

Reinforcement signaling in *Drosophila*.

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by

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ABSTRACT

An organism's ability to interact with the environment, evaluate choices and produce a behavioral output is critical for survival. Organisms of varying complexities from vertebrates to invertebrates are able to acquire information from their environment and express memories. Memory performance levels can reliably match the intensity / amount/ probability of reinforcement. Interestingly, experience with a reinforcer unpaired from any predictors can enhance or impair later associative learning. We have identified and characterized the reinforcement matching and the pre-exposure induced behavior in *Drosophila* spatial operant learning using high temperature as negative reinforcement.

Further we investigated the neural systems influencing matching and pre-exposure effect. Analysis of the *white* mutant implicates a role for serotonin and dopamine in memory matching. Using genetic and pharmacological manipulations, we found that serotonin but not dopamine plays a critical role in reinforcement matching. The serotonergic system is required for reinforcement processing in the heat box. Our results also show that the serotonergic reinforcement circuit is required for uncertainty bias in the heat box. Furthermore, altering the excitability of serotonergic neurons is sufficient in memory matching and inducing the pre-exposure effect. Another important finding is that the absence of a behavioral predictor is important for the pre-exposure effect and this effect can bias learning in a positive way.

Moreover, we also looked at the role of serotonergic signaling in aversive olfactory learning. Unlike the spatial learning where serotonin mediates the aversive reinforcing signal, serotonin enhances the olfactory memory 3 and 6 hrs post training.

Finally, serotonin and dopamine seem to have specific functions in two different aversive learning paradigms arguing against common negative reinforcing signals. Octopamine, known to mediate some positive reinforcing signals, seems dispensable for place learning. This either supports the conclusion of the absence of an appetitive component in place learning or the presence of one that is not dependent on octopamine.

CHAPTER 1. INTRODUCTION

An organism's ability to interact with the environment, evaluate choices and produce a behavioral output is critical for survival. Organisms of varying complexities from vertebrates to invertebrates are able to acquire information from their environment and express memories. For centuries, biologists and psychologists have been intrigued by the interactions occurring within the nervous system that makes an organism capable of learning. The complexity of learning and memory formation processes occurring in nature are often difficult to interpret and replicate. But the common theme is that all organisms in nature exhibit behaviors that maximize rewards and minimize punishments. Classical and operant conditioning paradigms in vertebrates and invertebrates have provided a wealth of information about the molecular and neural basis of learning. Associative learning tasks with stimuli and reinforcers that are not even typically found in nature have been used with great success indicating the plasticity in these processes. An important property of associative learning is the ability of organisms to match their behavior with the magnitude of reward and punishment obtainable from that behavior (Herrnstein, 1961). Reinforcement learning or processing describes the mechanisms that support this matching. Relatively little is known of how reinforcement information is processed within the nervous system. Evidence from invertebrates and vertebrates increasingly indicate the role of biogenic amines as the mediators of reinforcement information.

Mechanisms underlying learning and memory formation seem to recruit common signaling pathways that function in special structures. Additionally, learning processes are not fixed and seem to be highly plastic, catering to different learning situations. The processes of learning and memory have conventionally been studied in large experimental organisms such as *Aplysia*, mice, rats, monkeys, and humans with several well-characterized behaviors. More recently however with improved genetic tools *C. elegans* and *Drosophila* have been used with great success in elucidating molecular components of associative and non associative learning.

Drosophila, an invertebrate with nervous system of intermediate complexity and extensive genetic toolkit is an excellent model system to study this process. *Drosophila* has many behaviors that are plastic, this combined with the ability to manipulate specific neurons has been critical in further understanding of the learning processes. Using a spatial operant learning paradigm we will explore the molecular and neuronal basis of reinforcement processing critical to memory formation in *Drosophila*. Additionally, we are also interested in understanding the influence of unexpected aversive reinforcers on place learning at the behavioral and molecular level. Finally, we will test if the place learning reinforcement signal has a function in a second learning test, classically conditioned olfactory learning.

***Drosophila* as a model of learning and memory**

Drosophila as a genetic model organism has been at the forefront of learning and memory studies. The *Drosophila* nervous system exhibits intermediate complexity and evidence of conserved systems in learning across species, attributes that make it a promising model organism to study behavior and learning processes (Benzer, 1967; Quinn et al., 1974). The key players, thus far identified in learning and memory processes namely the neurotransmitters, ion channels, synaptic proteins, signaling molecules, and receptors are similar to those found in vertebrates. Therefore, it is expected that the way in which the learning circuits develop, connect and operate should be conserved at some level. Investigating the molecular mechanisms supporting memory formation in *Drosophila* offers several advantages. The *Drosophila* brain has an estimated 250,000 neurons (Heisenberg and Wolf, 1984) and easily identifiable anatomical structures. In addition to a short life cycle, the wide array of robust behaviors can be exploited in designing diverse learning paradigms to understand classical and operant conditioning mechanisms. The behaviors have been used in paradigms that include assays for appetitive and

aversive classical olfactory conditioning, operant visual learning, and operant spatial learning (Dubnau and Tully, 1998; Gerber et al., 2004; Putz and Heisenberg, 2002).

In Chapter 1 we will first introduce *Drosophila* as a model to study behavior, followed by the neuroanatomy of the fly brain. Thereafter, we will discuss the genetic tools that have been critical in understanding the neuronal circuitry of learning. Finally, we will focus on the previously known reinforcement mechanisms that support learning function in *Drosophila*.

Behavioral and learning assays in *Drosophila*

Several assays have been developed in *Drosophila* to study simple and complex behaviors and perceivable rewards and punishments. Larvae and adult flies exhibit several sensory behaviors such as phototaxis (movement towards light), gravitaxis (movement against gravity), taste and olfactory discrimination, audition (mainly courtship songs), nociception (a pain response), thermosensation, mechanosensation, optomotor response, and visual pattern discrimination (reviewed in (Simpson, 2009). Other innate behaviors involving integration of multiple motor and sensory modalities include courtship, aggression, and the proboscis extension reflex. The ability to execute complex behaviors has changed the notion of flies' ability to learn and form memories. For example, flies can remember the spatial position of an object that has been removed from their environment (Neuser et al., 2008). However, none of the neural circuits mediating these behaviors are completely understood, and most are only beginning to be investigated.

Of the associative learning paradigms the olfactory learning, spatial learning, visual pattern conditioning in the flight simulator, and courtship conditioning have been successful and addressed with genetic tools (Hall, 1994; Tully and Quinn, 1985; Wustmann et al., 1996). Each of these behaviors is shown to be modulated by both diverse and common signaling pathways. Of

these, classically conditioned olfactory learning and spatial learning in the heat box have been used in this dissertation.

Spatial operant learning in *Drosophila*

The heat box

Operant learning depends on association between a behavior and its consequences (Skinner, 1938). Operant learning allows the organisms to form a prediction of whether a behavior will be rewarded or punished. This association is made by previous experience or training. Examples from *Aplysia*, *Drosophila*, and *Lymnea* show that invertebrates are capable of operant learning (Brembs et al., 2002; Hawkins et al., 2006; Lukowiak et al., 1996). A spatial operant learning paradigm using the heat box (schematic shown in Figure 1-1 A) allows for a rapid and robust test of learning and memory in *Drosophila* (Wustmann et al., 1996). The heat box can be used to condition an individual fly by pairing elevated temperature with a spatial position in the chamber.

During an experiment, one-half of the chamber is defined as the "punished associated" side and the other as the "unpunished" side. There are three phases in a typical experiment. A fly is introduced into the chamber followed by a brief pre-test phase in which the chamber temperature is maintained at 24° C (Figure 1-1 B). Flies typically explore the environment by walking back and forth. During the training phase (Figure 1-1 C), the chamber heats up to a defined high temperature (>33° C) every time the fly crosses the midline to go to the "punished" side. A fly will usually avoid the punishment associated side of the chamber after only a few minutes of training. In the post-test phase the high-temperature spatial contingency is removed and chamber is maintained at 24° C (Figure 1-1 D). Flies continue to avoid the half of the chamber formerly associated with high temperature. In all the three phases, the position of the fly in the chamber is monitored. A performance index is calculated for quantitative analysis of memory performance levels (refer to materials and methods).

The pre-test activity measures the movement of the fly in the chamber that is not modulated by conditioning. A comparison of pre-test walking activity with memory performance in flies with several different mutations during the test phase so far shows no significant relationship (Diegelmann et al., 2006; Sitaraman et al., 2008). Therefore, the low and high pre-test activity itself does not pre-dispose the fly to perform better or worse. One of the pre-requisites of spatial conditioning is the locomotor ability of the tested flies. Therefore, while calculating performance index an additional contingency is added such that only flies that receive the heat strike once and have walked at least one chamber length are included in calculations.

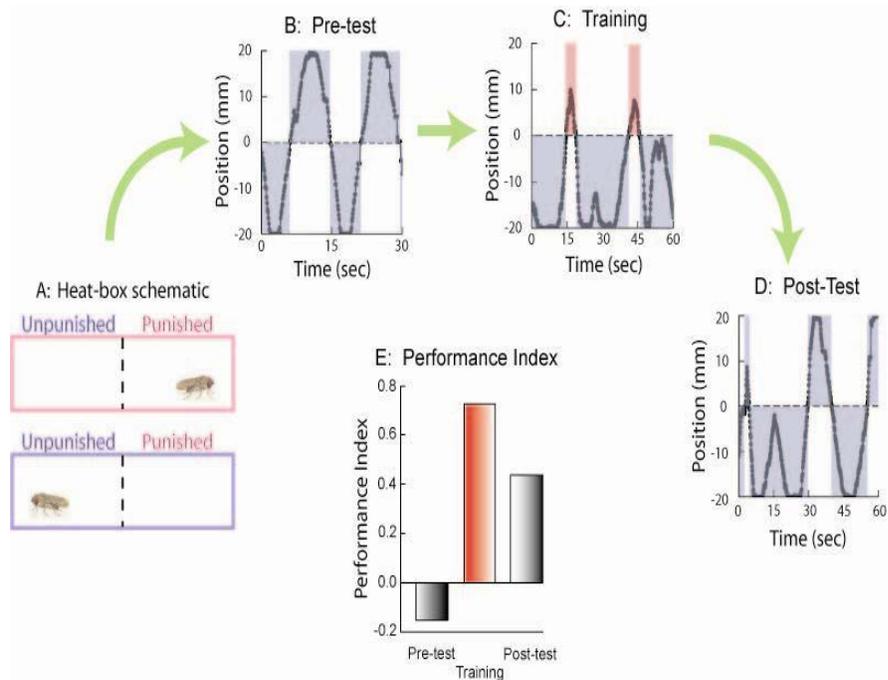


Figure 1-1. Learning in the heat box.

A) Schematic diagram of a heat box chamber. B, C, D) Position trace of an individual fly in a chamber. B) Typical pretest, training and post-test position traces of a fly; Y-axis represents the position in the chamber, time is represented on the X-axis. E) Performance index of different phases. Red and blue shading represent the heating and cooling of the chamber based on the spatial position of the fly.

Sensory requirements of learning in the heat box

In conditioning experiments, we use 24°C as the reference temperature (most preferred by adult flies) and 37 or 41°C as the reinforcing temperature. In *Drosophila* two distinct thermosensors have been identified, one in the fly antennae that is necessary for sensing temperatures below 30°C and a second one for temperatures above 31°C (Sayeed and Benzer, 1996). Control and antennae-less flies avoid temperatures above 31°C indicating that the antennal thermosensor is dispensable for detecting temperatures > 31°C. The presence of a high temperature thermosensor of unknown location has been hypothesized for higher temperatures (Sayeed and Benzer, 1996; Zars, 2001). Some members of the transient receptor potential channel (Trp's) have been implicated as thermosensors mediating avoidance of very specific temperatures. The nocifensive rolling and escape behavior is absent in *painless* which encodes a dTrpA and required for avoidance of 46°C (Tracey et al., 2003). Two other dTrpA channels, dTrpA1 and Pyrexia have been identified that mediate avoidance of 30-32°C and > 40 °C respectively (Lee et al., 2005; Rosenzweig et al., 2005).

To differentiate between conditioning and thermosensory deficits, a thermosensitivity assay has been developed in the lab (Zars and Zars, 2006; Zars, 2001). In this assay both chamber halves are maintained at 24°C for a minute followed by temperature change in one half to 27, 30, 33, 37, 41, and 45°C every consecutive minute. The chamber half that is associated with high temperatures is switched alternatively to ensure that thermopreference is not confounded by side preference (protocol outlined in material and methods section).

Conditioning in the heat box depends on reinforcement intensity and processing mechanisms

An important determinant of the levels of asymptotic memory performance level depends on the intensity of the reinforcer. That is, higher temperature leads to higher memory scores (Figure 1-2) (Diegelmann et al., 2006). Reinforcement processing is central to this relationship and the heat box can be used effectively to study these mechanisms.

Impaired place learning behavior reflected in low test performance indices could be due to a reduced memory acquisition rate or altered high-temperature reinforcement processing. Extended training experiments can differentiate between these possibilities. Mutation of *rutabaga* and *white* allow for a unique insight into distinct learning processes in the heat box. Both of these mutant flies lead to reduced memory scores after 4 minutes of training as compared to wild-type CS flies. Increasing the training time increases performance, reaching an asymptotic level between 10 and 20 minutes of training.

Mutation of the *white* gene restricts asymptotic performance (Figure 1-3, A and B) (Diegelmann et al., 2006). A reduction in asymptotic performance is phenotypically different from acquisition deficits (as in seen in *rut²⁰⁸⁰*) which shows a slower rise in performance but has a memory score similar to the wild-type with extended training (Figure 1-3,A). *white* mutant flies can avoid the temperatures used for conditioning, but the memory scores at 37 and 41°C are similar to wild-type flies conditioned with lower temperatures (<33°C). This inability of *white* flies to process the high temperature information in the context of the spatial position shows that altering training time in the heat box allows for a genetic dissection of acquisition and reinforcement processing deficits (Diegelmann et al., 2006). Mechanistically, how the *white* mutation influences conditioning of flies in the heat box is not known. The *white* gene was pursued in the dissertation as it provided insights into the reinforcement processing mechanisms in the heat box.

