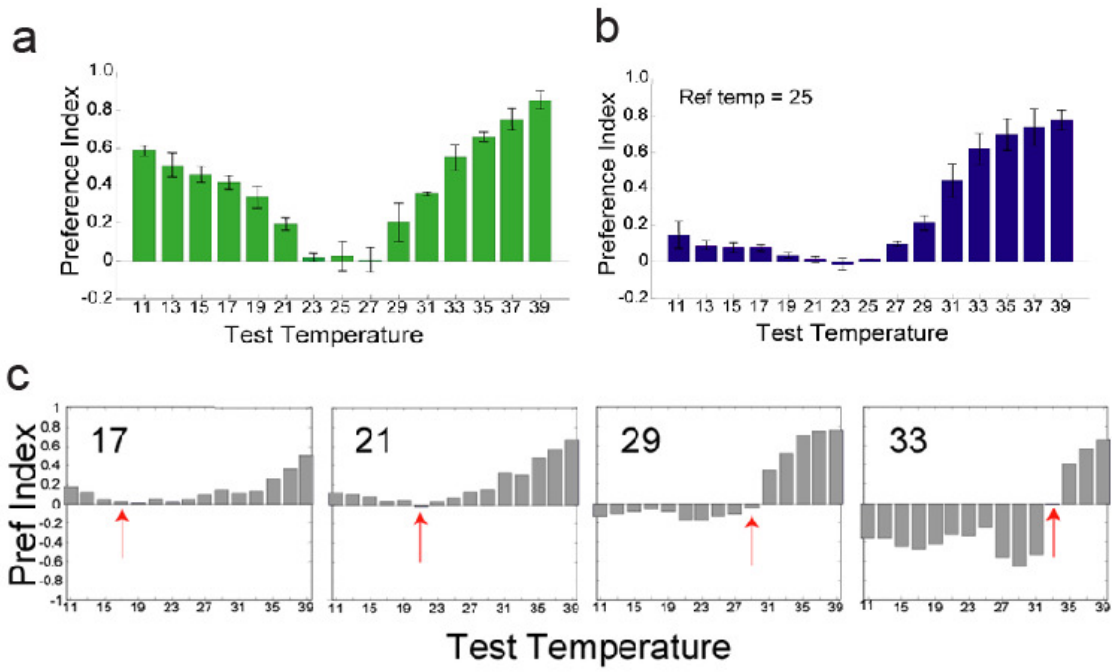

Figure 2-10: Effects of light on thermal aversion
Thermal aversion studies show that flies display stronger aversion to cool temperature when visual cues are present. (**a**) Shown is a thermal aversion curve for flies tested in the presence of visual cues (green bars). Visual cues (a uniform checker pattern) were displayed on a LED display encircling the arena. (**b**) Aversion to cool temperatures is significantly diminished when flies are tested in the dark (blue bars). (**c**) Flies tested with reference temperatures other than 25ºC (Ref. temp shown in top left of each panel) show stronger aversions to warm temperatures than to cool temperatures. Red arrow indicates comparisons where reference and test temperatures are equal. For **a** and **b**, n>5 experiments, 15 flies per experiment. Error bars are ± SEM. In **c**, n=1 experiment, 15 flies.

2.2.3 effect of rearing temperature and humidity on thermal aversion

What, if any, are the affects of rearing temperature on *Drosophila* thermal aversion? In *c. elegans*, it has been shown that worms will prefer temperatures at which they have been raised, and avoid temperatures at which they have been starved (Mori 1999). Is the same also true for *Drosophila* (i.e. is thermal preference fixed or is it adaptive)? To test this, we reared flies at 3 different temperatures, 18ºC, 25ºC,

and 30ºC. Our results (Figure 2-11) indicate little or no effect of rearing temperature on subsequent temperature preference.

Perception of temperature and thermal comfort in vertebrates is influenced by humidity as well as absolute temperature (Rohles et al. 1973). Is the same also true in flies? To test this possibility, thermal aversion experiments were performed at high (65%), medium (40%), and low (20%) relative humidity (Figure 2-11). Relative humidity was measured at 25ºC in the enclosure surrounding the thermal aversion assay. Flies tested at low humidity had thermal aversion curves shifted to the left (i.e. stronger aversion to warm temperature). Conversely, flies tested at high humidity had thermal aversion curves shifted to the right (i.e. more tolerant of high temperatures). Unfortunately, it is not immediately clear if humidity has an acute or prolonged impact on thermal aversion, and if the effects are mediated through the thermosensory system (i.e. does humidity affect the sensory representation of temperature), a separate dedicated hygrosensation system (Sayeed et al. 1996; Liu et al. 2007), or a through a non-specific effect on animal health and activity. In order to limit behavioral variability in all other behavior assays, we perform all of our tests in a room held at 25ºC and 40% RH. Additional discussion on thermosensation and experimental data demonstrating a labeled line logic with distinct anatomic substrates for hot and cold sensing in the fly can be found in our paper “The Coding of Temperature in the *Drosophila* Brain” (Gallio et al. 2011).

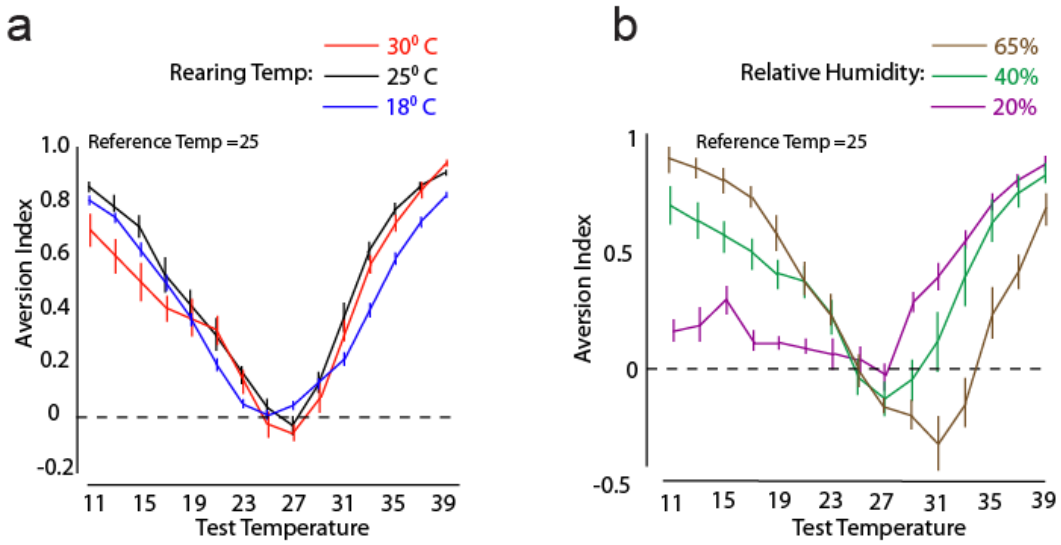

Figure 2-11: Effect of rearing temperature and humidity on thermal aversion

(**a**) To test the effect of rearing temperature on *Drosophila* thermal aversion, flies were reared at 18ºC (blue line), 25ºC (black line), or 30ºC (red line) and then tested in the thermal aversion assay. Rearing temperature has little effect on subsequent thermal aversion behavior. (**b**) To test the effect of relative humidity on thermal aversion, flies were tested in an enclosure at 20% (purple line), 40% (green line), and 65% (brown line) relative humidity. Low relative humidity results in a shift of the thermal aversion curve to the left (i.e. stronger warm aversion, weaker cool aversion). Conversely, high relative humidity shifts the thermal aversion curve to the right (i.e. stronger cool aversion, weaker warm aversion). Error bars are ± SEM. n>5 experiments, 15 flies per experiment.

2.3 Acknowledgements

I'd especially like to thank Marco Gallio for discussions and advice on *Drosophila* thermosensation and the establishment of the two-choice thermal aversion assay. Additionally, I'd like to thank Gus Lott, Jason Osborne, and Christopher Werner for help with the mechanical design and integration of the peltier array; Kristen Branson, Don Olbris andMark Bolstad for supporting Ctrax fly tracking software; and Michael Dickinson for discussions and advice on the development of the

fly arena. Chapter 2, in part, has been accepted for publication in Nature, Ofstad, Tyler A.; Zuker, Charles S.; Reiser, Michael B. (2011). The dissertation author was the primary investigator and author of this paper.

Chapter 3
Visual Place Learning in *Drosophila*

3.1 Visual Place Learning

Vision provides the richest source of information about the external world, and most seeing organisms devote enormous neural resources to visual processing. In addition to visual reflexes, many animals use visual features to recall specific routes and locations, such as the placement of a nest or food source. When leaving the nest, bees perform structured "orientation flights" to learn visual landmarks that will aid in returns. If subsequently displaced from their outbound flight, bees are capable of taking direct paths back to their nest using these learned visual cues (Thorpe 1949; Gould 1986; Capaldi et al. 1999; Menzel et al. 2005). How do insects, with relatively compact nervous systems, perform these navigational feats? In mammals, the identification of place (O'Keefe et al. 1971), grid (Hafting et al. 2005), and head direction cells (Taube et al. 1990) in the medial temporal lobe suggests the existence of a "cognitive map" (Moser et al. 2008). Unfortunately, little is known about the cellular basis of invertebrate visual place learning. In order to identify the neurons and dissect the circuits that underlie navigation, we studied place learning in a genetically tractable model organism, *Drosophila melanogaster*.

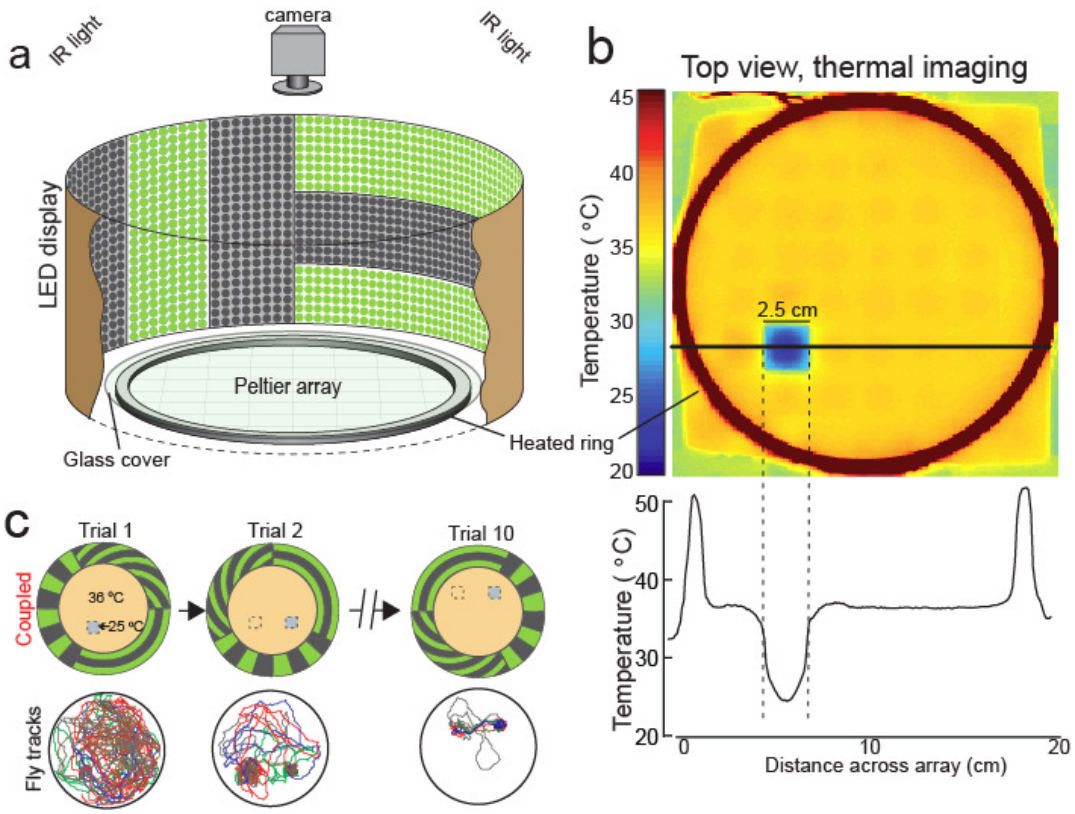

Figure 3-1: Schematic representation of the Visual Place Learning Arena. (**a**) Illustration of the behavior chamber inspired by the Morris Water Maze. The floor of the arena is composed of 64 peltier elements, the panorama is provided by a 24x192 LED display, and flies are recorded using a high-res CMOS camera under infrared (IR) illumination (see text for details). (**b**) Thermal imaging view of the arena's floor showing the uniformly warm surface with a single cool tile; also shown is the heated ring barrier. The bottom panel depicts the temperature readings across the arena (black line); note the sharpness of the temperature boundaries. (**c**) Trajectories of 4 representative flies from trials #1,2 and 10 are shown below a diagrammatic representation of the visual panorama denoting the location of the cool tile in the previous trial (dashed square), and the current location of the cool tile (blue square). In this *coupled* condition the position of the cool tile relative to the visual panorama remains constant even as its absolute position changes between trials.

To explicitly test for visual place learning in *Drosophila*, we developed a thermal-visual arena inspired by the Morris Water Maze (Morris 1981) and the Heat Maze of Mizunami et al. (1998) (Figure 3-1a, See also Chapter 2). In the *Drosophila*

place learning assay, flies must find a hidden "safe" target (i.e. a cool tile) in an otherwise unappealing warm environment (36^{o} C) (Figure 3-1b, Figure 2-3). Notably, there are no local cues that identify the cool tile. Flies cannot see, smell, hear, or follow temperature gradients to locate the cool spot. Rather, the only available spatial cues are provided by the surrounding electronic visual panorama that displays a pattern of evenly spaced bars in three orientations (vertical, diagonal, and horizontal; Figure 3-1a and c).

To assay spatial navigation and visual place memory, fifteen adult female flies are introduced in the arena and confined to the array surface by placing a glass disk on top of a 3mm high aluminum ring that encircles the outer perimeter of the arena. The array is set so that a single tile is cooled to 25^{o} C (the preferred temperature, (Sayeed et al. 1996; Hamada et al. 2008; Gallio et al. 2011)) while the rest of the arena is heated to 36^{o} C. During the first 5 minute trial, nearly all (94% of flies) eventually succeed in locating the cool target (Figure 3-3a). In subsequent trials, the cool tile and corresponding visual panorama are rapidly shifted to a new location (90^{o} clockwise or 90^{o} counterclockwise with the direction chosen at random). Importantly, the cool tile and visual panorama are *coupled* so that even though the absolute position of the cool tile changes, its location relative to the visual panorama remained constant (Figure 3-1c, Figure 3-4a). Our results (Figure 3-2a, red trace) demonstrate that over the course of 10 training trials flies improve dramatically in the time they require to locate the cool tile. This improvement is accomplished by taking a shorter (Figure 3-2b), more direct route to the target (Figure 3-2c Figure 3-4a), without noteworthy changes

in the mean walking velocity (Figure 3-2d). To ensure that social interactions between flies were not influencing place learning (e.g. flies following each other to the safe spot), we also trained single flies and found that flies tested individually show equivalent place learning abilities (Figure 3-6). As would be predicted for *bona-fide* visual place learning, the improvement in place memory is critically dependent on the visual panorama. Flies tested in the dark show no improvement in the time, path length, or directness of their routes to the target (Figure 3-2, black trace; Figure 3-4b).

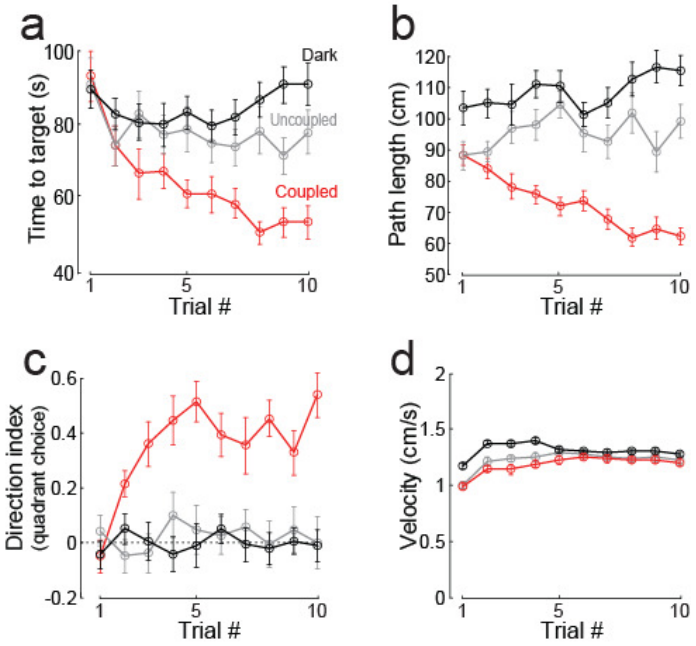

Figure 3-2: Flies use visual cues to improve in place learning task.
Flies were trained in the place learning arena with a *coupled* visual panorama (red, n=33 experiments, 495 flies) or with an *uncoupled* (gray, n=21 experiments, 315 flies) or *dark* (black n=23 experiments, 345 flies) visual surround. (**a**) When trained in the *coupled* condition (red) flies reduce the time to find the cool tile by nearly half, whereas flies trained with an *uncoupled* panorama (gray) or in the dark (black) show little or no improvement in time to locate the target. The improvement exhibited with the *coupled* visual panorama is due to flies taking (**b**) shorter, (**c**) more direct paths to the target (**d**) rather than simply increasing walking velocity. See section 3.3 for details of calculations. Values are mean ± SEM.

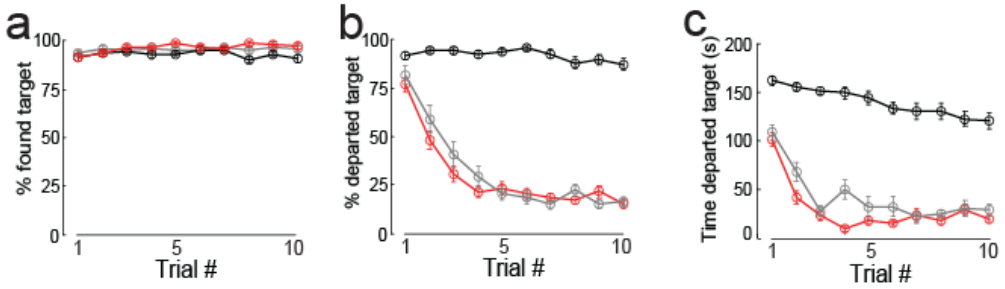

Figure 3-3: Flies learn to remain at the cool location
(**a**) Nearly all flies eventually locate the cool spot during each 5 minute trial under all three training conditions. (**b-c**) For flies that successfully find the cool tile, the fraction that later leave the tile (**b**), and the length of time they leave for (**c**) are shown. Flies trained with a visual panorama, either *coupled* (red) or *uncoupled* (gray), learn to remain at the cool tile after the first couple trials. Interestingly, flies trained in the dark fail to learn (or are unable) to remain at the cool tile. Values are ± SEM. n is as reported in Figure 3-2.

