

GENETIC AND NEURONAL SYSTEMS THAT ARE CRITICAL FOR HIGH TEMPERATURE  
AVOIDANCE IN REFLEXIVE AND LEARNED BEHAVIORS

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by

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

GENETIC AND NEURONAL SYSTEMS THAT ARE CRITICAL FOR HIGH TEMPERATURE AVOIDANCE  
IN REFLEXIVE AND LEARNED BEHAVIORS

presented by Elizabeth F. Kramer,

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## ABSTRACT

In a variable temperature world, there is selective advantage to being able to either tolerate or escape from bad conditions and learn from experience to avoid future bad events. Within species there are multiple strategies to survive and prevent future harmful events, but how organisms allocate genetic resources to tolerance and learning strategies is far from clear. High temperatures can be used to examine multiple genetic bases of tolerance and avoidance of places predictive of aversive high temperature exposure in *Drosophila*.

Natural variations at the *foraging* (*for*) locus provide flies with distinct behavioral strategies for survival. *Drosophila* with natural variants of the *for* locus are known to behave differently in the presence of food; the heat box was used to observe behavior in an aversive environment. I examined locomotion in *rover* (*for<sup>R</sup>*) and *sitter* (*for<sup>S</sup>*) flies for differences in high temperature induced incapacitation to test the idea that trade-offs in high temperature survival strategies might exist with key polymorphisms at this locus.

Serotonin is one of the primary biogenic amines in *Drosophila* implicated in operant place learning. Although serotonergic cell bodies and innervation patterns have been identified, relationships between cell bodies and the associated neurite projections are still not well established. Unambiguously assigning innervation patterns to specific neuronal cell bodies is the first step to understanding the anatomical organization of serotonergic neurons. Thus I have begun to identify subsystems of serotonergic systems by generating transcription activator and repressor elements.

I used the heat box paradigm to investigate both neuronal circuitry and genetic bases for operant conditioning in *Drosophila*, providing new insight into the fly's ability to learn and remember to avoid a high temperature-associated place in a simple environment. Using transcription activator and repressor elements, I have identified a subsystem of serotonergic neurons necessary and sufficient for place memory after direct conditioning but not after pre-exposure to aversive temperatures.

This dissertation work has served to identify both a subsystem of serotonergic neurons involved in learned avoidance of high temperature-associated places and tradeoffs in survival strategies employed by *Drosophila* when presented with a high temperature.

## CHAPTER 1: INTRODUCTION

In a variable temperature world, there is selective advantage to being able to either tolerate or escape from bad conditions and learn from experience to avoid future aversive events. That different animal species typically have multiple strategies for surviving environmental extremes, then, should be no surprise. For example, some hymenoptera use a regurgitation behavior and evaporation to control over-heating, while locusts invest in a physiological response to compensate for elevated body temperature (Heinrich, 1993; Newman et al., 2003). Even within species there are multiple strategies to survive and prevent future harmful events, but how organisms allocate genetic resources to tolerance and learning strategies is far from clear. I used high temperatures to examine multiple genetic bases for high-temperature tolerance and avoidance of places predictive of aversive high temperature exposure in *Drosophila*.

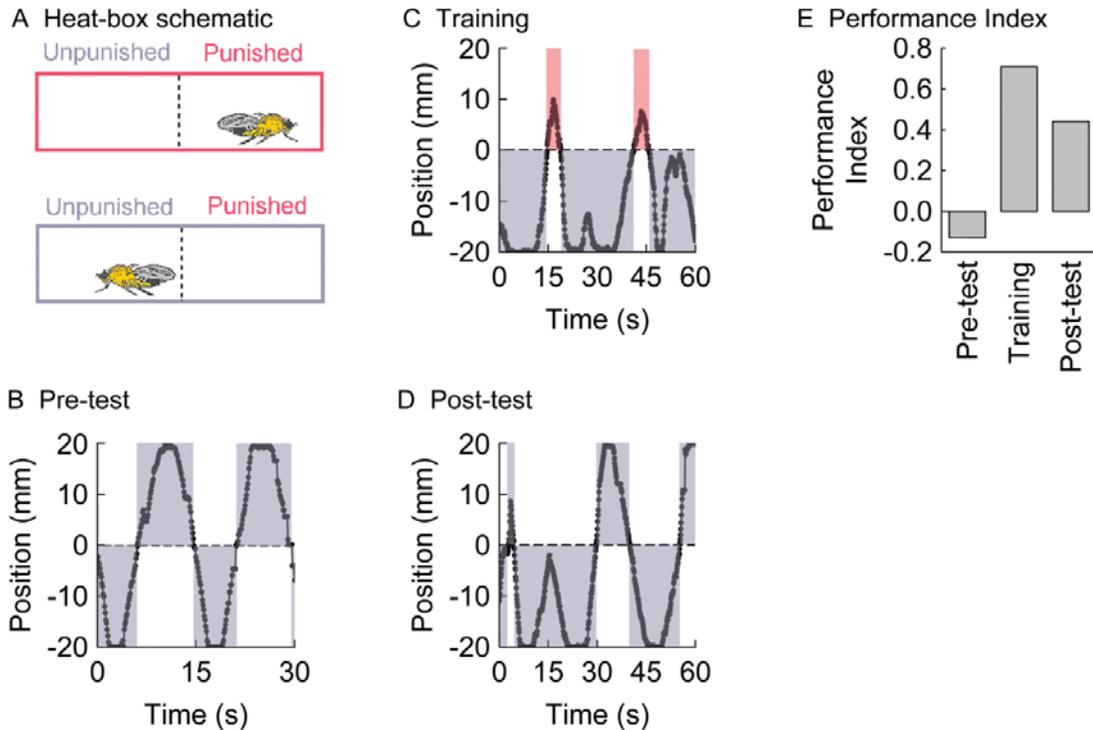
### **High temperature survival strategies in *Drosophila***

Flies become incapacitated with prolonged exposure to elevated temperatures and they become sterile over generations when continuously exposed to 29°C (Ashburner et al., 2005; Sayeed & Benzer, 1996; Zars, 2001). Therefore flies have a strong preference for 24°C over warmer temperatures, and when acutely exposed to temperature steps from 24°C to 30°C and higher temperatures, they try to avoid the aversive temperatures by initially increasing their locomotor activity and finally moving away from hot places (Sitaraman & Zars, 2010). If flies cannot eventually avoid increasing temperatures they become incapacitated; the higher the temperature, the shorter the time to locomotor cessation (Gioia & Zars, 2009).

Furthermore, physiological measures such as changes in heart rate and effects of multiple exposures (i.e. hardening or tolerance) as a response to high temperatures can be addressed

(Ashburner et al., 2005; Dahlggaard et al., 1998; Johnson et al., 1997; Levins, 1969; White et al., 1992). Intriguingly, a classic allele pair termed *rover* and *sitter* in the foraging cGMP-dependent protein kinase (PKG) gives rise to different physiological responses to high temperature exposure (Dawson-Scully et al., 2007; Gioia & Zars, 2009). I examined locomotion in *rover* and *sitter* flies for differences in high temperature incapacitation to test the idea that trade-offs in tolerance might exist with key polymorphisms at this locus (Chapter 3).

Flies can also actively avoid dangerous temperatures through both nonconditioned behavioral avoidance of a high temperature source and conditioned avoidance of part of a long narrow chamber. The heat box paradigm tests both of these avoidance behaviors (Figure 1.1). First, given a step gradient of a low and high temperature, flies will avoid the high temperature side of the chamber. This avoidance behavior represents both an ability of a fly to sense a particular temperature and a capability to move away from an aversive cue. The avoidance behavior in this ‘thermosensitivity’ assay is short-lived, lasting only seconds in the absence of the temperature differential (Diegelmann et al., 2006; Zars, 2001). Second, if flies are provided with a predictive cue for the rising temperature by place, they will avoid that place for minutes (Diegelmann et al., 2006; Putz & Heisenberg, 2002; Wustmann et al., 1996). In this second assay, when a fly crosses to the side associated with the aversive temperature, the entire chamber adjusts to the aversive temperature. Returning to the other side of the chamber restores the chamber to a temperature the flies prefer (Diegelmann et al., 2006; Zars, 2001; Zars et al., 2000). I used the heat box paradigm to investigate both neuronal circuitry and genetic bases for this operantly conditioned avoidance in *Drosophila*, providing new insight into the fly’s ability to learn and remember to avoid a high temperature associated place (Chapters 4-5).



**Figure 1.1** – Operant conditioning in the heat box.

A) Flies are trained to avoid one half of the chamber by associating that half with an aversive change in temperature. B) During a 30 s pretest, no aversive reinforcement is applied. Flies walk freely between sides of the chamber. The purpose of this test is to analyze the fly's initial locomotor activity and to account for any initial side preference the fly might exhibit. C) Training occurs with the application of an aversive temperature dependent on the fly's behavior (aversive exposure in red). When the fly crosses to the aversively-associated chamber half, the entire chamber heats to the aversive temperature. Returning to the unassociated side restores the preferred temperature. Training times and temperatures can be adjusted prior to each trial. D) The post-test phase monitors the fly's behavior once the aversive reinforcement is removed. E) The performance index (PI) quantifies conditioning as time spent on the non-aversive side less the time spent on the aversive side divided by the total time in the chamber. PIs range from -1 to 1. A PI of 0 indicates no side preference while a PI of 1 is perfect avoidance of the side associated with aversive reinforcement (Sitaraman et al., 2008).

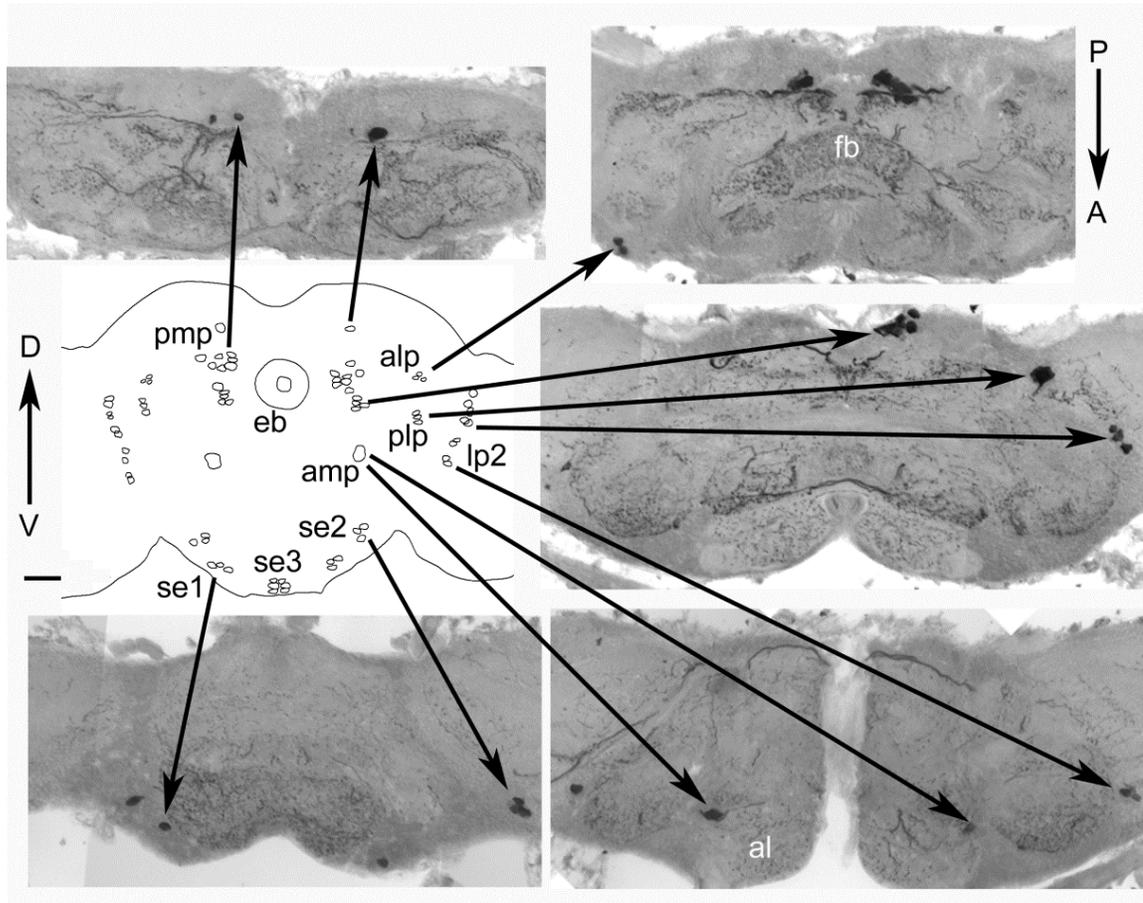
### **Biogenic amines in *Drosophila***

Biogenic amines, derivatives of amino acids, have many functions in both vertebrate and invertebrate species. In insects, they serve as neurotransmitters/neuromodulators, chemical messengers controlling or modulating neuronal activity for specific neurons in the central nervous system, as well as acting as neurohormones by transport through hemolymph to the site of action. These two functions result in behavioral changes, physiological changes, or both in the organism. Serotonin is one of the primary biogenic amines in *Drosophila* previously implicated in operant place learning mechanisms.

### **The serotonergic circuit in *Drosophila***

Serotonin is the end product of a two-step biosynthetic pathway. Tryptophan is hydroxylated to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase, identified in *Drosophila* as either TpH or TrH (Coleman & Neckameyer, 2005; Monastirioti, 1999). 5-HTP is then decarboxylated by dopa decarboxylase (DDC) to generate 5-hydroxytryptamine (5-HT or serotonin).

Immunohistochemistry and expression studies have identified and described ~40 serotonergic neurons in the adult central brain (Alekseyenko et al., 2010; Giang et al., 2011; Sitaraman et al., 2008; Valles & White, 1988). These neurons are found in eight clusters identified by their location in the central brain (Figure 1.2), though recent work has subdivided some of these clusters for a total of twelve (Giang et al., 2011; Sitaraman et al., 2008). Discrete regions of the brain are innervated by these neurons and include the subesophageal ganglion, ellipsoid body, fan-shaped body, antennal lobes, lateral protocerebrum, and mushroom bodies, similar to structural innervations found in moths and bees (Homberg & Hildebrand, 1989; Schurmann & Klemm, 1984; Sinakevitch et al., 2008).



**Figure 1.2** – The serotonergic system in the *Drosophila* central brain.

A schematic showing the location of the serotonergic cell bodies (Sitaraman et al., 2008). Eight serotonergic cell body clusters are distributed in characteristic positions (amp = anterior medial protocerebrum; alp = anterior lateral protocerebrum; se1-se3 = sub-esophageal groups 1 to 3; lp2 = lateral protocerebrum group 2; pip = posterior lateral protocerebrum; and pmp = posterior medial protocerebrum). The ellipsoid body (eb) is outlined for orientation; dorsal (D) is up. Examples of the serotonergic cell bodies are shown in frozen horizontal sections challenged with an anti-serotonin antibody (as in (Buchner et al., 1988)). The dorsal-most section is shown in the upper left and a subset of the serial sections progressing ventrally can be followed clockwise. Also evident is the discrete but widespread arborization of the serotonergic neurons in the central brain (e.g. the fan-shaped body (fb)). In the tissue sections anterior (A) is down. The scale bar = 50  $\mu$ m (Zars, 2009).

Although cell bodies and innervation patterns have been identified, previous relationships between cell bodies and the associated neurite projections are still not well established because all serotonergic neurons are labeled at one time and all neurites project into the neuropil. Unambiguously assigning innervation patterns to specific neuronal cell bodies is the first step to understanding the anatomical organization of serotonergic neurons. Toward this end, I have begun to identify subsystems of serotonergic systems by generating transcription activator and repressor elements which can be localized using immunohistochemical techniques (Chapter 4).

A clear understanding of the *Drosophila* serotonergic system requires not only a detailed description of anatomical relationships, but also an understanding of the many behaviors in which it functions. Place conditioning experiments demonstrate that flies with genetically or pharmacologically reduced serotonin levels have decreased memory performance when compared to controls (Sitaraman et al., 2008). Other studies using similar genetic and pharmacological approaches identify a role for the serotonergic system in aggression, olfaction, circadian entrainment, sleep behaviors, and appetitive olfactory memory (Dacks et al., 2009; Dierick & Greenspan, 2007; Johnson et al., 2009; Sitaraman et al., 2012; Yuan et al., 2006; Yuan et al., 2005). However, identification of neurons involved in these behaviors has been impeded by lack of suitable techniques that would allow for genetic manipulation of individual neurons or neuronal subsystems. In Chapter 5, I examine the function of subsystems of the serotonergic system in place memory by identifying a subsystem of neurons necessary and sufficient for place memory.

## **CHAPTER 2: GENERAL MATERIALS AND METHODS**

### **Fly stocks and crosses**

All fly stocks have been introgressed into a Canton-S (CS) background – all transgenic lines created are crossed to a Cantonized  $w^{1118}$  line for a minimum of six generations. Ultimately, the X-chromosomes are replaced with a wild-type version using balancer crosses. Generation of stocks with multiple genetic elements follow standard balancer crossing schemes.

### **Fly rearing**

*Drosophila melanogaster* are raised on a cornmeal media in a light, temperature, and humidity controlled chamber. Food is provided *ad libitum* and flies are maintained at 24°C and 60% humidity on a 12 hour light cycle. All behavioral experiments are conducted with three to six day old flies which have never been anesthetized.

### **Operant place conditioning**

The heat box consists of individually enclosed chambers measuring 33mm x 5mm x 2mm. Single flies are able to walk freely from one end of a chamber to the other. Each chamber is lined top and bottom with Peltier elements that can adjust the temperature of the chamber within seconds. A light-bar reader is positioned on the side of each chamber, detecting the fly's location within the chamber as a shadow. This is monitored continuously by a computer which then adjusts the temperature of the chamber according to the fly's position (Diegelmann et al., 2006; Wustmann et al., 1996; Zars et al., 2000).

### **Direct conditioning**

A typical direct conditioning protocol consists of a pre-training block, a training block, and a test block. During the pre-training phase, no aversive temperature is applied as flies acquaint themselves with the chamber. During training, flies are exposed to aversive temperature for the first time. This training time is of variable duration depending on the parameters of the experiment being performed. The test phase removes the aversive stimulus and monitors the fly's avoidance of the aversively-associated side. The subject's exposure to the aversive stimulus during training is the first and only time the subject is exposed to aversive reinforcement.