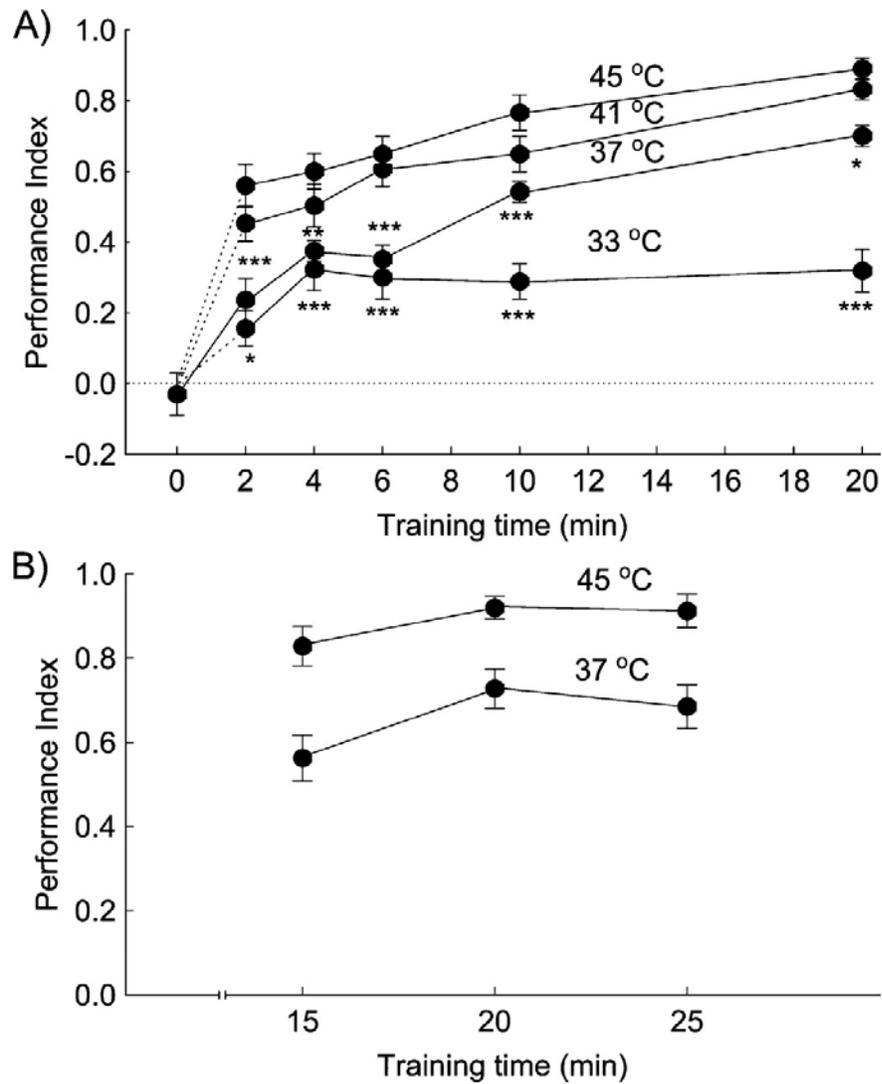


Figure 1-2. Higher reinforcing temperature increases memory strength in the heat-box. A) Wild-type CS flies were trained for increasingly longer periods at different reinforcing temperatures (33°, 37°, 41°, and 45°C). The 3-min memory was measured after training for 2, 4, 6, 10, or 20 min. B) Wild-type CS flies were trained for 15, 20, and 25 min to determine whether the 20-min training schedule induced asymptotic memory levels. p-values represented in this and following figures and tables are: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ (Diegelmann et al., 2006).

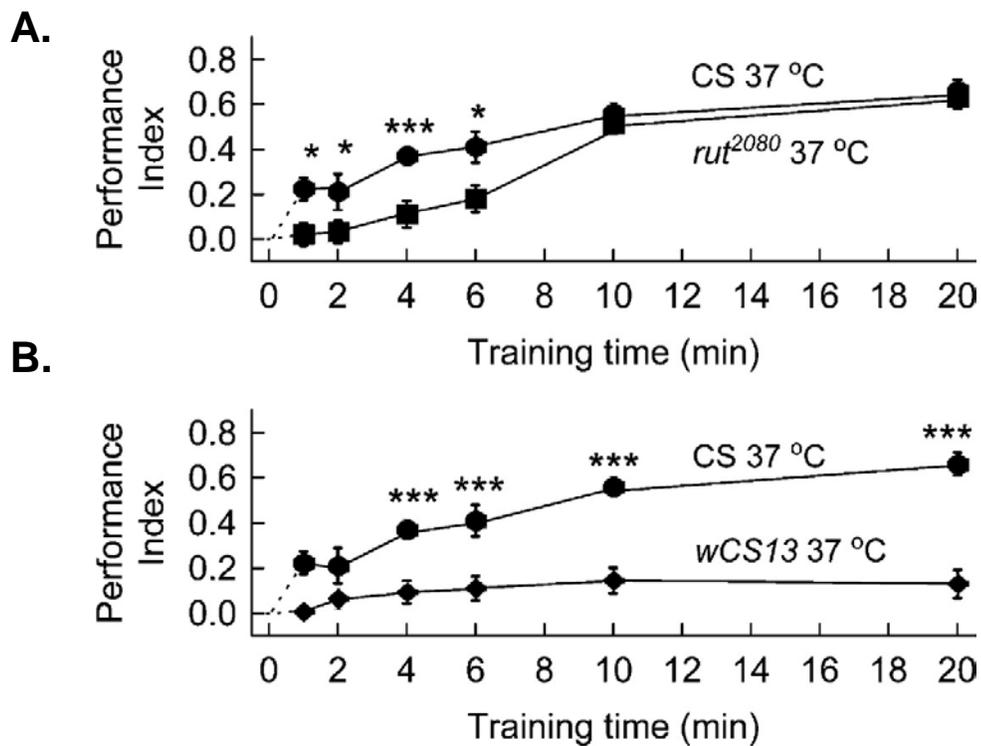


Figure 1-3. The *rut*²⁰⁸⁰ mutation affects acquisition rates, while the *wCS13* mutation influences asymptotic memory levels. In A and B wild-type CS, *rut*²⁰⁸⁰, and *wCS13* mutant flies were continuously trained from 1 to 20 min and tested directly afterward for continued side preference for 3 min. The 3-min memory score is presented. Interestingly, *rut*²⁰⁸⁰ mutant flies have indistinguishable memory scores from wild-type flies when trained for 10 or 20 min; all other training situations show deficits. The *wCS13* mutant flies have a different phenotype, having a significantly lower asymptote than wild-type flies. Values represent mean and SEMs.

Cellular / Molecular basis of spatial learning in *Drosophila*

How organisms learn about their spatial information is not well understood. Other spatial learning paradigms such as the Morris water maze task in rodents show that specific neurons in the hippocampus called place cells are critical for encoding the spatial information (Morris, 1984; O'Keefe, 1976). The heat box learning paradigm requires the flies to associate spatial position in the chamber to rising or falling temperature. How flies orient themselves in space and exhibit avoidance of punished side without any visual and olfactory cues is unknown. Tactile information and idiothetic cues are probably used by the flies to distinguish the chamber halves (Wustmann et al., 1996). Like rodents even flies could have specific neuronal structures that mediate the path integration information enabled by computations (counting steps, etc.) of movement.

Other than the heat box, another spatial memory task in *Drosophila* has been developed that uses target selection as spatial cues. Interestingly, the central complex region specifically the ellipsoid body (eb) seems to be the important structure that stores the spatial information (Neuser et al., 2008). Whether, the same candidate structures are important for spatial learning in the heat box is not known.

Classically conditioned olfactory memory

The second learning paradigm used in this dissertation is the olfactory conditioning. Flies can be trained with olfactory cues. In this form of learning, flies are exposed to one of two odors (conditioned stimulus-CS) at a time and one of the odors is paired with aversive electric shock (the unconditioned stimulus (US)) or sugar (appetitive-US) during training (Schwaerzel et al., 2003; Tempel et al., 1983; Tully and Quinn, 1985). Flies are then challenged with the two odors

during the test phase. Flies typically avoid the odor associated with shock or approach the odor paired with sugar. Aversive olfactory learning is robust and even a single training can form memories lasting hours.

Neuroanatomy and neuronal circuitry underlying learning in *Drosophila*

The *Drosophila* brain has several identifiable structures that are critical for the neuronal control of learning. In *Drosophila*, the cell bodies are located on the outside surface of the brain, the cortical rind, while the neurites project inside to form the synaptic neuropil. Candidate structures and neuronal subsets have been identified that can be activated or inhibited to influence learned behavior.

Of these the most prominent and well studied structure with a learning function are the mushroom bodies (mb) (Figure 1-4). The mushroom bodies are paired structures important for olfactory learning and memory formation (Heisenberg et al., 1985; Zars et al., 2000a). Chemical ablation of the mushroom bodies with hydroxyurea (HU) and mushroom body mutants such as *mbm* and *mbd* are not capable of classically conditioned olfactory memories (de Belle and Heisenberg, 1994; Heisenberg et al., 1985). Furthermore rescue experiments using the UAS-Gal4/Gal80ts system shows that the neuronal plasticity in the MB neurons is sufficient for memory formation (McGuire et al., 2001; Zars et al., 2000a). However, the mushroom bodies are not important for all learning and memory tasks in *Drosophila*, HU ablation and analysis of *mbm* or *mbd* mutation does not alter place memory (Putz and Heisenberg, 2002; Wolf et al., 1998). Another important structure in the brain is the central complex (Figure 1-4) which comprises the ellipsoid body, the fan shaped body, noduli, and the protocerebral bridge. The ellipsoid body (eb) is the anterior most part of the central complex and is a round, dough-nut shaped neuropil, composed of 16 radial segments representing the termini of neurons originating in the protocerebral bridge (pb). The fan

shaped body is a far more elaborate structure with arborizations and termini also originating from the pb. The nodule represents two ball shaped neuropils and show concentric layers. The pb is composed of commissural fibers connecting the two dorsal lobes of the protocerebrum (Hanesch et al., 1989). The central complex is critical for locomotion and spatial orientation (Martin et al., 1999; Neuser et al., 2008). The other functional centers of the fly brain that have been studied in depth are the suboesophageal ganglion important in taste and feeding behaviors and the antennal lobes that relays olfactory information from the antenna to the higher brain centers.

An effective way of identifying the minimum neural structures where a gene is required for a learning function is by expressing a UAS driven cDNA in different regions of the brain using multiple Gal4 lines to potentially rescue a poor learning phenotype. Such analysis has been effective in localizing *rut* dependant short term olfactory memory to the mushroom bodies. In the acquisition of spatial memory, the ventral ganglion, antennal lobes, and median bundle have been identified as likely candidate structures (Putz and Heisenberg, 2002; Zars et al., 2000a; Zars et al., 2000b).

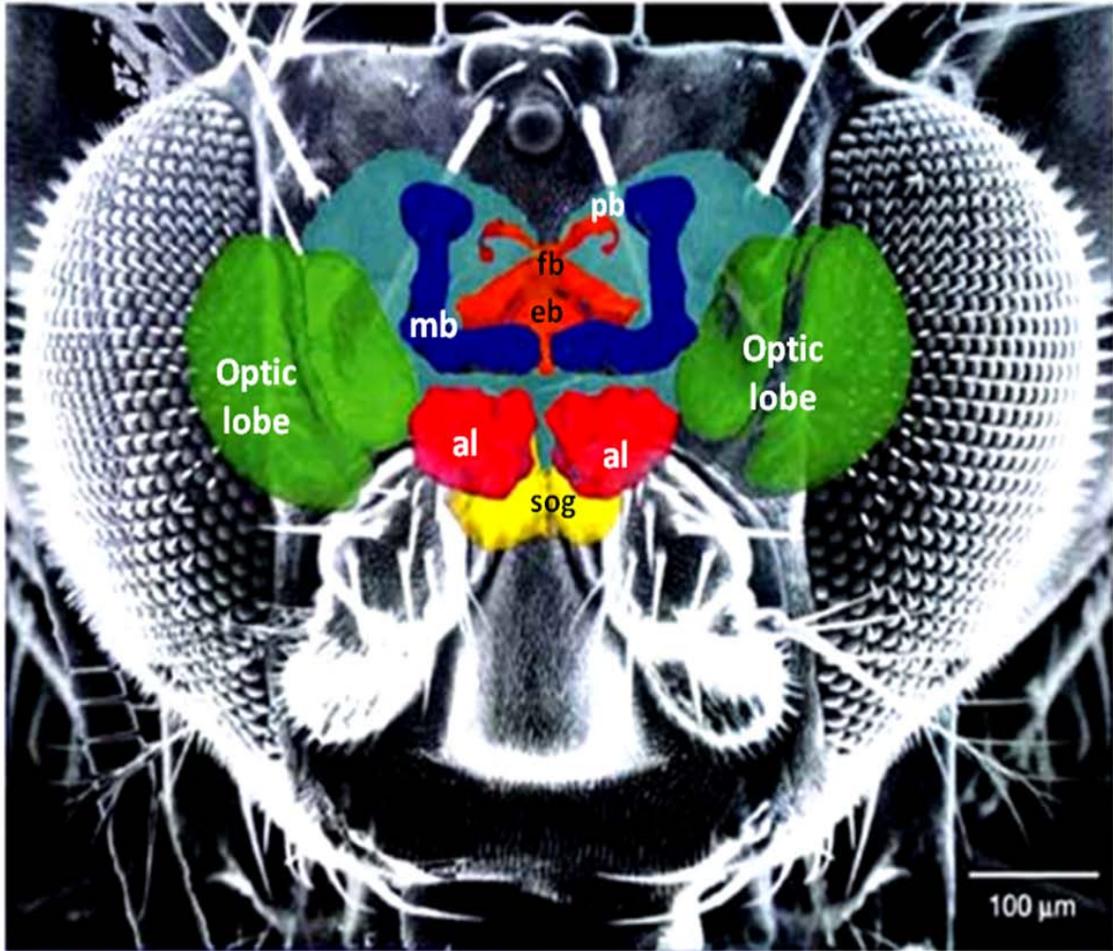


Figure 1-4. Schematic of a *Drosophila* brain. Major neuropils are color coded, green represents the optic lobes, red (ventral)- antennal lobes, yellow-suboesophageal ganglion, blue-mushroom bodies, red above mb(dorsal)- central complex (eb- ellipsoid body, fb- fan shaped body, pb- protocerebral bridge). Grey in the background represents neuropil surrounding mb and cc. Image adapted from (Heisenberg, 2003).

Genetic tools and approaches to study *Drosophila* neurobiology

A gene is considered necessary for a process if null or hypomorphic mutants disrupt, reduce or enhance performance in that process. While, a gene is thought to be sufficient for a process if expression of that gene in a select set of cells rescues the phenotype of a mutant fly. Sufficiency is also used in the context of a system constituting a neuron or a set of neurons. Such a system is thought to be sufficient when its activation alone produces a behavior. Genetic analysis in *Drosophila* has made it possible to identify the necessity and in some cases sufficiency of several genes and systems in innate and learned behaviors. Multiple genetic tools such as P-element insertions, mutations, deletions, genome sequence and UAS-Gal4/Gal80 have aided the understanding of mechanisms of learning and memory processes.