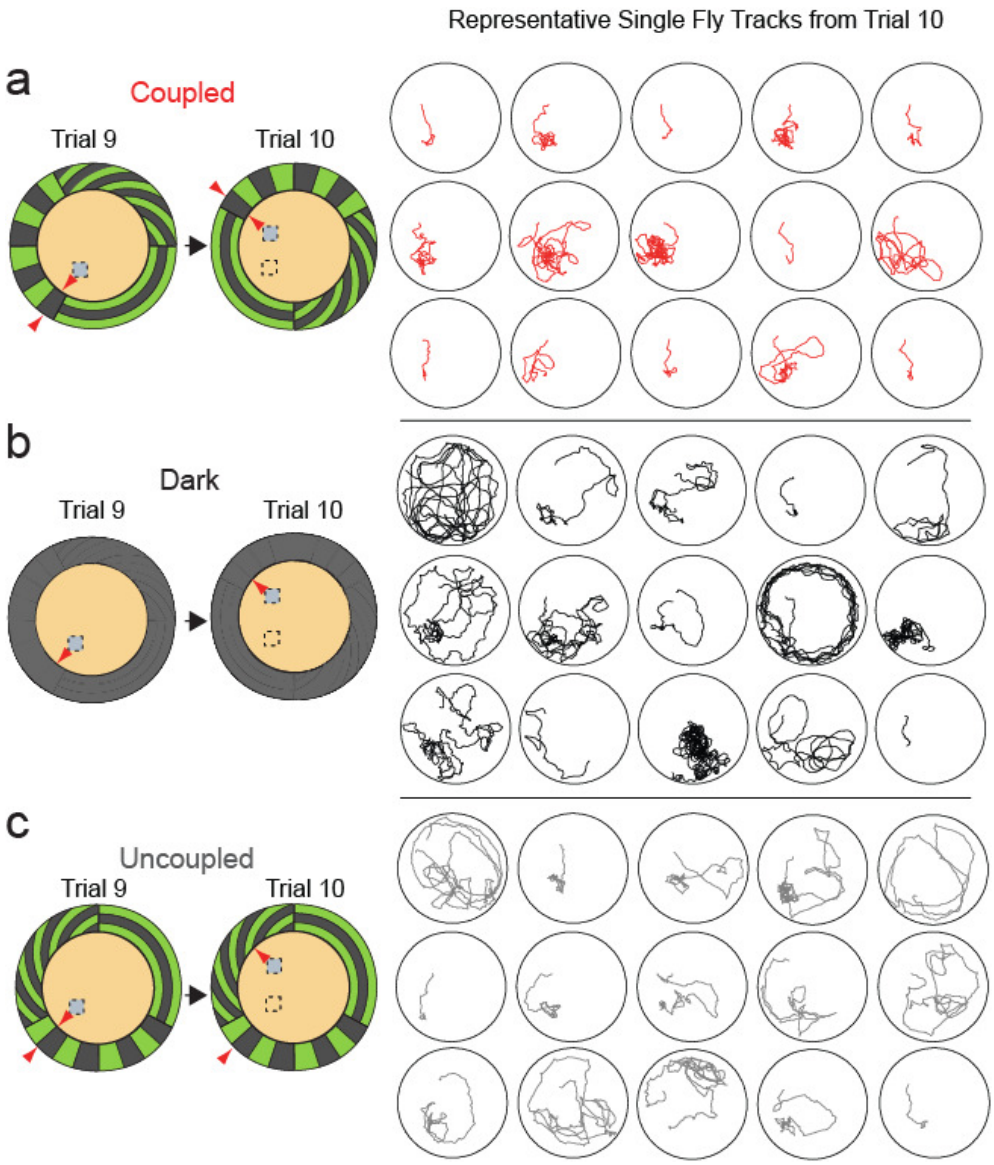

Figure 3-4: Representative single fly tracks from trial #10
(**a-c**) Diagrammatic representation of the visual display (gray and green) and cool spot (blue square) during trials 9 & 10 shown on left. Red arrows mark relative location of cool spot and visual display in each trial. Flies were trained with a (**a**) *coupled*, (**b**) *dark,* or (**c**) *uncoupled* visual panorama. Representative tracks from all 15 files in trial 10 plotted individually are shown on right.

To verify that flies are using the spatially distinct features of the visual panorama to direct navigation, we also tested flies using an *uncoupled* condition

whereby the cool tile was still randomly relocated for each trial, but now the display remained stationary throughout. With this training regime the visual panorama provides no consistent location cues, but weaker spatial cues such as distance and local orientation of the arena wall are still available to the flies. Our results (Figure 3-2, grey trace; Figure 3-4c) demonstrate that flies trained with the *uncoupled* visual panorama exhibit little improvement in the time to find the cool tile, and no improvement in the directness of their approaches. Thus, spatially relevant visual cues are required for flies to learn the location of the cool tile.

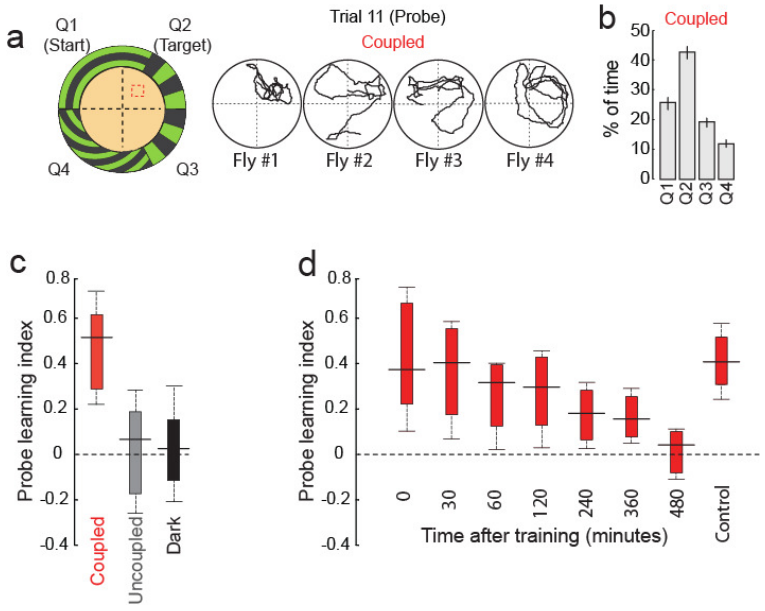

Figure 3-5: Following training flies exhibit a persistent search bias.
Flies are tested in a probe trial (#11) where the visual display is relocated as before but no cool tile is present. (**a**) Trajectories from four representative flies, each plotted for 60 s after leaving their starting quadrant. Flies start in the top-left quadrant (Q1, Start; see diagram); the small dashed square denotes the "expected" location of the cool tile (Q2, Target). (**b**) Flies preferentially search in the quadrant where they have been trained to find the cool spot (Q2), even when the cool spot is absent (see section 3.3 for details and statistics); values are mean ± SEM, n=33 experiments, 495 flies. (**c**) Probe learning index (see section 3.3 for details of calculation) is significantly greater than zero (indicating learning) when flies are trained with a *coupled* visual panorama (red, $p<0.0001$, n=33), but not when trained with an *uncoupled* (grey, $p=0.28$, n=21) or dark (black, $p=0.39$, n=23) visual panorama. p-values are calculated using one-tailed t-test. For additional statistical comparisons see section 3.3. (**d**) To test place memory retention, flies were tested in a probe trial at several time intervals following training ($n\geq5$). Flies retain visual place memories for 2 hours after training ($p<0.05$, one-way ANOVA with a Bonferroni correction for multiple comparisons and pairwise comparisons to the *uncoupled* condition in c). Although the Place learning index is not significantly different from the *uncoupled* control at 4 and 6 hours, they are significantly greater than 0 (one-tailed t-test, $p<0.05$). Since flies were left in the arena between training and testing (up to 8 hrs), *control* refers to siblings placed in the arena for an equivalent window of time (i.e. 8 hours) prior to training followed by immediate testing. Box plots indicate the median value (solid black line), 25 and 75 percentiles (box), and the data range (dashed whiskers).

As a further test of visual place memory, flies were challenged immediately after training with a probe trial (trial #11) where the visual landscape is relocated as usual, but no cool tile is provided (i.e. can the flies be fooled into going to the non-existent safe spot?). We hypothesized that if the flies learned to locate the cool tile by using the peripheral visual landmarks, then they should bias their searches to the area of the arena where the visual landscape indicates the cool tile should have been, even when the cool tile is absent. Indeed, flies preferentially search in the arena quadrant where they have been trained to locate the now "imaginary" cool tile (Figure 3-5, Figure 3-7a and d). Whereas, if flies were trained in the dark or with an *uncoupled* visual landscape, conditions that contain no specific information about the location of the cool tile, the flies instead search the arena uniformly during the probe trial (Figure 3-5c, Figure 3-7b, c and d). Together, these results demonstrate that fruit flies can learn spatial locations based on distal visual cues and use this memory to guide navigation. Interestingly, by varying the time between the end of a single round of training (10 trials) and testing during a probe trial, we could also show that flies retain these visual place memories for at least 2 hours (Figure 3-5d).

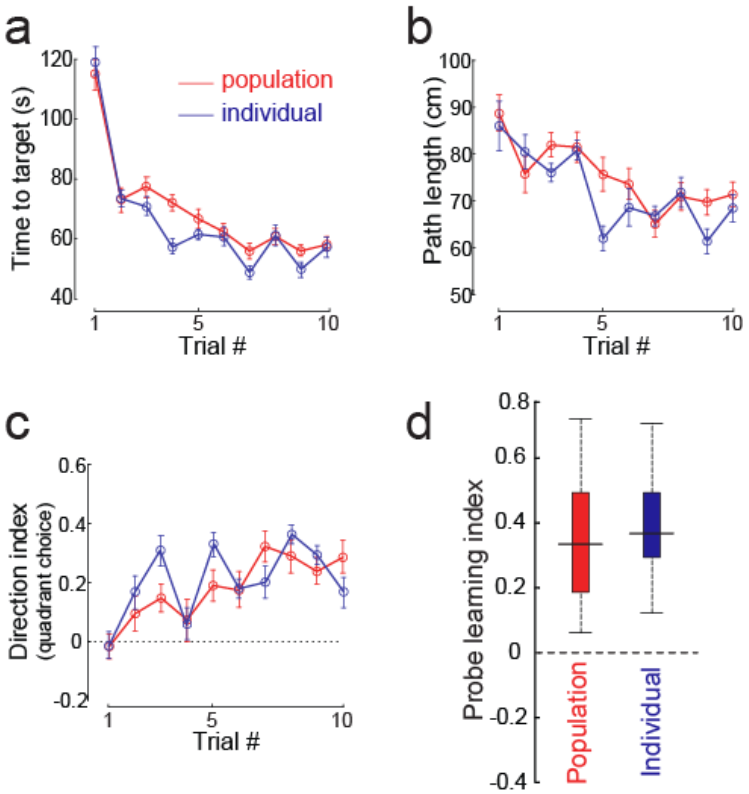

Figure 3-6: Single flies learn as well as populations of flies
(**a-d**) To assess the possibility of social interactions influencing place learning behavior, flies were tested either as a population (red, 25 experiment, 15 flies per experiment), or individually (blue, 30 flies). For comparison, the results from individual flies were randomly sampled to generate 25 groups composed of 15 flies each. (**a**) There is no noticeable difference in the time to target, (**b**) path length, (**c**) direction index, or (**d**) probe learning index when comparing flies tested individually to flies tested as a population. (**a-c**) Values represent mean ± SEM or (**d**) as described in Fig. 3.

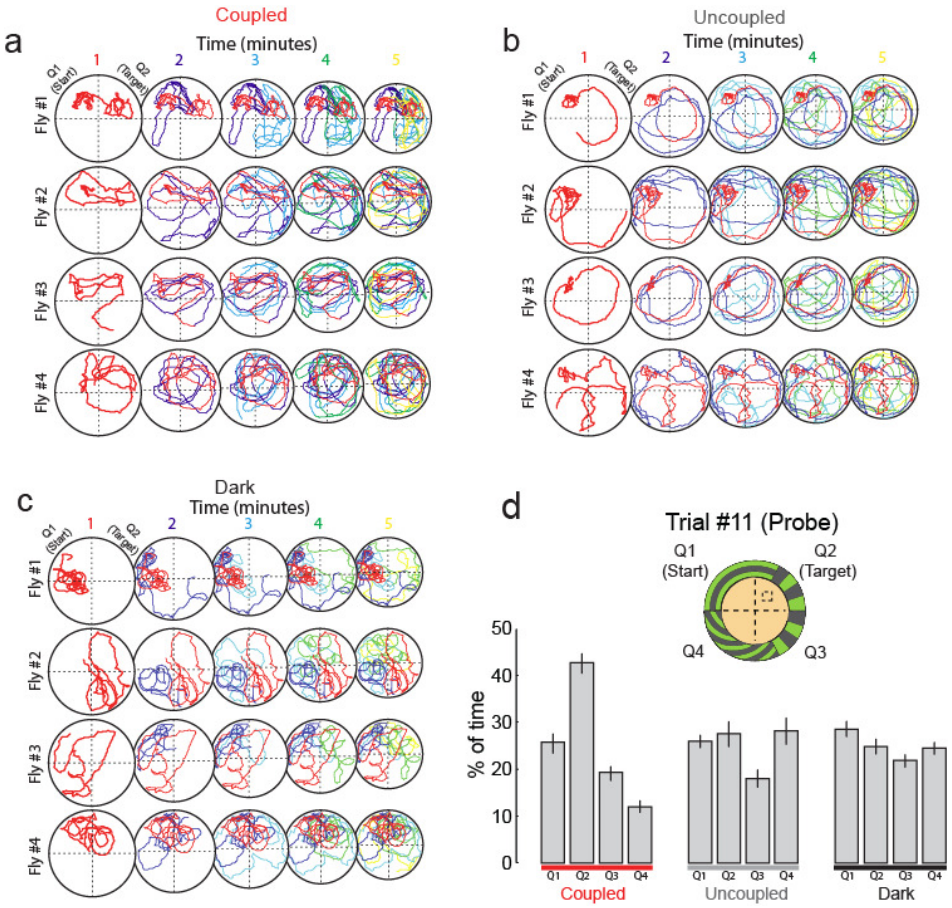

Figure 3-7: Representative fly tracks during the probe trial
(**a-c**) Trajectories of 4 representative wildtype flies during a probe trial after training with a (**a**) *coupled*, (**b**) *uncoupled*, or (**c**) *dark* visual panorama. Trajectories are color coded by minute (in order red, dark blue, light blue, green, yellow). Q1 (start) is the location of the cool tile in the preceding trial and Q2 (target) is the location that the cool spot would be (as indicated by the visual panorama). Note that in addition to preferentially searching in Q2 (the target quadrant), wildtype flies trained with a *coupled* visual panorama return repeatedly to Q1 (the location of the cool tile in the previous trial). (**d**) Wildtype flies trained with a *coupled* visual panorama (red, n=33) preferentially search Q2, the quadrant where they have been trained to locate the cool tile. When trained with an *uncoupled* panorama (gray, n=21) or in the dark (black, n=23), flies display no spatial search bias and explore the arena uniformly. Percentage of time is calculated during the first 60 seconds after flies leave Q1.

3.2 Non-visual Place Learning

Work from the Heisenberg (Wustmann et al. 1996; Wustmann et al. 1997; Putz et al. 2002), Zars (Diegelmann et al. 2006; Zars 2009), and Strauss (Neuser et al. 2008) groups have shown that flies can use idiothetic cues and path integration to navigate (in several forms of non-visual place memory tasks). To address whether idiothetic (i.e. non-visual) cues are sufficient to guide navigation in the thermal-visual arena we tested flies using a set of modified training protocols that included (a) keeping the cool tile stationary, (b) rotating the cool tile in a constant direction, and (c) randomly rotating the cool tile between trials, all in the dark. Next, we tested flies with (d) a stationary visual panorama and a randomly relocated cool tile, (e) with a randomly rotating visual panorama and a stationary cool tile, and (f) with random, and independent relocations of the visual panorama and cool tile (Figure 3-8). In essence, we hypothesized that if (non-visual) path integration was used by flies while navigating the large arena, we would expect the flies to improve in their ability to find the cool tile when trained with (i) the target consistently in the same physical location, or (ii) consistently rotated in the same direction. In order to disperse flies from the stationary cool tile between trials the entire array was heated to 36ºC for 60s prior to the start of each trial. When trained with the standard *coupled* visual panorama, this manipulation does not impair visual place learning (data not shown). We see no indication of spatial learning with flies trained using any of these experimental conditions (Figure 3-8). We note that flies may use idiothetic information while

navigating; however, this experience is not sufficient for the formation of a place memory.

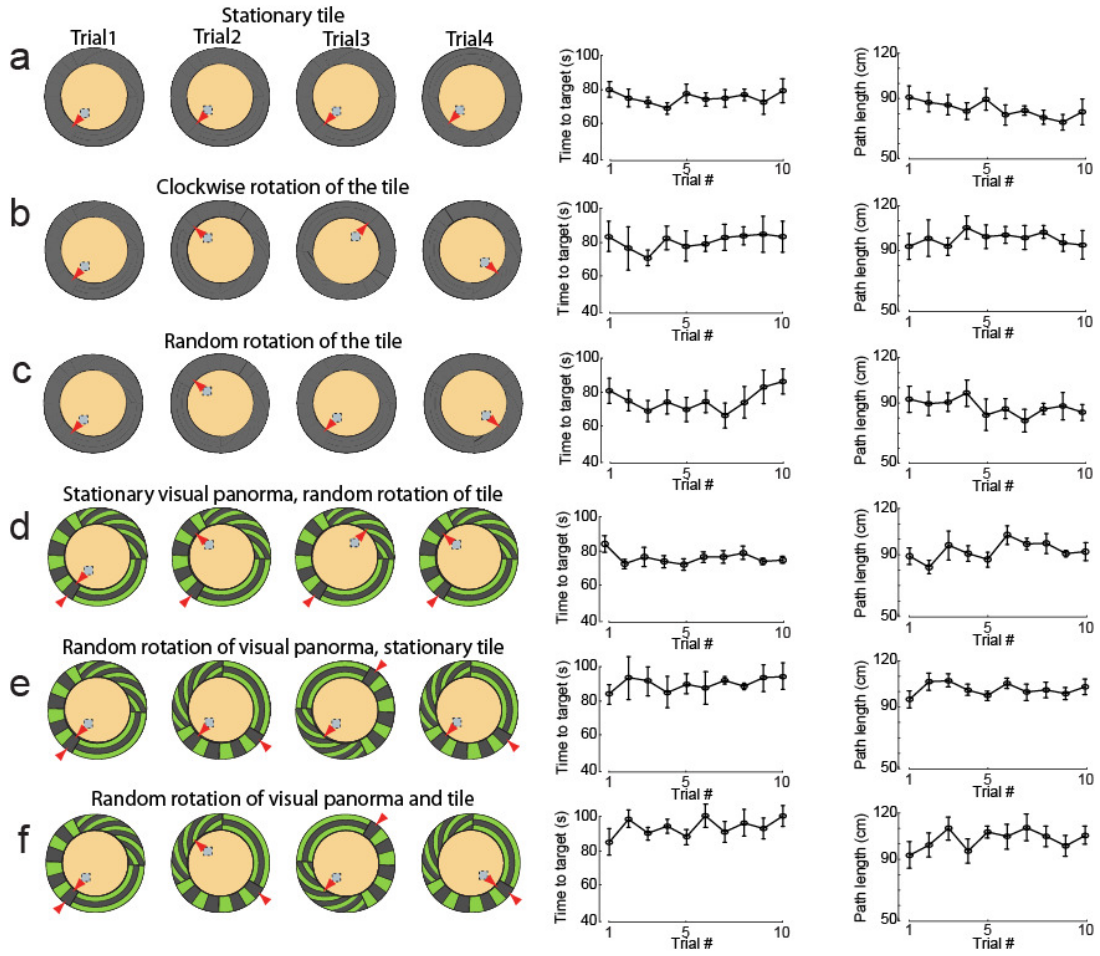

Figure 3-8: Idiothetic cues are not sufficient to guide flies to the cool spot
If (non-visual) path integration were used by flies while navigating the large arena, we expect that flies might improve in their ability to find the cool tile when trained in the dark with (**a**) the target consistently in the same physical location, or (**b**) consistently rotated in the same direction (e.g. relocated 90º clockwise). Flies were trained for 10 trials, with trials 1-4 shown in the illustrations (left panels). Also shown are "time to target" and "path length" for each condition. (**c**) The experiment was also performed with the tile randomly relocated in each trial. No notable spatial learning is observed with flies trained using any of these experimental paradigms. (**d-f**) In a parallel set of experiments the visual surround was active, but was *uncoupled* from the location of the cool tile. (**d**) The visual surround was held constant but the cool tile was moved randomly, (**e**) visual surround was moved randomly but the tile was held constant, (**f**) visual surround and cool tile were both moved randomly. For all conditions, n = 8 experiments; values are mean ± SEM.

3.3 Methods and Calculations

3.3.1 analysis

At the end of the experiment, flies were tracked off-line using Ctrax fly tracking software (Branson et al. 2009). Fly centroid data was imported into MATLAB (Mathworks, Natick, MA) and processed using custom scripts (appendix). In Figure 3-2, Time to target, Path length, and Velocity are calculated, per fly, for the time window from the start of the trial until each fly reaches the target tile. Direction index (quadrant choice) is calculated as the number of flies that first enter the quadrant containing the cool tile (# correct) minus the number of flies that first pass into the opposite quadrant (# incorrect) divided by the total number of flies. To test if flies exhibit a bias for certain quadrants or rotation directions during training in the visual place learning arena, we tested for the dependence of the time to target on the target quadrant and on the rotation direction (CW or CCW). This was accomplished by calculating the difference between the time to target for each trial (from each experiment) and the mean time to target for each trial across all experiments. The differences from mean (in seconds) for training to quadrant 1, 2, 3, or 4 are 1.7±2.3, -0.5±2.7, 0.0±2.2, and -1.2±2.2 seconds respectively. The differences from mean (in seconds) for clockwise and counterclockwise rotations are 0.6±1.5 and -0.6±1.8 seconds respectively. Error is reported as SEM. For both tests, there are no statistically significant comparisons using one-way ANOVA at $p<0.05$.