### **Pre-exposure training**

Pre-exposure differs from direct training because the flies receive more than one exposure to an aversive stimulus. Flies are exposed to a highly aversive temperature, e.g. 41°C, unrelated to their position in the heat box. After a pre-determined time, again specific to the experiment, flies are then run through a direct conditioning protocol with a less aversive temperature, for example 30°C, which produces a low memory score during direct training. In wild-type flies, pre-exposure to a higher temperature increases aversive operant learning at lower temperatures (Sitaraman et al., 2010; Sitaraman & Zars, 2010).

### **Thermosensitivity assay**

Thermosensitivity assays are designed to test for naive temperature preference behavior. One half of the chamber is programmed to heat to a defined temperature, the other part of the chamber is held at a preferred temperature. This provides a steep temperature gradient.

Alternate halves heat for the duration of the experiment and only one half heats at a time so the

fly's avoidance of increasingly higher temperatures can be quantified. The heat box is heated on alternate sides as follows: 24°C/27°C, 32°C/24°C, 24°C/37°C, 41°C/24°C.

### **Performance index**

The performance index (PI) for a conditioning trial is calculated as the amount of time flies spend on the non-aversive side of the heat box chamber less the amount of time flies spend receiving aversive stimulus divided by the total time spent in the chamber. The PI ranges from -1 to 1: a PI of 1 indicates perfect avoidance of aversive temperatures, a PI of -1 indicates no avoidance of aversive temperatures, and a PI of 0 indicates no side preference. The PI for a thermosensitivity assay is calculated in the same fashion.

### **Behavior statistics**

Tests of normality for statistical analysis of performance indices in the heat box give mixed results (Putz & Heisenberg, 2002). Therefore, parametric analysis cannot be used for heat box data. For comparison of two samples that are independent of each other and do not assume any normality, we used the Mann-Whitney U test. When more than two independent samples were compared, the non-parametric Kruskal-Wallis ANOVA and multiple comparisons of mean ranks for groups were performed (Putz & Heisenberg, 2002). All statistical analyses were performed using the STATISTICA program (StatSoft Inc., Tulsa, Oklahoma).

**CHAPTER 3: THE INFLUENCE OF NATURAL VARIATION AT THE *FORAGING* GENE ON THERMOTOLERANCE IN ADULT *DROSOPHILA* IN A NARROW TEMPERATURE RANGE.**

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<sup>1</sup> = equal contribution

**Introduction**

Natural variations at the *foraging* (*for*) locus of the fruit fly, *Drosophila melanogaster*, provide flies with distinct behavioral strategies for survival. Perhaps best known for its role influencing food searching behavior, variations at the *for* locus also provide stress protection (Dawson-Scully et al., 2007; Sokolowski, 1980). Although there is a clear role for natural *for* alleles in thermoprotection in the larval stage, a previous study in the adult fly did not find the same relationship (Dawson-Scully et al., 2007; Dawson-Scully et al., 2010; Gioia & Zars, 2009).

Experiments described here re-examine the function of natural *for* variants for thermoprotection in adult flies.

Natural *for* polymorphisms create an allele-dependent variation in cGMP-dependent protein kinase G (PKG) activity that influences several behaviors. Flies carrying the *rover* allele (*for<sup>R</sup>*) are associated with higher levels of PKG activity than the *sitter* (*for<sup>S</sup>*) counterparts (Osborne et al., 1997). This difference in PKG activity influences various phenotypic behaviors such as foraging and learning (de Belle et al., 1989; Kaun et al., 2007; Mery et al., 2007; Osborne et al., 1997). Higher levels of PKG activity are correlated with the increased locomotion observed in the

presence of food as well as increased olfactory learning (Kaun et al., 2007; Mery et al., 2007; Osborne et al., 1997). Similarly, high PKG flies perform better at high-temperature-reinforced visual learning tasks than low PKG flies (Wang et al., 2008). However, not all learning is affected by the *for* locus (Gioia & Zars, 2009; Zars, 2010). Adult *Drosophila* with *for* variations showed no difference in place memory when conditioned with high temperatures.

Variation in PKG activity also influences stress responses, such as hyperthermic and anoxic tolerance. Lower levels of PKG are associated with prolonged protection from acute hyperthermia (in larvae) and hypoxia (in adults) (Dawson-Scully et al., 2007; Dawson-Scully et al., 2010). Genetic and pharmacological manipulation of the PKG pathway has shown that *Drosophila* larvae with low levels of PKG activity have an increase in hyperthermic protection for locomotion and synaptic transmission at the neuromuscular junction. Critically, the central respiratory circuit of the locust is also protected by reducing PKG activity during acute hyperthermia, indicating that the PKG pathway has a conserved function in thermotolerance (Dawson-Scully et al., 2007).

Thermoprotection of locomotion appears to be differentially sensitive to variation of the *for* locus in adult and larval life stages in *Drosophila* (Dawson-Scully et al., 2007; Gioia & Zars, 2009). It was previously shown that there was no significant difference in time to incapacitation in adult flies having *for<sup>R</sup>*, *for<sup>S</sup>*, or *for<sup>S2</sup>* alleles across a broad range of temperatures in time-limited experiments (Gioia & Zars, 2009). In contrast, larval stage *for<sup>R</sup>*, *for<sup>S</sup>*, and *for<sup>S2</sup>* animals show differences in the time to incapacitation (Dawson-Scully et al., 2007). Close examination of incapacitation times in *for<sup>R</sup>*, *for<sup>S</sup>*, and *for<sup>S2</sup>* flies at the temperature of 39°C suggests that there might be an enhanced thermoprotective function for the *for<sup>S</sup>* or *for<sup>S2</sup>* flies with prolonged

exposure (Gioia & Zars, 2009). High temperature knockdown experiments were performed at 39°C to further explore variation at the *for* locus and thermoprotection in adults.

## Methods

### *Fly stocks*

Adult *Drosophila melanogaster* that expressed the *for<sup>R</sup>*, *for<sup>S</sup>*, and *for<sup>S2</sup>* (a mutant allele on *for<sup>R</sup>* genetic background) alleles were used for this study. Flies used for the heat box assay and walking speed analysis were 2-7 days old, raised on a 12 h L:D light cycle, fed a standard yeast-sucrose-agar medium, and incubated at 24 °C (Gioia & Zars, 2009). Flies used in the single- and multi-fly failure assays were reared in a similar fashion, except flies were 5-9 days old.

### *Behavioral experiments*

The chamber of the heat box (33 mm x 5mm x 2mm) was lined with Peltier heating elements along the floor and ceiling (Zars, 2001). Flies were detected by a bar-code reader, detection bins are spaced evenly into 126 units measuring 0.2 mm.

Flies were tested individually in the heat box chamber for 12 minutes. During the first 30 seconds of the experiment, the test temperature was kept constant at 24°C and then immediately increased to 39°C for the remainder of the trial. For control runs, the temperature remained at 24°C for the entire trial. Fly displacement was recorded every 0.1s and flies were assigned a 'change value' of either a 1 (inactive: moved four units [0.8mm] or less) or a 0 (active: moved more than four units). Time to fly incapacitation was defined as the first instance of inactivity for 60 seconds. This 60 second 'failure' criterion is equivalent to the fly being ascribed a 'change value' of 1 for 600 consecutive times; the first 0.1s of these 60 seconds of inactivity was recorded as the time of incapacitation. We also calculated speed for each 0.1s interval. The

mean speed was calculated for 30 seconds before the temperature increase (24°C), and 30 seconds after the shift (39°C).

A visual-based locomotor failure test examined the time to incapacitation in a group of 10 flies using a micro-hybridization incubator (Model 2000, Robbins Scientific, Sunnyvale, CA). Methods used were similar to those in Dawson-Scully et al. (2010) except a hyperthermic, rather than anoxic, environment was induced (Dawson-Scully et al., 2010). Ten flies of each strain (*for<sup>R</sup>*, *for<sup>S</sup>*, or *for<sup>S2</sup>*) were placed into three separate testing vials. A single vial of 10 flies was placed into the center of the incubator. Individual fly failure (cessation of movement) was timed and measured and the average time for failure of each genotype was calculated.

In a single-fly walking assay, individual flies were placed into a preheated 1.1 ml segment (~28 mm in length) of a Fisherbrand<sup>®</sup> 5mL polystyrene pipette, and two foam plugs were used to prevent the fly from escaping. The micro-hybridization incubator was again used to keep temperature at 39°C, and the apparatus was placed directly in the center of the incubator. Failure times were recorded at the instant of incapacitation onset.

### *Statistics*

A Kaplan-Meier (K-M) survival analysis was used for determining statistically significant differences in heat box incapacitation times across the three genotypes (*for<sup>R</sup>*, *for<sup>S</sup>*, and *for<sup>S2</sup>*). Flies that did not cease activity had their incapacitation time censored; as a result, the time to incapacitation equaled the length of the test (12 minutes). For the speed, group failure, and individual fly failure statistics, an ANOVA with multiple comparisons (Student-Newman-Keuls post-hoc) was used. Sigma Plot software was used for all statistical tests, and Multi-Arena-Multi-Event-Recorder (MAMER; created by Dr. Craig A.L. Riedl) was used to record and average

failure times in the population and individual failure assays (Dawson-Scully et al., 2010). SEM is standard error mean, and letters on graphs that differ show statistical significance within individual graphs.

## Results

### *Time to Incapacitation at 24°C and 39°C*

To test incapacitation of flies at 24°C and 39°C, individual flies were tested in single heat box chambers for 12 minutes. Measurement of the time to incapacitation between *for<sup>R</sup>*, *for<sup>S</sup>*, and *for<sup>S2</sup>* alleles under control conditions at 24°C (Figure 3.1a) revealed a significant difference in activity across the three genotypes (K-M, N=336,  $X^2=24.5$ ,  $p < 0.001$ ). The *for<sup>R</sup>* flies remained active for nearly the entire 12 minutes while the number of active *for<sup>S</sup>* and *for<sup>S2</sup>* flies decreased at approximately equal rates. In contrast, when the temperature was raised to 39°C *for<sup>R</sup>* flies became incapacitated first, followed by *for<sup>S</sup>*, then *for<sup>S2</sup>* flies (Figure 3.1b; K-M, N=336,  $X^2=10.6$ ,  $p=0.005$ ).

### *Walking speed*

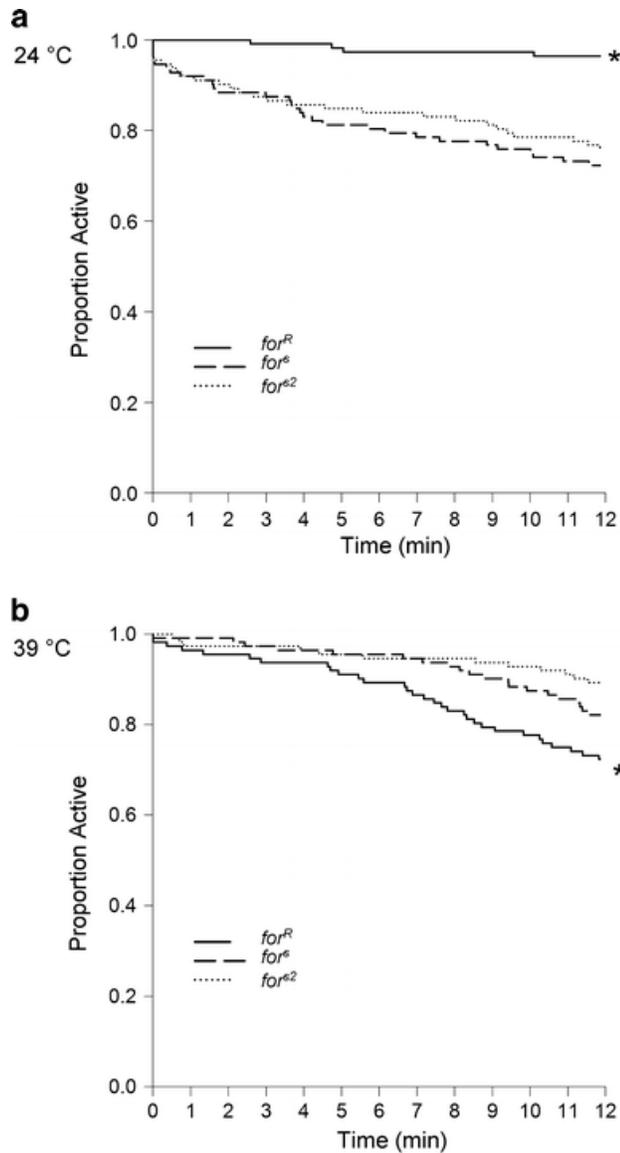
Walking speed was determined using position data from the heat box incapacitation tests. Positions were recorded and speeds (mm/s) were calculated for every position change over 30 seconds. The 30 second average speed analysis at 24°C revealed phenotypic differences among the *for<sup>R</sup>*, *for<sup>S</sup>*, and *for<sup>S2</sup>* flies. The *for<sup>R</sup>* flies had considerably higher speeds than flies with the *for<sup>S</sup>* and *for<sup>S2</sup>* alleles (Figure 3.2a; *for<sup>S</sup>*: N=224,  $p<0.001$ ,  $q=26.8$ ; *for<sup>S2</sup>*: N=224,  $p<0.001$ ,  $q=25.1$ ). At high temperature (39°C), average speed was again different between flies with the three *for* alleles (Figure 3.2b). Flies with the *for<sup>R</sup>* allele had higher walking speed than both *for<sup>S</sup>* alleles (*for<sup>S</sup>*: N=224,  $p<0.001$ ,  $q=24.2$ ; *for<sup>S2</sup>*: N=224,  $p<0.001$ ,  $q=45.1$ ), and overall an increase in

temperature caused an increase in speed ( $for^R$ : N=112,  $p<0.001$ ,  $q=16.7$ ;  $for^S$ : N=112,  $p<0.001$ ,  $q=19.3$ ;  $for^{S2}$ : N=112,  $p=0.003$ ,  $q=4.2$ ). Interestingly, there was actually a small decline in walking speed for the  $for^{S2}$  flies after the rise in temperature. No notable difference in average speed was evident between the  $for^S$  and  $for^{S2}$  mutants at 24°C (N=224,  $p=0.236$ ,  $q=1.7$ ). The temperature increase to 39°C resulted in a significant difference in speed between flies of these *sitter* genotypes, largely because the  $for^{S2}$  flies did not increase activity with the higher temperature (N=224,  $p<0.001$ ,  $q=21.9$ ).

#### *Locomotor failure testing of for flies*

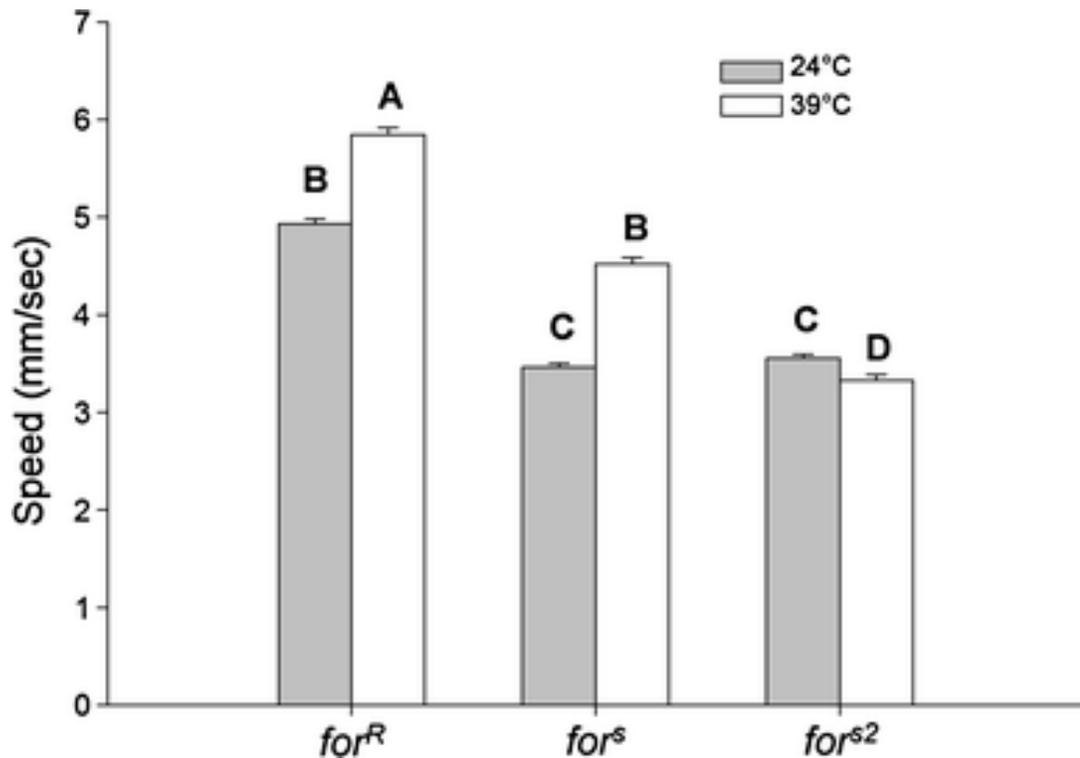
Groups of 10  $for^R$ ,  $for^S$ , and  $for^{S2}$  flies were placed into separate vials and then into a micro-hybridization incubator. Time to failure was visually determined and recorded using the software program MAMER (Dawson-Scully et al., 2010). Average time to failure of the  $for^{S2}$  mutant was significantly higher than all other genotypes ( $for^{S2}$  vs.  $for^S$ : N=14,  $p<0.001$ ,  $q=5.7$ ;  $for^{S2}$  vs.  $for^R$ : N=14,  $p<0.001$ ,  $q=11.0$ ) (Figure 3.3a).  $for^R$  flies had the lowest average failure time and  $for^S$  flies failed at an intermediate time ( $for^R$  vs.  $for^S$ : N=14,  $p<0.001$ ,  $q=5.4$ ). Fly thermotolerance for group failures was also tested at 41°C and 43°C; no significant differences were found between  $for^R$  and  $for^S$  at 41°C and no significant differences were shown across all genotypes at 43°C (Figure 3.3a), demonstrating the narrow range of temperature where observable differences in thermotolerance occurs in adults.