To control gene expression spatially and temporally in identified neurons and circuits the UAS-GAL4 expression system has been used extensively (Brand and Perrimon, 1993). GAL4 encodes a protein that activates transcription in the yeast *Saccharomyces cerevisiae* (Laughon et al., 1984; Laughon and Gesteland, 1984)(Figure 1-5). It directly binds to the Upstream Activating Sequence (UAS) in a sequence specific manner; the UAS is the promoter region from yeast analogous to the enhancer in multicellular organisms. In *Drosophila*, a promoter (or enhancer) directs the expression of the transcription activator Gal4 in a particular set of cells. Gal4 in turn directs transcription of the Gal4-responsive UAS-tagged gene in a cell and tissue specific manner (Brand and Perrimon, 1993). To achieve additional spatial and temporal control over the Gal4 driven UAS transgene expression, a Gal80 system from yeast has been developed for use in *Drosophila* (Lee and Luo, 1999). Gal80 encodes a transcriptional repressor and binds to Gal4 protein and prevents the expression of the UAS-transgene (Figure 1-5) (Lue et al., 1987). Fusing Gal80 with a promoter or enhancer further refines and restricts the expression of the Gal4 (Figure 1-5). To add temporal control to cell specific expression a temperature sensitive Gal80 protein (Gal80^{ts}) is expressed ubiquitously from the tubulin promoter. At a permissive temperature (22°C),

Gal80^{ts} binds Gal4 and inhibits gene expression, whereas at a non permissive temperature (30°C), the Gal80^{ts} protein misfolds and does not bind Gal4 and gene expression occurs (McGuire et al., 2003).

For effective mapping and manipulating of neural circuits targeted by promoter/ enhancer Gal4/Gal80 lines several UAS-transgenes are available. These transgenes alter neurons and neuronal circuits by blocking neurotransmitter release, reducing excitability or by killing them with a cell death gene. Transgenes like UAS-Tetanus toxin light chain (TNT) and UAS-*shibire*^{ts} (*shi*^{ts}) alter and disrupt chemical neurotransmission by targeting n-synaptobrevin (*syb*) and dynamin respectively (Kitamoto, 2001a; Scholz et al., 2000; Sweeney et al., 1995). However, they are not effective in circuits involving electrical synapses or signaling by hormones and some neuromodulatory factors (Bhattacharya et al., 2002; Thum et al., 2006). In the majority of the targeted neurons, UAS-TNT expression has been successful and provided useful insights into behavior, but in some cases it does not block neuronal output, which could be due to compensation from cellular synaptobrevin (Bhattacharya et al., 2002). Additional transgenes such as UAS-*hid* and UAS-*rpr* have been developed that can be expressed to genetically ablate targeted regions by inducing apoptosis (Zhou et al., 1997).

A second set of UAS-transgenes are used to alter the excitable properties of neurons. These alter membrane potential and hyperpolarize or partially depolarize the target neurons. Transgenes that reduce excitability of target neurons mainly involve over-expression of K⁺ channels that are open at resting membrane potential and lead to K⁺ efflux and membrane hyperpolarisation (Baines et al., 2001). Two of these, EKO and dORK encode a genetically engineered dominant negative Shaker potassium and an outward rectifying two pore leak potassium channel respectively (Nitabach et al., 2002; White et al., 2001b). The Kir2.1 transgene expresses an N-terminal GFP tagged human inwardly rectifying KCNJ2 potassium channel (Baines et al., 2001; Paradis et al.,

2001). These transgenes have the capability to electrically silence neurons by altering potassium conductances and are capable of inhibiting a wider set of neurons compared to TNT and *sh1^{ts}*.

Increasing excitability on the other hand is appealing to a neuroscientist as it allows identification of sufficient systems that can influence behavior. Excitability of target neurons can be increased by increasing sodium and calcium conductance (Nitabach et al., 2006; White et al., 2001a). Non-specific cation channels such as temperature sensitive trps, dTrpA1 and TrpM8 activate at different temperatures (Hamada et al., 2008; Peabody et al., 2009). dTRPA1 is the *Drosophila* ortholog of the mammalian transient receptor potential channel TrpA1 (Patapoutian et al., 2003). dTrpA1 is a temperature- and voltage-gated cation channel that regulates *Drosophila* thermotactic behavior. Although dTRPA1 is normally expressed in a small subset of fly neurons, previous work has shown that when dTRPA1 is expressed ectopically throughout the nervous system, dTRPA1 triggers paralysis in response to small temperature increases from 24°C (Hamada et al., 2008; Rosenzweig et al., 2005). Recently, dTRPA1 has been used as a tool to activate neural circuits in behaving flies using temperature stimulation (>28°C) (Parisky et al., 2008). On the other hand, Trpm8 is a mammalian trp channel that opens in response to cold temperatures (15-18°C) and is sufficient in executing wing expansion upon expression in specific neurons and activation (Peabody et al., 2009). A dominant negative ATPase pump has also been used recently in *Drosophila* carrying a point mutation in the ATP hydrolyzing site. The expression of dn-ATPase in target neurons increases the excitability without directly effecting ion conductances (Parisky et al., 2008; Sun et al., 2001). Other conditional effectors such as UAS-P2X2 and UAS-ChR2 activate, while UAS-NpHR inhibit target neurons upon stimulation by light of specific wavelengths (Arenkiel et al., 2007; Hwang et al., 2007; Lima and Miesenbock, 2005; Schroll et al., 2006). The most successful approaches in determining neural control of behavior use multiple approaches. With consistent results from different manipulations, one gains confidence with interpreting the data.

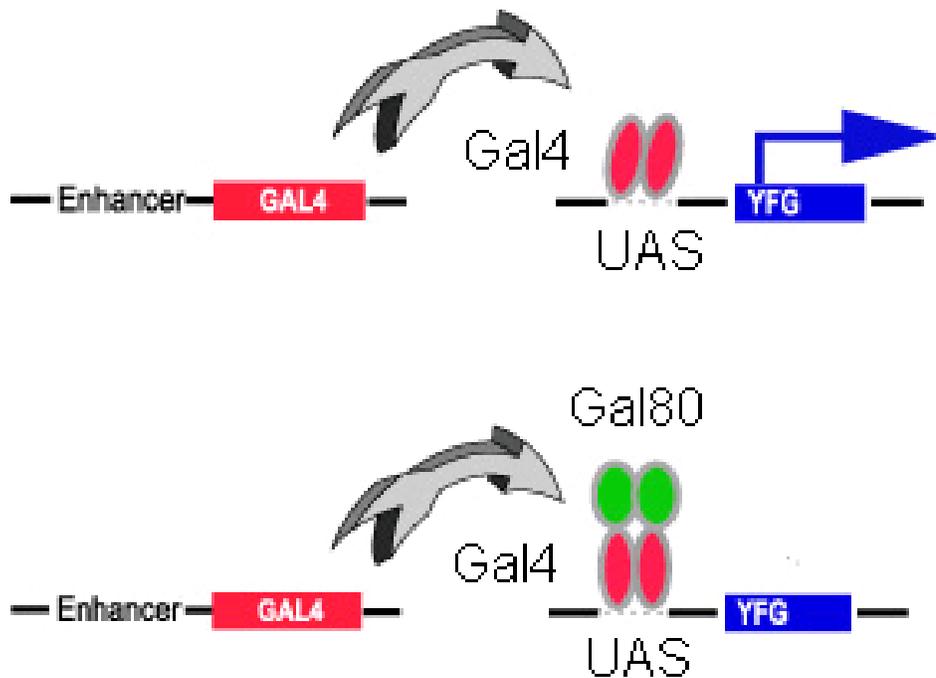


Figure1-5. The UAS-Gal4/Gal80 system. The top panel shows expression of Gal4 (red) under the control of a promoter and enhancer, binds to the UAS (upstream activating sequence) driving the expression of YFG (your favorite gene) in cell / tissue specific manner. The bottom panel shows Gal80 (green) driven by another specific enhancer binds to Gal4, preventing activation of UAS transgene. Image adapted from (McGuire et al., 2003).

Reinforcement signaling mechanisms in *Drosophila*

An important goal of this dissertation research is to identify and study the neural systems that are recruited for reinforcement of place learning. The genes encoding biogenic amine biosynthesis enzymes (Figure 1-6) provide the basis for manipulating these neurons to identify their role in reinforcement signaling in the heat box. In *Drosophila*, these genes have been targeted in designing Gal4 lines and the enzymes themselves can be inhibited pharmacologically. Five biogenic amines namely dopamine, octopamine, serotonin, tyramine, and histamine have a neural function in *Drosophila*. In classical olfactory conditioning the upstream processes mediating the electric shock and sugar reward information have been identified as being dopaminergic and octopaminergic respectively (Claridge-Chang et al., 2009; Schroll et al., 2006; Schwaerzel et al., 2003).

As part of this dissertation we have explored the reinforcement role of dopamine, serotonin, tyramine, and octopamine using pharmacological treatments and UAS-Gal4/ Gal80 system. Histamine has not been examined for place learning because of its function in temperature sensation (Hong et al., 2006). Biogenic amines exert their signaling effects by binding to specific membrane proteins that primarily belong to the superfamily of G protein-coupled receptors. Aminergic systems could be the upstream signals that feed into downstream associative processes involving cAMP/PKA or other molecular signals (Davis, 2004; Zars, 2010). Therefore these upstream processes could be functioning at a so-called comparator level (see Discussion) while the downstream processes are critical in associative learning and coincidence detection (Diegelmann et al., 2006).

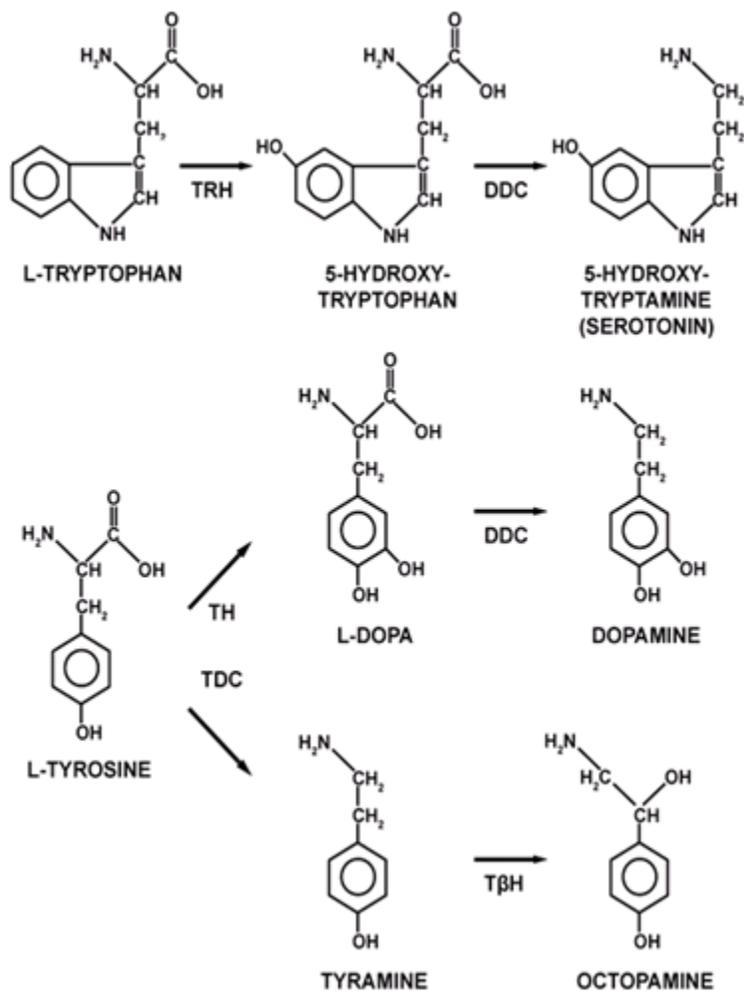


Figure 1-6. Biogenic amine biosynthesis pathway in *Drosophila*. Top panel shows enzymatic steps catalyzed by tryptophan hydroxylase and dopamine decarboxylase to convert tryptophan to 5-hydroxytryptophan (5HTP) and 5Hydroxytryptamine or serotonin (5HT). Bottom panel shows Dopamine and Octopamine synthesis. Dopamine is synthesized in two steps, Tyrosine to L-Dopa catalyzed by Tyrosine hydroxylase and L-Dopa to Dopamine mediated by dopamine decarboxylase. Octopamine and Tyramine are also synthesized from Tyrosine. Tyrosine is converted to Tyramine by Tyrosine decarboxylase followed by Tyramine β hydroxylase that converts Tyramine to Octopamine [Image adapted from (Cole et al., 2005)].

CHAPTER 2. MATERIALS AND METHODS

Fly rearing

All flies used were of the *Drosophila melanogaster* species. Flies were raised on cornmeal-based media in a light- and humidity-controlled chamber (12:12-hr light: dark cycle, 60% relative humidity). Flies used for behavioral experiments were <7 days old and were never anesthetized.

Fly stocks and crosses

All flies tested in the heat box paradigm were outcrossed with cantonized w^{1118} for a minimum of six generations and the X chromosome was replaced with wild-type using CS/Fm7;Cyo or CS/Fm7;TM3. The genotype of the flies used in the experiments is listed in Table 2-1.

Pharmacological Treatments

Two to four day old wild-type CS flies were exposed to drugs in 1% agarose and 1% sucrose for 48 h. The sham control flies were in identical vials except without drug. The drugs α -methyl tyrosine, am-Y (2 mM) and alpha-methyl tryptophan, am-W (20 mM) were mixed with cooling but melted agarose/sucrose solution and allowed to harden. Food coloring was added to the solution to monitor feeding behavior with the drugs. For pre-exposure experiments the fly food for pharmacological treatments was supplemented with 1% hydrolysable yeast (Ja et al., 2007).

Immunohistochemical analysis of the fly brain

Fly brains were dissected in Ringer's solution (130 mM NaCl, 0.7 mM KH_2PO_4 , 0.35 mM Na_2HPO_4 , 18 mM MgCl_2 and 4.7 mM KCl) by removing the proboscis and the eyes with fine forceps. The brains were then fixed in 2% formaldehyde for 8-10 hours at 4°C. Fixed brains were blocked for 2-3 hours in normal goat serum (3% in PAT, composition outlined below) followed by overnight incubation with a primary antibody (e.g. anti-5HT 1:10, nc82 1:10 or anti-GFP 1:100) at 4°C. The brains were then incubated with the secondary antibody (Alexa 647 goat anti-mouse 1:100 and Alexa 488 goat anti rabbit 1:100 or 1:250) for 4 hours at room temperature or overnight at 4°C. All antibody incubations were followed by three 10 minutes washes with PAT (100 ml 1XPBS, 1g BSA and 0.5 ml Triton X-100). All antibody solutions and dilutions were also made in PAT. The whole brain was mounted on Vectashield medium (1:3 Ringers: Vectashield) in a narrow well made from coverslip slides. The whole mount brains were visualized using LSM 510 NLO confocal microscope with either 20X or 40 X oil immersion objectives. Images were visualized using the LSM examiner software available on the Zeiss website (<http://www.zeiss.de>).