In Figure 3-5 b, percentage of time spent in each of the quadrants was tested for statistical significance using one-way ANOVA with a Bonferroni correction for

multiple comparisons. Flies spend significantly more time searching in Q2 when compared to Q1, Q3, or Q4 ($p<0.01$). Probe learning index in Figure 3-5 and Figure 3-6 is calculated from probe trial trajectories as the amount of time during the first 60 seconds after leaving the starting quadrant that flies spent searching in Q2 (the quadrant where they have been trained to locate the cool tile) minus the amount of time spent searching in Q4 (a quadrant that is the same distance from the starting quadrant, but the wrong direction) divided by the total time in both quadrants. In addition to statistical tests described in Figure 3-5 legend probe learning index scores reported in were also tested for statistical significance using one-way ANOVA with a Bonferroni correction for multiple comparisons. Flies trained in the *coupled* condition show significantly higher probe learning scores ($p<0.01$) when compared to flies trained in the *uncoupled* or dark conditions. There is no significant difference (at $p<0.05$ level) between *uncoupled* and dark.

3.3.2 experimental animals

All flies used were female and unless otherwise noted, are DL wildtype. This strain is a laboratory culture descended from interbreeding dozens of wild caught isofemale lines, established in 1995 and maintained by Michael Dickinson's laboratory (Dickinson 1999a). Flies were reared on standard media at 25^{o}C on a 16hr light/8hr dark cycle where hour 0 corresponds to the transition from dark to light. Visual place learning experiments were performed with 4 day old adult flies during hours 11-15 of the flies' subjective day in a room kept at 25^{o}C and 40% RH.

3.4 Additional Notes and Experiment Details

Many of the experimental details presented above are the results of months of careful calibration and optimization of the place learning paradigm. While most are not central to the experimental findings, they were nonetheless crucial to the success of the experiment. As such, they are documented here.

3.4.1 automated nature of our place learning assay.

We believe the automated design of our assay, which allows flies to be introduced into the arena and then trained and tested without further handling, is a significant factor in the success of our experiments. Other groups have attempted similar place learning experiments with flies. These previous attempts all entailed manually removing and re-introducing animals to the arena between trials, and have met with limited success (personal communications, (Foucaud et al. 2010)). In other learning paradigms, it has been shown that handling flies between trials disrupts memory formation (Wustmann et al. 1997). We believe that our ability to train and test flies without physical intervention during the experiment is critical to the robust place learning we observe.

3.4.2 choice of visual panorama

Our current wide field visual panorama (composed of evenly spaced vertical, diagonal, and horizontal bars) was chosen for several reasons. (i) Flies in the thermal-visual arena show no innate preference for any one portion of the visual panorama prior to training, (ii) previous work has demonstrated that flies can differentiate contour orientation (Liu et al. 2006), and (iii) flies are exquisitely sensitive to motion

and this pattern will differentially drive direction selective motion detectors as the fly traverses the arena. We believe that all of these factors contribute to the saliency of our visual panorama and the fly's ability to use these cues to remember spatial locations.

3.4.3 males vs. females

The careful reader may have noticed that we perform nearly all of our behavioral experiments with female flies. This is not to suggest that female flies are inherently "smarter" than male flies. In fact, we have tested male flies as well and find that they are just as capable of place learning as female flies. However, testing mixed populations of males and females, or populations of males alone proves problematic due to confounding social interactions. When males and females are tested together, males often spend a significant amount of time following a female (courtship) whereas males tested as a population display aggressive behaviors when clustered on the 1 inch cool tile[6] . Female only populations were chosen due to a lack of significant social interactions in our place learning paradigm.

3.4.4 genetic background

As has been previously reported for multiple *Drosophila* assays (de Belle et al. 1996; Anholt et al. 2004) and for many different species (Owen et al. 1997; Silve et al. 1997), genetic background has a significant effect on behavior. In optimizing our assay, we tested a wide range of genetic backgrounds. All wildtype backgrounds that

[6] Perhaps this could be an efficient means of studying aggression in the fly? (Baieret al. 2002; Chen et al. 2002; Kravitz et al. 2003; Yurkovic et al. 2006)

we tested showed the ability to learn spatial locations (Figure 3-9a). However, there was significant variability in the level of performance. The best performers were the "TS" and "DL" lines. TS flies were recently collected (<4 years ago) from a wild population of flies in Chicago. DL flies are a laboratory culture descended from interbreeding 50 wild caught isofemale lines, established in 1995 and maintained by Michael Dickinson's laboratory (Dickinson 1999b). We choose DL as the standard line for our behavioral studies because they (i) shows robust place learning capabilities, (ii) have been used extensively by other laboratories over the past 15 years and appear in dozens of publications, and (iii) are the preferred wildtype background for many of the behavioral studies occurring at Janelia. Interestingly, the performance of flies in the place learning assay is (roughly) inversely correlated with the length of time since the lines were collected and established as laboratory strains. The worst performers, CantonS (collected in Canton, Ohio by Calvin Bridges in the 1920's) and OregonR (collected in Roseburg, Oregon by D. Lancefield in 1925) have both existed for >2000 generations in a bottle whereas the best performers, DL and TS were collected very recently (1995 and 2008 respectively) and have existed as laboratory stocks for relatively few generations. This apparent correlation may reflect different selective pressures between flies in the wild and flies maintained in the laboratory.

Of significant concern, particularly for the experiments presented in the following chapter, is the performance of non-wildtype w1118 flies. w1118 flies contain a mutation in the white gene, an ABC transporter that is necessary for the

transport of guanine and tryptophan (both necessary for red pigmentation) into the pigment cells in *Drosophila* eyes (Sullivan et al. 1975). Flies with the w1118 allele have white eyes and as such, are the common genetic background used when generating transgenic fly stocks. By including mini-white (a portion of the full length white gene that provides pigmentation) in the DNA transgenic construct to be injected into *Drosophila* embryos, identifying successful transformants is greatly simplified by sorting for red-eyed flies (i.e. rescue of the white eye phenotype with the transgenic mini-white gene). Unfortunately, in addition to the white-eyed phenotype, w1118 flies have documented impairment in memory (Diegelmann et al. 2006; Cheng et al. 2008; Hoyer et al. 2008). When Rubin lab GAL4 lines (all in w1118 background) are tested in the visual place learning assay, we see no evidence of successful place learning (Figure 3-9b and c). Poor behavior in these driver lines is not specific to place learning as poor performance has also been noted in multiple other behavior paradigms, including tethered flight, gravitaxis and aggression (personal correspondence). As the white gene is located on the X chromosome and all of our GAL4 drivers are inserted into the 3rd chromosome, we tried rescuing place learning by replacing the X and 2nd chromosome in several Rubin w1118 GAL4 driver lines with corresponding chromosomes from DL wildtype flies. Replacing the X and second chromosomes in these stocks with DL equivalents partially restores place learning capabilities (Figure 3-9b). While this strategy works, it would be prohibitively time consuming and expensive to replace chromosomes in all, or even in a fraction of the 8000+ GAL4 driver lines being created by the Rubin lab. As an

alternate, more efficient means of rescuing behavior of the w1118 driver lines, we tried testing heterozygous flies created by crossing Rubin lab GAL4 driver lines to the DL wildtype strain. Crossing a Rubin GAL4 line to DL and testing the resulting heterozygous F1's results in near complete rescue of place learning capabilities (Figure 3-9c). Therefore, it is not necessary to "fix" all of the GAL4 driver lines; rather, it is only necessary to have the effector line in a suitable background. Tetanus (w+;UAS-tnt,tubP-GAL80ts) and Kir2.1 (w+;UAS-Kir2.1;tubp-GAL80ts) effector lines were backcrossed for 10 generations into the DL wildtype background. These backcrossed effector lines show place learning performance that is comparable to DL wildtype. Importantly, when crossed to the (poorly behaving) Rubin GAL4 lines, the resulting F1's show robust place learning abilities that are comparable to the parental DL line (Figure 3-9c).

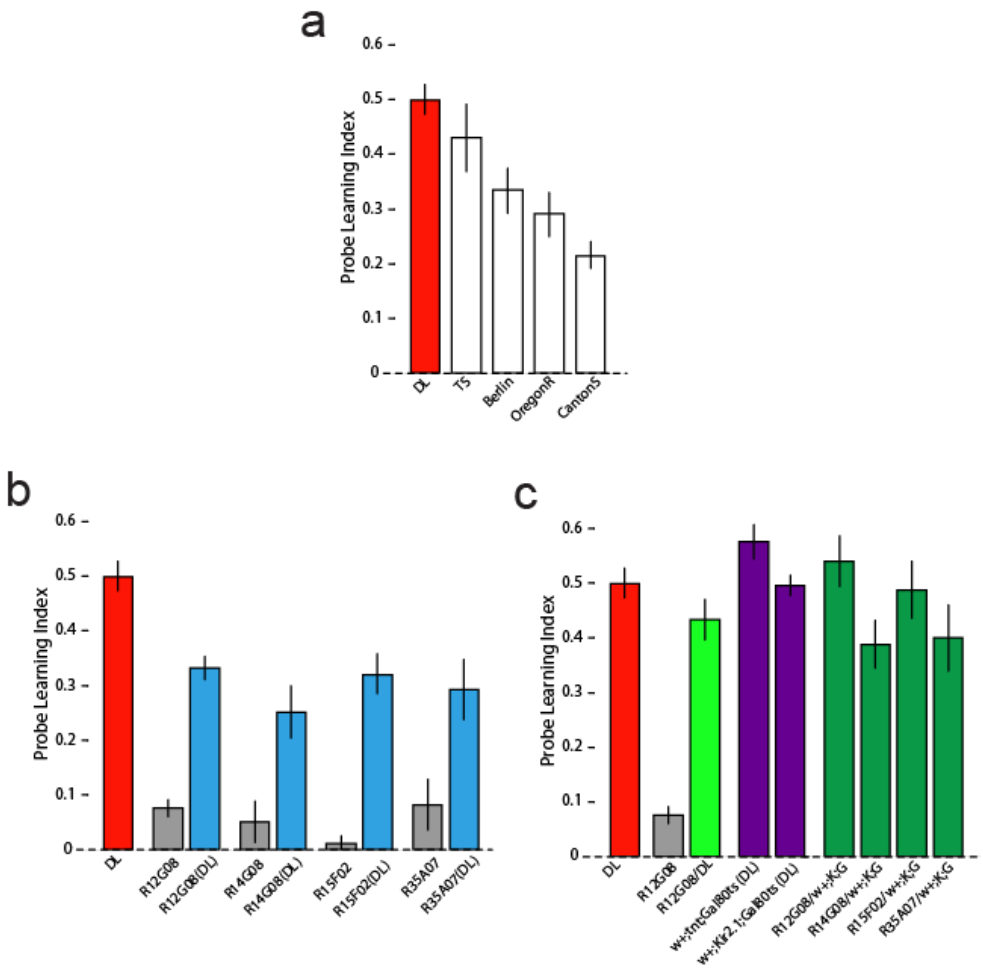

Figure 3-9: Effects of genetic background on place learning performance
(**a**) Flies from 5 different genetic backgrounds were tested in the visual place learning assay. DL (red bar) shows the strongest place learning capabilities and is the standard wildtype line for most behavior experiments in this dissertation. (**b**) GAL4 driver lines in the w1118 genetic background (gray bars) show poor place learning behavior. Replacing the X and 2nd chromosome in these lines with corresponding chromosomes from DL wildtype partially restores place learning capabilities (blue bars). (**c**) When GAL4 driver lines in the w1118 genetic background are crossed to DL wildtype and the F1 offspring are tested (light green bar), place learning behavior is comparable to DL wildtype. Place learning behavior in tetanus or Kir2.1 effector lines backcrossed for 10 generations into a DL background is comparable to DL wildtype (purple bars). Finally, crossing these backcrossed effector lines to GAL4 driver lines in the w1118 genetic background and testing the F1 offspring (dark green bars) restores place learning capabilities to that of DL wildtype. n≥4 experiments per condition, 15 flies per experiment. Error bars are ± SEM.

3.4.5 temperature and humidity

Selection of 36ºC as the aversive temperature required careful calibration and is a significant factor in the success of our experiments. At low temperatures, fed flies are largely stationary (~0.25 cm/s mean walking velocity at 27ºC, see Figure 2-7) and show little motivation for finding the cool spot. As temperatures are increase up to ~37ºC, there is a corresponding increase in fly walking velocity (~1.2 cm/s), drive to search for the cool tile, and place learning scores. Above 37ºC, mean walking velocity increases rapidly (> 2 cm/s), flies display a dramatic increase in jumps and wing extensions, and place learning scores drop to near zero. This is likely related to a switch from "warm" thermosensation to nociceptive (i.e. painful) thermosensation and the corresponding behavioral switch from guided navigation to activation of escape reflexes. Therefore, the challenge of achieving good place learning behavior was a matter of finding a temperature that was high enough to elicit strong place learning behavior, but not so high as to trigger escape responses. Finding the "ideal" temperature proved more difficult than initially thought as a fly's response to temperature is intimately tied to humidity (see Figure 2-11). This frustrated early experiments as an assay that was "optimized" would often stop working a month (or sometimes even a day) later as humidity fluctuated with daily and seasonal weather patterns. Once the contribution of humidity was identified, we put particular effort into maintaining a constant humidity in our fly incubators and behavior rooms. We currently keep the room at 25ºC, 40% RH and use 36ºC as the aversive warm temperature. Additionally, for each experiment, temperature and humidity readings are

recorded from 3 remote sensors; one in the behavior room and 2 mounted at different locations inside the enclosure surrounding the thermal-visual arena. As a side note and to give the reader a better frame of reference, it is worth pointing out that 36ºC is a temperature that may be encountered outside on a hot summer day, and 25ºC (the temperature we use for our cool spot) is similar to walking into an air-conditioned building.

3.5 Acknowledgements

Chapter 3, in part, has been accepted for publication in Nature, Ofstad, Tyler A.; Zuker, Charles S.; Reiser, Michael B. (2011). The dissertation author was the primary investigator and author of this paper.

Chapter 4
The Cells and Circuits for Visual Place Learning

4.1 Role of the Ellipsoid Body, but not the Mushroom Bodies in Place Learning

Where are spatial memories processed (or stored) in the *Drosophila* brain? The fly brain is organized in well defined neuropils, with many areas involved in distinct functions and behaviors. We reasoned that specific regions of the fly brain would function as the neuroanatomic substrate for visual place learning and therefore set out to engineer and test animals where different brain areas were selectively inactivated using the GAL4/UAS expression system. In essence, we conditionally silenced small subsets of neurons in adult flies by targeting expression of the inward rectifying potassium channel Kir2.1 (Baines et al. 2001); to limit potential side-effects of Kir2.1 expression during development, we used a temperature sensitive $GAL80^{ts}$ which blocks Kir2.1 expression when flies are reared at $18^{o}C$ but allows expression when flies are shifted to $30^{o}C$ prior to testing (McGuire et al. 2004). After silencing, flies were tested for place learning using the protocol described in Chapter 3. This protocol was composed of 10 training trials with a *coupled* visual landscape followed by testing in a probe trial (Figure 4-1). Following the probe trial, flies were tested for thermal preference by setting alternating tiles on the TEM array to either $25^{o}C$ or $36^{o}C$. Flies were allowed to distribute for 2 minutes before the cool and warm tiles were switched. The flies were then allowed another 2 minutes to re-distribute and the thermal aversion index was calculated as the amount of time flies spent at $25^{o}C$ minus the amount of time spent at $36^{o}C$ divided by the total time. Finally, flies were tested for normal optomotor responses (Strauss et al. 1997) by rotating a checkerboard pattern on the visual panorama clockwise and then counterclockwise at 90^{o} per second for 45

seconds. Optomotor responses are reported as the mean rotational velocity (in the direction of the stimulus) of the flies over the course of these trials.

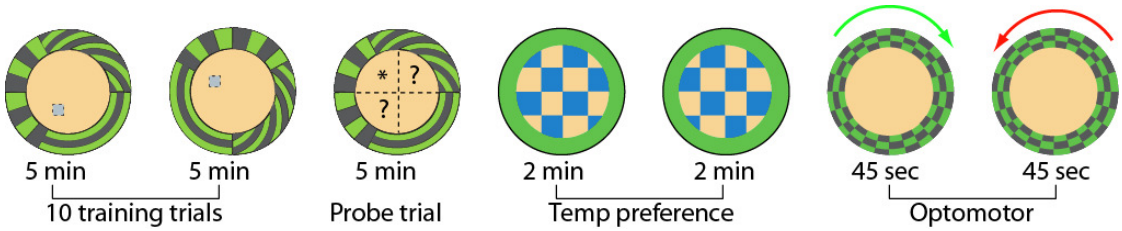

Figure 4-1: Diagram of the visual place learning screen.
Shown is a diagram of the visual place learning screen. Flies are trained for 10 trials with a *coupled* visual panorama followed by testing in a probe trial. At the end of the experiment, flies are tested for their ability to sense temperature (temperature preference) and their ability to respond to visual motion (optomotor).

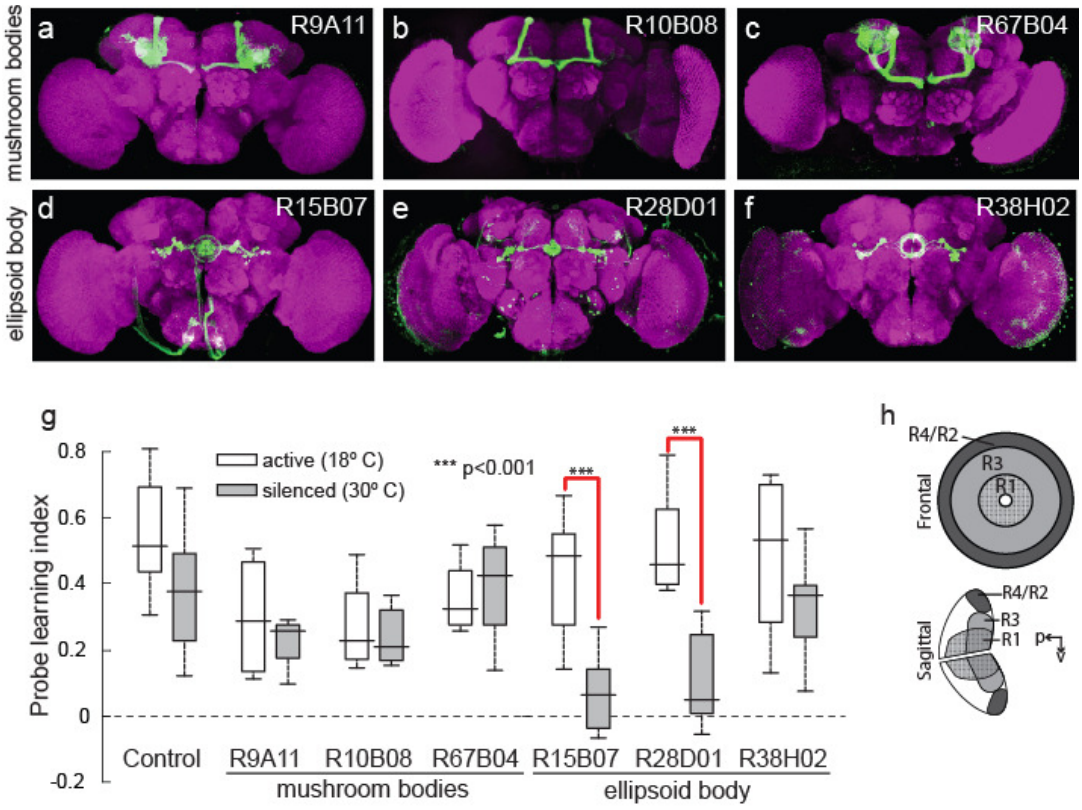

Figure 4-2: Subsets of ellipsoid-body ring neurons are required for place learning.
(**a-c**) GAL4 driver lines targeting subsets of cells in the mushroom body or (**d-f**) the ellipsoid body were used to conditionally silence neurons in the adult brain. Shown are the expression patterns for each driver following expression of a GFP reporter. (**g**) White boxes denote spatial learning performance of lines prior to Kir2.1 induction; grey boxes indicate the performance of the same lines following Kir2.1 expression. Silencing ellipsoid body neurons projecting to R1 and R4 (R15B07, **d**) or R1 (R28D01, **e**) severely impairs place learning ($p<.001$, one-tailed t-test red lines) while silencing mushroom body neurons (**a-c**), or a separate subset of eb neurons projecting to R4 alone (R38H02, **f**), leaves place learning intact (for additional statistical analysis, see section 3.3). Box plots are as described in Fig3, $n\geq8$. (**h**) Schematic representation of ellipsoid body ring neuron anatomy, illustrating the location of the R1-R4 structures (Hanesch et al. 1989).