Individual flies were also tested in a walking assay mimicking heat box assay experiments while only measuring absolute failure. Single flies were placed into a 1.1 ml portion of a preheated, polystyrene pipette and trapped using foam plugs. Flies were allowed to walk back and forth



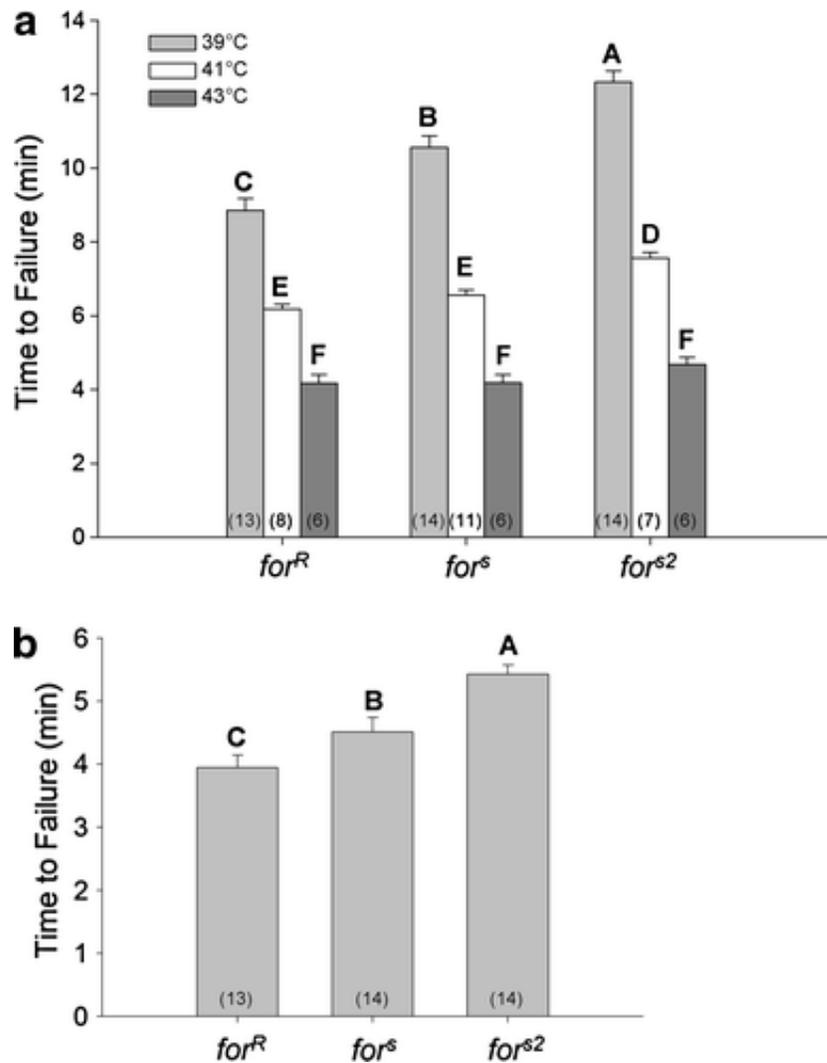
**Figure 3.1** – Incapacitation of  $for^R$ ,  $for^S$ , and  $for^{S2}$  at 24°C and 39°C

Proportions of active *Drosophila* carrying the  $for^R$ ,  $for^S$ , and  $for^{S2}$  mutant alleles were evaluated for incapacitation at 24°C (a; control temperature) and 39°C (b; hyperthermic temperature) for 12 minutes. Active proportions of  $for^R$ ,  $for^S$ , and  $for^{S2}$  flies were calculated based on the activity of the total population during the entire test. a)  $for^S$  and  $for^{S2}$  flies had a greater proportion of flies with less activity than  $for^R$  flies at 24°C. b)  $for^R$  flies had the fastest rate of incapacitation under hyperthermic conditions (39°C), followed by  $for^S$  and then by the  $for^{S2}$  flies. Asterisks represent significant differences between populations ( $p < 0.05$ ). N=112 animals per treatment group.



**Figure 3.2** – Walking speed in *for<sup>R</sup>*, *for<sup>S</sup>*, and *for<sup>S2</sup>* mutants at 24°C and 39°C.

Walking speed was measured in individual *for* flies for 30 seconds, and the mean was taken for each genotype. In control conditions, *for<sup>R</sup>* flies had a significantly higher walking speed than *for<sup>S</sup>* and *for<sup>S2</sup>* flies. No significant difference in speed was observed between *for<sup>S</sup>* and *for<sup>S2</sup>* flies. Examination of walking speed in hyperthermic conditions revealed that speed was largely increased compared control conditions. Also under high temperature conditions, *for<sup>S</sup>* flies had a higher walking speed than the *for<sup>S2</sup>* flies, while *for<sup>R</sup>* speed was faster than both *for<sup>S</sup>* and *for<sup>S2</sup>* flies. Values are means  $\pm$  SEMs. Bars containing different letters represent significantly different values. N=112 animals per treatment group.



**Figure 3.3** – Locomotor failure times from two independent assays using *for<sup>R</sup>*, *for<sup>S</sup>*, and *for<sup>S2</sup>* flies.

a) Groups of 10 flies were placed into the apparatus at 39°C, 41°C, and 43°C, and time to incapacitation was recorded. At 39°C *for<sup>R</sup>* flies were incapacitated significantly sooner than *for<sup>S</sup>* and *for<sup>S2</sup>*; *for<sup>S</sup>* failure time was between that of *for<sup>R</sup>* and *for<sup>S2</sup>* flies. At 41°C no significant difference was found between *for<sup>R</sup>* and *for<sup>S</sup>* flies, but *for<sup>S2</sup>* significantly differed from both. At 43°C, no significant differences were found between all three genotypes. b) Individual flies were placed into the apparatus at 39°C, and time to incapacitation was recorded. Failure trends resembled those in (a). Values represent means  $\pm$  SEMs. Bars containing different letters represent significantly different values. N= is signified by numbers in parentheses within each data group.

and typically continued to do so until they became incapacitated. Incapacitation was determined visually. Flies with the *for<sup>R</sup>* allele were the first to exhibit incapacitation (Figure 3.3b; *for<sup>R</sup>* vs. *for<sup>S2</sup>*: N=14, p<0.001, q=11.0; *for<sup>S</sup>* vs. *for<sup>S2</sup>*: N=14, p<0.001, q=5.7; *for<sup>R</sup>* vs. *for<sup>S</sup>*: N=14, p<0.001, q=5.4)

## Discussion

Natural variations at the *foraging* (*for*) locus of *Drosophila melanogaster* provide for different levels of cGMP-dependent protein kinase G (PKG) activity as well as variation of feeding behavior observed throughout development (Osborne et al., 1997; Pereira & Sokolowski, 1993; Sokolowski, 1985a, 1985b). In early life, *for<sup>R</sup>* larvae travel further vertically into food than *for<sup>S</sup>* larvae (Sokolowski, 1982). Fermenting fruits can reach temperatures of 50°C and variation at the *for* locus influences thermoprotection of larval feeding behavior (Dawson-Scully et al., 2007; Feder & Krebs, 1997). This suggests a trade-off in feeding strategies for larval flies. Likewise, adult *for<sup>R</sup>* will walk away from food in order to find multiple sources while *for<sup>S</sup>* remain close to the meal that they've found (Pereira & Sokolowski, 1993).

### *Differential proportions active between rover and sitter flies at 24°C*

Traditionally, *for* variants of *Drosophila* display different behaviors in the presence of food (Pereira & Sokolowski, 1993; Reaume & Sokolowski, 2006). For instance, *for<sup>R</sup>* will pupate away from their food, while *for<sup>S</sup>* pupate directly onto the fruit they use as a food source (Reaume & Sokolowski, 2006). Our control temperatures (24°C) 'time to incapacitation' data, analyzed via activity, is consistent with the differences seen in *for<sup>S</sup>* and *for<sup>R</sup>* locomotion in the presence of food (Pereira & Sokolowski, 1993).

From the start of the test, the proportion of *for<sup>s</sup>* and *for<sup>s2</sup>* flies that were active decreased at a faster rate than *for<sup>R</sup>* flies (Figure 3.1a). In addition, at the end of the 12 minute measuring period, approximately 20% of *for<sup>s</sup>* and *for<sup>s2</sup>* flies were inactive while nearly all of the *for<sup>R</sup>* population remained active. This suggests that *rover/sitter* locomotion modulation can be detected in multiple contexts. Since ‘incapacitation’ in this assay was defined as inactivity for 60 seconds, the accelerated failure of *for<sup>s</sup>* and *for<sup>s2</sup>* flies was due to their natural ‘sitting’ behavior being counted as inactivity.

#### *Speed differences between rovers and sitters under control (24°C) and elevated temperatures (39°C)*

Flies that have low levels of PKG activity (*for<sup>s</sup>* and *for<sup>s2</sup>*) were found to have a lower walking speed at control temperature (24°C) when compared to the high PKG strain flies (*for<sup>R</sup>*; Figure 3.2). These findings resemble activity trends of Figure 3.1a, and provide further support that variation in *for* locomotion behavior is not absolutely dependent on the presence of food. That *for<sup>R</sup>* flies walk faster than *for<sup>s</sup>* and *for<sup>s2</sup>* flies reveals a novel assay for behaviorally indentifying variants at the *for* locus.

Under elevated temperatures (39°C), walking speed increases in *for<sup>R</sup>* and *for<sup>s</sup>* flies, but not in *for<sup>s2</sup>* flies. Walking speed is highest in *for<sup>R</sup>* flies compared to both *for<sup>s</sup>* and *for<sup>s2</sup>* flies (Figure 3.2). Thus, elevated PKG activity is again correlated with higher walking speed at higher temperatures. Furthermore, the increase in walking speed caused by raising the temperature occurs in *for<sup>R</sup>* and *for<sup>s</sup>* flies. In contrast, the *for<sup>s2</sup>* flies do not seem to increase walking speed with increasing temperature, revealing a novel phenotype for these flies.

### *Differential proportions of active flies at 39°C in $for^R$ , $for^S$ , and $for^{S2}$ flies*

Inconsistent conclusions regarding  $for^R$ ,  $for^S$ , and  $for^{S2}$  and thermotolerance exist in previous works between larva and adult *Drosophila* (Dawson-Scully et al., 2007; Gioia & Zars, 2009). Tests of larvae with the *for* variants  $for^R$ ,  $for^S$ , and  $for^{S2}$  showed the fastest failure rates for  $for^R$  animals (Dawson-Scully et al., 2007). Thus, low PKG activity is correlated with elevated thermotolerance in the larval animal. However, it was previously shown that adult flies carrying the  $for^R$ ,  $for^S$ , and  $for^{S2}$  alleles have no significant difference in failure time when placed into high temperature conditions (39°C and higher)(Gioia & Zars, 2009). Interestingly, a small divergence of the  $for^R$  population from flies with  $for^S$  or  $for^{S2}$  alleles in the Gioia and Zars results (2009) showed that there might be an emerging difference between flies of the three genotypes with prolonged high temperature exposure. To further investigate this divergence, the exposure time test was extended by one third from 9 minutes to 12 minutes total.

Statistical analyses revealed that the proportion of active flies in the three populations diverge over time. First differences appear in the three groups at the 7 minute mark, and at 12 minutes approximately 25% of the  $for^R$  populations have become incapacitated while 95% of the  $for^{S2}$  flies remain active. Thus,  $for^R$  flies are more susceptible to high temperature incapacitation than  $for^S$  and  $for^{S2}$  flies at 39°C, as measured in the heat box.

### *Independent incapacitation assays*

Two independent visual-based incapacitation tests were used to measure failure times of  $for^R$ ,  $for^S$ , and  $for^{S2}$  flies at 39°C. In the first assay (measuring group failure), fly failure time was fastest in  $for^R$  flies, consistent with failure times as measured in the heat box. The second assay (testing individual fly failure time) found similar results as those gained from both the heat box and

population failure assay. Variation at the *for* locus has previously been described as not altering incapacitation times when tested at 41°C and 43°C (Gioia & Zars, 2009). Tests with the individual assay also failed to find a difference in incapacitation times between *for<sup>R</sup>* and *for<sup>S</sup>* at 41°C and across all three genotypes at 43°C (Figure 3.3a). Thus, lower PKG activity in *for<sup>S</sup>* and *for<sup>S2</sup>* flies is correlated with increased thermotolerance in adult flies in a narrow temperature range.

Interestingly, adult high temperature walking speed in *for* natural variants does not resemble behavioral incapacitation rates in these same flies and larvae (this paper and Dawson-Scully et al., 2007). Instead, an inverse relationship is observed between thermotolerance and walking speed. That is, *for<sup>R</sup>* flies are incapacitated most quickly but have the highest average walking speed at low and high temperatures. This suggests that *for<sup>R</sup>* flies depend more on locomotion to avoid hyperthermic stresses. The higher walking speed in *for<sup>R</sup>* flies might also influence feeding strategies. If a fruit becomes warm, the *for<sup>R</sup>* fly will perhaps more quickly move off of the food and travel to another source while *for<sup>S</sup>* flies might spend more time on the warm food and have physiological mechanisms to avoid heat stress. Nevertheless, the data presented here clearly demonstrate that variation at the *foraging* locus influences behavioral thermoprotection of adult flies in a limited temperature range. It would be of interest to determine why phenotypic differences due to the natural allele *for* occur in such a narrow range of temperature. One possible explanation is the environment in which these flies were initially collected. Previous work has shown that *Drosophila* collected from different latitudes exhibit differences in their thermotolerance (Garbuz et al., 2003). For instance, flies collected close to the equator exhibit increased thermotolerance compared to flies collected further North (Garbuz et al., 2003). The animals used in this study were found in Toronto, Ontario, Canada, where, on average, 37.9 days/year (between 1980-1996) temperatures are between 30-39.9°C (Smoyer-Tomic & Rainham, 2001). This is in contrast to the only 2.2 days/year where temperatures are between

40-45°C (Smoyer-Tomic & Rainham, 2001). There is the possibility that these populations used in this study are adapted to demonstrate such phenotypic differences at ~39°C. Future work on other populations adapted to either higher or lower temperatures would help resolve this interesting question.

## CHAPTER 4: IDENTIFYING SUBSETS OF SEROTONERGIC NEURONS BY GENERATING TRANSCRIPTION ACTIVATOR AND REPRESSOR ELEMENTS

This chapter contributes to a manuscript that is under revision as: Sitaraman, D., E.F. Kramer, D. Ostrowski, and T. Zars. *Current Biology*.

### Introduction

Serotonin acts as an important neuromodulator in many different organisms. It has been shown to influence various behaviors including modulation of gill withdrawal reflex in *Aplysia*, place learning in *Drosophila*, and impulsive behavior and aggression in humans (Kandel, 2001; Montoya et al., 2012; Sitaraman et al., 2008; van Honk et al., 2010). Although there may only be relatively small numbers of serotonergic neurons present in a system, innervation patterns can be quite broad, projecting to and affecting most parts of the nervous system as demonstrated in *Aplysia* and *Drosophila* (Marois & Carew, 1997; Valles & White, 1988). Current studies manipulate either the amount of serotonin or the ability of receptors to bind serotonin throughout the whole brain (Birzniece et al., 2001; Schweighofer et al., 2008). New methods would help to identify the role of individual serotonergic neurons or subsystems of neurons in behavior.

The serotonergic system of the *Drosophila* is relatively simple, with ~40 serotonergic neurons per hemisphere and discrete patterns of innervation in the neuropil (Sitaraman et al., 2008). If individual neurons or subsets of neurons can be identified, it may be possible to link separate neuronal subsets with independent behaviors. Identification is currently limited by the lack of effective methods to manipulate individual serotonergic neurons. The goal of the research

described in this chapter is to develop tools to manipulate subsystems of serotonergic neurons using the GAL4/UAS system.

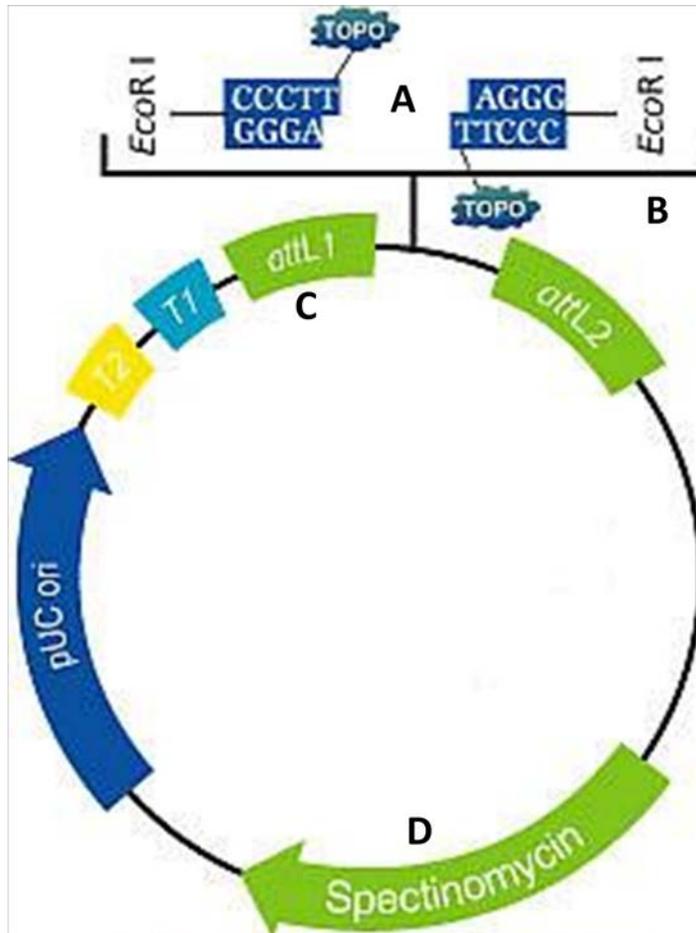
## **Methods**

### *PCR amplification*

Accuracy in amplification of potential regulatory domains was of paramount importance as even a single base-pair mutation can greatly affect transcription (Rosenberg & Court, 1979). Thus, PCR amplification was performed using *Pfu* for high-fidelity amplification (Lundberg et al., 1991). The PCR reaction was then amplified with *Taq* to add single 3'-A overhangs for ease of insertion into the pCR<sup>®</sup>8/GW/TOPO<sup>®</sup> donor vector (Invitrogen, 2012). PCR products were run on a 1% agarose gel to confirm size. Products of the correct size were then sent to the University of Missouri DNA Core Facility for sequencing. PCR products with the correct sequence were inserted into the pCR<sup>®</sup>8/GW/TOPO<sup>®</sup> vector.