Reverse Phase HPLC for quantitative estimation of biogenic amines

Fly heads or fly brains were isolated and homogenized in mobile phase using a plastic pestle [MDTM/ acetonitrile (85:15); (MDTM = 75 mM NaH_2PO_4 , 1.7 mM 1-octanesulfonic acid, 100 $\mu\text{l/liter}$ triethylamine, 25 mM EDTA, adjusted to pH 3 with phosphoric acid)]. To further extract the amines, samples were sonicated on ice for 1 minute. The lysate was centrifuged for 5 minutes at maximum speed using an Eppendorf table top centrifuge maintained at 4° C. The supernatant was used for HPLC analysis of amines. The HPLC set up included an ESA model 582 isocratic pump, a Thermoseparation AS3500 autosampler (20 μl) and detection with an ESA CoulArray detector with potentials set at 25, 500, 650, and 800 mV. A prodigy C_{18} 5 μm (250 × 4.6 mm) column (Phenomenex) and a Phenomenex Securityguard C_{18} precolumn were used with a

mobile phase pumped at 0.8 ml/min for the HPLC followed by ECL detection. Serotonin levels were quantified by using standard curves generated in parallel (serotonin >98% purity; Sigma). Octopamine levels were detected by using the same HPLC column, but with a mobile phase of 50 mM citrate acetate buffer, 11 mM octanesulfonic acid, pH 4.5/acetonitrile (80:20) and quantified by comparing peak areas to a standard curve (octopamine >95% purity; Sigma). The reverse phase HPLC experiments were conducted at the Veterinary Medical Diagnostic lab in association with Dr. Yin-Chieh Chen and Dr. George Rottinghaus.

Enzyme immunoassay for quantitative estimation of dopamine

Fly heads were isolated and homogenized in 0.01 N HCl using a plastic pestle. The lysate was centrifuged for 5 minutes at maximum speed using an Eppendorf table top centrifuge. The lysate was loaded onto a 96 well plate for extraction and acylation followed by enzyme immunoassay using dopamine anti-serum *as per* the instructions on LDN BA10-5300 dopamine EIA kit. The samples were compared to standards provided in the kit and 0.01 N HCl was used as blank to read the absorbance / optical density at 450 nm using a Tecan-Magellan plate reader. Data was analyzed using a plot of the linear mean absorbance readings of the standards. The analyte concentrations of the controls and unknowns were calculated from the standard curve.

Western Blotting for detection of proteins in fly heads

Proteins were extracted from fly head in 5% Laemelli buffer (62 mM Tris Buffer pH7, 2% SDS, 10% glycerol, 0.01% Bromophenol blue). The fly heads were homogenized using a plastic pestle in a 1.5 ml Eppendorff tube. The lysate was centrifuged at maximum speed for 5 minutes and the supernatant was run on 8% SDS-PAGE gel at 100V. The proteins separated on the gel were transferred onto a PVDF membrane for 4 hours at 25V using western transfer buffer [pH 8.3 (25

mM Tris base, 200 mM glycine, 20% Methanol and 0.037% SDS)]. The PVDF membrane was incubated with 5% non fat dry milk (NFM) for 5-30 minutes in TBST (10 mM Tris pH 8, 130 mM NaCl, 5 mM KCl, 0.1% Tween-20) followed by 5 minutes rinse with TBST. After the blocking step with NFM the immunoblot was incubated in primary antibody using 1:100 Anti-ABCG4 (Abcam, Inc) overnight at 4°C followed by three minutes TBST washes. The primary antibody incubation step was followed by secondary antibody incubation with HRP conjugated goat anti-mouse antibody for 60 minutes followed by three 5 minute TBST washes. To visualize the bands, the immunoblot was incubated with 1.5 ml of luminol and peroxide each (HRP tagged super signal) followed by visualization using X-ray film. Initial western blot analysis was carried out in collaboration with Dr. Per Stromhaug.

Table 2-1. Genotype and source of flies used in genetic and behavioral experiments

Fly strain and genotype	Donor	Reference
CS	Wild-type flies. Wuerzberg collection	
wCS 13	<i>w¹¹¹⁸</i> flies outcrossed with CS for 13 generations	(Dura et al., 1993)
Ddc-Gal4(X)	Bloomington stock collection	(Li et al., 2000)
Ddc-Gal4(II)		
Th-Gal4(III)	Bloomington stock collection	(Friggi-Grelin et al., 2003)
Th-Gal80(II)	Toshi Kitamoto	(Sitaraman et al., 2008)
UAS-RNAi- <i>white</i>	Dean Smith	(Kalidas and Smith, 2002)
w-adh;ry506(X)	James Birchler	(Pal-Bhadra et al., 1997)
Adh fn6;ry506(II)		
UAS-mCD8GFP(II)	Bloomington stock collection	(Lee and Luo, 1999)
UAS-nlsGFP(III)	Bloomington stock collection	
Trh-Gal4(II)	Serge Birman (ESPCI)	Unpublished
Tph-Gal4(II)	J. Kim (KAIST)	(Park et al., 2006)
UAS-Eag Δ 932(II)	Bloomington stock collection	(Broughton et al., 2004)
UAS-NaChBac(III)	Bloomington stock collection	(Nitabach et al., 2006)
UAS-Kir2.1(III)	B. H. white	(Baines et al., 2001)

5HT1b-Gal4(III)	Amita Sehgal	(Yuan et al., 2005)
UAS- RNAi- <i>5HT1b</i>	Amita Sehgal	
Gal80ts (III)	Bloomington Stock Collection	(McGuire et al., 2003)
UAS-TNT(II)	Henrike Scholz	(Scholz et al., 2000; Sweeney et al., 1995)
UAS-dTrp1a(II)	Leslie Griffith	(Hamada et al., 2008)
UAS-dnATPase	Leslie Griffith	(Parisky et al., 2008; Sun et al., 2001)
Tdc-Gal4(II)	Wuerzberg collection, Sarah Certel	(Cole et al., 2005)
<i>TβH^{nm18}</i> /Fm7	Wuerzberg collection	(Monastirioti et al., 1996)

Behavioral experiments in the heat box

Experimental protocols used in the heat box were of three types, direct conditioning, pre-exposure or thermosensitivity. For direct conditioning experiments a fly is allowed to run in a small chamber (29X4X2mm) that is heated or cooled to a defined temperature. A chamber half is associated with high aversive temperature ($>37^{\circ}\text{C}$) such that every time the fly goes to this so-called punished zone the chamber heats up to the aversive temperature (Wustmann et al., 1996; Zars et al., 2000b). This temperature change, requiring several seconds, occurs when the fly crosses an invisible midline. When the fly returns to the previous chamber half, the whole chamber quickly reverts to a defined baseline temperature. The baseline temperature of 24°C was used as flies have a strong preference for this temperature over both higher and lower temperatures (Sayeed and Benzer, 1996; Zars, 2001). A Performance index (PI) is calculated by subtracting the time spent on the side associated with reinforcement from the time spent on the non-reinforced side and dividing this by the total time. Thus, a scale of -1 to 1 is generated with a total preference for the reinforced side giving a -1 and for the non-reinforced side a 1 . In all direct conditioning experiments, a 30-s pre-test was followed by training of different lengths (0–20 min) and a 3-min post-test. Training periods of 4 minutes and 20 minutes are defined as short and long training protocols. The spatial task acquisition occurs within 4 minutes and an asymptote is reached at 10 minutes of training (Diegelmann et al., 2006). Reinforcement temperatures were changed as indicated in the figure legends, for most direct conditioning experiments 41°C was used as measured at the surface of the peltier elements with a thermocouple.

For pre-exposure experiments flies were individually trained in the heat box chambers. These flies were exposed to a high temperature (either 30 or 41°C) in 1-min blocks such that the entire chamber heated up to this exposure temperature. The number of blocks ranged from 0 to 3 followed by a 1 min pause, before the next block started. In the time between the pre-exposure and conditioning a gap of 1–20 min was allowed in which flies usually remained in the chamber

maintained at 24°C. Conditioning, as above, used either 30 or 41°C as negative reinforcement. Training duration was 4, 10, or 20 minutes and was followed by a 3 min memory post-test. The 3-min memory performance is presented as a Performance Index (PI). An equal number of experiments paired high temperature with the back or front half of the chamber, which makes the effect of any potential side preference in the chambers negligible.

The thermosensitivity assay used the same chambers but the temperature inside was altered independent of the flies' behavior. Individual flies were presented with a chamber that initially was at 24°C on both sides, but then on one side increased to a probe temperature of 24-45°C, while the other chamber half was kept at 24°C (Zars, 2001). Different flies were presented with opposite chamber halves at 24 and probe temperature (in the range of 24-45°C) to avoid the effects of potential spontaneous chamber half preferences. A PI was calculated for this assay as in the learning assay.

Olfactory conditioning

Classical olfactory conditioning paired one of two odorants (4-methylcyclohexanol and octanol) with an electric shock aversive reinforcer (100 V) (Tully and Quinn, 1985) and 1M sucrose appetitive reinforcer (Schwaerzel et al., 2003). Memory tests were performed 3 minutes, 3, or 6 hours after training. Changed olfactory preferences were tested in a T-maze in the memory test. For appetitive learning flies were starved for 16-20 hours with water before testing. During training for appetitive learning flies were exposed to odorants paired with water or 1M sucrose for 2 minutes while for aversive shock learning flies were exposed to odorant paired with 12, 1.2 sec 100V shock pulses for 1 min during training. Control experiments measured flies' avoidance of the odorants or shock used in the conditioning experiment. That is, an odorant at the same concentration used in the conditioning experiments was presented in one arm of the T-maze. The other arm of the T-maze had ambient air. In the shock test, two shock tubes were placed at the T-

maze choice point and one of these was pulsed with 100-V electric shocks. In both control experiments, flies were allowed to choose between the two tubes for 1 min (the same amount of decision time used in the learning experiments). A PI was calculated for the learning and control experiments and multiplied by 100. This scale ranges from –100 to 100, with 0 indicating no memory or avoidance behavior. This was calculated by subtracting the number of flies choosing the shock-associated odorant from the number of flies choosing the non-shock-associated odorant, divided by the total number of flies in a "half test." An average PI was calculated from a pair of half-test PIs, where each half came from conditioning of one of the two odors.

Statistical analysis

For statistical analysis of performance indices in the heat box tests of normality give mixed results (Putz and 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. For more than two independent samples the non-parametric Kruskal Wallis ANOVA and multiple comparisons of mean ranks for groups were done. On the other hand for olfactory conditioning data, tests for significant differences used a parametric ANOVA and Newman–Keuls *post hoc* tests (Zars et al., 2000a). All statistical analyses were performed using the Statistica program (StatSoft, Tulsa, Oklahoma).

CHAPTER 3: SEROTONIN MODULATION OF PLACE LEARNING

Introduction

Biogenic amines are important neuromodulators of learning and memory processes

Biogenic amines act as neurotransmitters and modulators of key signaling pathways that are highly conserved across species. Evidence from several vertebrates and invertebrates suggests recruitment of aminergic circuits in associative learning and behavioral plasticity. For example in monkeys dopaminergic neurons are activated preferentially in response to reward or reward predicting stimuli (Schultz, 2002). In honeybees, there is evidence that octopamine and dopamine are required for appetitive and aversive olfactory conditioning. Further, electrical stimulation of a single neuron VUMmx1 that belongs to a group of octopamine immunoreactive neurons can replace the reinforcing component of sucrose reward in olfactory conditioning (Hammer, 1993; Hammer and Menzel, 1998; Menzel and Muller, 1996). These results are similar to those in crickets, where dopamine and octopamine receptor antagonists impair aversive and appetitive olfactory learning. Serotonin, tested in honeybees impairs appetitive learning (Menzel et al., 1999). Thus far a clear role for serotonin in insect learning as a whole has not been explored.

Different aminergic systems are critical for appetitive and aversive memory in *Drosophila*

Classical olfactory conditioned memory in *Drosophila* can be distinguished by the requirement for different catecholamines: dopamine for aversive and octopamine for appetitive conditioning

(Schwaerzel et al., 2003). Recent evidence from *Drosophila* larvae and adult flies shows activation of dopaminergic neurons paired with an odor is sufficient to induce an aversive memory (Claridge-Chang et al., 2009; Schroll et al., 2006) and alternatively octopaminergic / tyraminergetic neuron activation induces appetitive memory in larvae (Schroll et al., 2006).

Because dopamine seems to be important in negative reinforcement learning in insects, we hypothesized that it is also important for place learning. The earliest evidence of mechanisms possibly involving amines in place learning came from previous work in the laboratory with the *white* gene. *white* mutants exhibit severe reinforcement processing deficits in the heat box (Diegelmann et al., 2006), (Figure 3-2). The *white* gene in *Drosophila* belongs to the ABC (ATP binding cassette) transporter super family (Dean et al., 2001). ABC transporters bind and hydrolyze ATP to power translocation of a wide variety of molecules across the cell membrane. The *white* gene encoded half size ABC transporter forms a dimer with either Scarlet or Brown. The White-Scarlet dimer and White-Brown dimer transport tryptophan and guanine respectively into at least some pigmented tissue (Sullivan and Sullivan, 1975). Tryptophan is an aromatic amino acid and a precursor of serotonin and kyneurine. Guanine is converted to a cofactor, tetrahydrobiopterin (BH4) in two steps; BH4 is essential for serotonin and dopamine synthesis reviewed in (Kaufman, 1993). Keeping with the role of White ABC transporter in making available these biogenic amine precursors to some cells in the fly brain, behavioral impairments in *white* mutants could result from lack of neuromodulation by dopamine or serotonin release. Mechanistically, how *white* mutation influences learned and perhaps other behaviors is not known (Campbell and Nash, 2001; Hing and Carlson, 1996; Hoyer et al., 2008; Lee et al., 2008).

Results

Biogenic amine regulation by *white* gene function

The *white*-ABC transporter is necessary for asymptotic memory performance in the heat-box. Multiple lines of evidence show that the *white*-ABC transporter is necessary for place learning in the heat-box (Diegelmann et al., 2006). *white* mutant flies show reduced asymptotic memory levels after long training, exhibiting reinforcement processing deficits (Figure 3-1 A). Since, *white* is important for availability of biogenic amine precursors this phenotype could result from impaired or reduced amine neuromodulation. To address this directly we measured the levels of dopamine, serotonin and another behaviorally relevant amine, octopamine in w^{1118} and CS fly heads (Figure 3-1 B, C, and D). Interestingly, we found that w^{1118} had severely reduced levels of serotonin and dopamine ($\approx 30\%$) as compared to wild-type CS flies (Sitaraman et al., 2008). Although the octopamine level in *white* mutant flies is $\approx 80\%$ of normal, this reduction does not reach significance. Thus, low serotonin and dopamine levels are correlated with abnormal place conditioning.