GAL4 driver lines were selected for expression in two areas: the mushroom bodies (Figure 4-2a-c; Figure 4-3) and the central complex (Figure 4-2d-f). The mushroom bodies have been the subject of extensive studies of learning and memory

in *Drosophila* (Waddell et al. 2001), and have been shown to be essential for associative olfactory conditioning (de Belle et al. 1994), but not for some other forms of learning like tactile, motor, and non-visually guided place learning (Wolf et al. 1998; Zars et al. 2000; Putz et al. 2002). In cockroaches, ants and bees, the mushroom bodies have been implicated in visual place learning (Bernstein et al. 1969; Fahrbach et al. 1995; Mizunami et al. 1998) . The central complex is hypothesized to be a site of orientation behavior, multisensory integration and other "high order" processes (Hanesch et al. 1989; Strauss 2002; Neuser et al. 2008). Remarkably, we see no evidence for involvement of the mushroom bodies (mb) in our assay. In fact, silencing mb intrinsic neurons using the GAL4 drivers R9A11, R10B08, R67B04 (Figure 4-2a-c, g), OK107 (Figure 4-3a and b) or even chemically ablating the mb (hydroxyurea (Truman et al. 1986; de Belle et al. 1994); Figure 4-3c) had no significant effect on the performance of flies in visual place learning. In sharp contrast, silencing subsets of neurons with projections to the central complex ellipsoid body (eb, lines R15B07 and R28D01) dramatically impaired visual place learning ability (Figure 4-2d-h). Notably, silencing a different subset of ring neurons with line R38H02 leaves visual place learning intact (Figure 4-2f-h). Thus, specific circuits within the eb (but not the entire structure) are necessary for visual place learning.

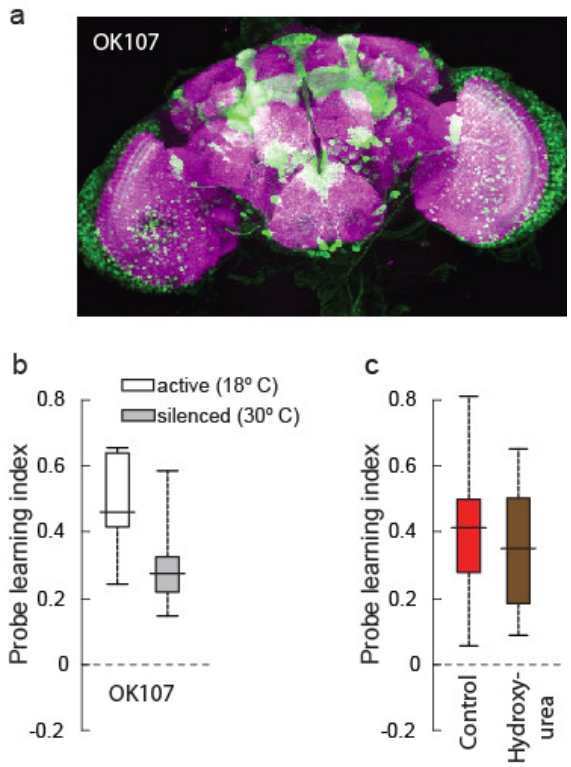

Figure 4-3: Mushroom bodies are not required for visual place learning
(a, b) The pan-mushroom body GAL4 driver line OK107 was used to conditionally silence neurons in the adult brain. **(a)** Shown is the expression pattern for the OK107 driver following expression of a GFP reporter. (**b)** White boxes denote spatial learning performance of the OK107 line prior to Kir2.1 induction; grey box indicates the performance of sibling flies following Kir2.1 expression. No significant place learning impairment is seen after Kir2.1 induction (one-tailed t-test, $p<0.05$, $n=10$ experiments, 150 flies for each condition). **(c)** Likewise, ablation of the mushroom bodies using hydroxyurea has no significant effect on place learning abilities when compared to sham treated siblings "Control" (one-tailed t-test, $p<0.05$, $n=5$ experiments, 75 flies for each condition). Box plots are as described in Figure 3-5.

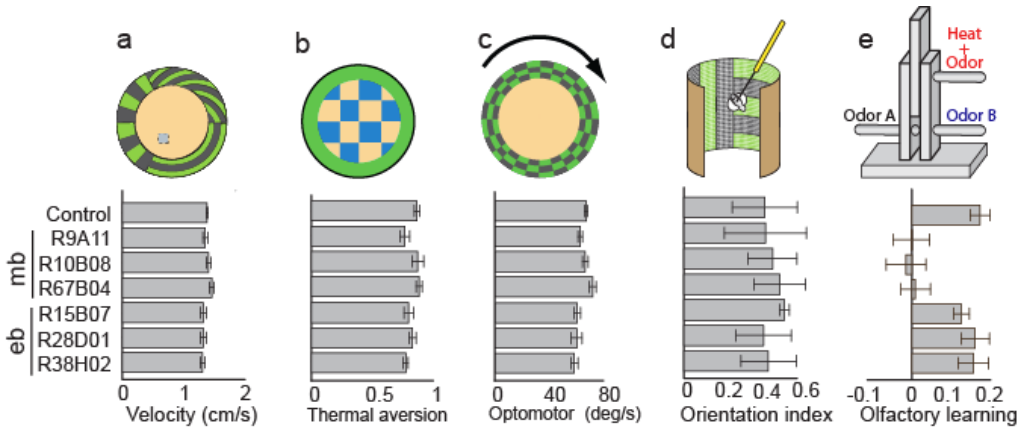

Figure 4-4: eb silenced flies are not impaired in sensory or locomotor behaviors. Flies with impaired place learning (expressing Kir2.1) show normal (**a**) walking velocity, (**b**) heat aversion, (**c**) optomotor response, (**d**) visual pattern discrimination during tethered flight, and (**e**) olfactory learning. For a-c (n≥8), values are mean ± SEM. For d (n≥6) & e (n=8), values are mean ± SD. See section 3.3 for details of calculations and statistical analysis.

To confirm that silencing the eb neurons in lines R15B07 and R28D01 produces a specific impairment in visual place memory, we tested these flies in a series of behavioral paradigms and showed they display normal locomotor (Figure 4-4a, Figure 4-5), optomotor (Figure 4-4c), thermosensory (Figure 4-4b) and visual pattern discrimination behaviors (Figure 4-4d). In addition, we reasoned that if these flies have a general defect in memory (or in processing thermally-driven learned behaviors), then they should exhibit impairment in multiple types of learning (or in using thermal signals to drive learning and memory). In what to our knowledge is the first use of thermal conditioning in a "T maze", we developed a novel olfactory conditioning paradigm using temperature (rather than electric shock (Tully et al. 1985)) as the unconditioned stimulus (Figure 4-4e, Figure 4-6). As expected, silencing

the mushroom bodies leads to a total loss of odor learning (Figure 4-4e). In contrast, silencing subsets of neurons in the ellipsoid body (eb) has no effect on olfactory learning, yet ablates visual place learning. Taken together, these results demonstrate that subsets of cells in the eb are specifically required for visual place learning, rather than just learning in general, and substantiate distinct neuroanatomical substrates for visually guided spatial (place) versus non-spatial (olfactory) learning in *Drosophila*.

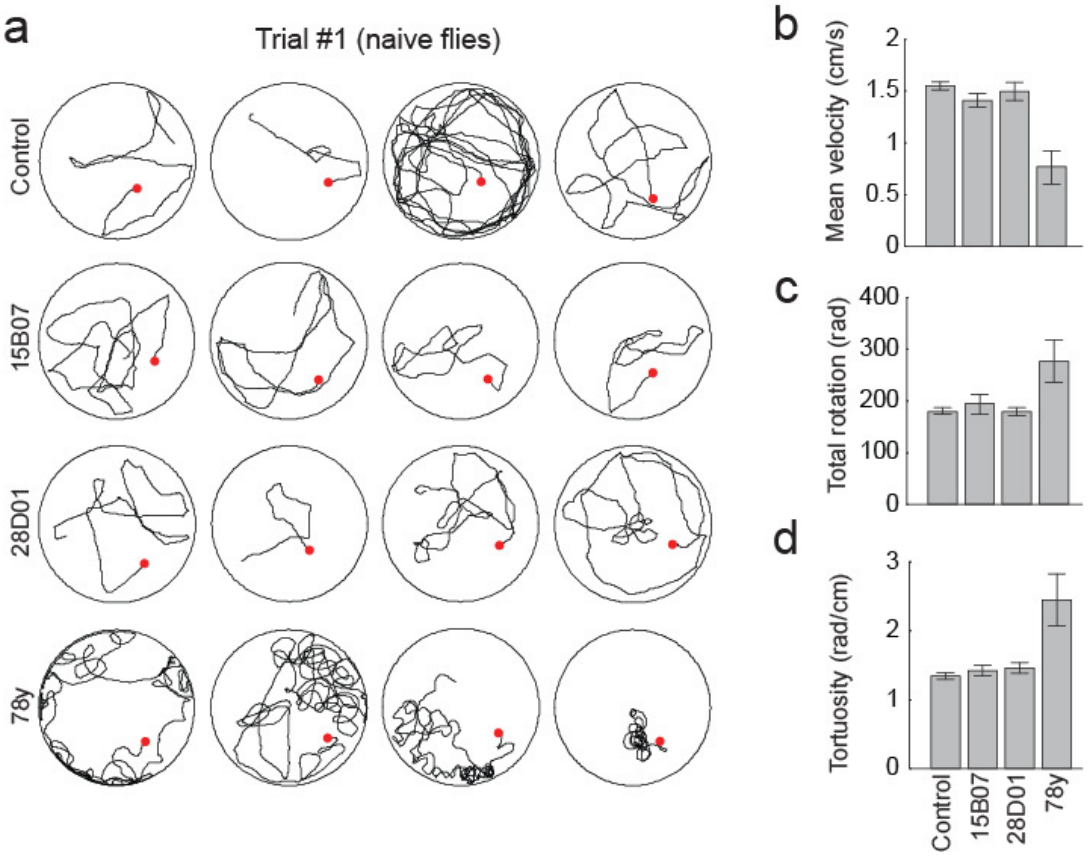

Figure 4-5: No locomotor abnormalities in lines R15B07 and R28D01.
(a) Trajectories from 4 representative naïve flies in trial #1 are shown for control flies, lines R15B07, R28D01, and 78y, all after induction of Kir2.1. The red dot denotes the point at which flies locate the cool spot. Note no apparent abnormalities in the trajectories of R15B07 and R28D01 when compared to control trajectories. For comparison, refer to line 78y which has documented locomotor impairment (Martin et al. 1999). **(b-d)** Likewise, lines R15B07 and R28D01 show no abnormalities in **(b)** walking velocity, **(c)** cumulative rotation, or **(d)** tortuosity (the ratio of the accumulated rotation over the accumulated distance traveled) of their paths when compared to control flies. Abnormalities in motor control are easily observable in line 78y using all of these metrics.

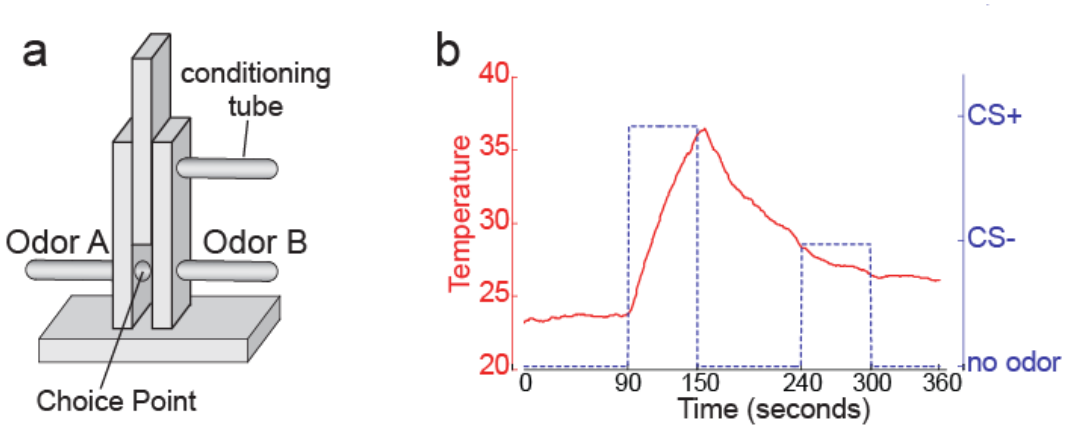

Figure 4-6: A novel olfactory conditioning paradigm using heat as the US.
(**a, b**) Flies were conditioned to avoid one of two odors using an elevated T maze and heat (rather than electric shock) as the unconditioned stimulus (See methods). (**a**) Flies were initially place in the conditioning tube and exposed to one of two odors, 4-Methylcyclohexanol (Odor A) or 3-octanol (Odor B), paired with heat. Following exposure to the first odor, flies were presented with the alternate odor in the absence of heat. After this training, flies were lowered into the choice point and allowed to distribute between a tube containing Odor A or Odor B. (**b**) Conditioning regime: flies are trained with the CS+ and CS- stimulus for a total of 6 min prior to testing; dashed blue lines show the time window of odor delivery (CS+ odor at 90 sec and CS- odor at 240 sec), red trace indicates the temperature in the conditioning tube during the training.

4.2 Beyond the Ellipsoid Body

To identify additional neural components of visual place learning, we expanded our GAL4 screen to include lines driving expression in additional structures in the central complex and surrounding neuropils. We have tested > 180 lines (as diagramed in Figure 4-1) and find that a significant number of the place learning "hits" are in lines that drive expression in the ellipsoid body (eb), fan shaped body (fsb), noduli (no) and/or the protocerebral bridge (pb) (see Table 4.1, Figure 4-7,

Figure 4-8, Figure 4-9, Figure 4-10). This is perhaps not surprising given our previous findings indicating a requirement for subsets of eb neurons and the documented reciprocal connectivity of these central complex structures (Hanesch et al. 1989; Renn et al. 1999; Young et al. 2010a; Young et al. 2010b). Notably, there appear to be specific subsets of cells in these structures that are required for visual place learning. In the eb, lines that show place learning impairment routinely show overlapping expression in the inner ring (R1) of the ellipsoid body (Figure 4-7a). In the fan shaped body, a common feature in impaired lines is expression in the dorsal layers of the fan shaped body (Figure 4-7b) and in lines with protocerebral bridge expression, there is commonly overlap in the pb, dorsal layers of the fsb, and nodule (Figure 4-7c). While we do not yet know the functional connectivity between these various cell types, it is likely that they are all part of a place learning circuit in the central complex. Future experiments are planned to establish the connectivity between these cells and better defining the role each component plays in processing spatial information.

What are the inputs into the central complex? As expected, flies with defects in photo transduction fail to learn in the visual place learning assay (NorpA mutants, data not shown). Additionally, using split GAL4 to specifically silencing lamina monopolar cell subtypes (the first synaptic inputs downstream of the photoreceptors) L1-L4 (PL338 ∩ PL309), L2 alone (PL453 ∩ PL429) or L3 alone (PL720 ∩ PL722), (but not L1 alone or L4 alone, PL648 ∩ PL666 and PL420 ∩ PL431 respectively)

leads to severe place learning impairment (See Table 4.1)[7]. As most of these manipulations also lead to impairments in optomotor response, we believe them to be primary sensory deficits affecting vision rather than having a specific role in encoding place. How is visual information transmitted from primary sensory neuropils (i.e. the optic lobes) into the central brain? We believe that projection neurons from the optic lobes to optic glomeruli (also termed optic foci) may serve this function. Despite their anatomic characterization (Otsuna et al. 2006), there is little evidence for the behavioral role of these visual projections neurons. It has been hypothesized that these neurons and local glomerular circuits may play a role in encoding higher order visual features. Additionally, single cells studies have identified neurons projecting from optic glomeruli to the central complex (Chiang et al. 2010). This may form a pathway for bridging the optic lobes and central complex in two synapses. When we silence these optic lobe projection neurons, we find certain types (but not all types) that are required for place learning in our thermal visual arena (Table 4.1 and Figure 4-11). Notably silencing these cells has no effect on optomotor performance, further reinforcing the idea that they are downstream from the cells and circuits required for more basic visual reflexes.

[7] Lamina monopolar cell types were specifically targeted using split GAL4 (Luan et al. 2006). By using two different enhancers to drive each half of GAL4, GAL4 activity is only reconstituted in cells with overlapping expression of the two driver lines (driver#1 ∩ driver#2). This intersectional strategy allows us to cleanly target only the cell type of interest. All split GAL4 lines were created and anatomically characterized by Dr. Aljoscha Nern.

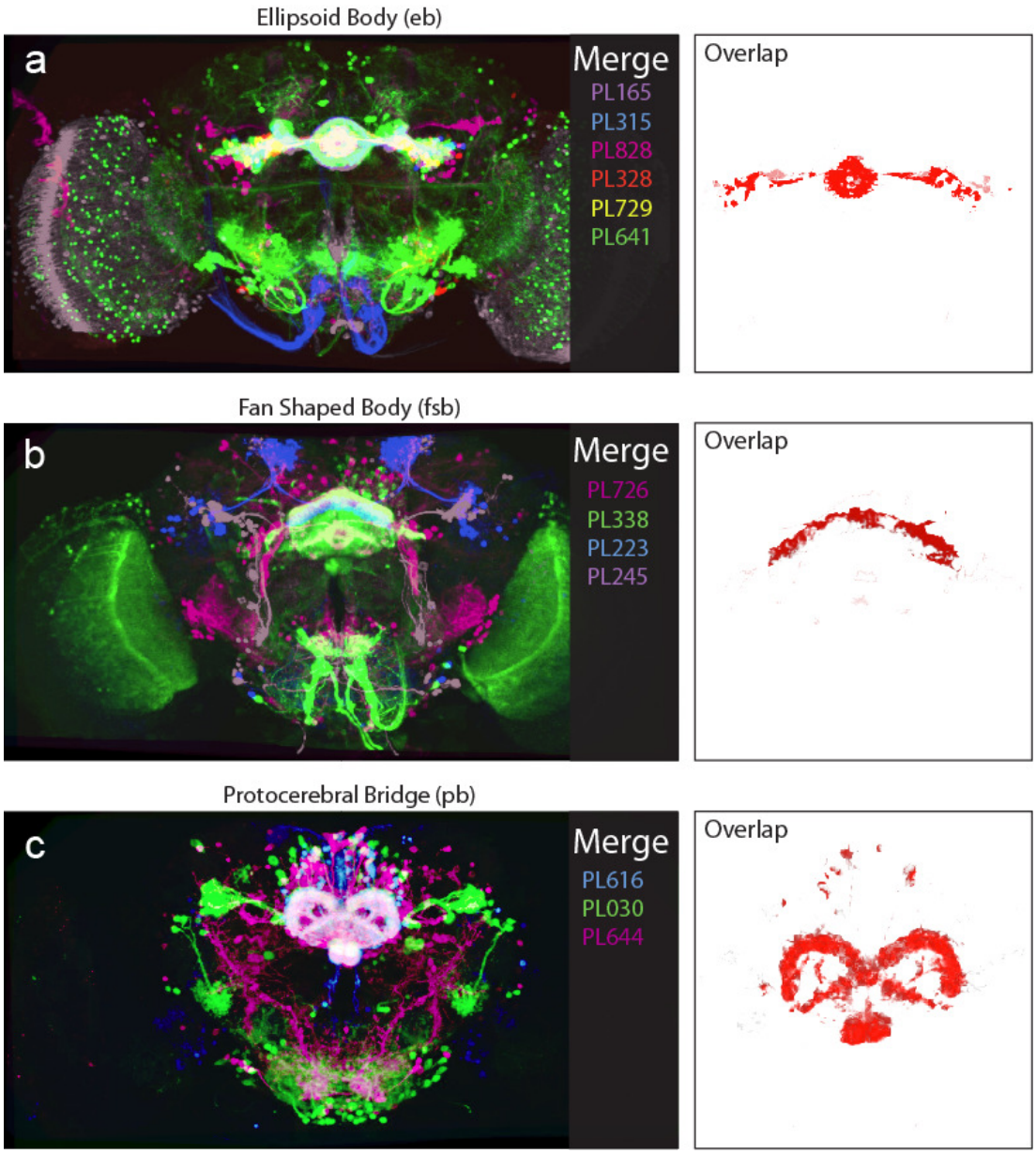

Figure 4-7: Overlapping expression in lines with place learning impairment. (**Left**) Shown are the computational alignments of GAL4 expression patterns for lines that were detected as place learning impaired. Lines are color coded according to the scheme indicated in the right margin of the *Merge* panel. White indicates areas where all lines overlap. (**Right**) Shown are the brain regions where all aligned expression patterns overlap (colored red). (**a**) In the eb, place learning impaired lines commonly show expression in R1 neurons. (**b**) In the fsb, expression is routinely seen in the dorsal layers. (**c**) pb lines with impaired place learning commonly show expression in the pb, dorsal layer of the fsb, and no.