### *TOPO-mediated insertion into the pCR<sup>®</sup>8/GW/TOPO<sup>®</sup> vector*

The PCR products generated were inserted into the pCR<sup>®</sup>8/GW/TOPO<sup>®</sup> vector (Invitrogen, 2012). The pCR<sup>®</sup>8/GW/TOPO<sup>®</sup> vector has four features that make it an ideal donor vector (Figure 4.1). First, the vector is linearized and has single 3'-T overhangs covalently bound to topoisomerase I. These overhangs allow efficient ligation of the *Pfu/Taq*-amplified PCR product to the vector. Second, *EcoRI* restriction sites are located on either side of the PCR product insertion site which allows for simple confirmation of sequence accuracy in transformed colonies. Third, the vector contains *attL1* and *attL2* sites for gene transfer to Gateway<sup>®</sup>



**Figure 4.1** – The pCR<sup>8</sup>/GW/TOPO<sup>®</sup> donor vector.

A) Topoisomerase I bound to 3'-T overhangs for simple ligation of *Pfu*/*Taq*-amplified PCR product into vector. B) *EcoRI* sites allow for quick confirmation of sequence size once inserted into the vector. C) LR-mediated exchange at *attL1* and *attL2* sites ensure accurate orientation and sequence when inserted into the destination vector. D) Spectinomycin resistance enables antibiotic-resistant selection of transformed colonies (Invitrogen, 2012).

destination vectors through recombination. Finally, the vector contains a spectinomycin-resistance marker, which allows for selection of successfully transformed *E.coli* using antibiotic growth medium.

Freshly amplified PCR product was mixed and incubated with the pCR<sup>®</sup>8/GW/TOPO<sup>®</sup> vector at room temperature for 5 minutes, then immediately transferred to ice for transformation of chemically competent *E. coli*. Successfully transformed cells were selected by plating the transformation reaction onto LB agar plates containing spectinomycin. Five colonies from each plate were selected for sequence confirmation. DNA was extracted using the boiling-lysis method (Sambrook & Russell, 2001). Restriction analysis was performed to confirm the size of the insert. Correct size inserts were sent to the University of Missouri DNA Core for analysis to determine the orientation of the insert as well as confirm the accuracy of the sequence. GW1 and GW2 primers, designed for use with the pCR<sup>®</sup>8/GW/TOPO<sup>®</sup> vector, were used to confirm accuracy and orientation (Invitrogen, 2012). Clones containing inserts with the correct sequence in the correct orientation were inserted into the Gateway<sup>®</sup>-compatible destination vector pBPGUw, Addgene plasmid 17575 (Pfeiffer et al., 2008)

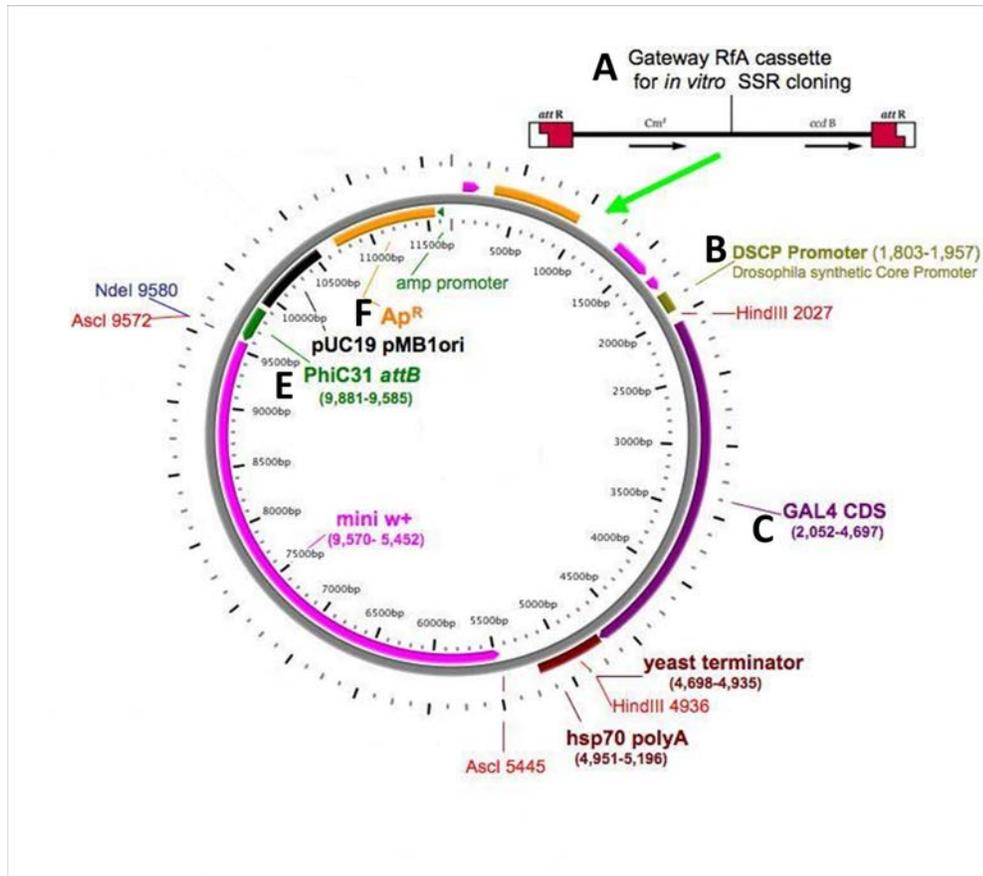
#### *Germline transformation vectors*

Three germline transformation vectors were used. The germline transformation vector pBPGUw (Figure 4.2) has many attributes which make it ideal for this work (Pfeiffer et al., 2008). First, subcloning potential enhancer elements into this plasmid from a donor vector uses the Gateway<sup>®</sup> cloning strategy based on bacteriophage  $\lambda$  site-specific recombination (Invitrogen, 2000). This is considerably faster than previous methods of finding or creating restriction enzyme sites unique to each potential enhancer for subsequent testing in a transformation vector. Second, this vector contains a *Drosophila* synthetic core promoter (DSCP) which drives

expression in near-identical patterns to an endogenous promoter and is an effective replacement for many different enhancers (Pfeiffer et al., 2008). Third, this vector contains the GAL4 positive transcriptional element which will drive an effector or a reporter when coupled with an appropriate Upstream Activation Sequence (UAS). Fourth, site-specific integration into transcriptionally silent regions of the genome has been engineered into this vector in the form of the  $\Phi C31$  *attB* sequence (Groth et al., 2004). This ensures that when reporter gene expression is detected, it is actually caused by the enhancer being investigated and not by hijacking of native transcription. Fifth, this vector contains ampicillin resistance, allowing for easy selection of transformed colonies by plating on LB-agar plates containing the antibiotic. Finally, the vector contains a copy of mini  $w^+$  which enables efficient selection by eye color of transformed *Drosophila*. A second GAL4 vector, pBPGw (Addgene plasmid 17574), which is identical to pBPGUw but does not contain the DSCP, was used for subcloning those regulatory regions of interest that contained endogenous promoters. A GAL80 vector, pBPGAL80Uw-6 (Addgene plasmid 26236), which uses the backbone of pBPGUw but contains the GAL80 repressor element rather than GAL4, was used to generate two repressor elements.

#### *LR-mediated exchange*

*Escherichia coli* bacteriophage  $\lambda$ -based site-specific recombination is a feature of Gateway<sup>®</sup> transformation vectors. The LR recombination reaction takes place between *attL* sites in the entry clone, pCR<sup>®</sup>8/GW/TOPO<sup>®</sup>, and *attR* sites in the destination vector, pBPGUw. Site-specific recombination is based on bacteriophage integration into a host genome during lysogeny and excision of the integrated prophage after phage induction (Fu et al., 2008; Landy, 1989). LR Clonase enzyme mix, available from Invitrogen, contains the bacterial recombination proteins Integrase (Int), Integration Host Factor (IHF), and Excisionase (Xis), which are essential for



**Figure 4.2** – Transformation vector pBPGUw

A) Gateway® cassette for site-specific recombinant cloning of regions of interest. Cassette is exchanged with PCR product between *attL* sites in donor vector. B) *Drosophila* synthetic core promoter drives expression of potential regulatory regions, thus insertion into the genome does not need to occur near an endogenous promoter. C) GAL4 positive transcriptional element allows determination of location and/or function of a region of interest when crossed to a UAS-coupled reporter or effector. D)  $\Phi$ C31 *attB* enables site-specific insertion into a transcriptionally silent region of the *Drosophila* genome. E) Ap<sup>R</sup> allows for ampicillin-resistant selection of transformed colonies. F) Mini *w*<sup>+</sup> allows for selection of transformed *Drosophila* based on eye-color – successful transformants in a *white* background will have yellow eyes (Pfeiffer et al., 2008).

mediating insertion and excision of DNA sequences (Invitrogen, 2000; Landy, 1989). This site-specific recombination preserves both the open reading frame and the orientation of the insert.

To carry out the LR recombination reaction, 4  $\mu$ l LR Clonase enzyme mix was added to a solution containing 4  $\mu$ l LR reaction buffer and  $\sim$ 300 ng of both the donor and the destination vector.

The reaction was incubated at 25°C for 60 min, then inactivated by incubation with 2  $\mu$ l of Proteinase K at 37°C for 10 min. The product of LR reaction was immediately used to transform DH5 $\alpha$  competent cells.

A single  $\mu$ l of the LR reaction was added to 25  $\mu$ l DH5 $\alpha$  cells and incubated on ice for 30 min. The cells were heat-shocked at 42°C for 30 sec. Next, 250  $\mu$ l S.O.C. medium was added to the cells and the whole tube was incubated at 37°C for 1 hr. The culture was plated on LB-agar plates containing 100  $\mu$ g/ml ampicillin. Successfully transformed bacterial colonies were isolated into 3 ml tubes of LB-ampicillin broth and incubated at 37°C shaking overnight. One hundred  $\mu$ l of this broth was then used to inoculate 50 ml flasks of LB-ampicillin broth which again incubated at 37°C shaking overnight. DNA was extracted using a QIAGEN Plasmid Midi Kit (QIAGEN) to maximize yield and purification for injection.

### *Injection*

Between 30 and 50  $\mu$ g of QIAGEN-purified DNA was sent to Genetic Services, Inc (Sudbury, MA) for injection. Injected embryos were raised to adulthood until they could be crossed with  $w^{1118}$  flies. Offspring containing the desired insert were selected by the yellow eye phenotype resulting from the mini  $w^+$  contained in the transformation vectors (Pfeiffer et al., 2008).

### *The GAL4 /UAS system*

Transcriptional activation elements were generated using the yeast GAL4 system which has been modified for use in *Drosophila* (Brand & Perrimon, 1993; Lee & Luo, 1999). GAL4 is a positive transcriptional element under the control of a driver gene, either from regulatory elements near a random insertion point or from a basal promoter cloned upstream in a GAL4-containing vector like pBPGUw. By itself GAL4 is innocuous, but when crossed to a UAS-containing fly line, the GAL4/UAS interaction drives expression of the UAS-coupled reporter in the GAL4-positive cells (Brand & Perrimon, 1993). GAL4 elements have made possible the study of neural systems thought to be important for many different fly behaviors (McGuire et al., 2005).

### *GAL80*

GAL80 is a GAL4 repressor and can be used to block GAL4-based transcription. The GAL80 protein binds to the activation domain of GAL4, blocking transcription and repressing reporter gene expression (Lee & Luo, 1999). Combinations of overlapping GAL4 and GAL80 elements allow reporter gene expression in neurons that are GAL4-positive only.

### *Immunohistochemistry*

Fly brains were dissected in Ringer's solution (130 mM NaCl, 0.7 mM  $\text{KH}_2\text{PO}_4$ , 0.35 mM  $\text{Na}_2\text{HPO}_4$ , 18 mM  $\text{MgCl}_2$  and 4.7 mM KCl) by removing the proboscis and the eyes with forceps. The brains were then fixed in 2% or 4% formaldehyde for 8-10 hours at 4°C. Fixed brains were blocked overnight at room temperature in normal goat serum (3% in PBST, composition outlined below) followed by two days incubation with a primary antibody (e.g., anti-5HT 1:25 and anti-GFP 1:100) at 4°C. The brains were then incubated with a secondary antibody (e.g., Alexa 546 goat

anti-mouse 1:500 and Alexa 488 goat anti-rabbit 1:500) for 4 hours at room temperature or overnight at 4°C. All antibody incubations were followed by three 15 minutes washes with PBST (100 ml 1XPBS and 0.5 ml Triton X-100). All antibody solutions and dilutions were made in either PBST or 3% normal goat serum in PBST. The whole brain was mounted in 20% glycerol/80% PBST in a narrow well made from coverslip slides. The whole mount brains were visualized using LSM 510 NLO confocal microscope with a 20X objective. Images were visualized using the LSM examiner software available on the Zeiss website (<http://www.zeiss.de>).

## Results

Potential genes of interest were identified by their importance in the synthesis or function of serotonin. Tryptophan/phenylalanine hydroxylase (T<sub>PH</sub>), tryptophan hydroxylase (T<sub>H</sub>), and the serotonin transporter (SerT) were selected as biochemical activity and experimental evidence suggest that they are expressed in serotonergic neurons (Coleman & Neckameyer, 2005; Demchyshyn et al., 1994; Neckameyer et al., 2007). Regions of interest in these three genes were selected from the 5' and intronic regions based on two criteria. First, only regions larger than 300 bp were selected to search for potential regulatory regions, limiting the number of potential regions to a manageable number (Pfeiffer et al., 2008). Second, genomic comparisons were performed between *Drosophila melanogaster*, *Drosophila pseudoobscura*, and *Drosophila simulans*. Regions with significant homology were selected as likely having regulatory functions, as the sequence was conserved over time. These two criteria led to identification of a small set of potential regulatory regions from these three genes (Table 4.1).

Potential serotonergic regulatory regions were cloned and transgenic animals were generated. Using primers specific to each potential regulatory region, all four candidate regions were amplified with *Pfu* polymerase, cloned into the pCR<sup>®</sup>8/GW/TOPO<sup>®</sup> vector, confirmed by

sequencing, and further subcloned into the germline transformation vectors. Transgenic lines were established and stable lines were generated with standard *Drosophila* genetic approaches (not shown). Each potential serotonergic regulatory region was examined for expression in serotonin neurons with double labeling of GFP and serotonin and confocal microscopy. The results for each region will be described in turn.

**Table 4.1 – Potential regulatory regions from serotonin-enhancer genes of interest**

Gene	Target Region	Conserved Regions	Size (bp)	Primer sequences	Restriction Enzyme
TrH	5' (w/ promoter)*	6	1433	<i>f</i> - CTGCACTTGGTAGCTACTCGTTTT <i>r</i> - GTACGTTCTTAAAGCAGACACATG	<i>PvuII</i>
TpH	5' (w/ promoter)*	2	1393	<i>f</i> - ATTCTCCGCTGAAGACAAATC <i>r</i> - AAGATTCTCGGGTTTCTCCA	<i>KpnI</i>
SerT	5' (w/o promoter)	1	1016	<i>f</i> - GATTTGTCGTCGGCAGGC <i>r</i> - AAATATCGCGCCACCTATT	<i>SalI</i>
	6 <sup>th</sup> intron	4	536	<i>f</i> - GCTTTATTAAATTCCAATCCCA <i>r</i> - TTCGGTTAATTAACCTAAGCA	<i>NheI/ClaI</i>

Four potential regulatory domains were PCR amplified with the primer sequences listed. PCR products were cloned first into the pCR<sup>®</sup>8/GW/TOPO<sup>®</sup> donor vector (Invitrogen) and then into the pBPGUw or pBPGw (indicated by \*) transformation vector. Restriction enzymes were used to confirm orientation in the pCR<sup>®</sup>8/GW/TOPO<sup>®</sup> donor vector.

TrH is thought to be the primary enzyme responsible for hydroxylating tryptophan in the fly brain *in vivo* (Coleman & Neckameyer, 2005; Neckameyer et al., 2007; Neckameyer & White, 1992). The expression pattern of the potential regulatory region from the 5' end of the *TrH* gene was determined (Table 4.2). Our name for this region is TrH5p-GAL4. The 'p' represents the fact that the endogenous promoter from the *TrH* gene was used. From double labeling experiments, the so-called SP1 and SP2 clusters, components of what we term the larger PMP cluster, were labeled. Furthermore, the AMP neurons, also identified by the Scholz group as CSD neurons, showed strong expression (Giang et al., 2011). GAL4-driven GFP expression was also detected in all three SE clusters and the LP2 cluster.

From the *TpH* gene, TpH5p-GAL4 showed expression in all eight clusters of serotonergic neurons, though not in all neurons in each cluster (Table 4.2). All SE1 and SE3 neurons as well as the AMP neurons were labeled. All neurons in the ALP and LP2 clusters also expressed GFP. Finally, expression was seen in the posterior of the brain in both the PLP cluster and the SP1 and SP2 clusters of the PMP. Although it has been claimed TpH has only peripheral function, TpH5p-GAL4 showed a broad expression pattern similar to that of TrH5p-GAL4 (Coleman & Neckameyer, 2005; Neckameyer et al., 2007).