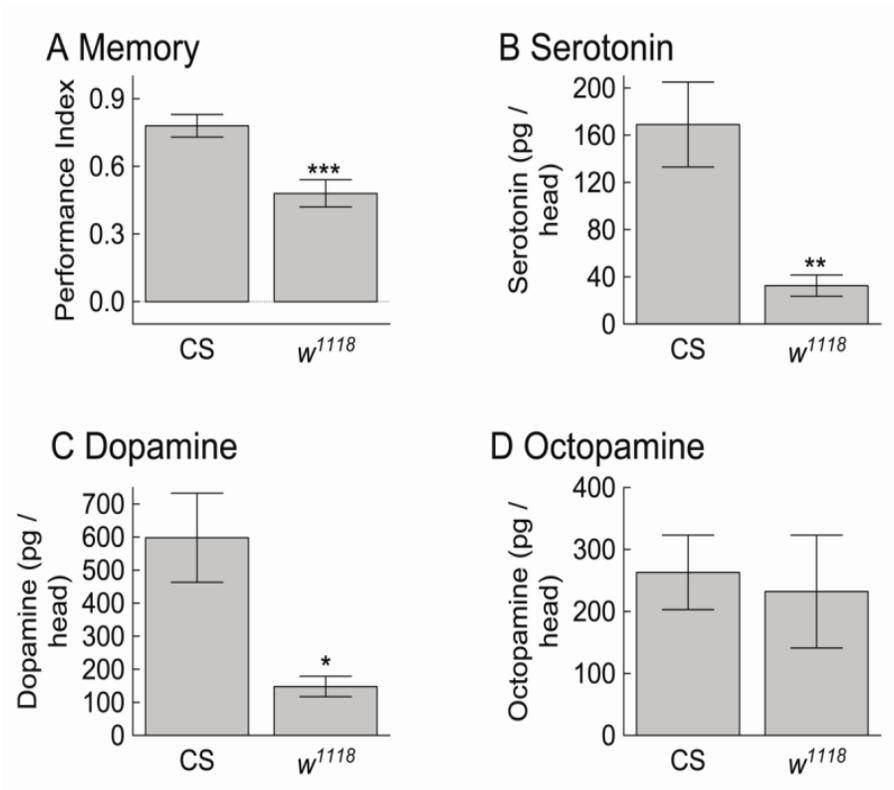


Figure 3-1. Comparison of memory and biogenic amine levels in CS and w^{1118} flies. A) w^{1118} flies have reduced memory levels after long training (20 minutes) protocol as compared to CS flies. B) Serotonin levels are significantly reduced in w^{1118} as compared to CS flies. Serotonin and Octopamine levels (D) were measured using a C18 RP-HPLC followed by electrochemical detection. C) Dopamine levels measured using an enzyme immunoassay and are significantly reduced in w^{1118} flies as compared to wild-type CS flies. Bars represent are means \pm SEMs marked significant as * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ (Sitaraman et al., 2008).

The Serotonin / dopamine system is necessary for conditioning

A direct way of identifying a role for *white* in dopaminergic and serotonergic neurons would be to decrease levels of *white* transcripts *in vivo* and then ask if this manipulation negatively influences learning in the heat box. Previously, it has been shown that the White-ABC transporter levels can be reduced using an RNAi-*white* transgene under UAS control (Kalidas and Smith, 2002). Expression of UAS- RNAi-*white* construct driven by GMR-Gal4 in the retina reduces eye pigmentation in flies carrying four mini *white* genes (Kalidas and Smith, 2002) or with a wild-type *white* gene at the normal gene position (Figure 3-2).

White transporter functions in serotonergic and dopaminergic neurons to modulate place learning

After testing the efficacy in reducing *white* transcripts in the retina, we next expressed the RNAi-*white* transgene in the serotonergic / dopaminergic system using Ddc-Gal4 and the dopaminergic system using Th-Gal4 (Friggi-Grelin et al., 2003; Li et al., 2000). Place memory was tested after 20 minutes of training using 41 °C as the negative reinforcer. This temperature and training time was chosen as it conditions wild-type flies to have maximal performance index (Diegelmann et al., 2006). Only flies expressing the RNAi-*white* in Ddc-Gal4 positive neurons had conditioned memory deficits as compared to the genetic controls (Figure 3-3 A). The Th-Gal4 driven expression of the RNAi-*white* had no effect on conditioned behavior. In all genetic manipulations, there were no significant differences in the ability of flies to sense and avoid a 41 °C temperature source (Figure 3-3 B). These results indicate that either proper *white* expression in both systems is necessary for normal memory strength or that it may be required only in the serotonergic system.

A. GMR-Gal4

B. GMR-Gal4/RNAi-*white*

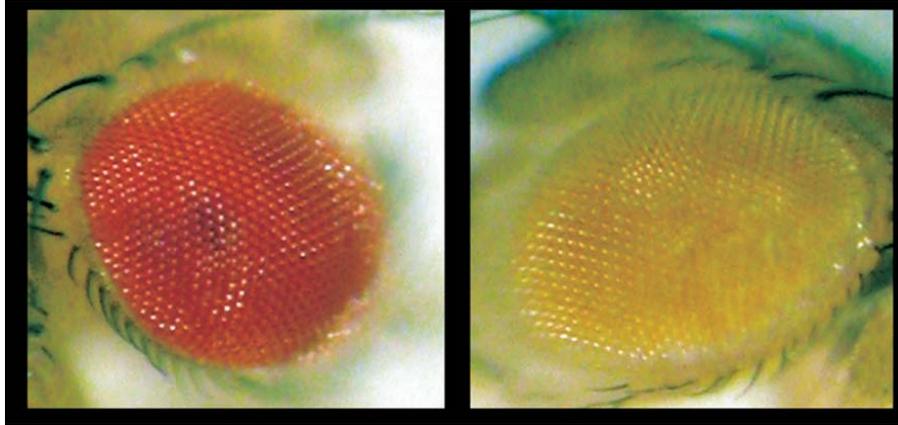


Figure 3-2. The UAS-RNAi-*white* transgene can effectively reduce *white* gene. A. The eye color of a fly with a wild-type allele of the *white* gene carrying the GMR-Gal4 driver alone is the typical brick-red like wild-type flies' eyes. B. Expression of UAS-RNAi-*white* in retina with GMR-Gal4 leads to a strong reduction in pigmentation of the eye, strongly supporting the conclusion that this transgene can reduce *white* gene expression.

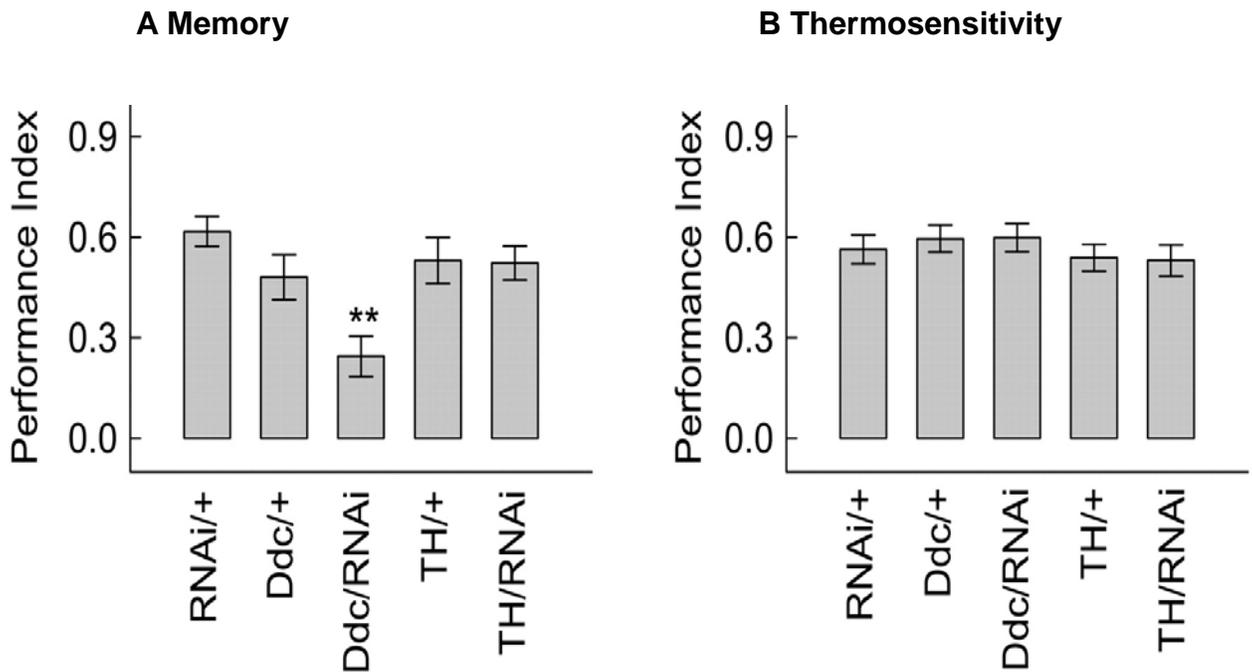


Figure 3-3. Reduced expression of the *white*-ABC transporter expression in the serotonergic / dopaminergic neurons reduces memory performance. When the UAS-RNAi-*white* transgene was expressed in the serotonergic / dopaminergic system with Ddc-GAL4 driver, flies' memory performance was strongly reduced compared to all control genotypes ($H(4, N = 643) = 28.3, p = 0.0000$). This was in contrast to Th-GAL4 driven UAS-RNAi-*white* expression, where no deficits were found. Significant differences after multiple comparisons are presented, Ddc-GAL4 / UAS-RNAi-*white* with UAS-RNAi-*white* and Ddc / +. Tests for the ability of flies with different genotypes to sense and avoid a 41 °C temperature source did not find significant differences between genotypes ($H(4, N = 382) = 3.37, p = 0.50$). The values represent means and error bars are SEMs, marked significant **= $p < 0.01$.

***white* and serotonin show co-localization in the fly brain**

One prediction of the Ddc-Gal4/ UAS-RNAi-*white* experiment is that the White-ABC transporter is expressed in the serotonin cells. To test this idea we determined the expression pattern of the *white* gene in the fly brain. *white* transcripts have been detected in fly heads that do not contain visual tissue using RT-PCR, but anti-serum against the protein has been difficult to work with (Campbell and Nash, 2001). Therefore, an indirect measure of *white* expression is required. One approach is to probe a functionally unrelated protein fused to the *white* promoter. We used this strategy to probe flies expressing Adh (Alcohol dehydrogenase) under the *white* promoter. *w*-Adh flies in an Adh null background (*adh^{fm6}*) were used to ensure detection of only the *white* promoter driven expression of Alcohol dehydrogenase (Pal-Bhadra et al., 1997). Double immunostaining of the adult fly brain using anti-ADH and anti-serotonin shows regions of colocalization of *white* and serotonin (n=5, Figure 3-4).

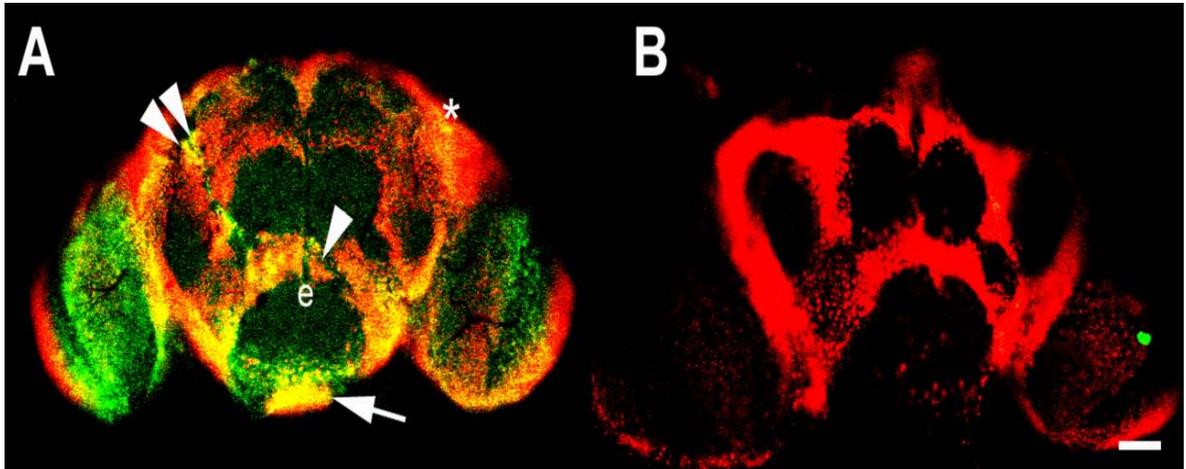


Figure 3-4. A *white*-promoter driving the expression of the *alcohol dehydrogenase* (*Adh*) gene indicative of *white* gene expression in the brain. A) Only in flies carrying the *w-Adh* transgene could *Adh* and serotonin co-expression be detected. These include the areas se1 and ip (arrowhead), se3 (arrow), lp1 (double arrowhead), and alp (asterisk) neurons. "e" marks the esophagus. The serotonin / *Adh* expression in the superior lateral protocerebrum likely corresponds to innervations sites of some of the serotonergic neurons. B) No *Adh* expression was detected in *adh^{fn6}* null mutant fly brains, although serotonin expression was detected in a similar pattern to that found in *w-Adh; adh^{fn6}* fly brains. The scale bar represents 50 μm .

Genetic manipulation of dopaminergic and serotonergic neurons

A second way of addressing the role of dopaminergic and serotonergic neurons in place learning is to alter the synaptic transmission of Ddc and Th neurons and ask if it affects performance in the heat box. Blocking synaptic activity by expressing tetanus toxin light chain (TNT) in dopaminergic neurons did not affect performance in the heat box while flies expressing TNT in both dopaminergic and serotonergic neurons had low performance (Figure 3-5). The pattern of heat box conditioning found in Ddc-Gal4 / TNT flies was similar to that observed in Ddc-Gal4 / RNAi-*white* flies. Further, the tested genotypes were not significantly different in their ability to sense and avoid 41° C (Figure 3-5).