4.2.1 place learning screen results

Table 4.1: Place Learning Screen Results
Shown are the Place Learning Indices (L.I.), Mean Velocity (Vel., cm/s), Thermal Aversion Index (Temp), and Optomotor responses (Opto, deg/s) from the visual place learning screen. All driver lines were crossed to the w+; tubP-Gal80ts;Kir2.1 effector line. Adult flies were temperature shifted to 30ºC for 40 hours then returned to 18ºC for 2 hours prior to testing. p values (p) for each metric are calculated using a student t-test and are reported relative to attP2 control flies (highlighted in yellow). Results are color coded red for lines showing statistically significant impairment specific to place learning, purple for lines showing place learning and additional behavioral deficits, and black for lines with no significant place learning impairment. Lines are sorted according to Place Learning Index. Representative GAL4 expression patterns for place learning specific hits (red) are shown in Figure 4-8 (eb), Figure 4-9 (fsb), Figure 4-10 (pb) and Figure 4-11 (visual projection). n = number of experiments performed, 15 flies tested per experiment.

Line	L.I.	SEM	p	Vel	SEM	p	Temp	SEM	p	Opto	SEM	p
PL215 n=3	-.08	0.01	0.00	1.4	0.0	0.14	0.68	0.01	0.09	43.2	0.9	0.00
PL542 n=2	-.07	0.01	0.00	1.7	0.0	0.89	0.78	0.00	0.45	1.0	0.4	0.00
PL749 n=4	-.03	0.02	0.00	1.6	0.0	0.62	0.72	0.06	0.20	63.9	2.5	0.93
PL227 n=4	-.03	0.03	0.00	1.6	0.1	0.85	0.85	0.03	0.79	64.3	1.2	0.96
PL314 n=4	-.03	0.03	0.00	1.6	0.0	0.87	0.80	0.04	0.55	51.6	1.6	0.07
PL323 n=4	-.02	0.05	0.00	1.6	0.1	0.66	0.81	0.02	0.62	57.4	2.2	0.50
PL126 n=4	-.02	0.05	0.00	1.4	0.0	0.07	0.70	0.05	0.13	45.5	2.3	0.00
PL210 n=4	-.01	0.02	0.00	1.8	0.0	0.99	0.84	0.03	0.75	2.9	0.9	0.00
PL643 n=2	0.00	0.08	0.03	1.5	0.1	0.52	0.77	0.01	0.39	55.6	0.3	0.36
PL624 n=4	0.00	0.04	0.00	1.5	0.0	0.33	0.76	0.04	0.31	60.3	1.9	0.77
PL647 n=8	0.00	0.05	0.00	1.6	0.0	0.87	0.81	0.04	0.64	59.3	1.3	0.75
PL018 n=4	0.00	0.02	0.00	1.7	0.0	0.91	0.71	0.04	0.15	64.1	1.0	0.96
PL153 n=4	0.01	0.01	0.00	1.8	0.0	0.99	0.87	0.00	0.86	58.8	1.2	0.65
PL834 n=4	0.01	0.03	0.00	1.6	0.0	0.88	0.77	0.01	0.39	58.1	1.2	0.57
PL119 n=4	0.02	0.07	0.01	1.7	0.1	0.89	0.92	0.02	0.96	62.7	2.2	0.90
PL616 n=8	0.02	0.02	0.00	1.5	0.0	0.37	0.79	0.03	0.46	53.5	4.1	0.19
PL647 n=4	0.02	0.03	0.01	1.5	0.0	0.36	0.86	0.05	0.79	70.1	1.0	1.00
PL566 n=2	0.03	0.02	0.03	1.6	0.0	0.75	0.90	0.01	0.86	65.2	0.3	0.93
PL519 n=4	0.03	0.03	0.01	1.5	0.1	0.30	0.82	0.03	0.67	57.7	1.9	0.53
PL140 n=6	0.03	0.04	0.00	1.5	0.1	0.22	0.73	0.03	0.16	52.3	1.2	0.05
PL533 n=4	0.04	0.03	0.01	1.7	0.0	0.98	0.89	0.02	0.90	68.7	2.1	0.99
PL030 n=9	0.04	0.03	0.00	1.6	0.1	0.91	0.84	0.03	0.81	63.9	1.5	0.99
PL727 n=4	0.04	0.05	0.02	1.5	0.0	0.38	0.80	0.02	0.55	64.7	1.1	0.97
PL485 n=4	0.04	0.04	0.01	1.6	0.0	0.86	0.84	0.02	0.74	64.2	0.8	0.96
PL856 n=2	0.05	0.01	0.04	1.3	0.0	0.05	0.71	0.02	0.19	54.2	1.2	0.27
PL423 n=6	0.05	0.05	0.01	1.7	0.0	0.96	0.85	0.04	0.84	73.6	1.2	1.00
PL948 n=8	0.05	0.03	0.00	1.5	0.1	0.62	0.80	0.04	0.57	61.4	1.8	0.91
PL467 n=8	0.05	0.02	0.00	1.6	0.1	0.73	0.80	0.04	0.53	56.3	2.3	0.36
PL644 n=4	0.05	0.02	0.01	1.6	0.0	0.79	0.82	0.01	0.66	54.9	1.3	0.25

Table 4.1: Place Learning Screen Results, continued

Line	L.I.	SEM	p	Vel	SEM	p	Temp	SEM	p	Opto	SEM	p
PL152 n=6	0.06	0.04	0.01	1.5	0.0	0.35	0.80	0.05	0.57	50.9	1.6	0.03
PL238 n=4	0.06	0.02	0.01	1.6	0.1	0.86	0.87	0.03	0.84	52.7	2.1	0.12
PL828 n=12	0.06	0.05	0.01	1.4	0.0	0.16	0.80	0.04	0.58	50.2	5.6	0.13
PL328 n=4	0.06	0.05	0.03	1.7	0.0	0.91	0.86	0.02	0.84	63.4	0.8	0.94
PL839 n=4	0.06	0.04	0.02	1.6	0.1	0.76	0.85	0.01	0.79	63.0	1.0	0.93
PL829 n=4	0.06	0.04	0.02	1.7	0.1	0.96	0.76	0.06	0.33	68.1	2.0	0.99
PL611 n=4	0.07	0.02	0.02	1.5	0.0	0.24	0.89	0.01	0.91	54.7	2.2	0.25
PL641 n=4	0.07	0.02	0.01	1.5	0.0	0.46	0.79	0.04	0.50	56.6	0.5	0.41
PL284 n=4	0.07	0.02	0.01	1.6	0.1	0.77	0.83	0.05	0.69	65.9	0.5	0.99
PL334 ∩ PL309 n=8	0.07	0.05	0.01	1.7	0.1	0.98	0.76	0.04	0.30	20.0	4.1	0.00
PL549 n=4	0.07	0.03	0.02	1.4	0.1	0.14	0.84	0.03	0.74	57.0	2.2	0.46
PL152 n=6	0.07	0.05	0.02	1.5	0.1	0.26	0.87	0.03	0.90	58.4	0.8	0.63
PL315 n=12	0.07	0.04	0.01	1.5	0.1	0.33	0.84	0.02	0.83	60.2	2.4	0.81
PL549 n=4	0.08	0.05	0.04	1.5	0.1	0.35	0.77	0.04	0.39	60.4	1.4	0.78
PL548 n=4	0.08	0.03	0.03	1.6	0.0	0.78	0.76	0.04	0.35	64.8	1.0	0.97
PL729 n=4	0.08	0.05	0.04	1.7	0.0	0.94	0.85	0.05	0.75	69.2	0.9	1.00
PL409 n=8	0.08	0.04	0.01	1.8	0.0	1.00	0.80	0.05	0.53	65.5	2.5	0.99
PL958 n=3	0.08	0.01	0.04	1.5	0.0	0.51	0.89	0.01	0.89	61.1	1.3	0.81
PL433 n=4	0.08	0.03	0.03	1.4	0.0	0.23	0.78	0.05	0.44	55.2	0.8	0.27
PL717 n=4	0.08	0.01	0.02	1.7	0.0	0.96	0.86	0.04	0.80	74.8	2.0	1.00
PL149 n=4	0.08	0.02	0.03	1.7	0.0	0.92	0.78	0.07	0.46	76.6	0.8	1.00
PL924 n=4	0.08	0.00	0.02	1.8	0.0	1.00	0.85	0.04	0.76	69.2	2.3	1.00
PL125 n=4	0.09	0.04	0.04	1.5	0.0	0.58	0.78	0.01	0.40	66.6	0.8	0.99
PL820 n=4	0.09	0.02	0.03	1.5	0.0	0.59	0.77	0.04	0.37	59.3	0.7	0.70
PL726 n=4	0.09	0.03	0.04	1.6	0.0	0.81	0.88	0.03	0.89	59.4	1.6	0.70
PL240 n=6	0.09	0.03	0.02	1.4	0.1	0.13	0.88	0.02	0.94	61.1	1.9	0.87
PL035 n=2	0.09	0.05	0.10	1.1	0.0	0.01	0.62	0.05	0.06	49.4	1.1	0.07
PL610 n=8	0.09	0.03	0.01	1.7	0.1	0.98	0.84	0.05	0.78	62.1	1.3	0.95
PL165 n=8	0.09	0.04	0.02	1.6	0.1	0.90	0.86	0.02	0.91	63.8	2.6	0.97
PL812 n=2	0.09	0.01	0.09	1.2	0.0	0.01	0.81	0.06	0.57	57.1	3.1	0.48
PL430 n=4	0.10	0.02	0.04	1.5	0.0	0.58	0.73	0.03	0.21	68.7	1.0	1.00
PL913 n=10	0.10	0.04	0.02	1.4	0.1	0.08	0.83	0.02	0.81	62.7	1.4	0.97
PL720 ∩ PL722 n=5	0.10	0.06	0.04	1.6	0.0	0.68	0.77	0.05	0.38	63.3	2.9	0.93
PL338 n=4	0.10	0.03	0.05	1.7	0.0	0.96	0.92	0.02	0.97	63.6	2.0	0.94
PL717 n=8	0.10	0.05	0.04	1.5	0.0	0.56	0.89	0.03	0.96	57.3	2.3	0.49
PL323 n=4	0.10	0.04	0.06	1.5	0.0	0.32	0.71	0.03	0.12	55.7	2.1	0.34
PL453 ∩ PL429 n=5	0.10	0.04	0.04	2.0	0.0	1.00	0.76	0.06	0.34	47.4	3.3	0.01
PL548 n=2	0.11	0.01	0.11	1.6	0.0	0.80	0.74	0.01	0.28	58.6	1.7	0.59
PL338 n=4	0.11	0.02	0.04	1.7	0.0	0.97	0.80	0.04	0.52	75.0	0.5	1.00
PL347 n=6	0.11	0.04	0.04	1.8	0.0	1.00	0.80	0.05	0.54	73.3	5.8	1.00
PL720 n=4	0.11	0.04	0.06	1.6	0.0	0.84	0.73	0.05	0.21	66.2	2.6	0.98
PL726 n=4	0.11	0.05	0.08	1.5	0.0	0.29	0.84	0.03	0.73	63.2	1.6	0.93
PL250 n=4	0.11	0.03	0.06	1.5	0.0	0.26	0.74	0.03	0.25	56.6	1.3	0.41
PL372 n=4	0.11	0.02	0.06	1.6	0.0	0.85	0.91	0.02	0.95	61.1	2.2	0.82
PL648 n=6	0.11	0.03	0.03	1.7	0.1	0.93	0.83	0.04	0.72	65.8	0.8	0.99

Table 4.1: Place Learning Screen Results, continued

Line	L.I.	SEM	p	Vel	SEM	p	Temp	SEM	p	Opto	SEM	p
PL245 n=8	0.11	0.04	0.04	1.6	0.0	0.85	0.81	0.03	0.61	65.6	2.3	0.99
PL454 n=2	0.12	0.00	0.14	1.7	0.0	0.88	0.74	0.04	0.29	63.5	0.1	0.88
PL145 n=8	0.12	0.04	0.04	1.8	0.0	1.00	0.85	0.02	0.87	69.5	1.2	1.00
PL223 n=4	0.12	0.04	0.10	1.7	0.0	0.92	0.87	0.02	0.88	60.6	1.8	0.79
PL567 n=4	0.12	0.03	0.08	1.5	0.0	0.28	0.85	0.05	0.78	64.9	1.4	0.97
PL327 n=4	0.12	0.02	0.07	1.8	0.0	0.99	0.83	0.01	0.73	56.0	2.5	0.37
PL245 n=8	0.12	0.04	0.04	1.6	0.0	0.92	0.88	0.03	0.95	62.8	2.4	0.94
PL744 n=4	0.12	0.04	0.10	1.5	0.0	0.26	0.70	0.07	0.15	51.5	2.6	0.08
PL237 n=4	0.12	0.04	0.10	1.7	0.0	0.97	0.87	0.00	0.87	60.2	1.5	0.77
PL725 n=4	0.12	0.02	0.07	1.5	0.1	0.38	0.82	0.04	0.66	58.2	1.9	0.58
PL311 n=8	0.12	0.03	0.04	1.7	0.0	1.00	0.83	0.03	0.78	59.0	3.0	0.68
PL524 n=4	0.12	0.02	0.08	1.7	0.0	0.96	0.74	0.03	0.23	59.4	2.4	0.69
PL347 n=8	0.13	0.04	0.06	1.2	0.0	0.00	0.73	0.04	0.14	50.7	2.6	0.03
PL545 n=4	0.13	0.04	0.12	1.6	0.0	0.61	0.87	0.03	0.86	67.2	0.7	0.99
PL238 n=4	0.13	0.06	0.15	1.7	0.0	0.95	0.81	0.05	0.58	53.8	2.1	0.19
PL771 n=2	0.13	0.01	0.18	1.2	0.0	0.01	0.68	0.01	0.12	51.8	2.6	0.16
PL442 n=4	0.13	0.02	0.11	1.6	0.0	0.84	0.72	0.02	0.14	64.0	0.8	0.96
PL322 n=8	0.13	0.05	0.09	1.2	0.0	0.00	0.77	0.05	0.38	49.1	2.2	0.01
PL147 n=4	0.14	0.04	0.12	1.7	0.0	0.97	0.83	0.02	0.68	73.9	1.2	1.00
PL712 n=6	0.14	0.03	0.09	1.6	0.0	0.71	0.82	0.05	0.67	53.6	2.5	0.14
PL924 n=4	0.14	0.01	0.11	1.6	0.0	0.88	0.88	0.02	0.88	67.4	2.0	0.99
PL765 n=4	0.14	0.03	0.12	1.6	0.0	0.67	0.81	0.03	0.57	60.1	1.2	0.77
PL945 n=8	0.14	0.04	0.08	1.6	0.1	0.78	0.79	0.03	0.52	60.6	2.2	0.84
PL737 n=4	0.14	0.02	0.12	1.7	0.0	0.94	0.85	0.02	0.80	61.7	1.3	0.87
PL537 n=4	0.14	0.02	0.13	1.3	0.1	0.05	0.84	0.03	0.75	39.1	1.4	0.00
PL814 n=4	0.14	0.06	0.17	1.6	0.1	0.76	0.90	0.03	0.92	69.0	1.9	1.00
PL614 n=4	0.14	0.01	0.13	1.7	0.0	0.97	0.82	0.06	0.63	65.9	1.1	0.98
PL517 n=4	0.15	0.03	0.14	1.7	0.0	0.98	0.82	0.08	0.62	68.2	2.4	0.99
PL741 n=2	0.15	0.02	0.23	1.4	0.0	0.19	0.73	0.04	0.27	63.4	0.1	0.87
PL612 n=4	0.15	0.03	0.17	1.7	0.0	0.98	0.87	0.03	0.85	61.0	1.6	0.83
PL822 n=4	0.15	0.02	0.16	1.6	0.0	0.88	0.89	0.03	0.90	57.4	2.0	0.50
PL914 n=4	0.16	0.02	0.17	1.7	0.0	0.96	0.83	0.02	0.71	64.1	2.0	0.95
PL415 n=8	0.16	0.04	0.12	1.7	0.0	0.98	0.90	0.01	0.98	70.7	2.5	1.00
PL510 n=4	0.16	0.03	0.18	1.6	0.0	0.81	0.58	0.04	0.01	58.6	1.8	0.62
PL618 n=4	0.16	0.05	0.22	1.7	0.0	0.98	0.74	0.04	0.23	72.2	1.1	1.00
PL524 n=6	0.16	0.04	0.17	1.6	0.0	0.85	0.87	0.02	0.91	69.2	2.4	1.00
PL930 n=4	0.16	0.06	0.25	1.7	0.0	0.94	0.84	0.04	0.74	71.9	0.5	1.00
PL571 n=2	0.17	0.03	0.29	1.6	0.0	0.65	0.83	0.02	0.65	72.6	1.6	0.99
PL846 n=8	0.17	0.05	0.20	1.7	0.0	1.00	0.83	0.02	0.78	68.5	1.8	1.00
PL746 n=8	0.17	0.04	0.18	1.5	0.0	0.35	0.81	0.04	0.61	61.0	2.9	0.84
PL912 n=8	0.17	0.06	0.23	1.7	0.0	0.99	0.88	0.03	0.94	67.5	1.7	1.00
PL326 n=8	0.17	0.05	0.20	1.5	0.0	0.63	0.80	0.05	0.57	64.3	2.2	0.98
PL014 n=7	0.17	0.05	0.21	1.7	0.0	0.94	0.81	0.03	0.65	67.6	2.1	1.00
PL947 n=8	0.17	0.06	0.24	1.4	0.1	0.12	0.75	0.06	0.28	58.0	2.1	0.58
PL122 n=4	0.17	0.03	0.25	1.6	0.1	0.78	0.85	0.02	0.80	61.7	2.2	0.85
PL347 n=10	0.18	0.03	0.18	1.6	0.0	0.81	0.86	0.03	0.92	60.1	2.1	0.81
PL958 n=2	0.18	0.01	0.32	1.6	0.1	0.67	0.82	0.03	0.61	54.5	1.0	0.29