The S5np-GAL4 driver created using the *SerT* gene drove GFP expression in multiple serotonergic neurons. These include the PMP neurons (SP1, SP2, IP, and LP3 neuron clusters) (Giang et al., 2011). The SE clusters 1-3, LP2, and AMP neurons showed GFP expression as well (Table 4.2). It has been previously suggested that the presence of the serotonin transporter is a better indicator for serotonergic neurons than the presence of enzymes active in serotonin synthesis (Nielsen et al., 2006). In our case, expression patterns of S5np-GAL4 are as broad as expression

**Table 4.2 – Neuronal expression patterns from GAL4 drivers created with serotonin enhancers.**

GAL4	Serotonergic clusters
TrH5p <sup>1</sup>	SE1, SE2, SE3, AMP, LP2, PMP
TpH5p <sup>1</sup>	SE1, SE3, ALP, AMP, LP2, PLP, PMP
S5np <sup>1</sup>	SE1, SE2, SE3, ALP, AMP, LP2, PLP, PMP
Si6 <sup>1</sup>	SE2, SE3, PMP
SerT 38749 <sup>2</sup>	SE1, SE2, SE3, AMP
SerT 38764 <sup>2</sup>	SE1, SE2, LP2, PMP
TrH 46910 <sup>2</sup>	SE2, SE3, LP2
TrH 247 <sup>3</sup>	SE3, LP2, PMP
TrH 493 <sup>3</sup>	SE3, LP2, PMP
TrH 819 <sup>3</sup>	SE1, SE2, SE3, AMP, LP2, PMP

<sup>1</sup>Fly lines are first described here. <sup>2</sup>Fly lines from Pfeiffer et al. were received from Bloomington *Drosophila* Stock Center and are identified by stock number (Pfeiffer et al., 2011). <sup>3</sup>Fly lines received from Jay Hirsh are identified by the remaining number of 5' flanking bases (Lee et al., 2011). SE1-3 – sub-esophageal; ALP – anterior lateral protocerebrum; AMP – anterior medial protocerebrum; LP2 – lateral protocerebrum; PLP – posterior lateral protocerebrum; PMP – posterior medial protocerebrum.

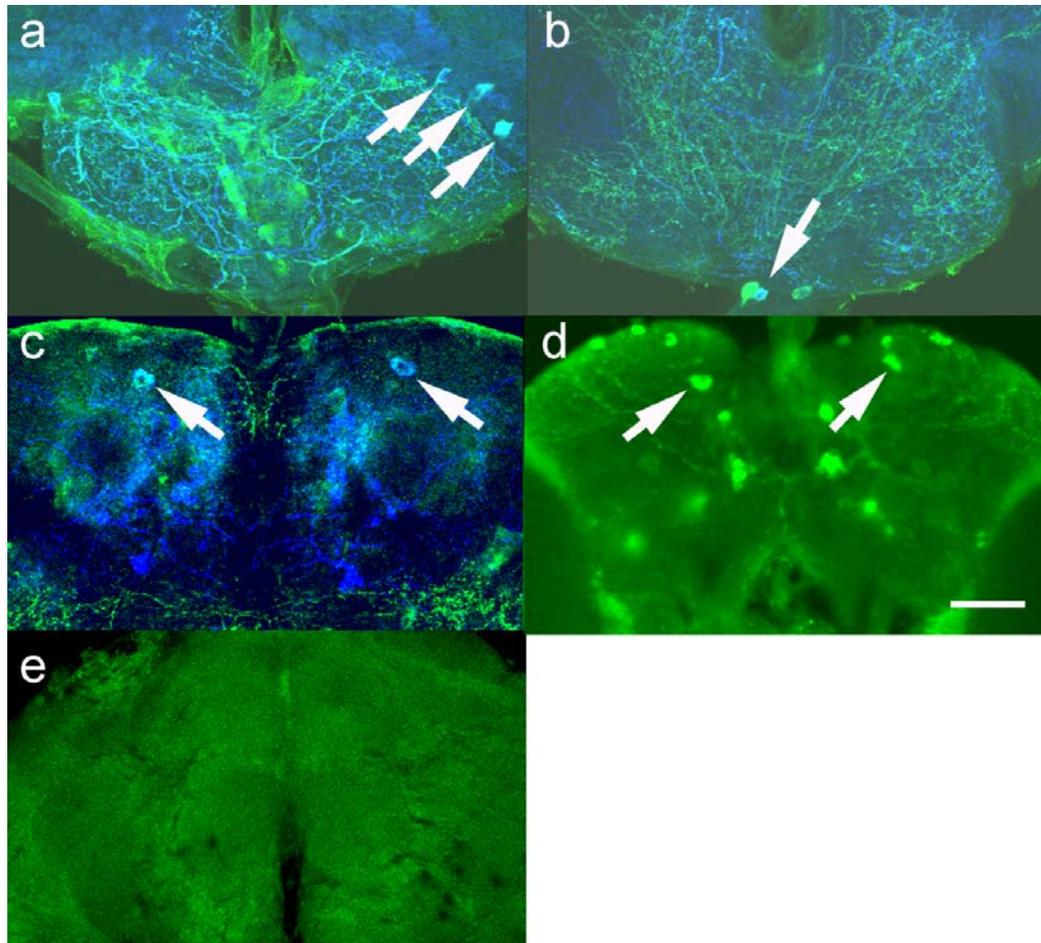
patterns generated with TrH5p-GAL4 and TpH5p-GAL4. S5np-GAL4 shares almost identical expression patterns with the other broad serotonergic drivers.

We also examined an enhancer cloned from the sixth intron of the *SerT* gene. The expression pattern of the Si6-GAL4 driver was a focus of attention because of the effects of altering these neurons on place learning behavior (Chapter 5). From double labeling experiments, Si6-GAL4 drives expression in five serotonergic neurons. These include neurons in the SE2 and SE3 clusters and one neuron in the PMP cluster, a dorsal SP1 neuron of (Giang et al., 2011) (Table 4.2, Figure 4.3). The Si6-GAL4 serotonergic neuron innervation pattern includes the sub-oesophageal ganglion, the median bundle, and discrete parts of the superior medial protocerebrum (Figure 4.3a-c). When Si6-GAL4 was crossed to Si6-GAL80, all GFP expression in Si6-GAL4-positive neurons was blocked (Figure 4.3e).

It is important to note that in the case of every GAL4 driver generated, expression was not limited to serotonergic neurons. There was also some expression in other neurons of the brain. Additionally, while GFP was detected in serotonergic clusters, it was rarely detected in all neurons in a cluster. This indicates that genetic serotonergic subsets consist of neurons distributed across clusters rather than restricted to a single cluster in or innervating any one particular neuropil. All lines described in Table 4.2 were used for behavioral analysis.

## **Discussion**

Four GAL4 drivers and one GAL80 driver were generated with predicted enhancers. No predicted enhancer drove expression in all serotonergic neurons but all drivers generated and tested showed at least some expression in the serotonergic system. GFP expression was also observed in non-serotonergic neurons in all enhancer lines examined. Previous similar studies have shown GFP expression in cells that do not produce the driver gene endogenously and given



**Figure 4.3** – Expression pattern of the serotonin driver Si6-GAL4 and repressor Si6-GAL80.

Brains were co-labeled with anti-GFP (green) and anti-serotonin (blue). a) In an anterior ventral region, three serotonergic neurons are co-labeled (white arrows). A few other small GFP-positive but serotonin negative neurons can also be seen in this region. These SE neurons appear to densely innervate the sub-esophageal ganglion here. b) Multiple GFP neurons are again labeled in this ventral but less anterior section, only one unpaired neuron appears to be co-labeled with anti-serotonin (arrow). This serotonergic neuron appears to also innervate the sub-esophageal ganglion. c & d) In a dorsal posterior section, one pair of large PMP neurons is co-labeled with GFP and anti-serotonin (arrows, c) or with anti-serotonin only (arrows, d). It is not clear what these neurons innervate. e) Addition of a Si6-GAL80 element to the Si6-GAL4 driver suppresses UAS-GFP expression. This is an anterior frontal optical section. Scale bar represents 20  $\mu\text{m}$  in a-c, and 50  $\mu\text{m}$  in d & e.

the many interactions between transcriptional activators, enhancers, and repressors, it is not surprising to see expression from a gene fragment in cells that at first glance appear to be unrelated to the gene of interest (Pfeiffer et al., 2008). The spurious expression probably reflects disentangling of enhancer and repressor elements from the cloned genomic fragments.

The broad expression pattern of TpH5p-GAL4 is interesting as, although *in vitro* studies have demonstrated that both TrH and TpH can hydroxylate tryptophan, it has previously been postulated that TpH functions primarily in the peripheral nervous system of *Drosophila* (Coleman & Neckameyer, 2005; Neckameyer et al., 2007). However, our work demonstrates that TpH5p-GAL4 drives expression more broadly throughout the central brain than does TrH-GAL4. In both drivers, the number of neurons expressing GFP is so large that closely linking any neuron or cluster with a behavior may prove challenging without an adequate GAL80 repressor element to limit expression.

A previous co-labeling experiment prepared with antibodies to *SerT* and 5-HT showed that all somata positive for one antibody were also positive for the other antibody (Giang et al., 2011). Our results support this previous result as the driver line expressing in the largest number of neurons is S5np-GAL4, a fragment from the 5' end of the serotonin transporter gene. GFP was observed in at least one neuron in all serotonergic clusters but not all serotonergic neurons were labeled. This is likely because only a portion of the *SerT* 5' region was used to create this driver line; this fragment is no longer under the control of native enhancers and repressors present in the entire gene. Because of its broad expression pattern, S5np-GAL4 may also not be very effective for identifying neuronal subsystems by itself. Again, effective use of the S5np-GAL4 driver will most likely require a different but also broadly-expressing GAL80 repressor element in order to identify a subset of neurons that can be linked with behavior.

In contrast, the driver expressing in the smallest number of neurons, Si6-GAL4, is a fragment from the sixth intronic region of the serotonin transporter gene. As demonstrated in Figure 4.3e, transcription of the GFP reporter gene is blocked entirely when the Si6-GAL80 element is crossed with the Si6-GAL4 driver. This makes Si6-GAL4 and Si6-GAL80 excellent tools for behavioral studies.

The serotonergic system as a whole has been shown to be necessary for place learning, but can an individual subsystem that is necessary, sufficient, or both for learning and memory be identified (Sitaraman et al., 2008)? This question is addressed in Chapter 5 using a combination of GAL4 and GAL80 drivers with UAS-coupled effectors.

## **CHAPTER 5: SEPARATE ROLES FOR SEROTONIN SYSTEMS IN AN INNATE BEHAVIORAL RESPONSE AND OPERANT FEEDBACK IN *DROSOPHILA* LEARNING.**

This chapter contributes to a manuscript that is under revision as: Sitaraman, D., E.F. Kramer, D. Ostrowski, and T. Zars. Current Biology.

### **Introduction**

In operant learning, feedback mechanisms are critical for an animal to select a potential behavior, increasing reward or reducing punishment (Skinner, 1950; Wolf & Heisenberg, 1991). Furthermore, multiple behaviors, including learning, are altered when animals experience unpredictable aversive events (Maier & Watkins, 2005). Understanding the mechanisms that link unexpected events with learning may provide insights into the origins of operant feedback. In a specially designed operant place memory paradigm termed the heat box, individual *Drosophila* develop memories by associating a high temperature with the behavioral routines that bring the fly to a part of a long narrow chamber (Wustmann et al., 1996; Zars, 2010; Zars et al., 2000). Exposure to unpredicted high temperature enhances place learning (Kahsai & Zars, 2011; Sitaraman et al., 2007; Sitaraman & Zars, 2010). Here we use multiple methods to examine the necessity and sufficiency of the serotonergic neurons for both learning effects. We show that the serotonergic system and sub-components in *Drosophila* mediate aversive feedback. Interestingly, it is only with activation of the whole system that the memory can be enhanced with unexpected aversive temperature exposure. Thus, the serotonergic system uses a subsystem to match operant memory levels to aversive high temperature but needs the whole set to induce the memory enhancing effect of unexpected aversive experience. The results argue that a key feature of operant learning, a feedback system that impinges on a behavioral routine, is a subsystem of one that influences behavior after unexpected experiences.

## Methods

### *Behavior*

Individual flies were conditioned using long but narrow chambers termed the heat box. A single fly was allowed to roam in the chamber, and they usually walked from chamber end to chamber end (Zars et al., 2000). During training, one half of the chamber was associated with aversive temperatures with a pre-determined maximum or minimum. Flies typically avoid the chamber half associated with aversive temperature, and continue to do so even after the chamber temperature is reset to the preferred 24°C (Kahsai & Zars, 2011; Wustmann et al., 1996). Unexpected exposures were presented as three 1-min exposures to temperatures of 41°C for normal flies, or other temperatures as indicated for the TrpA1 and TrpM8 experiments. Flies were allowed a rest after the unexpected exposure of four min, and then conditioned with a mid-level temperature of 30°C. The tests for thermosensitivity provided flies with a 1-min choice between the reference temperature and 30 or 41°C (Zars, 2001). Largely equal numbers of flies from all genotypes were tested in parallel over several weeks. The number of flies from each experiment is listed in the H-statistics. While the behavioral experiments were not performed blind to genotype, data were objectively collected by use of an automated conditioning apparatus and analytic software.

### *Drosophila husbandry*

Genetic crosses followed typical methods. The GAL4 and effector lines were introgressed with a Cantonized white strain (*wCS10*) and then the X-chromosomes were replaced with a wild-type version in some lines to prevent *white*-mutant effects on learning behavior (Diegelmann et al.,

2006). Flies tested for behavior were 2-7 days old, and raised on cornmeal food in an insectary at 25°C, unless otherwise noted, and 60% humidity.

### *Statistics*

Statistical comparisons used non-parametric tests with a Kruskal-Wallis ANOVA and multiple comparisons, when P-values were less than 0.05 (Putz & Heisenberg, 2002) . All data were compared with STATISTICA software (StatSoft Inc., Tulsa, Oklahoma).

## **Results**

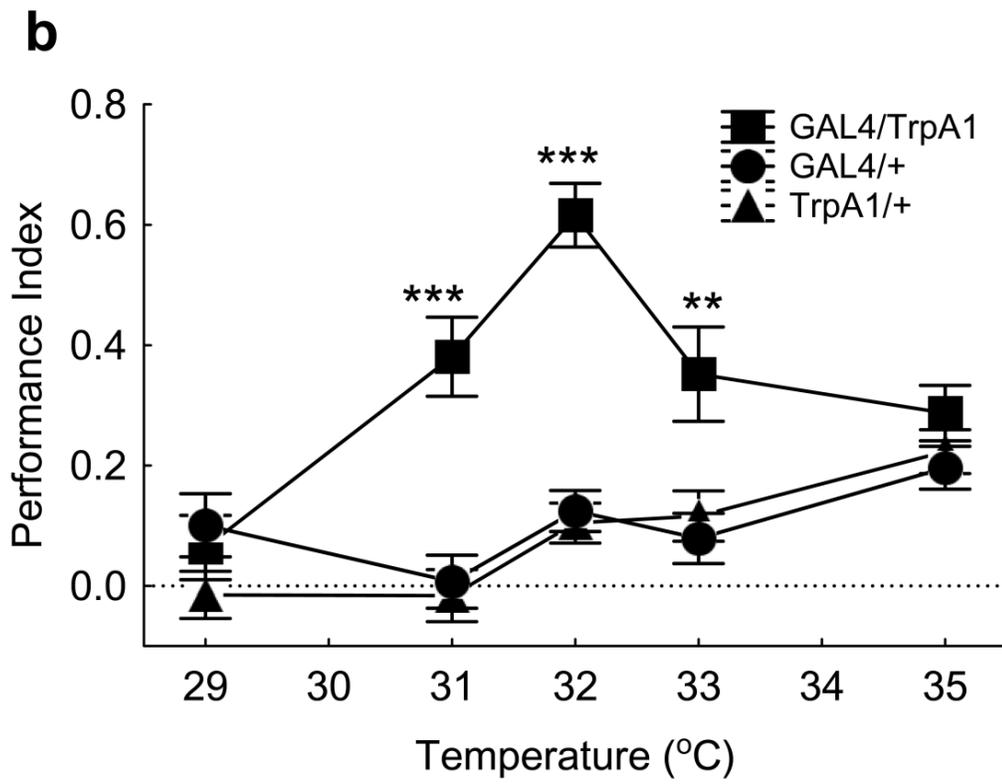
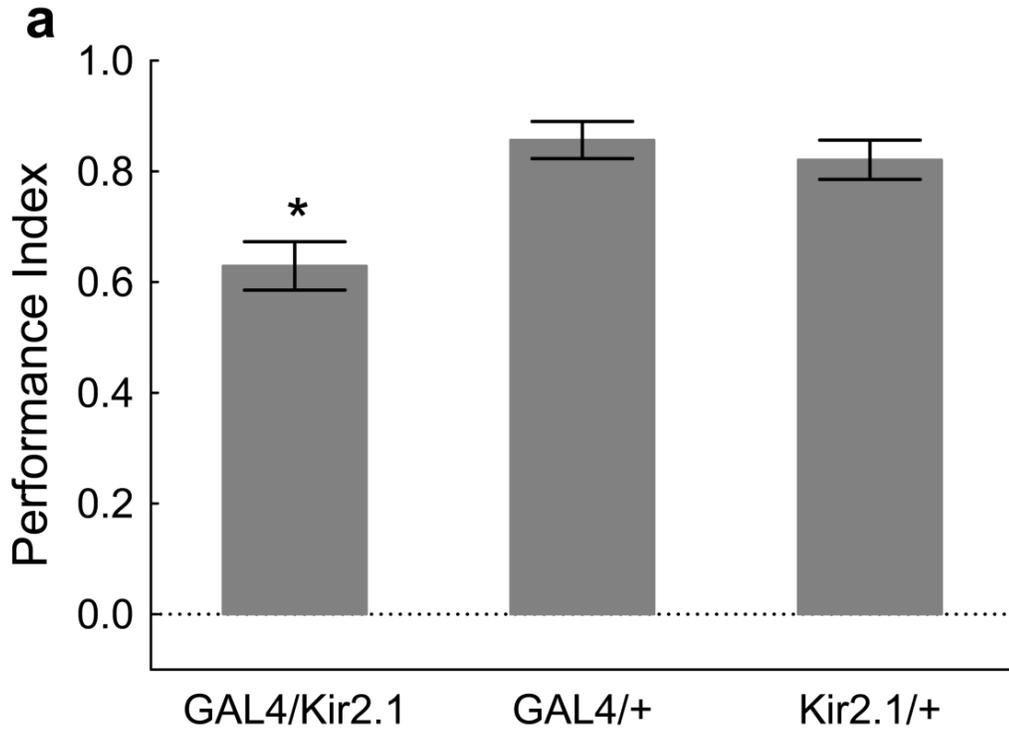
### *Serotonergic neurons are necessary and sufficient for normal operant feedback of place memory*

The serotonergic system is the only biogenic amine known to be necessary for *Drosophila* place memory (Kahsai & Zars, 2011; Sitaraman et al., 2008). In the *Drosophila* brain the serotonergic system is comprised of ~ 40 neurons per hemisphere, and these neurons broadly innervate the central brain (Lee et al., 2011; Sitaraman et al., 2008). To manipulate all or nearly all of these neurons, a TrH-GAL4 driver together with a ubiquitous GAL80<sup>ts</sup> was used to restrict expression of a *Kir2.1* channel to adult flies and reduce serotonergic neuron activity (Lee et al., 2011; McGuire et al., 2003; Paradis et al., 2001; Sitaraman et al., 2012). Flies expressing *Kir2.1* in TrH-GAL4-positive neurons have lower memory scores compared to control flies (Figure 5.1a). As a behavioral control, flies from all the genotypes were examined for the ability to sense and avoid the mid- and high-temperatures of 30 and 41°C. There were no significant differences in this behavior between these flies (Table 5.1). Reducing the excitability of serotonergic neurons reduces memory performance levels.