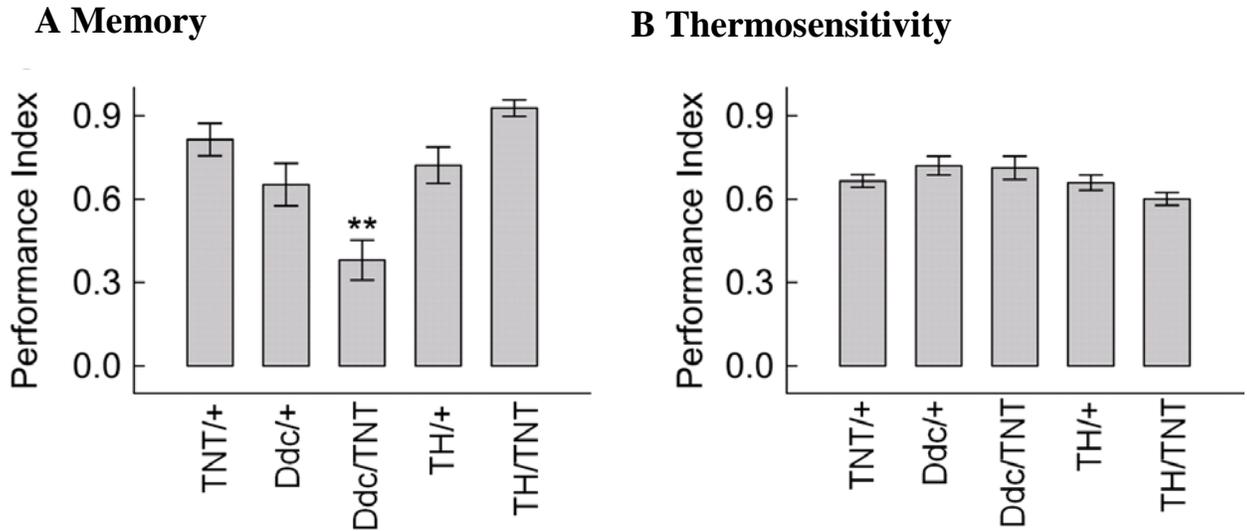


Figure 3-5. Serotonin and dopamine but not dopamine neurons are necessary for conditioning in the heat box. A) Ddc-Gal4 / TNT and Th-Gal4 / TNT flies expressing TNT in dopaminergic/serotonergic and dopaminergic neurons respectively were tested in the heat box. TNT/+, Ddc/+ and TH/+ represent the driver and transgene alone in a wild-type background. Only flies expressing TNT with the Ddc-Gal4 driver had a deficit in conditioned memory performance, multiple comparisons indicate significant differences between Ddc-Gal4 / UAS-TNT and both Ddc-GAL4 / + and UAS-TNT / + performances ([H (4, N = 643) = 28.3, $p < 0.0001$]). B) Tests for the ability of flies with different genotypes to sense and avoid a 41°C temperature source did not find significant differences between genotypes [H (4, N = 382) = 3.37, $p = 0.50$]. Values are means and error bars are SEMs, marked significant as **= $p < 0.01$.

Serotonergic requirement in spatial learning is not dependent on development

The genetic manipulation of the Ddc neurons thus far described affects the serotonergic and dopaminergic system through development. Any effect on conditioning could therefore be a combination of developmental and adult effects. The developmental and adult role of serotonergic and dopaminergic neurons in conditioning can be dissected by pharmacological manipulations that reduce the levels of dopamine and serotonin. Adult flies were fed α -methyl tryptophan (am-W), which competes with tryptophan for the active site of the enzyme Tryptophan hydroxylases (Trh), preventing normal serotonin synthesis (Dierick and Greenspan, 2007). Alternatively, flies were fed α -methyl tyrosine (am-Y) to inhibit TH activity and dopamine biosynthesis (Marican et al., 2004). Flies treated with am-W, but not am-Y, for 2 days as adult animals had memory performance deficits (Figure 3-6). We measured dopamine levels in fly heads to test the effectiveness of our am-Y feeding protocol. Flies fed am-Y had strongly reduced dopamine levels [am-Y: 29.9 ± 2.7 , sham: 507.4 ± 109.5 pg dopamine per head; $F(1, 10) = 19.0$, $p = 0.001$; $n = 6$ for each group]. Thus, our am-Y feeding reduces dopamine levels to 6% of normal, which does not alter memory performance. Also noted here is a reduced memory score in sham- and am-Y-treated flies compared with wild-type fly performance with a similar conditioning protocol but fed on cornmeal media (Figure 3-5 and 3-6). These differences could be caused by variation in flies performance over time or effects of the nutritionally restricted sugar-agarose media used for drug administration (Figure 3-6 A). Regardless of this somewhat reduced memory background, feeding am-W to adult flies has a dramatic effect on memory performance levels. Finally, feeding flies am-W had no effect on temperature avoidance behavior (Figure 3-6 B). These results support the conclusion that serotonin, but not dopamine, is necessary for place memory formation. Reducing serotonergic synthesis or output inhibits learning with the long training (20 minutes) but does not alter the ability of these flies to sense this temperature suggesting that serotonin is necessary for reinforcement processing in place learning.

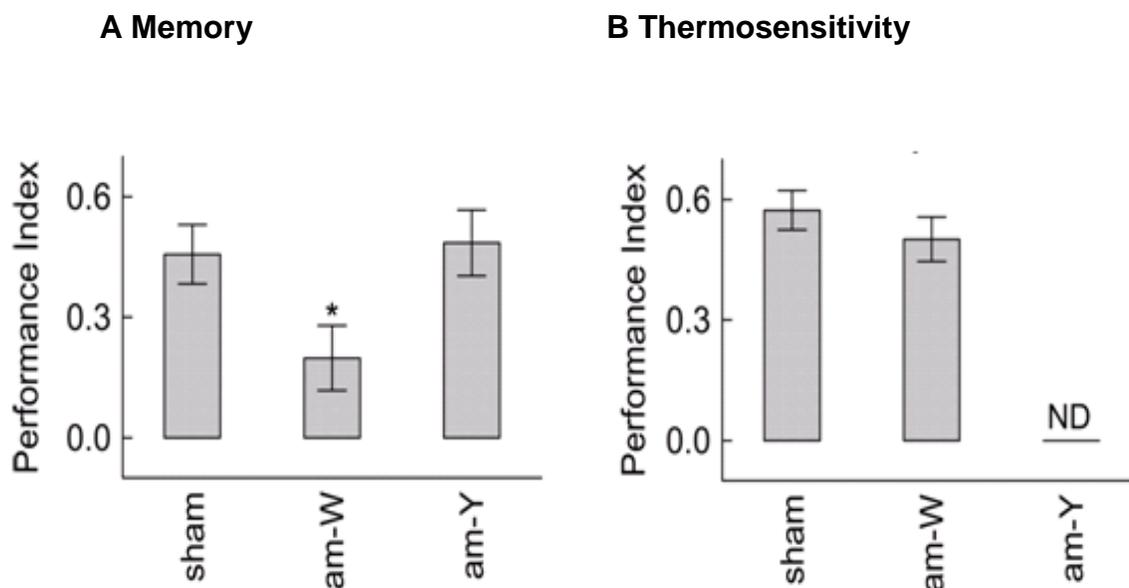


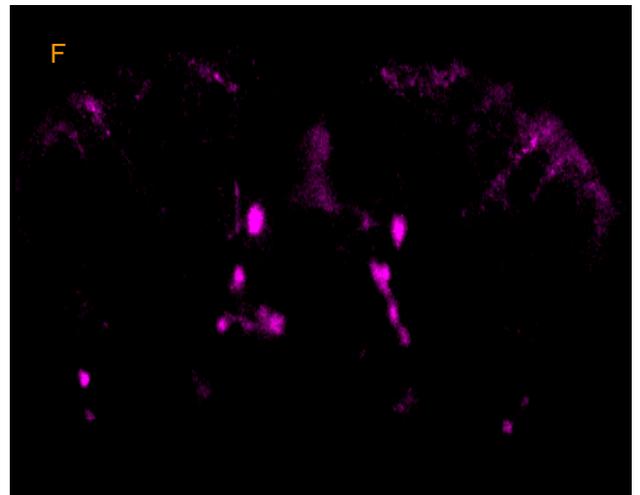
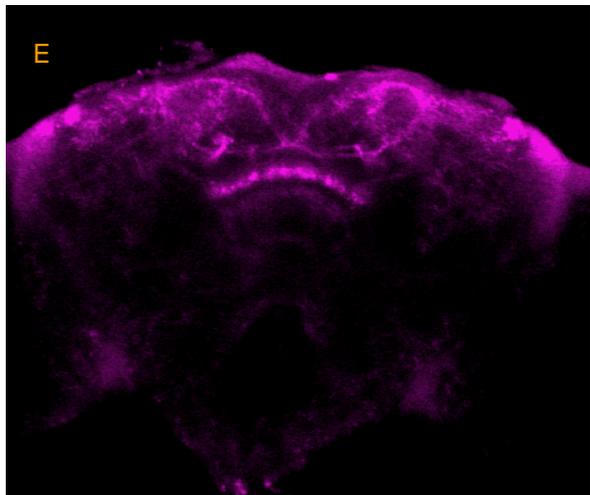
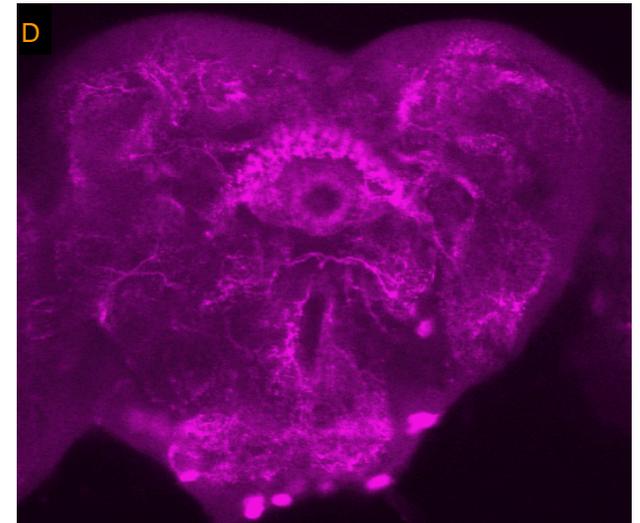
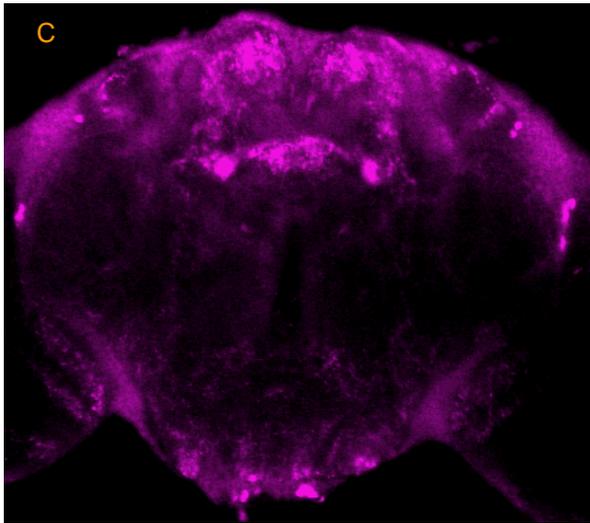
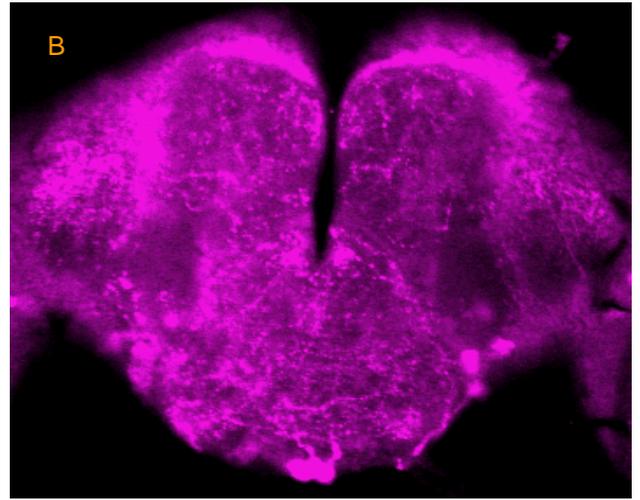
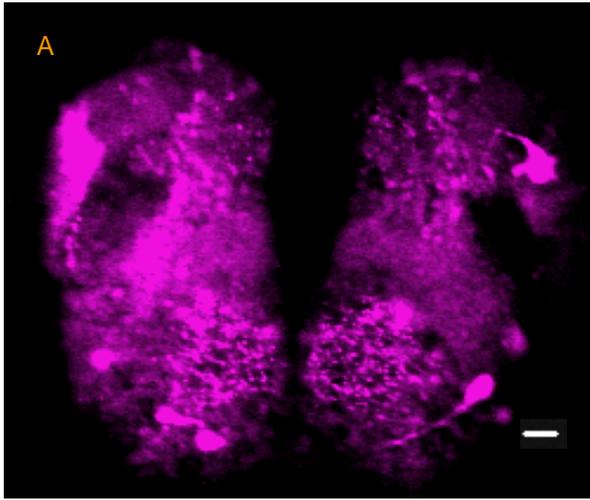
Figure 3-6. Pharmacological inhibition of serotonin but not dopamine synthesis leads to restricted memory performance levels. A) Adult flies fed am-W, reducing serotonin levels, had a significant difference in memory performance compared with flies fed on the media alone (sham) and flies fed am-Y, altering dopamine levels [H (2, n= 316) = 10.9, p = 0.004; multiple comparisons indicate significant differences between am-W-treated and both am-Y- and sham-treated flies (*, p < 0.05)]. B) Tests for the ability to sense and avoid a 41°C high-temperature source in the thermosensitivity assay found no significant differences between the treatment groups (U test: Z = 0.77, p = 0.44, n = 190). Flies fed am-Y were not tested (ND) in the thermosensitivity assay. Values are means and error bars are SEMs marked significant as * = p < 0.05.

Characterization of serotonergic neurons

The genetic and pharmacological approaches point to a function for serotonin but not dopamine in place learning. The serotonergic neurons in *Drosophila* have not been very well characterized and the ability of Ddc-Gal4 in targeting these neurons hasn't been well tested in adult flies. The identification and localization of these neurons is critical in understanding the neural circuit supporting place learning. To this end we used a specific monoclonal anti-serotonin antibody to characterize the serotonergic neurons. Similar to previous reports (Valles and White, 1988) we identified 38-40 cell bodies per central brain hemisphere and labeled them based on their position in the fly brain (Figure 3-7 and Table 3-1).

We followed the analysis of wild-type brain with anti-serotonin by identification of Ddc-Gal4 positive serotonergic neurons. Whole mount analysis with Ddc-Gal4/ UAS-GFP brains were carried out with brains immunostained for anti-serotonin and anti-GFP (Figure 3-8). 15 whole mounts were analyzed and neuronal cell bodies were counted based on fluorescence and morphology.