Table 4.1: Place Learning Screen Results, continued

Line	L.I.	SEM	p	Vel	SEM	p	Temp	SEM	p	Opto	SEM	p
PL884 n=4	0.18	0.03	0.28	1.3	0.0	0.01	0.88	0.04	0.87	56.8	1.6	0.43
PL415 n=8	0.18	0.02	0.21	1.8	0.0	1.00	0.93	0.01	1.00	71.2	2.2	1.00
PL113 n=4	0.18	0.05	0.32	1.7	0.1	0.93	0.86	0.03	0.82	62.1	1.2	0.90
PL855 n=6	0.19	0.06	0.33	1.5	0.1	0.60	0.84	0.04	0.78	61.4	1.7	0.89
PL123 n=4	0.19	0.07	0.37	1.7	0.0	0.97	0.94	0.01	0.98	68.4	2.3	0.99
PL319 n=4	0.19	0.04	0.37	1.7	0.1	0.89	0.92	0.04	0.96	72.9	1.5	1.00
PL787 n=3	0.20	0.04	0.38	1.6	0.0	0.86	0.89	0.01	0.89	65.6	1.8	0.96
PL521 n=6	0.20	0.02	0.34	1.8	0.0	1.00	0.92	0.03	0.98	67.1	1.3	1.00
PL642 n=4	0.20	0.04	0.37	1.6	0.0	0.65	0.79	0.04	0.50	61.2	1.8	0.84
PL259 n=8	0.20	0.04	0.34	1.5	0.0	0.20	0.86	0.02	0.91	62.7	0.7	0.98
PL312 n=9	0.20	0.04	0.36	1.6	0.0	0.73	0.76	0.05	0.32	66.2	1.8	1.00
PL532 n=4	0.20	0.03	0.41	1.8	0.0	1.00	0.78	0.04	0.44	70.6	1.2	1.00
PL535 n=6	0.20	0.03	0.40	1.7	0.1	0.98	0.82	0.03	0.69	63.9	1.7	0.97
PL567 n=2	0.21	0.01	0.44	1.6	0.0	0.73	0.87	0.02	0.77	61.8	0.5	0.80
PL656 n=8	0.21	0.02	0.39	1.7	0.1	0.98	0.88	0.03	0.95	67.5	1.4	1.00
PL340 n=4	0.21	0.02	0.44	1.6	0.0	0.87	0.87	0.03	0.85	63.7	2.4	0.93
PL179 n=4	0.21	0.03	0.44	1.5	0.0	0.50	0.84	0.04	0.74	62.1	0.8	0.90
PL110 n=4	0.21	0.03	0.45	1.9	0.1	1.00	0.92	0.01	0.96	67.7	0.5	0.99
PL638 n=3	0.22	0.12	0.49	1.7	0.0	0.89	0.85	0.03	0.75	67.4	1.3	0.98
attP2 n=12	0.22	0.03	0.50	1.5	0.1	0.50	0.79	0.04	0.50	57.4	1.9	0.50
PL938 n=9	0.22	0.02	0.51	1.3	0.0	0.01	0.65	0.04	0.01	54.6	1.1	0.14
PL312 n=7	0.22	0.03	0.51	1.6	0.0	0.73	0.90	0.02	0.98	66.4	2.5	0.99
PL383 n=6	0.22	0.06	0.53	1.4	0.1	0.06	0.74	0.04	0.23	55.9	1.9	0.32
PL746 n=4	0.22	0.05	0.53	1.7	0.0	0.97	0.83	0.03	0.72	62.3	1.7	0.89
PL446 n=8	0.23	0.06	0.54	1.6	0.1	0.83	0.87	0.02	0.92	61.7	2.7	0.89
PL611 n=4	0.23	0.03	0.55	1.7	0.1	0.89	0.82	0.03	0.63	79.4	1.1	1.00
PL453 n=4	0.23	0.01	0.55	1.6	0.0	0.66	0.85	0.03	0.77	68.3	1.7	0.99
PL649 n=2	0.23	0.01	0.56	1.6	0.0	0.58	0.82	0.02	0.59	57.1	0.3	0.48
PL547 n=8	0.24	0.03	0.63	1.7	0.0	0.95	0.86	0.04	0.89	63.6	2.0	0.98
PL967 n=3	0.24	0.02	0.59	1.8	0.0	0.98	0.85	0.02	0.76	68.8	0.4	0.99
PL209 n=4	0.24	0.04	0.60	1.6	0.1	0.83	0.91	0.01	0.95	57.8	1.5	0.55
PL738 n=4	0.24	0.00	0.62	1.6	0.0	0.64	0.87	0.02	0.86	58.3	1.0	0.60
PL645 n=4	0.25	0.06	0.64	1.7	0.1	0.94	0.84	0.03	0.76	66.1	2.3	0.98
PL622 n=6	0.25	0.06	0.67	1.4	0.0	0.10	0.92	0.02	0.98	52.6	2.3	0.09
PL846 n=4	0.25	0.02	0.69	1.7	0.0	0.89	0.89	0.02	0.92	71.4	3.0	1.00
PL770 n=4	0.26	0.04	0.69	1.4	0.0	0.18	0.81	0.03	0.57	60.8	1.8	0.81
PL029 n=4	0.26	0.03	0.72	1.7	0.0	0.92	0.89	0.02	0.92	69.2	1.5	1.00
PL116 n=4	0.26	0.04	0.71	1.6	0.0	0.72	0.86	0.02	0.84	67.4	1.7	0.99
PL446 n=4	0.26	0.01	0.75	1.4	0.0	0.06	0.86	0.04	0.82	57.3	1.2	0.49
PL083 n=4	0.27	0.01	0.80	1.3	0.1	0.04	0.76	0.04	0.34	44.4	1.1	0.00
PL846 n=4	0.28	0.02	0.84	1.7	0.0	0.92	0.85	0.03	0.78	70.2	1.4	1.00
PL215 n=8	0.28	0.06	0.84	1.4	0.0	0.07	0.75	0.03	0.20	51.1	1.8	0.02
PL410 n=4	0.29	0.05	0.81	1.5	0.0	0.59	0.89	0.04	0.90	72.2	0.5	1.00
PL524 n=4	0.29	0.03	0.85	1.5	0.0	0.29	0.86	0.04	0.83	65.2	1.3	0.97
PL630 n=4	0.30	0.04	0.88	1.6	0.1	0.70	0.88	0.03	0.89	64.2	1.5	0.96
PL311 ∩ PL319 n=5	0.30	0.08	0.83	1.5	0.0	0.52	0.83	0.04	0.70	67.9	1.6	1.00

Table 4.1: Place Learning Screen Results, continued

Line	L.I.	SEM	p	Vel	SEM	p	Temp	SEM	p	Opto	SEM	p
PL138 n=3	0.31	0.04	0.86	1.7	0.0	0.93	0.77	0.06	0.40	65.3	1.4	0.96
PL165 n=10	0.31	0.06	0.90	1.5	0.0	0.27	0.84	0.03	0.84	57.5	1.5	0.51
PL920 n=4	0.32	0.02	0.93	1.6	0.0	0.70	0.91	0.04	0.94	62.1	1.7	0.89
PL225 n=4	0.33	0.03	0.94	1.6	0.0	0.89	0.87	0.01	0.87	61.4	2.3	0.83
PL835 ∩ PL829 n=2	0.33	0.01	0.89	1.5	0.0	0.56	0.68	0.06	0.16	75.1	0.9	1.00
PL423 n=4	0.33	0.03	0.95	1.7	0.0	0.97	0.90	0.03	0.93	57.7	0.6	0.54
PL546 n=4	0.34	0.05	0.94	1.7	0.0	0.96	0.89	0.02	0.92	71.4	1.8	1.00
PL420 ∩ PL431 n=4	0.39	0.03	0.99	1.6	0.1	0.82	0.77	0.06	0.39	70.8	0.8	1.00
PL745 n=8	0.40	0.05	1.00	1.5	0.1	0.57	0.92	0.02	0.99	64.5	1.9	0.99
PL648 ∩ PL666 n=4	0.41	0.04	0.99	1.6	0.0	0.88	0.84	0.04	0.72	51.6	3.0	0.09
PL682 n=4	0.43	0.05	0.99	1.5	0.0	0.49	0.73	0.02	0.19	62.7	1.5	0.91

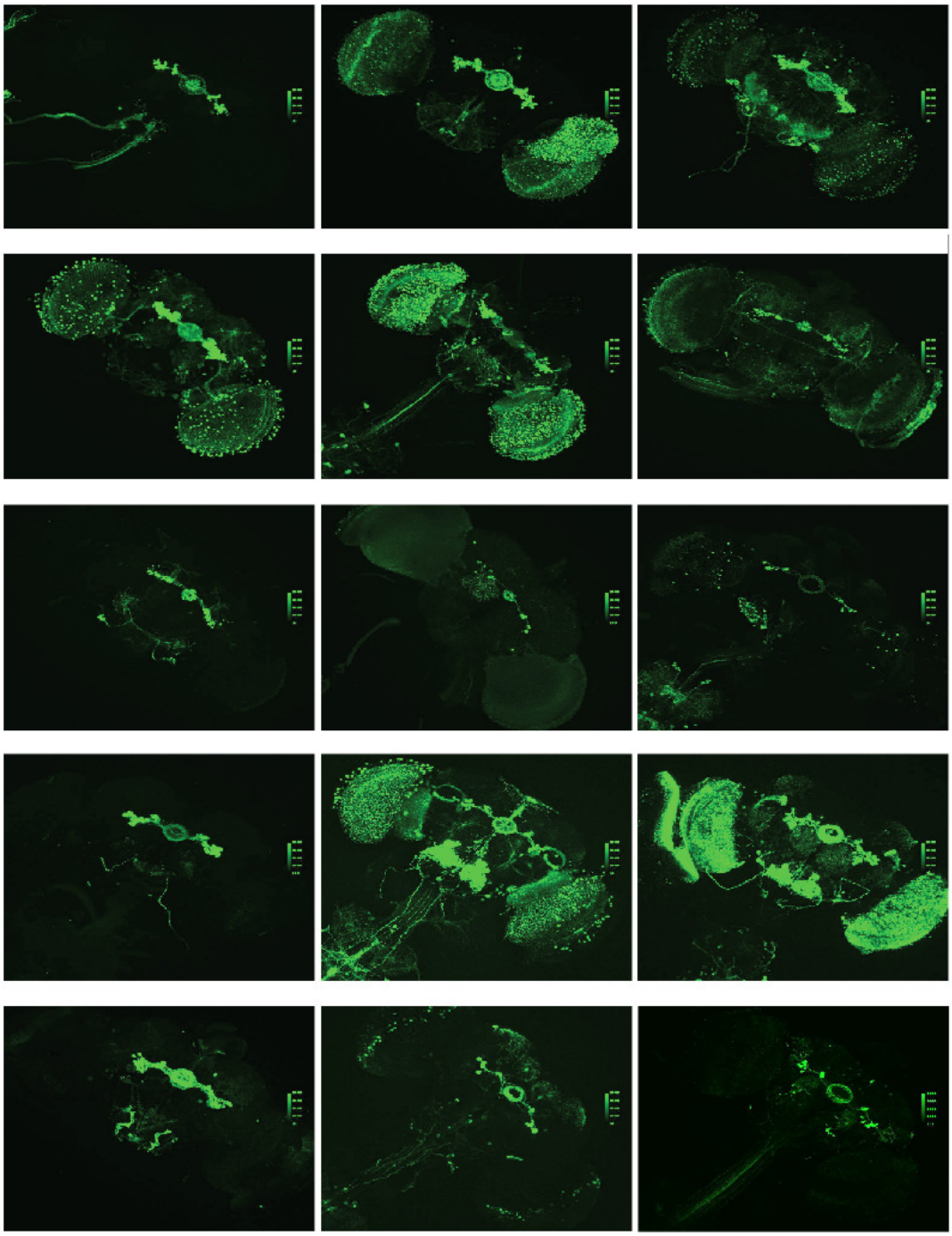

Figure 4-8: Place learning hits with expression in the eb
Shown are the expression patterns for all driver lines that shows place learning impairment in the visual place learning screen and have expression in the eb.

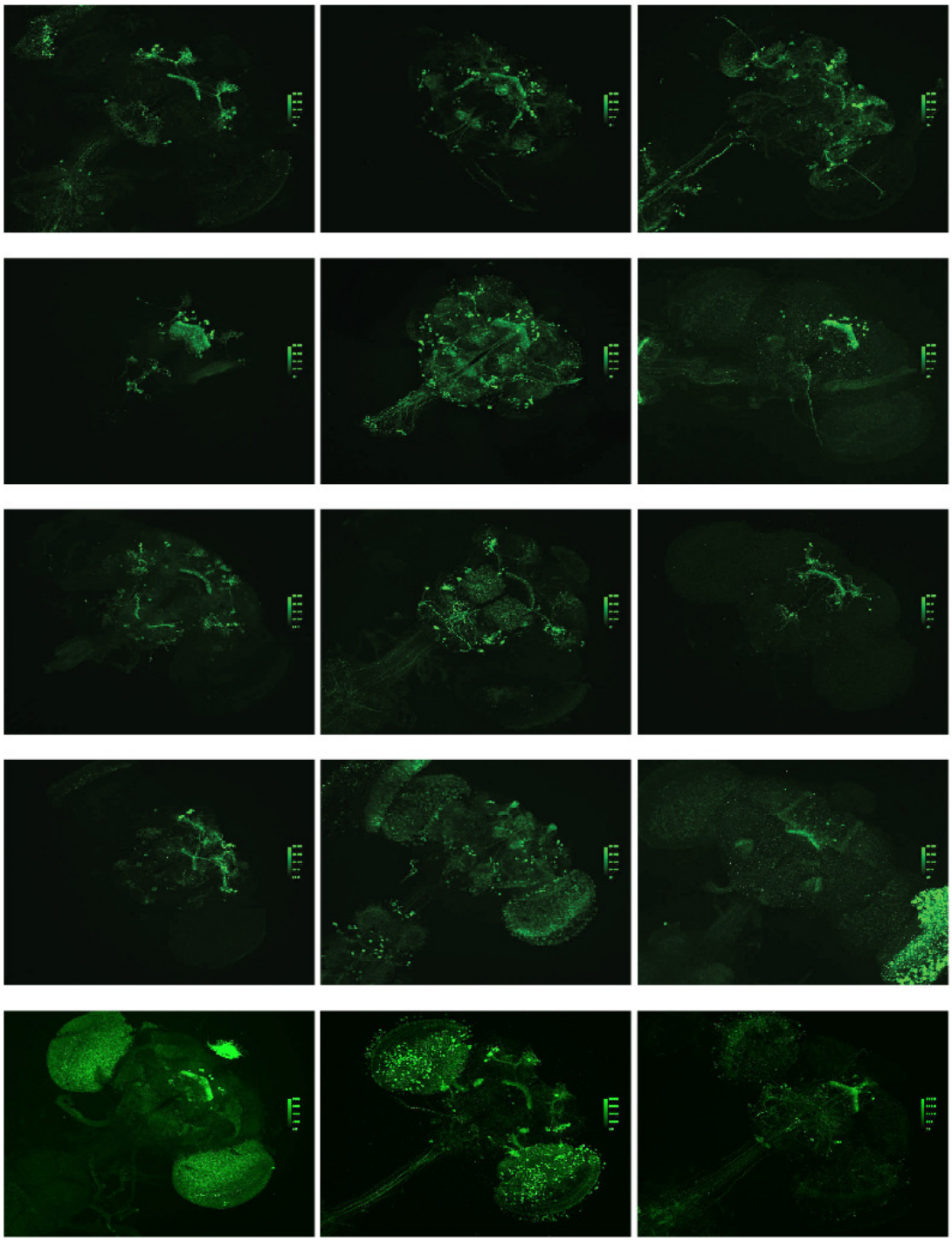

Figure 4-9: Place learning hits with expression in the fsb.
Shown are the expression patterns for all driver lines that shows place learning impairment in the visual place learning screen and have expression in the fsb.

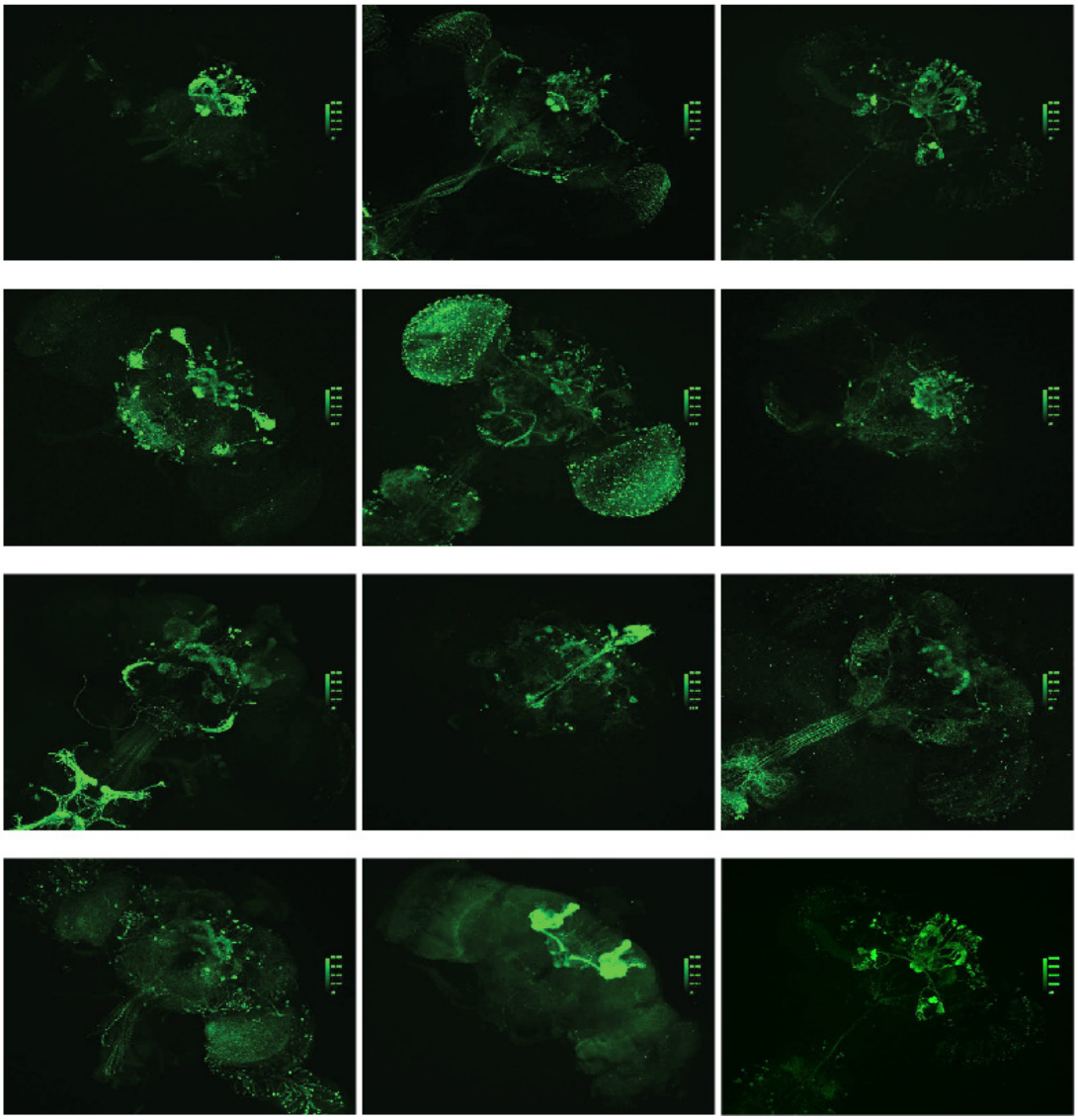

Figure 4-10: Place learning hits with expression in the pb
Shown are the expression patterns for all driver lines that shows place learning impairment in the visual place learning screen and have expression in the pb.

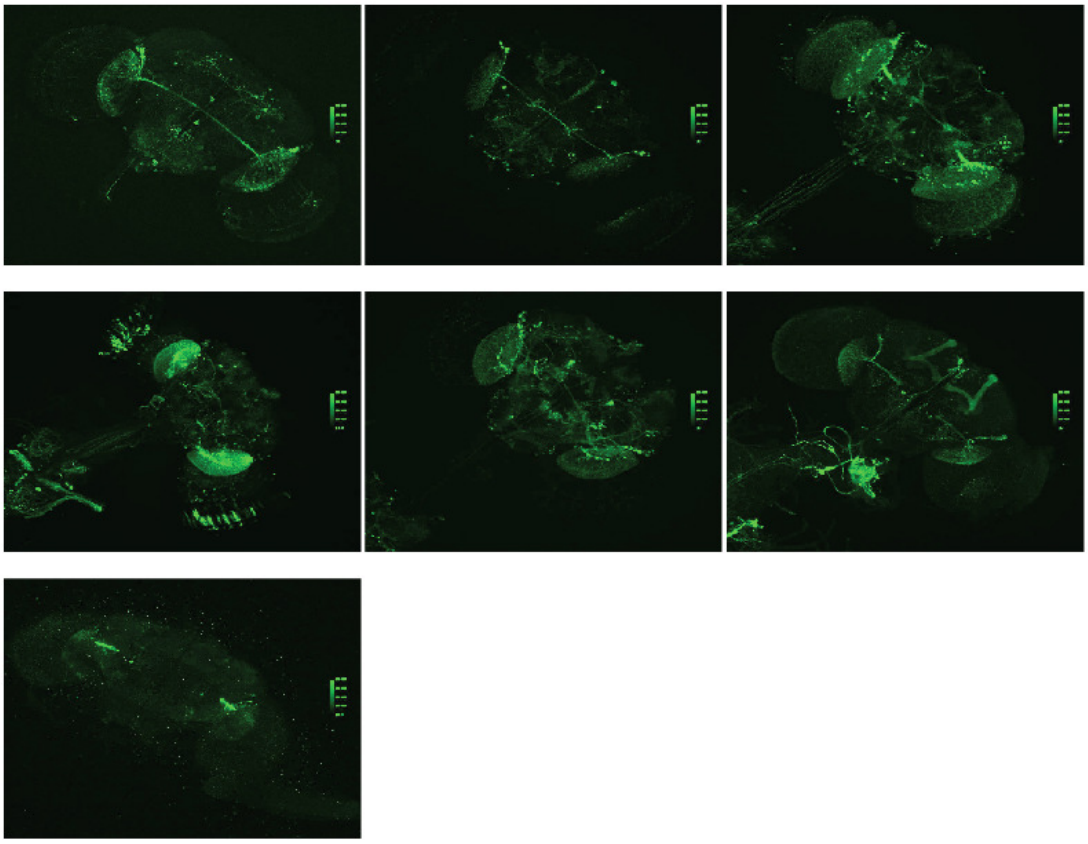

Figure 4-11: Place learning hits with expression in optic projection neurons
Shown are the expression patterns for all driver lines that shows place learning impairment in the visual place learning screen and have expression in visual projection neurons.