To address the sufficiency of the serotonergic system in providing operant feedback, extrinsic activation of serotonergic neurons was paired with a behavioral routine. The *Drosophila* thermogenetic effector TrpA1 can modify neuronal activity with high temporal precision (Pulver et al., 2009). The aversive high temperature feedback was replaced by temperatures that activate TrpA1 in serotonergic neurons. The TrH-GAL4/TrpA1 flies conditioned with 31, 32, and 33°C, temperatures that induce TrpA1 activation, had drastically higher memory levels compared to control flies (Figure 5.1b). Temperatures outside of the activation range of TrpA1 did not support memory formation (Figure 5.1b). Behavioral control experiments did not detect differences between flies of the different genotypes (Table 5.1). Activation of a large portion of the serotonergic system paired with operant behavior provides sufficient feedback for a robust place memory.

*Serotonergic neurons are necessary and sufficient for an unexpected exposure enhancement of place memory*

After unexpected high temperature exposure normal flies increase memory levels under moderate training conditions (Figure 5.2a) (Sitaraman et al., 2007; Sitaraman & Zars, 2010). Synaptic output from serotonergic neurons was blocked using both a DDC-GAL4; TH-GAL80 driver combination and the TrH-GAL4 driver with the tetanus toxin light chain (TNT) (Sweeney et al., 1995). Flies with blocked serotonergic synaptic transmission had a dampened memory level after unexpected exposure, even after extended training (Figure 5.2b). Memory performance in TrH-GAL4 / TNT flies and flies fed the drug alpha-methyl tryptophan (am-W) to reduce serotonin synthesis was also reduced (Figure 5.2c and 5.2d)(Lee et al., 2011). Flies from the tested genotypes had no significant changes in control behaviors (Table 5.1). The serotonergic system is necessary for the unexpected exposure enhancement of place memory.



**Figure 5.1** – The serotonergic neurons are necessary and sufficient for normal operant feedback in aversive place memory.

a) Silencing of serotonergic neurons labeled by TrH-GAL4 during adulthood reduces place memory performance [H(2, N= 477) =25.7,  $p < 0.0001$ ]. b) Activation of serotonergic neurons substitutes for high temperature feedback. GAL4/TrpA1 (■), GAL4 /+ (●) and TrpA1/+ (▲) flies were conditioned using 29 to 35°C temperatures. Experimental flies had statistically significantly higher place memories than control flies at three temperatures (29°C [H(2, N=274) =5.14,  $p=0.08$ ; 31°C [H(2, N=273) =27.22,  $p < 0.0001$ ; 32°C [H(2,N= 419)=70.25, $p < 0.0001$ ; 33°C [H(2, N=234) =12.84,  $p=0.0016$ ; 35°C [H(2, N=574) =2.64,  $p=0.27$ ]. Values represent mean and SEMs, marked significant  $*=p<0.05$ ,  $**=p<0.01$ , and  $***=p<0.001$  in all figures.

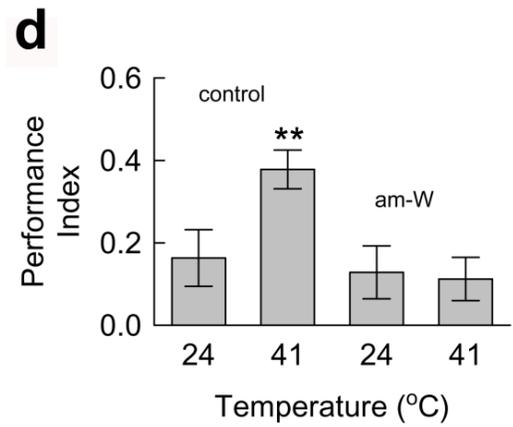
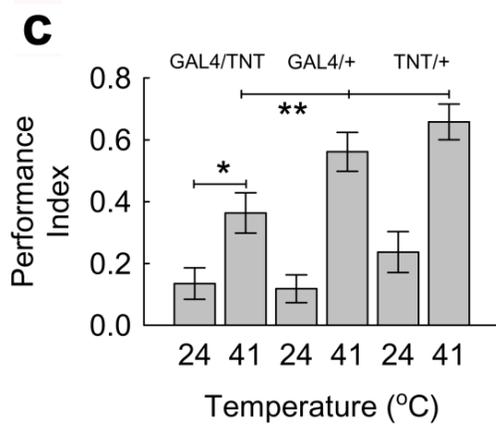
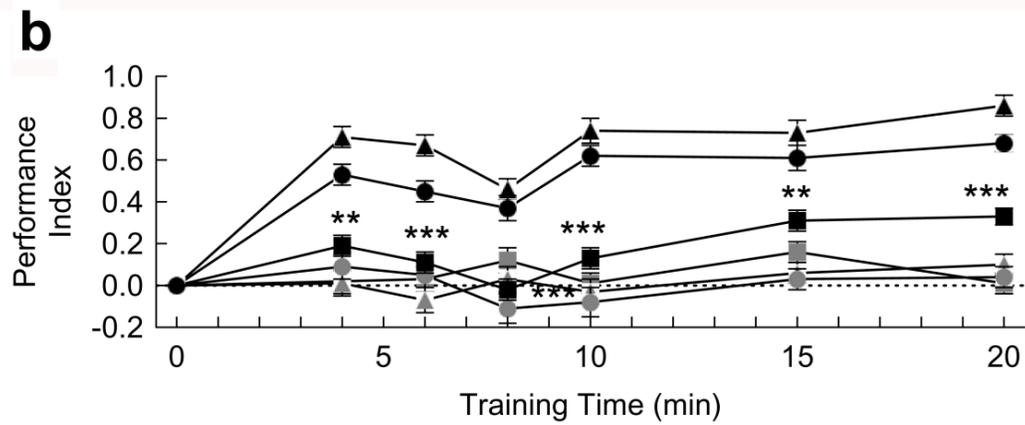
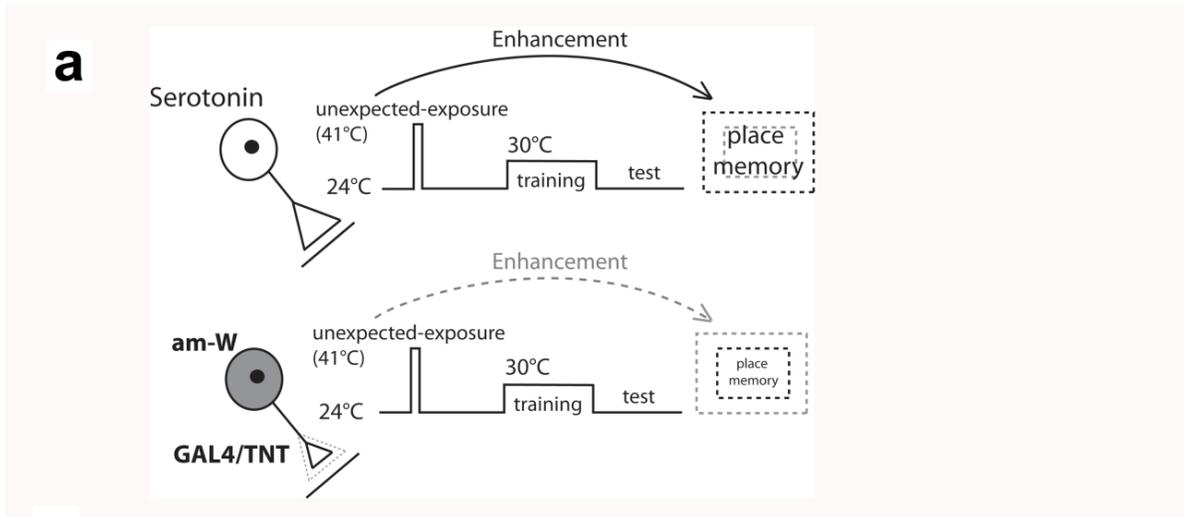
**Table 5.1 Control avoidance behavior in serotonin-altered flies.**

Genotype	N	24/30°C	24/41°C
TrH-GAL4/ <i>Kir2.1</i> *	96	0.25±0.05	0.76±0.04
TrH-GAL4/+*	90	0.19±0.05	0.71±0.05
<i>Kir2.1</i> /+*	93	0.33±0.04	0.74±0.05
TrH-GAL4/TrpA1	93	0.14±0.05	0.66±0.05
TrH-GAL4/+	93	0.20±0.05	0.72±0.03
TrpA1/+	99	0.16±0.05	0.71±0.04
DDC-GAL4, TH-GAL80/TNT	74	0.13±0.08	0.71±0.05
DDC-GAL4, TH-GAL80/+	70	0.14±0.08	0.71±0.03
TNT/+	68	0.16±0.06	0.70±0.06
TrH-GAL4/TNT	81	0.11±0.08	0.54±0.06
TrH-GAL4/+	87	0.14±0.07	0.60±0.06
TNT/+	81	0.19±0.07	0.58±0.06
TrH-GAL4/TrpM8	125	0.16±0.07	0.68±0.06
TrH-GAL4/+	129	0.20±0.06	0.73±0.05
TrpM8/+	133	0.19±0.05	0.65±0.05
Pharm Control	101	0.30±0.05	0.70±0.04
Am-W	101	0.26±0.08	0.50±0.07

\* indicates that test was done without unexpected exposure.

#### Statistics

TrH-GAL4/*Kir2.1*, 30°C, H(2, N=279) =4.6, p=0.1; 41°C =5.8, p=0.054. TrH-GAL4 / TrpA1, 30°C, H(2, N=285) =1.9, p=0.37; 41°C =0.28, p=0.87. DDC-GAL4, TH-GAL80/TNT, 30°C, H(2, N=208) =0.66, p=0.72, 41°C =2.43, p=0.29. TrH-GAL4/TNT, 30°C, H(2, N=249) =0.81, p=0.6, 41°C =2.75, p=0.25. TrH/Trpm8, 30°C, H(2, N=387) =0.107, p=0.95, 41°C =3.99, p=0.14. Control and am-W, 30°C, H(3, N=384) =4.91, p =0.18, 41°C =5.75, p=0.12



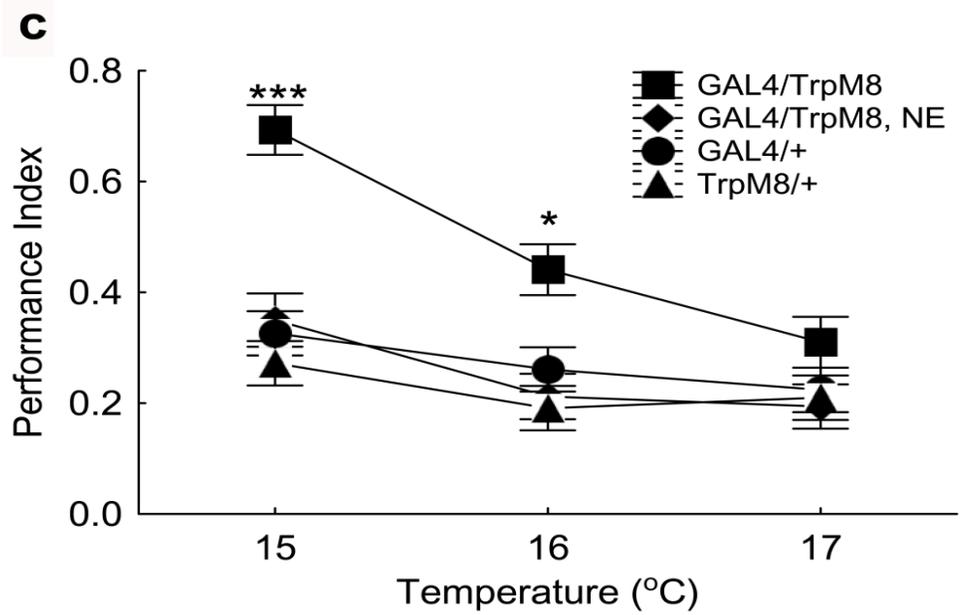
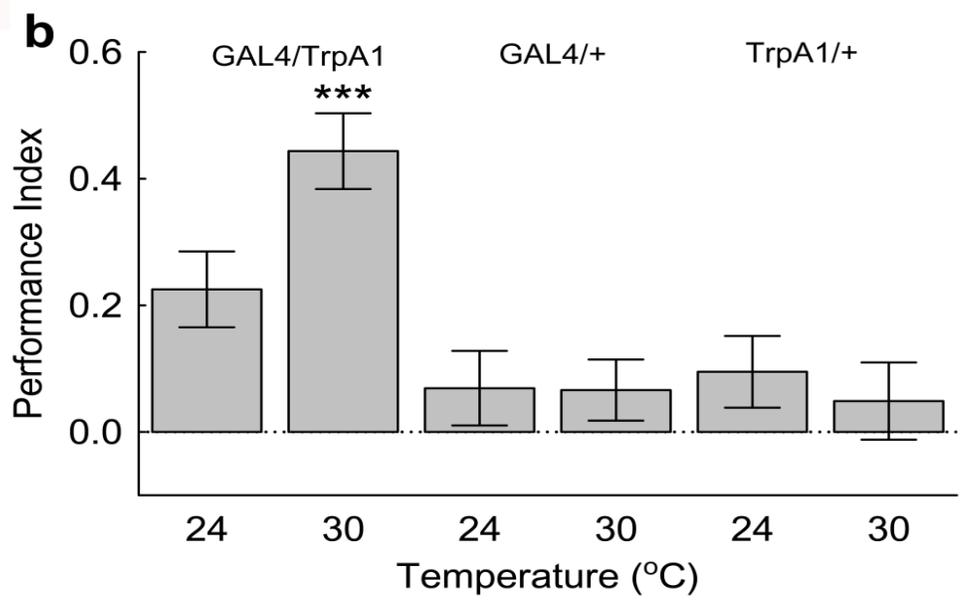
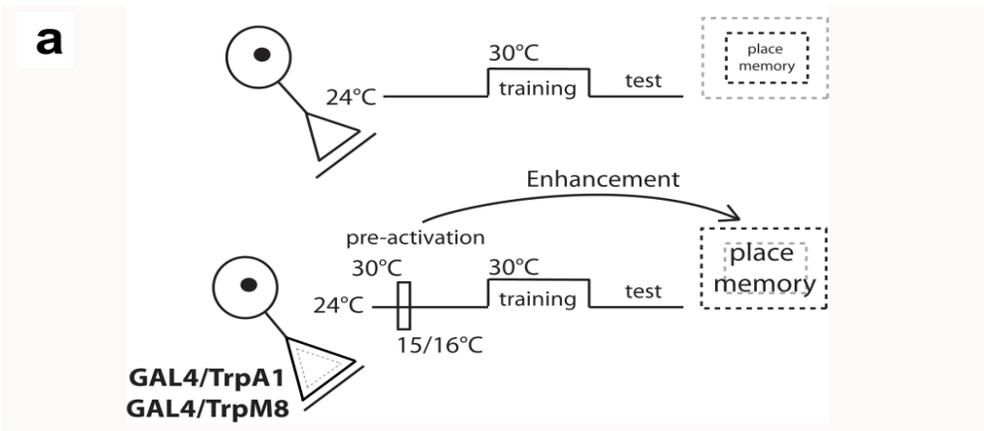
**Figure 5.2** – The serotonergic neurons are necessary for the unexpected exposure enhancement of place memory.

a) Reducing serotonin activity reduces unexpected exposure enhancement of place memory performance. b) Flies with the tetanus toxin light chain (TNT) expressed with DDC-GAL4; TH-GAL80 (GAL4) positive serotonergic neurons (GAL4/TNT ( ■ )) and control genotypes (GAL4/+ (●) and TNT/+ ( ▲ )) were conditioned for 4 to 20 minutes at 30°C with (dark symbols) or without (light symbols) unexpected exposure. Expression of TNT in the serotonergic neurons reduces the enhancement of place memory (4 min, H(5, N=553) =132.25, p<0.0001; 6 min, H(5, N=486) =124.05, p<0.0001; 8 min, H(5, N=428) =57.85, p<0.0001; 10 min, H(5, N=433) =125.41, p<0.0001, 15 min, H(5, N=477) =103.74, p<0.0001; 20 min, H(5, N=615) =212.66, p<0.0001). c) Normal unexpected enhancement of place memory requires TrH-GAL4 neurons (H(5, N=734) =97.6, p<0.0001). d) Inhibition of serotonin synthesis impairs unexpected exposure enhancement of place memory (H(3, N=566) =19.2, p<0.0001).

Is this large set of serotonergic neurons also sufficient for the unexpected exposure effects on memory? Flies expressing TrpA1 or the cool-responsive TrpM8 in the serotonergic neurons were exposed to activating temperatures (Figure 5.3a) (Peabody et al., 2009). Activation of serotonergic neurons with TrpA1 nearly doubled place memory compared to genetic and conditioning control flies (Figure 5.3b). Similarly, flies expressing TrpM8 in the serotonergic neurons had strongly enhanced memory with unexpected exposure to 15 and 16°C, but not 17°C (Figure 5.3c), consistent with the temperature range that opens these channels. Flies from the different genotypes did not have altered control behaviors (Table 5.1).