We found that Ddc-Gal4 drives expression of UAS-GFP in ≈ 220 neurons per hemisphere (excluding the optic lobes). We defined three broad classes of Ddc-Gal4-positive neurons: (i) serotonergic neurons, (ii) distributed nonserotonergic neurons, and (iii) a cluster of nonserotonergic neurons in the anterior superior medial part of the cell body rind. First, the Ddc-Gal4-positive serotonergic neurons number between 26 and 29 (Table 3-1 and Figure 3-8). Only a fraction of the lp2 and pmp neurons do not overlap with the Ddc-Gal4 positive neurons. Second, the ≈ 100 Ddc-Gal 4-positive distributed nonserotonergic neurons, presumably including the dopaminergic neurons, are found in several regions of the cellular rind. Third, ≈ 100 nonserotonergic Ddc-Gal 4-positive cell bodies are found in a cluster in the frontal cell rind. A predominant component of these cells includes what appear to be extrinsic mushroom body neurons (Sitaraman et al., 2008).



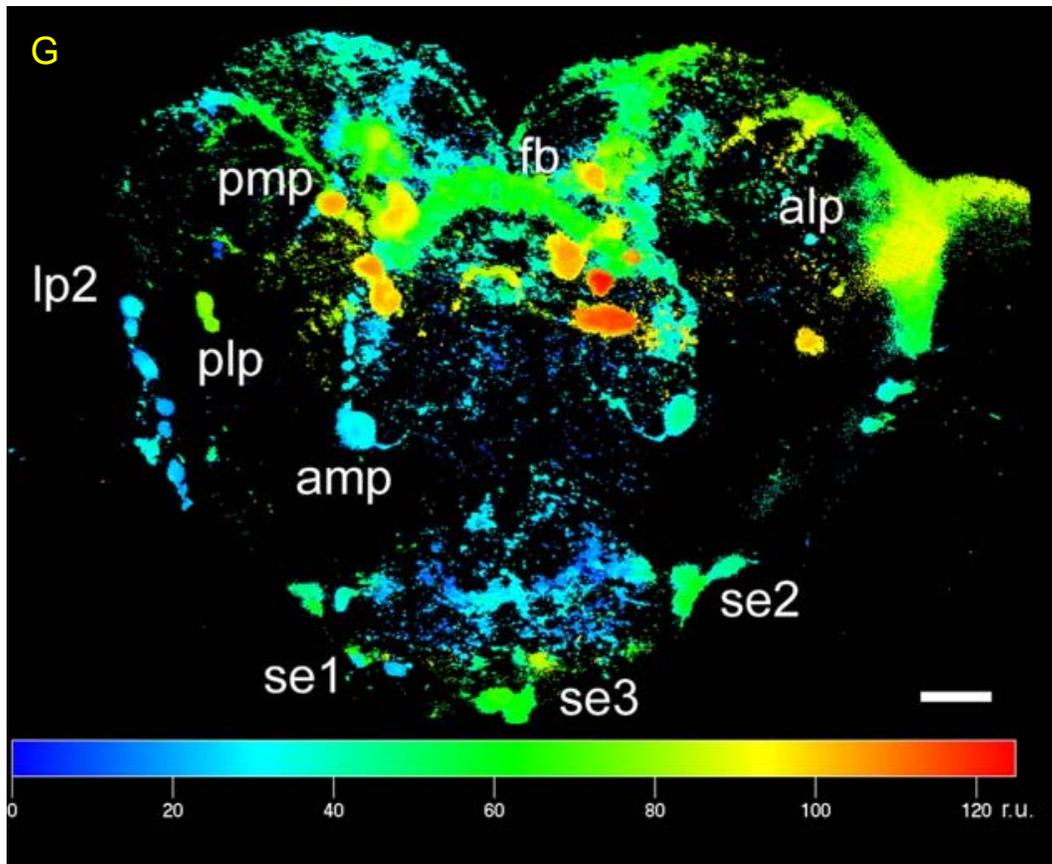


Figure 3-7. Identification and characterization of the serotonergic neurons in the fly brain. (A-F) Whole mount of wild-type CS brains from anterior (A and B), to medial (C, D and E) and posterior sections (F) showing serotonin immunoreactive neurons labeled by anti-serotonin. Cell bodies from fly brains (n=13) were identified based on size and morphology. Scale bar in A: 50 μ m; applies to A-F. G) A depth coded image of the fly brain from anterior to posterior as blue to red in 100 steps (0–125 relative units). Scale bar represents 50 μ m. The neurons are labeled based on their localization and relative position in the fly brains (refer to Table 3-1).

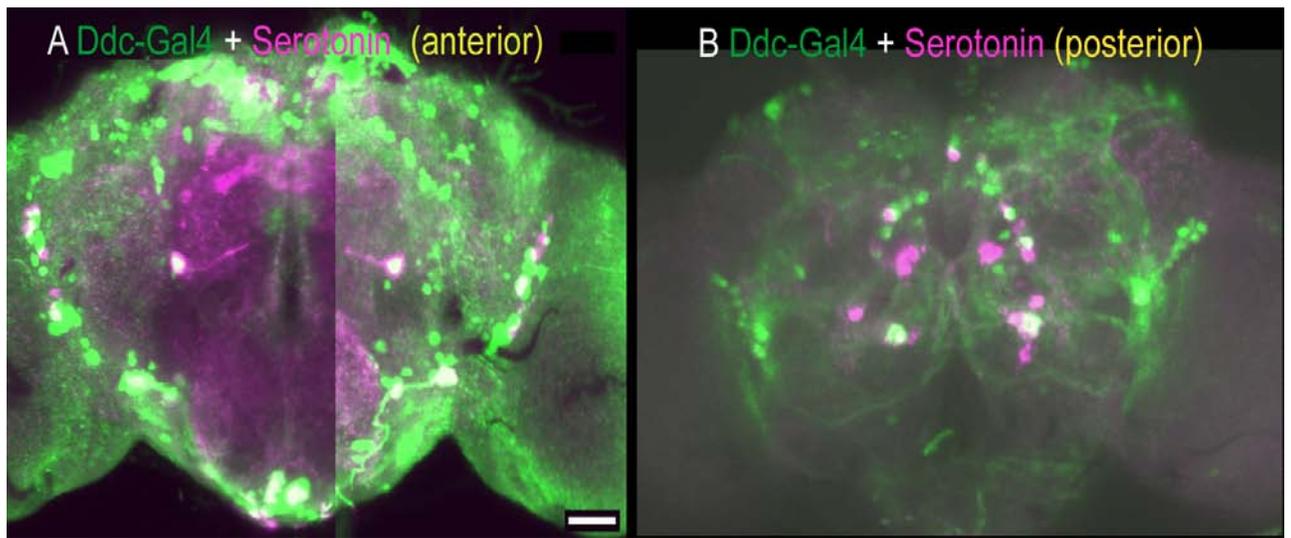


Figure 3-8. Ddc-Gal4 drives expression of UAS-GFP in a subset of the serotonergic neurons and two groups of non-serotonergic cells. Panel A and B represent the anterior and posterior structures of the brain. Magenta represents serotonin-IR neurons while GFP is shown in green. A summary of the co-localization and innervation pattern is shown in table 3-1. Scale bar in A: 50 μm .

Table 3-1. Location and number of serotonergic neurons

Group	Cell body/hemisphere	No. of DdcGal4 positive neurons	Location
alp	3	3	Extreme anterior cell body rind, lateral to midline
amp	1	1	Single large cell body (~ 10 μ m diameter) lateral to antennal lobe and dorsal to the ammc
lp2	9-11	3-4	3 - 4 cell cluster between medulla and central neuropil
pmp	13-14	8-10	Cell cluster in the posterior cell body rind, medial to the calyx and running dorsoventral
plp	3	3	Between medulla and central neuropil in posterior brain
se1	3	2	Most anterior sub-esophageal neurons, lateral to midline
se2	3	3	Posterior to se1, lateral to midline
se3	3	3	Posterior to se1, at the midline

Table 3-1. Location and number of serotonergic neurons identified using anti-serotonin in wild-types brains and Ddc-Gal4; Th-Gal80 brains counter stained with anti-GFP.

Specific targeting of serotonergic neurons

The ability to specifically label and identify serotonergic neurons based on morphology and localization can be helpful in finding Gal4 lines that target these neurons in transgenic approaches. Ddc-Gal4 drives expression of transgenes under UAS control in both dopaminergic and serotonergic neurons. In the previous experiments, we address serotonin function if we find an effect with Ddc-Gal4 but not with Th-Gal4. This conclusion however assumes that the two systems are not acting together to produce an observed phenotype. Clearly, a more specific serotonin driver would be an advantage in interpreting the conditioning data. The UAS-Gal4/Gal80 system can be exploited to express different constructs specifically in serotonergic neurons. To refine Ddc-Gal4 driven expression of transgenes to serotonergic neurons we used a Th-Gal80 line (Sitaraman et al., 2008). The expression of Th-Gal80 in flies expressing GFP under the Th-Gal4 control completely abolishes GFP expression, demonstrating its ability to effectively silence transgene expression in dopaminergic neurons (Figure 3-9, n=5). We also looked at Th-Gal4 driven UAS-GFP expression and found ≈ 75 neurons per hemisphere, none of which overlap with the serotonergic signal (Figure 3-9). Flies expressing GFP in Ddc-Gal4; Th-Gal80 neurons were also examined. The flies have an expression pattern similar to the Ddc-Gal4; GFP flies, but with suppression of Gal4 activity in the dopaminergic neurons. Since, there is no overlap between Th-Gal4; GFP neurons with serotonin, adding Th-Gal80 did not likely lead to loss of expression in any of the identified serotonergic neurons.

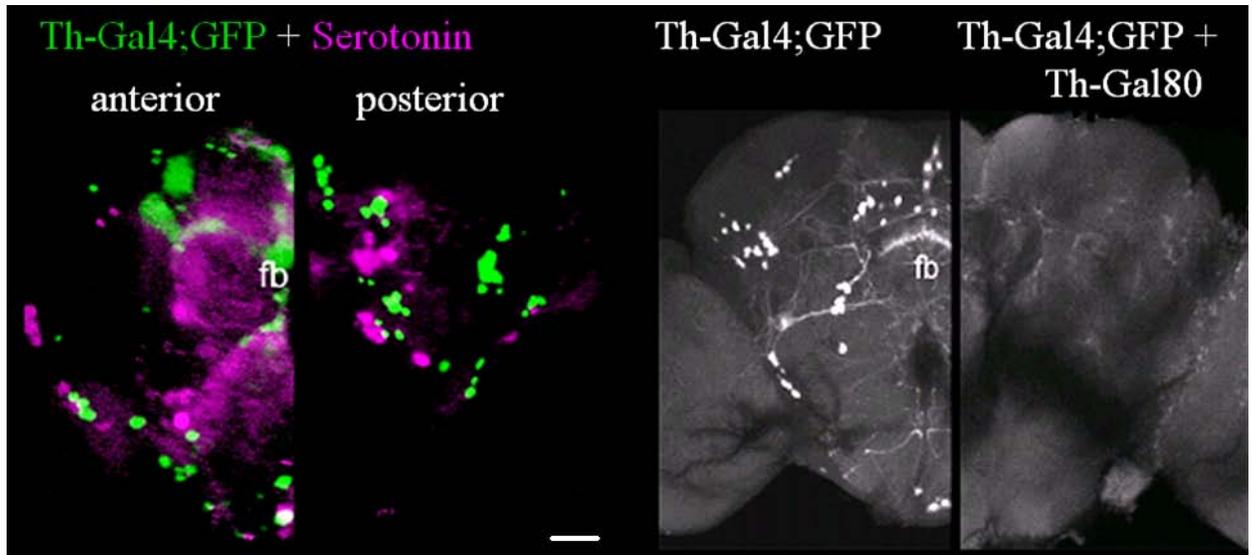


Figure 3-9. Th-Gal80 expression silences Th-Gal4 dependent dopaminergic neurons expression. A) TH-Gal 4 drives UAS-GFP expression in a distributed set of cells that do not overlap in expression with the serotonergic neurons. B) Th-Gal80 can effectively suppress Th-Gal4-driven UAS-GFP expression. Left panel- Th-Gal4 / GFP brain showing expression of dopaminergic neurons in green, counterstained with anti-serotonin in magenta. Right panel- Th-Gal4; GFP/Th-Gal80 brains shows no observable expression of the GFP labeling dopaminergic system in the presence of Th-Gal80. The white colocalization signal in the anterior medial ventral brain is an artifact of flattening these optical sections. Scale bar represents 50 μm .

Ddc-Gal4; Th-Gal80 system is necessary for reinforcement processing

We next used the Ddc-GAL4; Th-Gal80 driver combination to manipulate synaptic transmission. Flies that expressed the UAS-TNT with the Ddc-Gal4; Th-Gal80 driver combination had a strongly reduced memory level compared with genetic controls (Figure 3-10 A). This manipulation did not alter the ability of these flies to sense and avoid the high temperatures used as a negative reinforcer (Figure 3-10 B). Thus, the serotonergic system can be manipulated independent of the dopaminergic system to elucidate the molecular mechanisms underlying reinforcement processing.

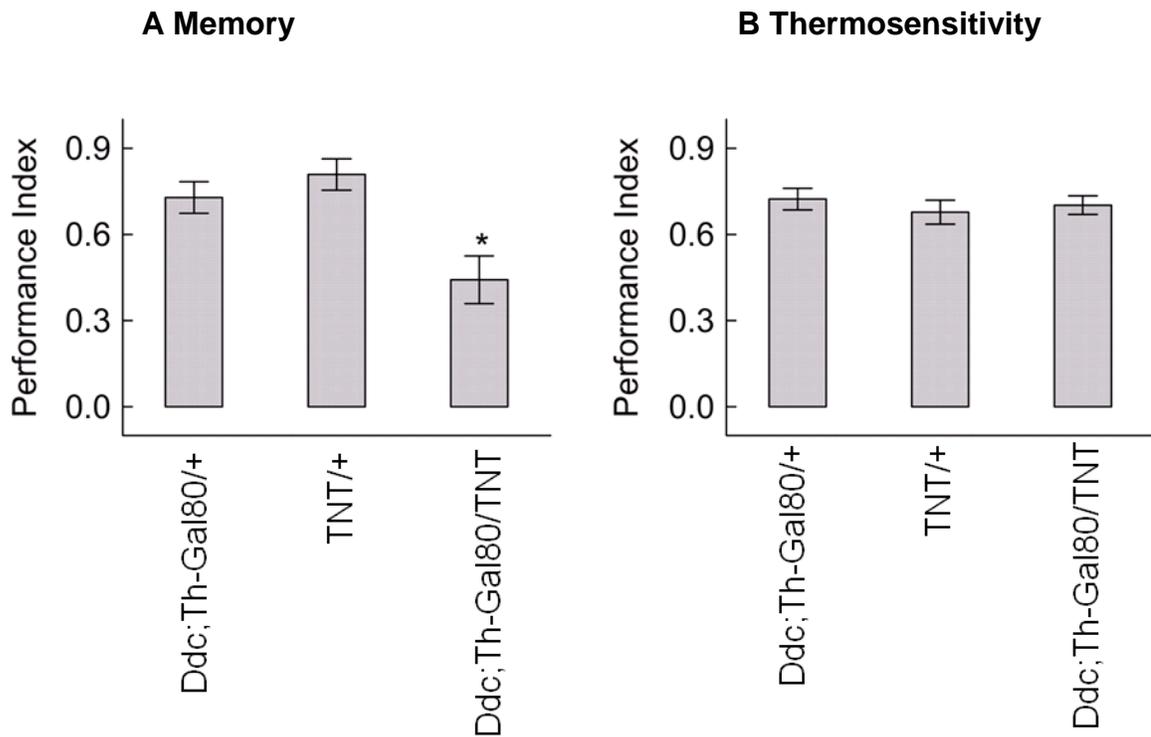


Figure 3-10. Manipulating the serotonergic system reduces memory performance. (A) Flies in which the TNT was expressed in a set of CNS neurons, including the serotonergic neurons, showed a strong reduction in place memory [$H(2, n = 286) = 14.5, p = 0.0007$, multiple comparisons indicate significant differences between Ddc-GAL4; Th-Gal80/UAS-TNT and both Ddc-GAL4; Th-Gal80/+ and UAS-TNT/+ performances (*, $p < 0.05$)]. (B) Tests for the ability to sense and avoid a 41°C high-temperature source in the thermosensitivity assay found no significant differences between the genotypes tested [$H(2, n = 210) = 1.20, p = 0.55$]. Values represent means and error bars are SEMs marked significant as * = $p < 0.05$.