4.3 Methods and Calculations

Unless noted otherwise, calculations are as described in Chapter 3.

4.3.1 visual place learning protocol and analysis

The experimental protocol included 10 training trials (5 minutes each) followed by a probe trial (trial 11) where the visual display was relocated in the absence of a cool spot. At the end of the experiment, flies were tested in a temperature

preference trial (Sayeed et al. 1996; Zars 2001) and an optomotor trial (Strauss et al. 1997) to measure normal thermal and visual responses.

In addition to statistical tests described in Figure 4-2 legend, the probe learning index scores reported were also tested for statistical significance using one-way ANOVA with a Bonferroni correction for multiple comparisons. R15B07 and R28D01 shifted to 30^{o}C are significant at $p<0.01$ when compared to control flies. No other comparisons to control flies are significant at $p<0.05$.

Following the probe trial, flies were tested for thermal preference by setting alternating tiles on the TEM array to either 25^{o}C or 36^{o}C. Flies were allowed to distribute for 2 minutes before the cool and warm tiles were switched. The flies were then allowed another 2 minutes to re-distribute and the thermal aversion index was calculated as the amount of time flies spent at 25^{o}C minus the amount of time spent at 36^{o}C divided by the total time. Finally, flies were tested for normal optomotor responses by rotating a checkerboard pattern on the visual panorama clockwise and then counterclockwise at 90^{o} per second for 45 seconds. Optomotor responses are reported as the mean rotational velocity (in the direction of the stimulus) of the flies over the course of these trials. No significant differences are observed in thermal aversion or optomotor response at $p<0.05$ using one-way ANOVA with a Bonferroni correction for multiple comparisons.

4.3.2 olfactory conditioning

Olfactory conditioning experiments were based on experiments using an elevated T-maze as described in Tully and Quinn (1985). The conditioning protocol

was modified to use temperature as the unconditioned stimulus rather than electric shock (Figure 4-5, Figure 4-6). During conditioning, the training tube was heated to 36^{o} C concurrent with delivery of the first odor by passing a 5V, 0.43A current through a custom built insulated resistance wire mesh (Pelican Wire, 29 AWG Nichrome 60 w/Kapton) inserted into the training tube. Odors were delivered by bubbling an air stream through a vial containing odorant diluted in paraffin oil. Odors used were 5% 4-methylcyclohexanol (MCH), flow rate 128ml/min and 5% 3-octanol (OCT), flow rate 60 ml/min. Flow rate through training and testing tubes was normalized to 800ml/min by combining the odorant stream with a humidified clean air stream. ~200 flies were tested in each experiment, ½ conditioned to MCH, ½ conditioned to OCT. Learning indexes were calculated as the average learning index of the two groups. All mushroom body lines (R9A11, R10B08, and R67B04) are significantly impaired in olfactory learning when compared to control flies ($p<0.05$ using one-way ANOVA with a Bonferroni correction for multiple comparisons). No eb lines are significantly different from control.

4.3.3 tethered flight experiments

Closed loop tethered flight experiments were performed as described in Reiser et al.(2008) using a cylindrical LED display and an optical wing beat analyzer to measure fly responses. To test whether flies were capable of discriminating the visual features of the panoramic pattern in the visual place learning arena, we examined the orientation preference of flying flies for a flight arena pattern that was composed of 4 quadrants that display 15^{o} wide bar gratings, in either a vertical (quadrants 1, 3) or

horizontal (quadrants 2, 4) direction. Each fly was allowed to selectively orient under behavioral closed loop with this pattern for 5 trials of 50 seconds each, as part of an experimental series consisting of other closed and open loop trials, for which no further data is shown. Flies showed a clear preference for the vertical bars, and so we quantified the behavior with an orientation index that was calculated as the amount of time flies oriented towards vertical bars minus the amount of time orienting towards horizontal bars divided by the total time. No significant differences are observed in the orientation index at $p<0.05$ using one-way ANOVA with a Bonferroni correction for multiple comparisons.

4.3.4 experimental animals

For neural silencing experiments, w+; tubP-GAL80^{ts}; UAS-Kir2.1 flies (backcrossed 10 generations into DL wildtype genetic background to control for the effects of genetic background (de Belle et al. 1996) and known behavioral deficits with flies homozygous for w1118 (Diegelmann et al. 2006)) were crossed to GAL4 driver lines and reared at 18^{o}C. Two day old adult females were temperature shifted to 30^{o}C for 40 hours and then returned to 18^{o}C for 2 hours prior to testing. GAL4 driver lines were constructed as described in Pfeiffer et al. (Pfeiffer et al. 2008) and provided by Gerry Rubin. Control flies are w^{1118}; attP2 (the same genetic background as the GAL4 lines) crossed to w+; tubP-GAL80^{ts}; UAS-Kir2.1.

4.4 Acknowledgements

I'd especially like to thank Gerry Rubin for access to GAL4 driver lines prior to their publication; aRnim Jenett and Aljoscha Nern for their initial anatomic

annotation and advice in choosing lines to screen; and Laura Henderson her help in testing fly lines in our *Drosophila* place learning screen. Brain images were provided by the Janelia Fly Light Project. Todd Laverty and the Janelia Fly Core assisted in *Drosophila* stock maintenance.

Chapter 4, in part, has been accepted for publication in Nature, Ofstad, Tyler A.; Zuker, Charles S.; Reiser, Michael B. (2011). The dissertation author was the primary investigator and author of this paper.

Chapter 5
Discussion

5.1 Role of the Central Complex

Mammals likely use place, grid, and head direction cells to solve and perform navigational tasks (reviewed in Moser et al. 2008). The tight correlation between place cell activity (i.e. firing rate) and an animal's position in space has established the hippocampus as the substrate for a cognitive map (O'Keefe et al. 1971). This map is likely informed by head directions cells (Taube et al. 1990) (indicating an animal's orientation) and grid cells (Hafting et al. 2005) which tile the surrounding environment and could support path integration. While it is not known if there are direct correlates to these cells in flies, invertebrates are capable of solving similarly challenging navigational feats and do so using significantly smaller brains. Indeed, flies are able to use idiothetic cues, like path integration, to aid navigation (Wustmann et al. 1996; Putz et al. 2002; Neuser et al. 2008; Zars 2009). Now, our studies demonstrate that *Drosophila* can learn and recall spatial locations in a complex visual arena, and do so with remarkable efficacy.

Here, we also show that subsets of neurons in the fly central complex are critical for visual place learning, likely by implementing, storing or reading spatial information. Interestingly, the mushroom bodies appear dispensable in this task. This apparent discrepancy between our findings and those in the cockroach (Mizunami et al. 1998) may be related to differences in the sensory inputs to the mb between these two species. In the cockroach, there are known visual afferents to the mb (Strausfeld et al. 1999) while inputs in *Drosophila* are predominantly olfactory *(Stocker 1994).*

The identification of the ellipsoid body as a necessary component of visual place learning is intriguing. Neuser et al. (2008) identified subsets of eb neurons that are required for flies to re-orient towards a hidden target after being temporarily lured in an alternate direction. This may indicate a role for the eb in an allocentric direction representation. In mammals, head direction cells in the temporal lobes are maximally activated when an animal's head is pointed in a specific direction. Adding credibility to the hypothesis that subsets of eb ring neurons function in an analogous manner, Heinze et al. have recorded from neurons in the locust and butterfly CBL (the non-dipteran equivalent of the eb) that are tuned to specific e-vectors of polarized light (Heinze et al. 2009a; Heinze et al. 2011). It is suggested that these neurons may play a role in encoding locust head direction with respect to a celestial compass.

What about the rest of the central complex? In *Drosophila* specific layers of the fsb have been shown to be involved in the memory trace for specific visual features. Liu et al. (2006) show that dorsal layers of the fsb are required for a fly to remember *elevation* of a visual cue while the ventral layers are required for memory of *contour orientation* (Liu et al. 2006). This role of the fsb in the memory trace for visual features is likely related to the place learning impairment we see when we silence subsets of fsb neurons. In *Drosophila*, there is little evidence for the function of the pb. However, in the locust and butterfly, electrophysiologic recordings from the pb show directionally tuned response to polarized light (Heinze et al. 2007; Heinze et al. 2008; Heinze et al. 2009a). The pb is divided into 16 columns (this columnar organization is maintained throughout most of the central complex) and Heinze et al.

find that each column responds to a specific e-vector orientation of polarized light resulting in a map like representation of e-vector orientation in the pb (Heinze et al. 2007)[8]. These recordings further support the notion of a role of the central complex in spatial navigation.

Do vertebrates and invertebrates utilize similar place learning strategies? Strikingly, flies in which we silenced eb neurons exhibit a basic "circling" search routine (Figure 5-1) that is reminiscent of the behavior displayed by rats with hippocampal lesions (Morris et al. 1990). When the hippocampus is lesioned, rats lose the ability to swim directly to the hidden platform in the Morris Water Maze. However, rats retain the ability to improve in the task by adopting a "circling" search strategy where they learn to circle the arena at the correct distance from the wall. It is only when larger lesions are made to both the hippocampus and entorhinal cortex that rats revert to randomized searches. The similar circling behavior seen in eb silenced flies suggest that like in rats, there are distinct neural substrates required for directed vs. circling search strategies. It will be interesting to look for the cells responsible for this circling behavior as we continue to screen GAL4 lines driving expression in additional subsets of neurons.

[8] Polarization sensitive neurons in the central complex also respond to many other experimentally defined and undefined stimuli further supporting their hypothesized role as multisensory integrators (personal communications with members of the Homberg lab).

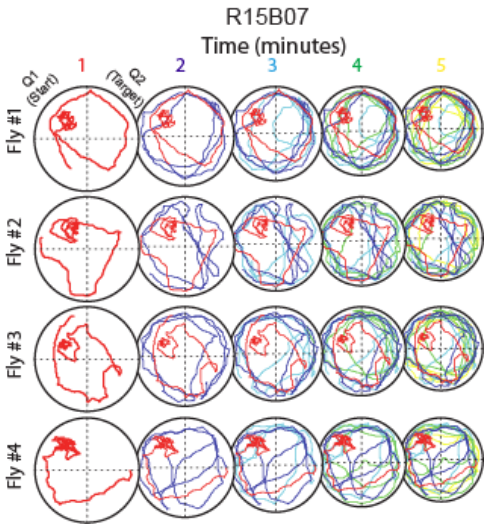

Figure 5-1: Representative tracks for an eb silenced fly in the probe trial
Shown are trajectories plotted as in Figure 3-7 for 4 representative eb silenced flies (line R15B07) trained with a *coupled* visual panorama. Note the prominent circling behavior. Circling is also seen in wildtype flies trained with an *uncoupled* visual panorama but not in flies trained in the dark (compare with Figure 3-7b and c).

5.2 Does the Central Complex Contain the Place Memory Trace?

The question of whether the central complex contains the memory trace for "place" arises often. While our behavioral assay depends on flies forming and recalling a memory of "place", the central complex (cc) could conceivably lie at several locations along the learning-memory-recall pathway. (i)The cc could be required for navigation independent of memory; (ii) it could be upstream of the memory trace and required for reinforcement; (iii) the cc could itself be the anatomic substrate for the place memory trace; (iv) the cc could be required for the retrieval and execution of place memories. These possibilities could conceivably be investigated by silencing neurons at specific times during training and/or testing (for example silencing neurons only during the probe trial after animals have already been trained).

Unfortunately, the practical aspect of this experiment presents several challenges. As the GAL80ts/Kir2.1 effector we use for our standard neural silencing experiments has onset/offset kinetics on the order of days, it is not suited for acute silencing studies. Shi^{ts} and dTRPA1 (for silencing and activating neurons respectively) have temporal dynamics that are much faster (seconds to minutes); however, as they are both gated by temperatures used in our thermal-visual arena and so they too are not suited for the proposed experiments. Channelrhodopsin and Halorhodopsin are the only other effectors currently available that have rapid onset and offset kinetics and could be used for the proposed experiments. Since both of these are gated by visible light (and our assay depends of visual navigation), using them in a freely walking assay is probably not feasible. However, if a fly could be tethered (i.e. head fixed) and taught to navigate a "virtual" thermal-visual landscape, then light could be directed to only the channelrhodopsin/halorhodopsin expressing cells and neural activity could be actively modulated as a fly performs different phases of the visual place learning task. An additional benefit of a head fixed visual place learning assay (and the most direct way to address the role of identified neurons in visual place learning) is the ability to optically and/or electrophysiologically monitor neuron activity in a behaving animal (Chiappe et al. 2010; Dombeck et al. 2010; Maimon et al. 2010; Seelig et al. 2010). Preliminary data for visual place learning in a tethered fly navigating a virtual thermal-visual arena is presented below (Section 5.3).

An alternative but less direct method for addressing the role of cc neurons in memory is the use of RNAi (Ni et al. 2009) to knock down the expression of proteins

that are required for memory formation (but not general neuron function). We find one such protein, Pumilio (Dubnau et al. 2003; Chen et al. 2008), that when knocked down pan-neuronally using the $C155^{elav}$-GAL4 driver shows severe place learning impairment. Place learning impairment is also evident when pumilio knock-down is confined to the ellipsoid body using C232-GAL4 (data not shown). This is consistent with the eb playing a role in the place memory trace. We are currently involved in experiments using RNAi against additional learning and memory related genes (Waddell et al. 2001) targeted to the neuron subsets identified in the visual place learning screen (Section 4.2.1). We hope that these experiments further clarify the role of the central complex in place memory.

5.3 Place Learning With a Head Fixed Fly in a Virtual World

What is the logic for spatial coding in the insect brain? Unfortunately, there are very few studies that have successfully recorded from neurons in the central complex (and to our knowledge, none of these are in *Drosophila*). Most recordings show complex responses that are difficult to correlate with a specific sensory stimulus. The best evidence coupling a calibrated sensory stimulus to a specific neural response come from the polarized light studies in the locust and butterfly discussed above (Heinze et al. 2007; Heinze et al. 2009a; Heinze et al. 2009b; Heinze et al. 2011). In the cockroach, lesions of the central complex result in complex motor impairments (Harley et al. 2010), while recording from central complex cells show a correlation with walking activity, but also other multisensory stimuli (notably vision) (Ritzmann et al. 2008; Bender et al. 2010). These studies are all consistent with the central

complex playing a role in navigation. However, the complex multisensory responses, and difficulties in correlating these responses with behavioral responses in an animal leave the precise role of the central complex unclear.

Imaging of neuronal activity in the fly brain while the animal is executing a navigation task should help further clarify the role of the central complex, and eb neurons in particular, in spatial memory (for example in a head-fixed preparation with a virtual reality arena (Dombeck et al. 2010; Seelig et al. 2010)). To assay visual place learning in a head-fixed fly, we developed a virtual place learning arena based on the fly treadmill described in Seelig et al. (2010). Briefly, a fly is tethered to a tungsten pin and positioned on a 9mm foam ball floating on a column of air. As the fly walks, rotations of the ball are tracked at high resolution. The accumulated rotation of the ball is converted to rotation and translation of the fly in a virtual arena. Custom Matlab scripts use this translation and rotation to calculate a virtual visual landscape (displayed on a LED display surrounding the treadmill) and to control an infrared laser for heating the fly. Both the LED display and infrared laser are updated at 30 Hz. In this way, the fly has closed loop control of a virtual thermal-visual arena analogous to the free walking thermal-visual arena described previously.

To confirm that flies show normal responses to temperature in this virtual arena, we tested flies for thermal aversion using the same protocol used for free walking flies (see Chapter 2.2.2). The virtual arena is divided into 4 quadrants and the fly is allowed to freely navigate in closed loop. To keep the fly within the virtual boarders of the arena, the infrared laser is used to heat the fly as it approaches the

arena edge. When the fly is in 2 of the quadrants (diagonally opposite from each other), the laser is switched off and the fly is at 25ºC (i.e. room temperature). When the fly is in the other 2 quadrants, the laser turns on and the fly is heated. Laser power is adjusted between trials so that flies are tested for their preference between the reference temperature (25ºC) and test temperatures between 25ºC and 39ºC. The time spent at each temperature is used to calculate an aversion index as described in Chapter 2.2.2. Flies tested in the virtual arena show thermal aversion behavior that is nearly identical to flies tested in the free walking arena (Figure 5-2).

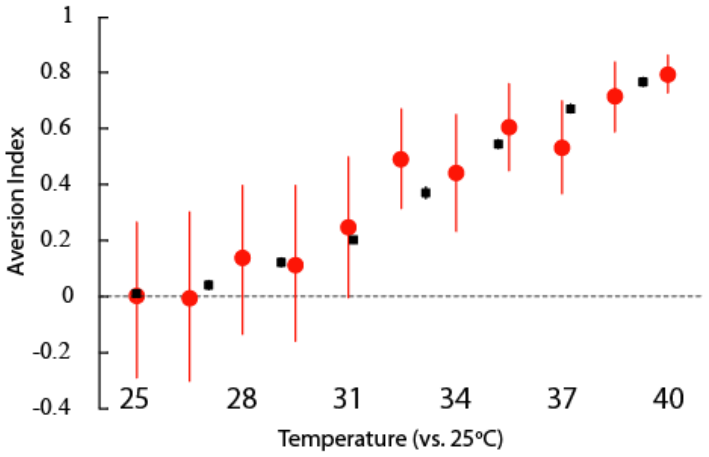

Figure 5-2: Virtual thermal aversion assay
Tethered flies tested in a virtual thermal aversion assay (red circles) show avoidance of warm temperatures that is comparable to flies tested in a free walking (i.e untethered) thermal aversion assay (black squares).

To test if flies are also able form visual place memories when navigating in the virtual arena, we modified the place learning protocol described in Chapter 3.1. In a virtual place learning experiment, flies are first placed on the ball for a 20 minute acclimation period. During this acclimation period, a uniform checker pattern is used for the visual panorama and flies are only heated (50º C) when they approach the edge

of the virtual arena. Following this acclimation period, the visual panorama is changed to a pattern composed of horizontal, vertical, and diagonal bars. Flies are trained using a protocol similar to that used in free walking (i.e. untethered) experiments. The fly starts each trial in the bottom right corner of the virtual arena and is heated to ~38 degrees using an infrared laser. When the fly reaches the "safe" location in the upper right corner of the virtual arena, the laser shuts off. Each trial lasts for 60 seconds after the fly locates the safe spot, or a maximum of 10 minutes. Over the course of multiple training trials, tethered flies improve significantly in their ability to locate the safe spot (Figure 5-3d and e). Following these training trials, flies are tested with a probe trial where no cool spot is present. Much like flies trained in the free walking arena, tethered flies preferential search in the virtual arena location where they have been trained to locate the safe spot (Figure 5-3f). These pilot studies demonstrate that flies are capable of visual place learning in a virtual thermal-visual landscape. We look forward to using this tethered fly preparation to further explore spatial orientation and to investigate the neural mechanisms that underlie navigation and place memories in *Drosophila*.