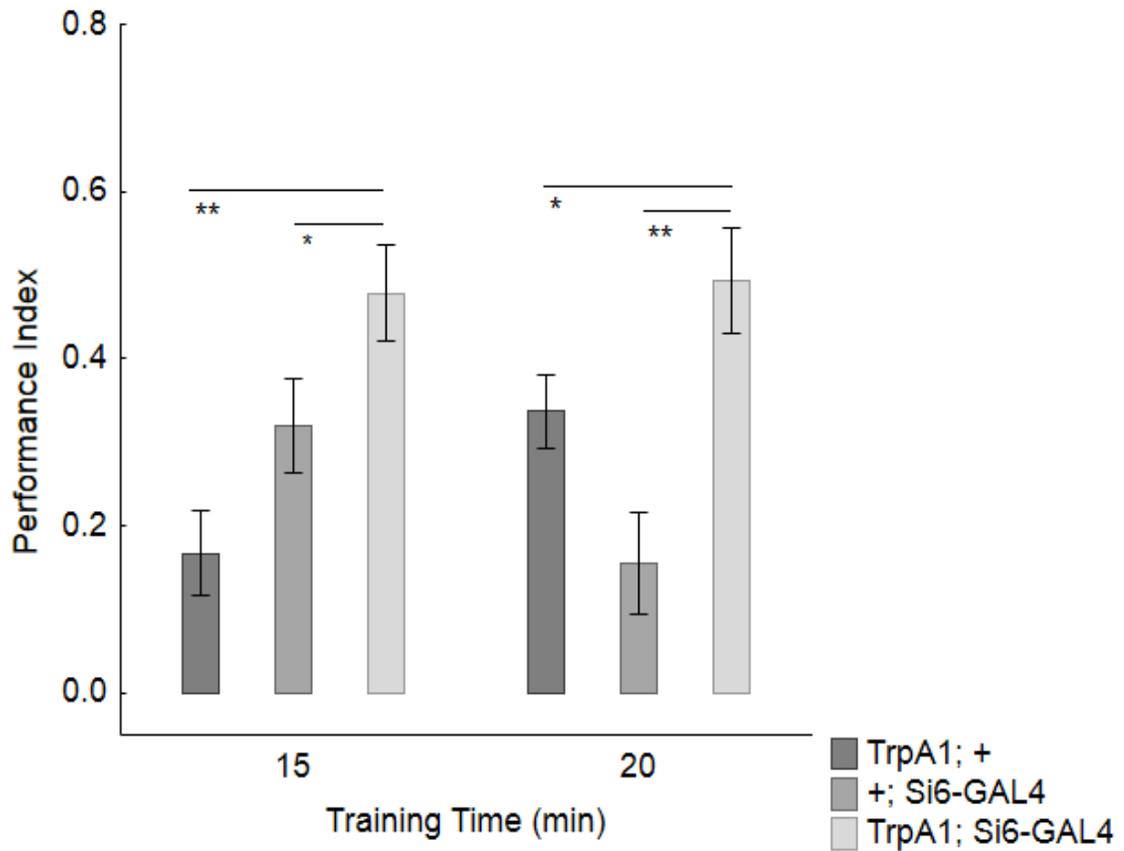
We next sought whether a subset of the serotonergic system could be found sufficient for operant feedback in place memory. Ten existing and newly made GAL4 drivers that express in subsets of the serotonergic system were tested for direct conditioning effects (Chapter 4). Of these, an enhancer from the sixth intron of the SerT gene (Si6-GAL4) was the only one to identify a subsystem of the serotonergic system sufficient for operant feedback (Table 5.2). When TrpA1 was expressed in the neurons from Si6-GAL4 and flies were trained with 32°C, place memory after training with either 15 or 20 min was significantly higher in the experimental flies compared to flies from the control genotypes (Figure 5. 4). Flies from the different genotypes did not have altered control behaviors (Table 5.3).

With the identification of the Si6-GAL4 neurons as sufficient for operant feedback, we next sought to determine if these neurons are also necessary for serotonergic feedback in place memory. To do this, we made an Si6-GAL80 line to suppress the potential activity of GAL4 in these neurons (Chapter 4). Combining the Si6-GAL80 element with TrH-GAL4 and the TrpA1 transgene led to a partial but significant reduction in the place memory that is formed with



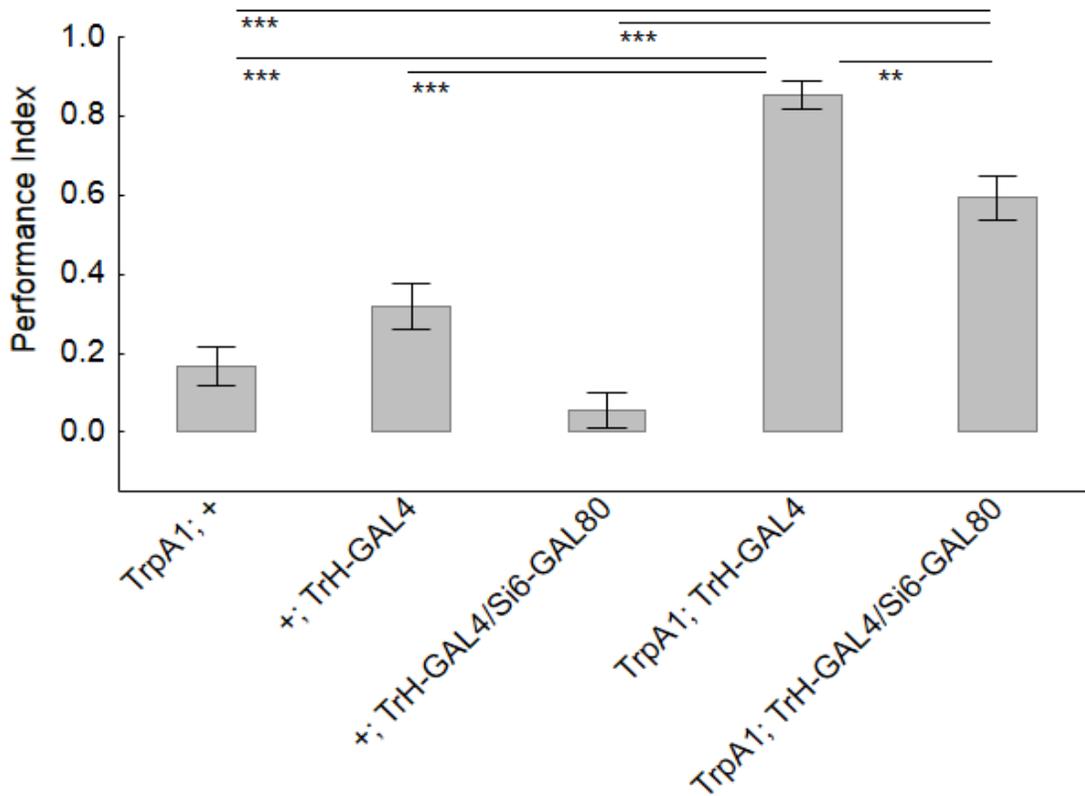
**Figure 5.3** – Serotonergic neurons mediate unexpected exposure enhancement of place memory.

a) Flies were exposed to several temperatures that either activate the TrpA1 or TrpM8 effector proteins or act as control temperatures. b) Flies expressing the warm-responsive TrpA1 in serotonergic neurons with the TrH-GAL4 driver (GAL4/TrpA1) had an enhanced memory after exposure to 30°C compared to genetic control flies ( $H(5, N=737) = 31.9, p < 0.0001$ ). c) Flies expressing the cool responsive TrpM8 with the TrH-GAL4 driver (GAL4/TrpM8 (■)) had enhanced memory levels compared to genetic control flies (GAL4/+ (●) and TrpM8/+ (▲)) and GAL4/TrpM8 flies not exposed to a low temperature (◆) (15°C,  $H(3, N=516) = 48.1, p < 0.0001$ ; 16°C,  $H(3, N=536) = 17.5, p = 0.0006$ ; 17°C,  $H(3, N=514) = 3.6, p = 0.3$ )



**Figure 5.4** – A subsystem of serotonergic neurons is sufficient for conditioning an operant place memory.

Flies were exposed to TrpA1-activating temperatures for either 15 or 20 minutes. Flies expressing TrpA1 in serotonergic neurons with the Si6-GAL4 driver (TrpA1; Si6-GAL4) had an enhanced memory after conditioning with 32°C compared to genetic control flies (15 min:  $H(2, N=370) = 20.69, p < 0.0001$ ; 20 min:  $H(2, N=396) = 19.32, p = 0.0001$ ).



**Figure 5.5** – The Si6 subsystem of serotonergic neurons is necessary for normal place memory.

Flies were conditioned with a TrpA1-activating temperature for 15 minutes. TrpA1; TrH-GAL4 flies showed place memory scores similar to those achieved with high-temperature training and significantly different from genetic controls ( $H(2, N=358) = 95.29, p < 0.0001$ ). Blocking a subsystem of serotonergic neurons resulted in a significant decrease in place memory scores (TrpA1; TrH-GAL4/Si6-GAL80,  $Z=3.42, p=0.00021$ . TrpA1; TrH-GAL4/Si6-GAL80: genetic controls,  $H(2, N=363) = 50.62, p < 0.0001$ ).

**Table 5.2 – Performance index after direct conditioning at 32°C**

Genotype	15 min		20 min	
	N	Performance Index	N	Performance Index
TrpA1; +	140	0.17 ± 0.05	166	0.35 ± 0.05
+; TrH5p-GAL4	116	0.31 ± 0.06	119	0.32 ± 0.06
TrpA1; TrH5p-GAL4	119	0.36 ± 0.06	119	0.25 ± 0.06
+; TpH5p-GAL4	115	0.51 ± 0.06	117	0.61 ± 0.05
TrpA1; TpH5p-GAL4	105	0.35 ± 0.07	112	0.37 ± 0.07
+; S5np-GAL4	119	0.43 ± 0.06	115	0.48 ± 0.06
TrpA1; S5np-GAL4	118	0.43 ± 0.06	117	0.51 ± 0.06
+; SerT 38749-GAL4	117	0.27 ± 0.06	118	0.51 ± 0.06
TrpA1; SerT 38749-GAL4	119	0.18 ± 0.06	118	0.39 ± 0.06
+; SerT 38764-GAL4	113	0.26 ± 0.07	113	0.37 ± 0.07
TrpA1; SerT 38764-GAL4	116	0.47 ± 0.06	116	0.51 ± 0.06
+; TrH 46910-GAL4	116	0.24 ± 0.06	118	0.44 ± 0.06
TrpA1; TrH 46910-GAL4	116	0.23 ± 0.06	116	0.36 ± 0.06
+; TrH 247-GAL4	117	0.20 ± 0.06	118	0.36 ± 0.06
TrpA1; TrH 247-GAL4	110	0.30 ± 0.07	115	0.36 ± 0.06
+; TrH 493-GAL4	115	0.27 ± 0.07	113	0.36 ± 0.06
TrpA1; TrH 493-GAL4	115	0.22 ± 0.07	115	0.34 ± 0.06
+; TrH 819-GAL4	112	0.34 ± 0.07	115	0.41 ± 0.06
TrpA1; TrH 819-GAL4	209	0.04 ± 0.04	161	0.28 ± 0.05

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Statistics:

TrpA1; TrH5p-GAL4: 15m, H(2, N=372) =8.2, p=0.017; 20m, H(2, N=404) =1.0, p=0.612.

TrpA1; TpH5p-GAL4: 15m, H(2, N=352) =19.2, p=0.000; 20m, H(2, N=390) =13.1, p=0.001.

TrpA1; S5np-GAL4: 15m, H(2, N=376) =17.5, p=0.000; 20m, H(2, N=395) =8.8, p=0.012.

TrpA1; SerT 38749-GAL4: 15m, H(2, N=375) =2.3, p=0.312; 20m, H(2, N=402) =5.8, p=0.054.

TrpA1; SerT 38764-GAL4: 15m, H(2, N=368) =17.2, p=0.000; 20m, H(2, N=395) =5.2, p=0.076.

TrpA1; TrH 46910-GAL4: 15m, H(2, N=372) =1.1, p=0.567; 20m, H(2, N=400) =1.9, p=0.384.

TrpA1; TrH 247-GAL4: 15m, H(2, N=366) =3.4, p=0.181; 20m, H(2, N=399) =0.3, p=0.862.

TrpA1; TrH 493-GAL4: 15m, H(2, N=369) =2.5, p=0.285; 20m, H(2, N=394) =0.1, p=0.969.

TrpA1; TrH 819-GAL4: 15m, H(2, N=460) =16.6, p=0.000; 20m, H(2, N=442) =3.2, p=0.202.

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**Table 5.3 – Control high temperature avoidance behavior in serotonin-altered flies expressing the thermosensitive effector, TrpA1**

Genotype	N	24/32°C	24/41°C
TrpA1; +	113	0.39 ± 0.04	0.75 ± 0.02
+; TrH-GAL4	110	0.43 ± 0.04	0.75 ± 0.02
TrpA1; TrH-GAL4	108	0.46 ± 0.03	0.72 ± 0.04
+; Si6-GAL4	120	0.69 ± 0.05	0.82 ± 0.03
TrpA1; Si6-GAL4	102	0.40 ± 0.06	0.61 ± 0.05
+; TrH-GAL4/Si6-GAL80	150	0.54 ± 0.04	0.77 ± 0.02
TrpA1; TrH-GAL4/Si6-GAL80	148	0.40 ± 0.03	0.71 ± 0.02

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Statistics

TrpA1; TrH-GAL4: 32°C, H(2, N=359) =0.2, p=0.921; 41°C =11.1, p=0.004.

TrpA1; Si6-GAL4: 32°C, H(2, N=363) =39.4, p=0.000; 41°C =10.4, p=0.000.

TrpA1; TrH-GAL4/Si6-GAL80: 32°C, H(2, N=408) =13.0, p=0.002; 41°C =1.2, p=0.545.

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**Table 5.4 – Performance Index after 20 minutes pre-exposure to 32°C**

Genotype	N	Performance Index
TrpA1; +	201	-0.04 ± 0.04
+; TrH5p-GAL4	131	0.05 ± 0.06
TrpA1; TrH5p-GAL4	135	0.10 ± 0.05
+; TpH5p-GAL4	110	0.19 ± 0.07
TrpA1; TpH5p-GAL4	100	-0.03 ± 0.07
+; S5np-GAL4	107	-0.03 ± 0.06
TrpA1; S5np-GAL4	110	0.02 ± 0.06
+; Si6-GAL4	132	0.13 ± 0.06
TrpA1; Si6-GAL4	123	0.13 ± 0.06
+; SerT 38749-GAL4	112	0.17 ± 0.07
TrpA1; SerT 38749-GAL4	127	0.12 ± 0.06
+; SerT 38764-GAL4	124	0.17 ± 0.07
TrpA1; SerT 38764-GAL4	111	0.20 ± 0.06
+; TrH 46910-GAL4	124	0.17 ± 0.07
TrpA1; TrH 46910-GAL4	138	-0.02 ± 0.05
+; TrH 247-GAL4	116	-0.03 ± 0.05
TrpA1; TrH 247-GAL4	110	0.06 ± 0.06
+; TrH 493-GAL4	105	0.12 ± 0.08
TrpA1; TrH 493-GAL4	114	-0.04 ± 0.06
+; TrH 819-GAL4	105	0.39 ± 0.07
TrpA1; TrH 819-GAL4	123	0.08 ± 0.07

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Statistics:

TrpA1; TrH5p-GAL4: H(2, N=467) =4.8, p=0.089. TrpA1; TpH5p-GAL4: H(2, N=411) =9.2, p=0.010. TrpA1; S5np-GAL4: H(2, N=418) =0.7, p=0.689. TrpA1; Si6-GAL4: H(2, N=456) =8.5, p=0.014. TrpA1; SerT 38749-GAL4: H(2, N=440) =9.7, p=0.008. TrpA1; SerT 38764-GAL4: H(2, N=436) =13.7, p=0.001. TrpA1; TrH 46910-GAL4: H(2, N=463) =7.3, p=0.026. TrpA1; TrH 247-GAL4: H(2, N=427) =2.5, p=0.285. TrpA1; TrH 493-GAL4: H(2, N=420) =5.6, p=0.059. TrpA1; TrH 819-GAL4: H(2, N=429) =29.2, p=0.000.

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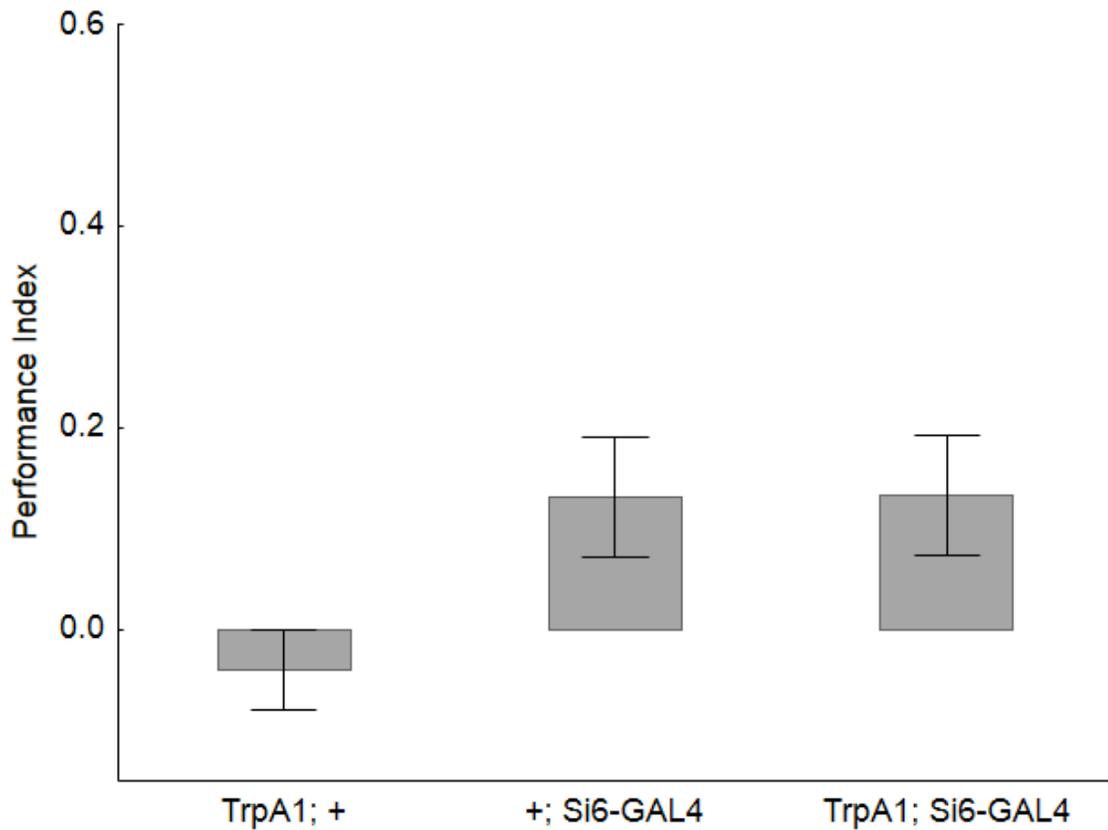
activation of all of the serotonergic system using the TrH-GAL4 driver (Figure 5.5). Again, flies from the different genotypes did not have altered control behaviors (Table 5.3).

We then explored whether or not the same or different subsets of serotonergic neurons can induce the unexpected exposure enhancement of place memory. We tested ten GAL4 lines, with expression in as little as a few neurons to nearly 70% of the whole serotonergic system.

Expression in a subsystem of the serotonergic system failed to enhance place memory with any of the ten GAL4 lines, including Si6-GAL4 (Table 5.4, Figure 5.6). Thus, it seems that only activation of the whole system can induce this effect.

## **Discussion**

The results show that the whole serotonergic set in *Drosophila* mediates (is necessary and sufficient for) both the aversive operant feedback effects of rising temperatures and the place memory enhancing effects of unexpected exposure to high temperature. One subsystem of the serotonergic system, defined by the Si6-GAL4 driver, is also necessary and sufficient for normal operant feedback in place memory. However, all attempts to identify a subsystem for the unexpected exposure effect failed. Thus, while a single biogenic amine system is used for both



**Figure 5.6** – The Si6 subsystem of neurons does not induce pre-exposure enhancement.

Flies were exposed to TrpA1-activating temperature for 20 minutes, then trained with a lower temperature. *Post-hoc* statistical analysis showed a difference between the TrpA1; + flies and TrpA1; Si6-GAL4 flies; however, there was no statistical difference between +; Si6-GAL4 and TrpA1; Si6-GAL4. Thus, TrpA1; Si6-GAL4 did not induce any pre-exposure enhancement (TrpA1; Si6-GAL4:  $H(2, N=456) = 8.51, p=0.014$ ).

learning mechanisms, one requires a larger proportion of the system. It appears then that the whole serotonergic set provides a slow and lasting change to buffer the aversive high-temperature information with the unexpected exposures until flies find control in an operant learning context. The output from some of these neurons then has a larger than expected effect on operant learning. Remarkably, unexpected exposure experience influences this operantly learned behavior.

Only the Si6 subsystem of serotonergic neurons demonstrated any effect on place memory. Double-labeling experiments showed Si6-GAL4 driving expression in both the SE2 and SE3 clusters as well as one neuron in the PMP cluster. The sub-esophageal neurons have been previously described as motor neurons activated by sugar and inhibited by bitter that are involved in the proboscis extension reflex (Alekseyenko et al., 2010; Gordon & Scott, 2009). While the possibility exists that these neurons are modulating place learning, they are more likely involved in gustation and motor control of the proboscis. Thus, we believe the effect on place learning is caused by activation of the dorsal neuron located in the PMP cluster, possibly from the SP1 cluster described in (Giang et al., 2011). Experiments using GAL80 repressors that block expression from SE2 and SE3 neurons, but not the dorsal PMP/SP1 neuron, can elucidate the role that these five neurons play in place learning and operant conditioning.

It is a consistent feature that control flies carrying only the GAL4 element have higher performance scores, but not significantly so, than their experimental counterparts carrying both the GAL4 element and the UAS element (Table 5.2). There should be little difference in the genetic background of the flies used for these conditioning experiments as all fly lines were introgressed with Cantonized  $w^{1118}$  and had the X-chromosome replaced, thus although genetic modifiers could be a factor, it is unlikely. It is also possible that the GAL4 transcription factor is

binding in the genome in an unpredicted and unexpected way, although the *attP2* site was selected because this region of the *Drosophila* genome is thought to be transcriptionally silent (Groth et al., 2004). Whatever the cause may be, the only fly line that showed significant difference between both controls and the experimental group was TrpA1; Si6-GAL4. Si6-GAL4 also showed some interesting behaviors in the thermosensitivity assay. At both 32°C and 41°C, the control genotype +; Si6-GAL4 showed better avoidance of higher temperature than did either TrpA1; + or TrpA1; Si6-GAL4. It is possible that flies with reduced avoidance in a thermosensitivity assay would also have reduced memory scores after conditioning; however, this is not what was observed. Although the TrpA1; Si6-GAL4 flies had lower thermosensitivity assay scores than did one control counterpart, they had a significant *improvement* in place memory after direct conditioning, which is the opposite direction than expected if thermosensitivity were influencing the conditioned behavior.

It is likely that the ability to initiate a behavior in response to an unexpected aversive experience provided an early selective advantage. Closing the loop between behavior initiation and sensory input via operant feedback would be the next great advantage. In *Drosophila* place memory both the unexpected exposure pathway and an aversive feedback pathway are part of a single neural system. Intriguingly, the operant feedback component is a subset of the whole serotonergic system. This smaller system suggests that an operant feedback circuit evolved from a slower state signal. Finally, flies have recently been shown to have some attributes of learned helplessness stemming from unexpected high temperature exposures, and serotonin has been shown to be important in learned helplessness across species (Maier & Watkins, 2005; Yang et al., 2013). It may be that the *Drosophila* unexpected exposure model will provide novel insights into the mechanisms of operant feedback and learned helplessness.

## CHAPTER 6: DISCUSSION

In a variable temperature world, animals that are either able to tolerate bad conditions or able to learn from experience to avoid future bad conditions have a selective advantage. This advantage is likely increased if organisms can employ multiple strategies to avoid or learn. My investigation sought to examine multiple genetic bases for the ability to tolerate or escape high temperatures and the ability to predict and avoid place-associated aversive temperatures. First, the genetic tradeoff between immediate survival (thermotolerance and walking speed) and future avoidance (learning and memory) as components of high temperature-exposure survival strategies will be discussed in relation to two natural alleles at the *foraging* (*for*) locus. Next, the role of serotonin, specifically the Si6 subsystem, in place learning will be discussed with reference to both direct conditioning and the pre-exposure effect (Sitaraman et al., 2008; Sitaraman et al., 2007). Finally, thermotolerance and serotonin-modulated learning will be discussed as a possible direction for future research.

### **Immediate high temperature-exposure survival strategies**

One way to view thermoprotective strategies is as a triad: in one corner is the ability to tolerate increasing temperatures, in another, the ability to immediately avoid increasing temperatures, and in the third, the ability to learn to avoid high temperature. Most organisms probably use all three strategies. Nevertheless, it may be that species or individual animals invest more heavily in one strategy over another. For example, if avoidance behavior is improved, less thermotolerance may be required, and vice versa. Learning to avoid places associated with high temperature may be the most efficient, if costly, strategy as memory formation and maintenance is associated with a reduced life-span (Mery & Kawecki, 2005). Is it then possible

that increased thermotolerance might correlate with decreased locomotion or memory as a genetic tradeoff of sorts? Two alleles of the *for* locus, *rover* and *sitter*, provide an excellent opportunity to examine the genetic bases for evolutionary tradeoffs between thermotolerance, locomotor activity, and memory.

Previous studies with *rover* (*for<sup>R</sup>*) and *sitter* (*for<sup>S</sup>*) revealed distinct behavioral differences between the two alleles in food environments (Kaun et al., 2007; Sokolowski, 1980, 1982; Sokolowski, 1985a, 1985b). As larvae, *for<sup>R</sup>* move about a larger area and dig deeper while foraging than do *for<sup>S</sup>* (Sokolowski, 1980, 1982). *for<sup>R</sup>* larvae demonstrate higher short-term memory scores in classical conditioning paradigms in which a sugar reward is paired with either an odor or light while *for<sup>S</sup>* show higher long-term memory (Kaun et al., 2007). *for<sup>S</sup>* pupate directly onto their food source while *for<sup>R</sup>* pupate further away from food (Sokolowski, 1985b). *for<sup>S</sup>* and *for<sup>R</sup>* flies also exhibit differences: *for<sup>S</sup>* flies spend more time circling a sucrose drop after feeding from it, remaining around the food source, while *for<sup>R</sup>* flies walk away from the sucrose drop (Pereira & Sokolowski, 1993). Each allele can provide an advantage, depending on the food environment. If food is found in small separated patches, *for<sup>R</sup>* flies have an advantage because after feeding they will seek more food in a different location. However, if food is concentrated in one location, *for<sup>S</sup>* flies have an advantage because they remain around the existing food and spend less energy seeking novel food sources.

*for<sup>R</sup>* flies and *for<sup>S</sup>* flies also show phenotypic differences under aversive conditions (Dawson-Scully et al., 2007; Dawson-Scully et al., 2010; Mery et al., 2007; Wang et al., 2008). Larval feeding behavior is altered as the temperature of the food environment increases: mouth hook failure occurs at lower temperatures in *for<sup>R</sup>* larvae than in *for<sup>S</sup>* larvae (Dawson-Scully et al., 2007). Under anoxic conditions *for<sup>R</sup>* flies enter anoxic coma faster than *for<sup>S</sup>* flies but have twice

the survival rate after recovery (Dawson-Scully et al., 2010). When trained in a classical aversive olfactory paradigm, *for<sup>R</sup>* flies have higher short-term but lower long-term memory scores than *for<sup>S</sup>* flies and in a high-temperature-reinforced visual learning paradigm, *for<sup>S</sup>* flies have lower memory performance (Mery et al., 2007; Wang et al., 2008).

A previous study using the heat box conditioning paradigm examined a potential tradeoff between thermotolerance and place memory in flies but did not detect any significant differences in the time to incapacitation for several temperatures in time-limited experiments or in place memory (Gioia & Zars, 2009). My studies showed that upon closer examination, a potential difference became clear once exposure times were increased from 9 minutes to 12 minutes. At 39°C, *for<sup>S</sup>* flies had a longer time to incapacitation than *for<sup>R</sup>* flies while *for<sup>R</sup>* flies had a faster walking speed than *for<sup>S</sup>* flies (Figures 3.1 and 3.2). When exposed to a high temperature, *for<sup>R</sup>* flies try to move away from the high temperature faster and exhibit locomotor failure sooner than the slower moving *for<sup>S</sup>* flies. Selective advantages may again apply to both alleles depending on the environment in which the flies are. We speculate that if food is plentiful, the faster locomotion demonstrated by *for<sup>R</sup>* flies may be beneficial; flies can move away from increasing temperatures faster and leave dangerous conditions before they become fatal in favor of other food. If food is scarce, *for<sup>S</sup>* have a distinct advantage by being able to tolerate, within narrow limits, the high temperature of decaying fruit, which can reach up to 50°C (Feder & Krebs, 1997).

We have added to the understanding of foraging function by examining *for<sup>R</sup>* and *for<sup>S</sup>* flies in an aversive environment. Previous results showed a direct correlation between short-term memory and locomotion as well as between long-term memory and tolerance of stressful environments. However, a correlation with place memory was not observed in the heat box.

An inverse relation between locomotor activity and tolerance was observed in high temperature environments and, in previous studies, in anoxic environments, supporting the hypothesis of a genetic tradeoff related to survival strategies.

### **Serotonin and memory formation**

One of the best, although costly, strategies an organism can use is to learn to avoid aversive stimuli or approach appetitive stimuli. The heat box is an operant place memory paradigm that involves an association between an aversive high temperature stimulus and walking behavior in a narrow chamber (Wustmann et al., 1996; Zars, 2001). When flies cross to a predetermined location, the entire chamber heats and when they move away from that location, the entire chamber cools. Serotonin was previously shown to be necessary in this operant paradigm: when the serotonergic system is blocked either genetically or pharmacologically, place memory scores are greatly reduced (Sitaraman et al., 2008).

Serotonin has also been found to be critical in both aversive and appetitive classical olfactory learning paradigms in which either shock or sugar is paired with one of two odorants (Johnson et al., 2011; Lee et al., 2011; Sitaraman et al., 2012). Historically, the requirement of serotonin in both aversive and appetitive paradigms was unique among the biogenic amines; dopamine was thought to be critical strictly for aversive olfactory learning while octopamine was strictly necessary for appetitive olfactory learning (Schwaerzel et al., 2003; Sitaraman et al., 2010). New results indicate that dopamine plays an appetitive role as well: blocking expression from a small group of dopaminergic neurons disrupts appetitive learning and activation of these same neurons is sufficient to produce an appetitive memory, even in the absence of octopamine (Waddell, 2013). Parsing of the serotonergic system into genetic subsets may allow one to

identify serotonergic subsystems necessary, sufficient, or both for appetitive, aversive, classical, operant, olfactory, visual, and place learning.

My results described multiple subsets of serotonergic neurons and expression patterns ranging from only a few serotonergic neurons in the fly brain to most of the serotonergic neurons (Table 4.2, Figure 4.3). Having so many genetically defined subsets with such varied expression patterns will surely lead to a better understanding of the role the serotonergic system plays in place learning as well as other learning contexts.

Of the ten serotonergic transgenic *Drosophila* lines tested in the heat box, only one, Si6-GAL4, showed a change in place memory scores when the GAL4-positive neurons were active (Figure 5.4). Memory scores were significantly higher in those flies expressing *TrpA1* under the control of the Si6-GAL4 driver. Blocking expression of the Si6 subsystem with TrH-GAL4 and an Si6-GAL80 repressor decreased place memory (Figure 5.5). Thus, the Si6 subsystem is demonstrated to be both necessary and sufficient for operant place learning.

Expression patterns of Si6-GAL4 showed neurons co-labeled for serotonin and Si6 in the subesophageal ganglion (SOG), specifically in the SE1 and SE3 clusters, and in the posterior medial protocerebral (PMP) neuron cluster, possibly in the SP1 subcluster (Figure 4.3). Serotonergic neurons in two SOG clusters, SE1 and SE2, have been implicated in the proboscis extension reflex (Gordon & Scott, 2009). When output from these neuronal clusters is blocked, *Drosophila* respond to sucrose stimulation but fail to lift the rostrum from the head. Two neurons from the same SE1 cluster project down into the ventral nerve cord in a pattern similar to that observed in the blowfly, and when these neurons are ablated, flies again demonstrate difficulty with the proboscis extension behavior (Alekseyenko et al., 2010; Nässel & Elekes, 1985). If these neurons are motor neurons, the Si6-GAL4 positive neurons in the SOG are less likely the candidates

affecting place learning and memory. While it is possible that learning and memory are being modulated by the single unpaired neuron from the SE3 cluster, it is more likely the paired Si6 neurons in the SP1 subcluster of the PMP cluster are modulating learning and memory.

### **Learned helplessness and the pre-exposure effect as a response to aversive temperature exposure**

A typical yoking experimental setup consists of two groups of animals, one “master” and one “yoked,” exposed to a series of aversive stimuli (Maier & Seligman, 1976; Seligman & Maier, 1967; Sitaraman & Zars, 2010). The master can stop the stimulus with their behavior while the yoked group experiences inescapable and uncontrollable stimulus simultaneously with the master. When yoked animals are then exposed to the same stimulus, this time with the ability to stop it, they respond much more slowly to try and stop the stimulus than do naïve or master animals. The yoked group learns that their behavior and the aversive stimulus are independent. Yoked flies exposed to an uncontrollable inescapable aversive stimulus not only suppressed escape responses during exposure to the stimulus but were also significantly less active and slower than master flies when the aversive stimulus was removed (Yang et al., 2013). This combination of passivity during exposure and activity reduction after exposure is described as “learned helplessness” (Maier & Seligman, 1976; Seligman & Maier, 1967; Yang et al., 2013). Studies in mammals have indicated an increase in serotonin levels during inescapable stimuli (Amat et al., 1998a, 1998b; Bland et al., 2003; Grahn et al., 1999; Maier & Watkins, 2005). Flies pre-exposed directly or through yoking experiments to an aversive and inescapable 41°C show enhanced memory levels after conditioning with a weaker reinforcing temperature (Sitaraman et al., 2007; Sitaraman & Zars, 2010). This memory enhancement may be due to a commonality between yoking and pre-exposure – both expose flies to an inescapable stimulus. Blocking the output of serotonergic neurons either genetically or pharmacologically resulted in a

reduced place memory level in pre-exposed flies after unexpected exposure to 41°C, indicating the necessity of serotonin for a fly to develop increased aversion to less aversive temperatures (Figure 5.2). Flies expressing TrpA1 or TrpM8 with a TrH-GAL4 driver also demonstrated increased avoidance of less aversive temperatures after unexpected activation of these neurons (Figure 5.3). We propose that an inescapable stimulus, e.g., a large temperature change, will result in the release of serotonin. Serotonin then modifies the response of the organism to potential predictors of future aversive stimuli, e.g., mild temperature changes, to increase avoidance. Is there a subsystem that governs this pre-exposure modulatory effect of serotonin? The pre-exposure effect demonstrated in Figure 5.2 was not induced by activation of the Si6 subsystem only (Figure 5.6). Thus, the pre-exposure effect requires either a different serotonergic subsystem or the entire serotonergic system.

### **Thermotolerance and serotonin-modulated learning**

Is there a tradeoff between serotonin-modulated learning and thermotolerance? My results strengthen the correlations between serotonin, inescapable stimuli, and learning. If slightly aversive conditions are indicators of more aversive conditions to come, then serotonin plays a critical role in ensuring organisms learn from experience to avoid similar situations.

Experiments with *for* flies demonstrate a tradeoff between thermotolerance and learning.

Many experiments have also demonstrated the necessity of serotonin in learning and memory across paradigms and species. Anecdotally, the transgenic fly line TrpA1; SerT 38750-GAL4 was paralyzed within a couple minutes when exposed to TrpA1-activating temperatures and recovered completely once high temperature was removed, indicating a tentative connection between serotonin and thermotolerance. Future studies into the role of serotonin in thermotolerance should provide insights into the inverse relation between thermotolerance and memory formation as well.

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