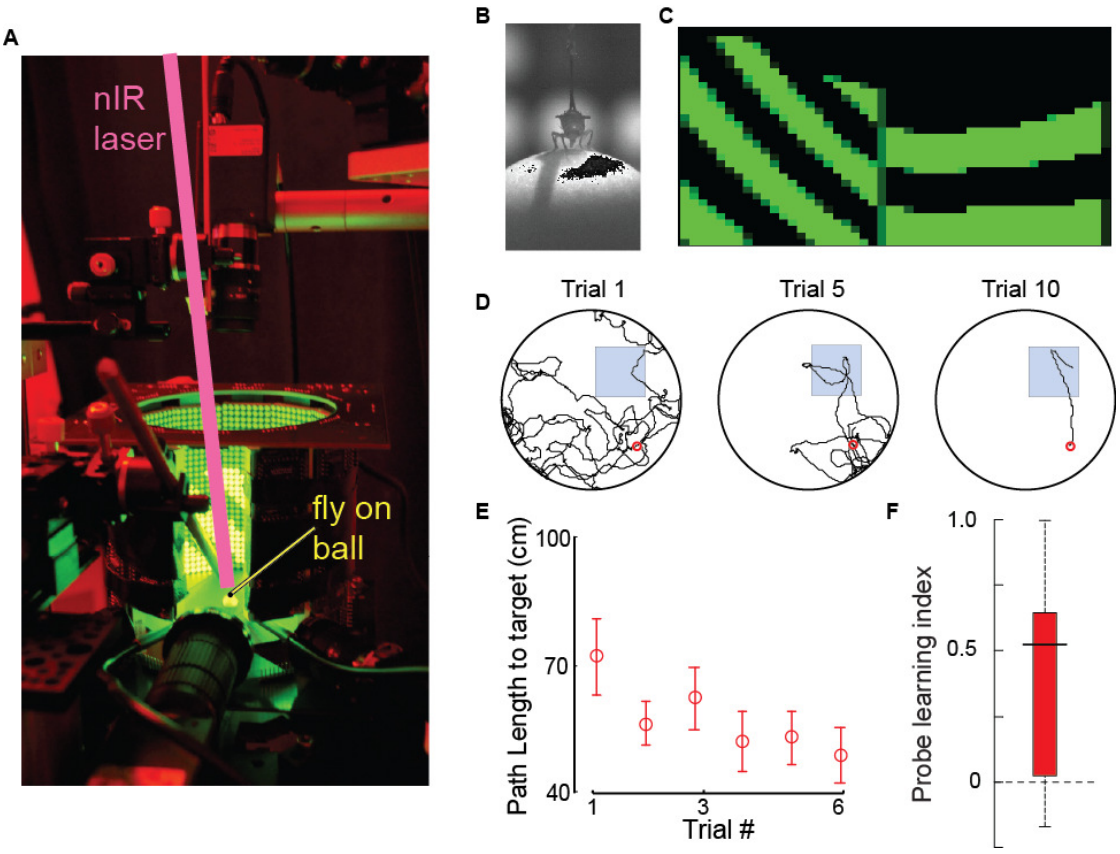

Figure 5-3: Tethered place learning in a virtual arena.
(**a**) Shown is a picture of the "virtual" place learning arena. Flies are glued to the end of a tungsten wire (shown in **b**) and positioned on top a foam ball suspended on a column of air. Cameras track the rotation of the ball. Rotation of the ball is converted to a virtual translation and rotation of the fly in a virtual coordinate system and used to update the visual display (shown in **c**). An infrared laser (pink line) is used to heat the fly. (**d**) Shown are representative tracks in the virtual arena from a single fly in trial 1, 5, and 10. The flies starting location (red circle) and location of the cool target (blue square) are illustrated. (**e**) Over the course of multiple training trials, flies take significantly shorter paths to the cool spot. (**f**) When tested in a probe trial after training, flies preferentially search where they have been trained to find the cool spot, even when the cool spot is not present. For a comparison to free walking flies, see Figure 3-5 and Figure 3-6. In (e), n = 30 flies, error bars are ± SEM. In (f) n = 11 flies, box plots are as described in Figure 3-5.

5.4 Summary of Dissertation and Concluding Remarks

In this dissertation, I have described the development of a novel *Drosophila* visual place learning assay. Using this assay, I show that flies are capable forming and recalling visual place memories. Subsets of cells in the central complex are specifically involved in this task, as silencing them leads to severe impairment in visual place learning without affecting other sensory (vision, olfaction, thermosensation), motor (locomotor and tethered flight), or learning (olfactory conditioning) behaviors. Notably, we see no role for the mushroom bodies (structures classically thought to be the substrates of learning and memory) in visual place learning. These findings substantiate distinct anatomic sites for spatial (place) vs. non-spatial (olfactory) learning in *Drosophila*.

In the vertebrate place learning field, the identification of a cognitive map in the medial temporal lobes (i.e. place, grid, and head direction cells) has created much excitement and led to many proposed theories for how these cells man encode space. However, despite these findings and their (seemingly) obvious implications, the complexity of the vertebrate brain has presented significant challenges in understanding how place fields are established, and how they are subsequently read out to support navigation. Despite their small size and compact nervous systems, insects also posses robust navigating capabilities. The addition of *Drosophila melanogaster* to the list of visual place learners brings a wealth of molecular genetic tools to the field of insect navigation. Through the use of these tools and new technologies (for example a head fixed virtual place learning assay) that will allow the

fly brain to be manipulated and observed in a behaving animal, we hope to gain fundamental insight into the neural representation of “place”. Ultimately, elucidating the cellular basis for place learning in the fruit fly will help uncover fundamental principles in the organization and implementation of spatial memories in general.

Chapter 6 Appendix

6.1 TEM Array Control and Calibration

The Matlab code in this section is used to control and calibrate the TEM arrays.

6.1.1 TEM_initialize

This code opens the serial port communication between the TEM arrays and the computer.

```
A = serial('COM4', 'BaudRate', 234000, 'Terminator', {94 13} );
fopen(A)
B = serial('COM5', 'BaudRate', 234000, 'Terminator', {94 13} );
fopen(B)
C = serial('COM6', 'BaudRate', 234000, 'Terminator', {94 13} );
fopen(C)
D = serial('COM7', 'BaudRate', 234000, 'Terminator', {94 13} );
fopen(D)

disp ('initialization finished');
```

6.1.2 send_TEM_serial_command

This code sends serial commands to the array controller. It is called by set_TEM_output_8x8, send_TEM_temps_8x8, and grab_all_TEM_temps_8x8.

```
function send_TEM_serial_command(s, Address, command_code, val)

if nargin == 3
    val = '';
else
    if (length(val) ~= 4)
        warning('val input is incorrect and will be ignored')
        val = '';
    end
end
str = [Address command_code val];

% compute checksum:
tmp_sum = 0;
for j = 1:length(str)
   tmp_sum = tmp_sum + double(str(j));
end
tmp_sum = mod(tmp_sum, 256);

send_str = ['*' str lower(dec2hex(tmp_sum))];
fprintf(s,send_str);
```

6.1.3 read_TEM_serial_command

This code sends serial commands to the array controller. It is called by set_TEM_output_8x8, send_TEM_temps_8x8, and grab_all_TEM_temps_8x8.

```
function Num_resp = read_TEM_serial_command(s, Address, command_code, val)


if nargin == 3
    val = '';
else
    if (length(val) ~= 4)
        warning('val input is incorrect and will be ignored')
        val = '';
    end
end
str = [Address command_code val];

% compute checksum:
tmp_sum = 0;
for j = 1:length(str)
   tmp_sum = tmp_sum + double(str(j));
end
tmp_sum = mod(tmp_sum, 256);

send_str = ['*' str lower(dec2hex(tmp_sum))];
[out,count,msg] = fgets(s);

if ((out(1) ~= '*')|(length(out) ~=8))
    warning(['return value not as expected: ' out])

else
     Num_resp = nhex2dec(out(2:5),32);
end
end
```

6.1.4 set_TEM_output_8x8

This code turns the power to the array on or off.

```
function [Array_output]=set_TEM_output_8x8_V2(A,B,C,D, Power_states)
% A,B,C,& D are serial objects opened with TEM_initialize. Power_states
%sets the output status of each of the 4 controllers, Power_states =
%[0 0 0 0] turns all off; [0 1 1 0] sets 2 and 3 on, 0 and 4 off; etc.

%% sends power output command to array
for ctr_ind = 1:4


        str_Add = lower(ndec2hex(ctr_ind,8));

         if Power_states(ctr_ind) == 0
            val = '0000';
         else
            val = '0001';
         end
         send_TEM_serial_command(A, str_Add, '0f', val);
         send_TEM_serial_command(B, str_Add, '0f', val);
         send_TEM_serial_command(C, str_Add, '0f', val);
         send_TEM_serial_command(D, str_Add, '0f', val);
end

 %% reads power output command from array
Pow_A=zeros(1,4);
Pow_B=zeros(1,4);
Pow_C=zeros(1,4);
Pow_D=zeros(1,4);

for ctr_ind = 1:4

      out=fgets(A);
      Pow_A(ctr_ind) = nhex2dec(out(2:5),32);

      out=fgets(B);
      Pow_B(ctr_ind) = nhex2dec(out(2:5),32);

      out=fgets(C);
      Pow_C(ctr_ind) = nhex2dec(out(2:5),32);

      out=fgets(D);
      Pow_D(ctr_ind) = nhex2dec(out(2:5),32);

 end

Array_output = [Pow_A,Pow_B,Pow_C,Pow_D];
```

6.1.5 send_TEM_temps_8x8

This code sends the temperature settings to the array.

```
function [Temps] = send_TEM_temps_8x8_V2(A,B,C,D, Temp_set)
% A,B,C,& D are serial objects opened with TEM_initialize.
% here we send the set temps to the entire array. Temp_set is an 8x8
% matrix where each number in the matrix is the set temperature for
%the corresponding TEM tile

Temp_set=[Temp_set(1:4,1:4);Temp_set(5:8,1:4);rot90(Temp_set(1:4,5:8),2);rot90(Temp_set(5:8,5:8),
2)];

if ((size(Temp_set) ~= [16 4]) | all(all(Temp_set < 2)) | all(all(Temp_set > 50)))
    warning('Temp_set is the wrong size, or out of range');

else

%% sends temps to array
   for ctr_ind = 1:4

      for panel_ind = 1:4

         str_Add = lower(ndec2hex(ctr_ind,8));
         str_tem_cmd = num2str(9 + panel_ind, '%02g');  %'10' is code for first addr.
         val_A = lower(dec2hex(round(Temp_set(ctr_ind, panel_ind)*100), 4));
         val_B = lower(dec2hex(round(Temp_set(ctr_ind+4, panel_ind)*100), 4));
         val_C = lower(dec2hex(round(Temp_set(ctr_ind+8, panel_ind)*100), 4));
         val_D = lower(dec2hex(round(Temp_set(ctr_ind+12, panel_ind)*100), 4));

            send_TEM_serial_command(A, str_Add, str_tem_cmd, val_A);
            send_TEM_serial_command(B, str_Add, str_tem_cmd, val_B);
            send_TEM_serial_command(C, str_Add, str_tem_cmd, val_C);
            send_TEM_serial_command(D, str_Add, str_tem_cmd, val_D);

      end
   end

 %% reads temps from array
Temps_A=zeros(4,4);
Temps_B=zeros(4,4);
Temps_C=zeros(4,4);
Temps_D=zeros(4,4);

for ctr_ind = 1:4
   for panel_ind = 1:4

      out=fgets(A);
      Temps_A(ctr_ind, panel_ind) = nhex2dec(out(2:5),32)/100;
```

```
        out=fgets(B);
        Temps_B(ctr_ind, panel_ind) = nhex2dec(out(2:5),32)/100;

        out=fgets(C);
        Temps_C(ctr_ind, panel_ind) = nhex2dec(out(2:5),32)/100;

        out=fgets(D);
        Temps_D(ctr_ind, panel_ind) = nhex2dec(out(2:5),32)/100;

    end
end

Temps=[Temps_A(1:4,1:4),rot90(Temps_C(1:4,1:4),2);Temps_B(1:4,1:4),rot90(Temps_D(1:4,1:4),2)];
end
```

6.1.6 grab_all_TEM_temps_8x8

This code queries the array for temperature and power readings.

```
function [Temps Pow_out] = grab_all_TEM_temps_8x8(A,B,C,D)
% A,B,C,& D are serial objects opened with TEM_initialize.
% here we grab the temps and power output from the entire array

Temps_A=zeros(4,4);
Pow_out_A=zeros(4,4);
Temps_B=zeros(4,4);
Pow_out_B=zeros(4,4);
Temps_C=zeros(4,4);
Pow_out_C=zeros(4,4);
Temps_D=zeros(4,4);
Pow_out_D=zeros(4,4);


for ctr_ind = 1:4
   for panel_ind = 1:4

      str_Add = lower(ndec2hex(ctr_ind,8));
      str_tem_cmd = lower(ndec2hex(panel_ind,8));
      str_pow_cmd = lower(ndec2hex(4+panel_ind,8));



      send_TEM_serial_command(A, str_Add, str_tem_cmd);
      out=fgets(A);
      Temps_A(ctr_ind, panel_ind) = nhex2dec(out(2:5),32)/100;
      send_TEM_serial_command(A, str_Add, str_pow_cmd);
      out=fgets(A);

      if strcmp(out(2),'V');
         p_out=hex2dec(['f',out(3:5)]);
         Pow_out_A(ctr_ind, panel_ind) = (p_out-65535)/5.11;
      else
      Pow_out_A(ctr_ind, panel_ind) = hex2dec(out(2:5))/5.11;
      end

      send_TEM_serial_command(B, str_Add, str_tem_cmd);
      out=fgets(B);
      Temps_B(ctr_ind, panel_ind) = nhex2dec(out(2:5),32)/100;
      send_TEM_serial_command(B, str_Add, str_pow_cmd);
      out=fgets(B);
```

```
        if strcmp(out(2),'V');
           p_out=hex2dec(['f',out(3:5)]);
           Pow_out_B(ctr_ind, panel_ind) = (p_out-65535)/5.11;
        else
        Pow_out_B(ctr_ind, panel_ind) = hex2dec(out(2:5))/5.11;
        end

        send_TEM_serial_command(C, str_Add, str_tem_cmd);
        out=fgets(C);
        Temps_C(ctr_ind, panel_ind) = nhex2dec(out(2:5),32)/100;
        send_TEM_serial_command(C, str_Add, str_pow_cmd);
        out=fgets(C);

        if strcmp(out(2),'V');
           p_out=hex2dec(['f',out(3:5)]);
           Pow_out_C(ctr_ind, panel_ind) = (p_out-65535)/5.11;
        else
        Pow_out_C(ctr_ind, panel_ind) = hex2dec(out(2:5))/5.11;
        end

        send_TEM_serial_command(D, str_Add, str_tem_cmd);
        out=fgets(D);
        Temps_D(ctr_ind, panel_ind) = nhex2dec(out(2:5),32)/100;
        send_TEM_serial_command(D, str_Add, str_pow_cmd);
        out=fgets(D);

        if strcmp(out(2),'V');
           p_out=hex2dec(['f',out(3:5)]);
           Pow_out_D(ctr_ind, panel_ind) = (p_out-65535)/5.11;
        else
        Pow_out_D(ctr_ind, panel_ind) = hex2dec(out(2:5))/5.11;
        end

    end

end

Pow_out=[Pow_out_A(1:4,1:4),rot90(Pow_out_C(1:4,1:4),2);Pow_out_B(1:4,1:4),rot90(Pow_out_D(1:
4,1:4),2)];
Temps=[Temps_A(1:4,1:4),rot90(Temps_C(1:4,1:4),2);Temps_B(1:4,1:4),rot90(Temps_D(1:4,1:4),2)];
```

6.1.7 retrieve_IR_temps

This code retrieves a thermal image and calculates an average temperature for each tile in the 64 tile TEM array. This function is called by TEM_calibrate

```
function [IR_tile_temps]=retrieve_IR_temps(ignore_threshold, Array_version)
%here we grab the latest IR image and convert it to an average tile temperature.
%ignore threshold sets temperatures to be ignored (for example if using a heated
%aluminum ring on top of the TEM array.

%set the tile edges in the TEM image
if Array_version==1
left=30;
right=220;
top=18;
bottom=205;
roi_offset=4;
elseif Array_version==2
left=37;
right=227;
top=3;
bottom=195;
roi_offset=4;
end

%this opens the latest file from thermalyze. Directories should be set to the thermalyze autosave file
if Array_version==1
  cd 'B:\Program Files\Thermalyze\Images\AutoSave';
elseif Array_version==2
  cd 'c:\Program Files\Thermalyze\Images\AutoSave';
else
    error ('Array_version does not match');
end

files=dir('*.imb');
file=[files(end).name];
data=fopen(file);
f_header=fread(data, 10, 'int16');
f_data=fread(data, [320 240], 'int16');
IR_temp = (f_data+18000)/100;
[r,c]=find(IR_temp>ignore_threshold);
for v=1:length(r)
    IR_temp(r(v),c(v))=nan;
end
fclose(data);
delete(files.name);
cd 'c:\MatlabRoot';

IR_temp=flipud(IR_temp);
```

```
tile_width=(right-left)/8;
tile_height=(bottom-top)/8;

close
figure ('Position',[600,10,640,512])
imagesc (IR_temp);
hold all;

for c=1:8
   for r=1:8
      tile(c,r).corners=round([top+tile_height*(c-1), top+tile_height*c, top+tile_height*c,
top+tile_height*(c-1), top+tile_height*(c-1);
                  left+tile_width*(r-1),left+tile_width*(r-
1),left+tile_width*(r),left+tile_width*(r),left+tile_width*(r-1)]);
                  plot(tile(c,r).corners(2,1:5),tile(c,r).corners(1,1:5));

                  tile(c,r).roi=round([top+tile_height*(c-1)+roi_offset, top+tile_height*c-roi_offset,
top+tile_height*c-roi_offset, top+tile_height*(c-1)+roi_offset, top+tile_height*(c-1)+roi_offset;
                  left+tile_width*(r-1)+roi_offset,left+tile_width*(r-1)+roi_offset,left+tile_width*(r)-
roi_offset,left+tile_width*(r)-roi_offset,left+tile_width*(r-1)+roi_offset]);
                  plot(tile(c,r).roi(2,1:5),tile(c,r).roi(1,1:5));

   end
end
axis ([0,320,0,240]);
hold off;

IR_tile_temps=(nan(8,8));
for c=1:8
   for r=1:8
roi=IR_temp((round(top+(tile_height*(c-1))+roi_offset)):(round(top+(tile_height*c)-
roi_offset)),(round(left+(tile_width*(r-1))+roi_offset)):(round(left+(tile_width*(r))-roi_offset)));
roi=nanmedian(roi);
IR_tile_temps(c,r)=nanmedian(roi,2);

   end
end
```

6.1.8 TEM_calibrate

This function automatically calibrates the TEM array for a particular set of TEM setpoints using a thermal imaging camera.

```
function [temp_calibrated]=TEM_calibrate(A,B,C,D,temp_condition,ignore_threshold, Array_version)
%A,B,C, and D are the serial objects opened in TEM_initialize.  This function calibrates the TEM array
% to the setpoints specified in temp_conditions.
%ignore threshold sets temperatures to be ignored (for example if using a heated
%aluminum ring on top of the TEM array.


%turns on the TEM array
   [Array_output]=set_TEM_output_8x8(A,B,C,D, [1,1,1,1])


 %sends temperature settings to the array
[Temps] = send_TEM_temps_8x8(A,B,C,D, temp_condition)


      target_temp=temp_condition;
      pause (40);
      calib_temp=temp_condition;
      [IR_tile_temps]=retrieve_IR_temps(ignore_threshold, Array_version); % grabs temperature from
IR camera
      calib_error=calib_temp-IR_tile_temps % calculates the error between the sent temp and the IR
measured temp



      while max(abs((calib_error(:))))>0.10 % may want to adjust this to increase or decrease accuracy
         calib_temp=(calib_temp)+(calib_error);

          [Temps] = send_TEM_temps_8x8(A,B,C,D, calib_temp)    %sends the new temperature
         settings

          pause(50);
          [IR_tile_temps]=retrieve_IR_temps(ignore_threshold, Array_version);
          calib_error=target_temp-IR_tile_temps

         % safety to shut down array if limits are exceeded
          if max(abs((calib_error(:))))>10 %adjust this to set max calibration error
            [Array_output]=set_TEM_output_8x8_V2(A,B,C,D, [0,0,0,0])
             error('ERROR: calib_error > max allowed')
          elseif min(calib_temp(:))<17
                [Array_output]=set_TEM_output_8x8(A,B,C,D, [0,0,0,0])
                error('ERROR: calib_temp < min allowed')
          elseif max(calib_temp(:))>47
```

```
            [Array_output]=set_TEM_output_8x8_V2(A,B,C,D, [0,0,0,0])
            error('ERROR: calib temp > max allowed')
        end

    temp_calibrated=calib_temp;    %this outputs the calibrated array settings

end
```

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