Targeting of the *Drosophila* Tryptophan hydroxylase (Trh) gene to generate a more specific serotonergic driver

To better elucidate the function of serotonin neurons in place learning, tools must be developed which target other genes involved in serotonergic synthesis and signaling. This allows for the stronger conclusions, when multiple Gal4 drivers show the same phenotype. This also provides a starting point for further dissection of serotonergic neuron systems. Interestingly, Tryptophan / phenylalanine hydroxylase (Trh or Tph) is an enzyme unique to serotonin and phenylalanine biosynthesis and not directly involved in other biogenic amine synthesis (Ichiyama et al., 1970). Gal4 lines made using the enhancer elements of this gene could be used effectively to target serotonergic neurons.

The *Drosophila* genome encodes two *trh* genes with the ability to hydroxylate tryptophan and phenylalanine. While the expression of one *trh* gene (CG9122) is seen mainly in the central nervous system the second isoform (CG7399) is thought to function in non neuronal tissue (Coleman and Neckameyer, 2005). There is also evidence that the putative neuronal Trh protein has higher enzymatic activity in hydroxylating tryptophan as compared to phenylalanine (Coleman and Neckameyer, 2004). However, a comprehensive expression pattern using specific antibodies or biochemical characterization of the two *trh* gene products has yet to be carried out.

A new serotonin Gal4 line generated by Dr. Serge Birman (ESPCI, Paris) has a Gal4 insertion in the 1st exon of *trh* (CG9122). This line includes the 5' region thought to be important for regulating expression of several genes (Friggi-Grelin et al., 2003). As a first step in characterization of behavioral phenotypes arising from this serotonergic Gal4 line we looked at the expression patterns with a UAS-GFP. Whole mounts of Trh-Gal4/ UAS-GFP flies were stained with anti-GFP and anti-serotonin (n=14, Figure 3-11). Trh-Gal4 effectively labels the se 1-3, amp, plp, pmp, and lp 2 neurons and has extensive innervation patterns in the central complex (Table 3-1, n=14). Parts of the serotonin projections also innervate the mushroom bodies and antennal lobes by Trh-

Gal4. The Trh-Gal4 provides an additional tool to gain confidence in identification of specific serotonergic neuron function in place learning.

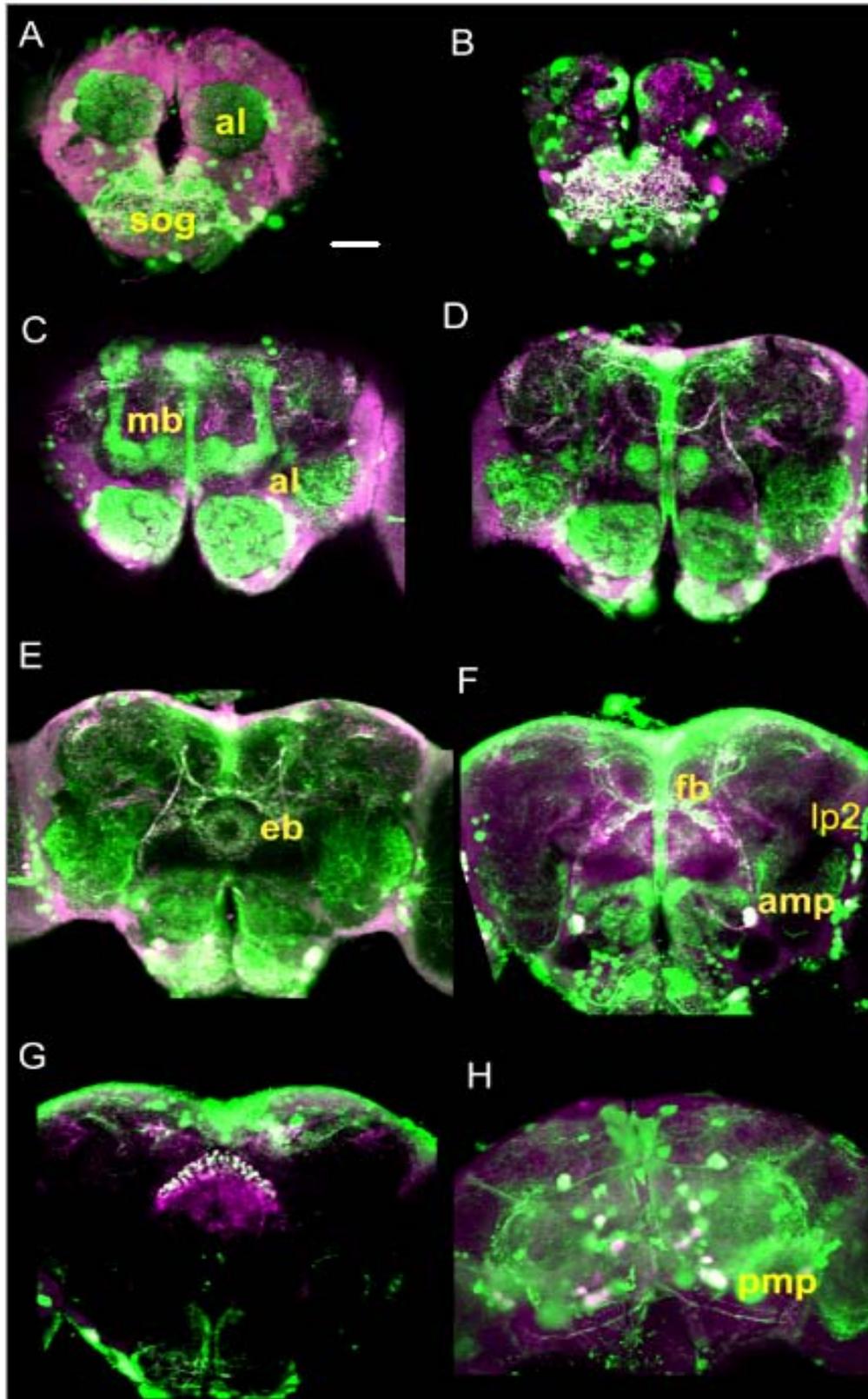


Figure 3-11. Identification and characterization of Trh-Gal4 as a serotonergic driver. Anterior to posterior section of Trh-Gal4; UAS-GFP brains stained with anti-serotonin (magenta) and anti-GFP (green). A, B and C: Anterior sections of the fly brain showing significant colocalization in serotonergic neurons in the sog (subesophageal ganglion- se 1, 2 and 3 cluster). Extensive innervations patterns of GFP are seen in the sog (subesophageal ganglion), antennal lobes (al) and mushroom bodies (mb). C, D, E and F) Mid-level sections of the fly brains with co-expression of GFP and serotonin in amp, lp2 neurons and central complex (eb- ellipsoid body and fb- fan shaped body). G and H) Posterior sections with colocalization in fan shaped body, plp and pmp neurons. Scale bar in A: 50 μ m applicable to all images A-H.

Intrinsic properties of serotonergic neurons targeted by Trh-Gal4 regulate memory strength

Blocking serotonin synthesis and chemical neurotransmission from serotonergic neurons shows the necessity of serotonergic system in place learning. Biogenic amines like dopamine have been shown to be sufficient mediators of negative reinforcement in olfactory learning in larvae and adult *Drosophila* (Claridge-Chang et al., 2009; Schroll et al., 2006). We hypothesize that reinforcement signaling in place learning is mediated by the serotonergic neurons. To test the hypothesis we increased and decreased the excitability of Trh positive neurons and asked if this modulation directly influenced the place memory levels.

Electrical silencing of Trh-Gal4 positive neurons during adulthood impairs memory formation

Electrical activity of neurons can be partially suppressed by altering the resting membrane potential. This can be accomplished by increasing potassium or chloride hyperpolarizing conductances, or by decreasing depolarizing sodium or calcium conductances. Of the available potassium channels that reduce excitability of targeted neurons, Kir2.1 is found to have the most severe effect in electrical silencing of neurons (Thum et al., 2006). In our first attempts to identify the function of serotonin neurons targeted by Trh-Gal4 and to address the role of excitability of these neurons in place learning, we expressed human inward rectifying potassium channel Kir2.1 and tested place memories. Expression of these channels in Trh-Gal4 positive neurons during development leads to lethality with significantly fewer larvae reaching the pupae stage (not shown). The few pupae that develop don't eclose. To study the effects of electrical silencing mediated by these channels in adult flies we used a Gal80^{ts} in combination with Trh-Gal4 / Kir2.1. Flies were raised at permissive temperature of 24°C at which Gal80^{ts} is synthesized and is functional and inhibits Gal4 activation of UAS transgenes. 2-3 day old adult flies were then placed

at 30°C for 36 hours to inhibit Gal80^{ts} activity and allow for Kir2.1 expression in Trh positive neurons. Flies expressing Kir2.1 in Trh neurons have lower memory scores compared to the flies of genotypic controls. All the tested genotypes sense and avoid the reinforcing temperature of 41°C (Figure 3-12). Thus, reducing the excitability of serotonergic neurons by increasing potassium conductance reduces memory performance levels.

Increasing excitability of serotonergic neurons increases memory performance levels

Multiple lines of evidence with two distinct Gal4 lines and results from electrical silencing of serotonergic by Kir2.1 clearly indicate that decreasing serotonin output reduces memory performance levels. To address the sufficiency of serotonergic system we asked if an increase in activity of serotonergic neurons increases memory levels. Modifying excitability of target neurons with temporal precision of milliseconds has been achieved by optogenetic (Channelrhodopsin, halorhodopsin, P2X2 receptor) and temperature sensitive ion channels (dTrpA1, Trpm8) (Deisseroth et al., 2006; Hamada et al., 2008; Miesenbock and Kevrekidis, 2005; Peabody et al., 2009). The expression of temperature-activated members of the Transient Receptor Potential (TRP) family (thermoTRPs) can make cell excitability very sensitive to temperature. Normally, dTrpA1 is expressed in the *Drosophila* brain in a set of anterior neurons (AC) and required for avoidance of 30-32° C. Expression of this channel in motor neurons leads to complete paralysis in response to increase in temperature (>27° C) (Hamada et al., 2008; Pulver et al., 2009). Mis-expression of dTrpA1 under the UAS control, in serotonergic neurons might activate these neurons independent of the 41° C place learning reinforcer. We asked if release of serotonin in a place learning context but in the absence of punishment itself might lead to memory formation.

We replaced the high temperature aversive reinforcer with a stimulus that activates the serotonergic neurons and tested place memory. To achieve this we misexpressed the warm sensitive dTrpA1 channels in serotonergic neurons targeted by Trh-Gal4 and conditioned them

with temperatures 29-35°C. dTrpA1 channels open in a narrow and specific temperature range (>27°C) in larvae and show robust activity at slightly higher temperatures (>30°C) in adult flies (Parisky et al., 2008; Pulver et al., 2009).

Interestingly, increasing temperatures within a narrow activation range could result in graded activation of serotonergic neurons based on probability of opening of the cation channels. It would be fascinating to correlate this graded activation of serotonergic neurons to memory levels in the heat box. To test this experimentally Trh-Gal4 / dTrpA1 flies were conditioned with temperatures between 29 and 35°C (Figure 3-13). We found Trh-Gal4/dTrpA1 flies avoided the side associated with temperatures that induce TrpA1 activation (31, 32, and 33°C) at significantly higher levels as compared to the controls (Figure 3-13). The asymptotic memory levels of Trh-Gal4 / dTrpA1 flies were at least four times greater than that from flies of genotypic controls. Remarkably, the temperatures outside of the activation range of dTrpA1 (29 and 35 °C) did not produce a memory enhancement, and all the tested groups performed at a similar level (Figure 3-13). Trh-Gal4 / dTrpA1 flies conditioned with 31, 32, and 33°C performed at memory levels expected of high temperature reinforcers (> 37°C) showing that the serotonergic system mediated reinforcement processing. Keeping with the role of dTrpA1 in thermotactic behaviors in *Drosophila* we carried out a thermosensitivity assay to test if Trh-Gal4/dTrpA1 flies can sense and avoid temperatures in the range of 24- 45° C (Table 3-2).

To gain further confidence of the specific role for serotonergic activation in substituting the negative reinforcer we expressed dTrpA1 channels using a Gal4 that traps the *tetraspanin 42E1* gene (Grumblin and Strelets, 2006). This allele has been tested previously and does not have a function in place learning (LaFerriere et al., 2008). We used 32°C for conditioning as it results in maximal activation of dTrpA1 with Trh-Gal4 (Figure 3-13). We found that flies with activation of neurons targeted by this second Gal4 does not increase memory levels (Figure 3-14).

These results clearly show that pairing excitation of serotonergic neurons with spatial position can induce a place memory. These results also support the finding that specific activation of serotonergic neurons in specific spatial position (punished side) of the chamber leads to avoidance of that position substituting the role of high temperature reinforcer. Conditioning in the heat box is largely mediated by high temperature aversive reinforcer but the preference of flies for 24° C argues in favor of an appetitive component that mediates the approach to the cool side of the chamber. The current findings suggest that serotonin activation mediates avoidance of a chamber half. Therefore, serotonin signals the aversive reinforcement information to condition flies in the heat box. This however does not rule out a positive component in place learning. We will explore further the role of appetitive component in place learning by identifying the function of octopamine that is thought be involved in mediating appetitive reinforcement in insect learning. These results combined with the earlier Kir2.1 expression shows that artificial inhibition or activation of a single system is sufficient in reinforcing a memory.

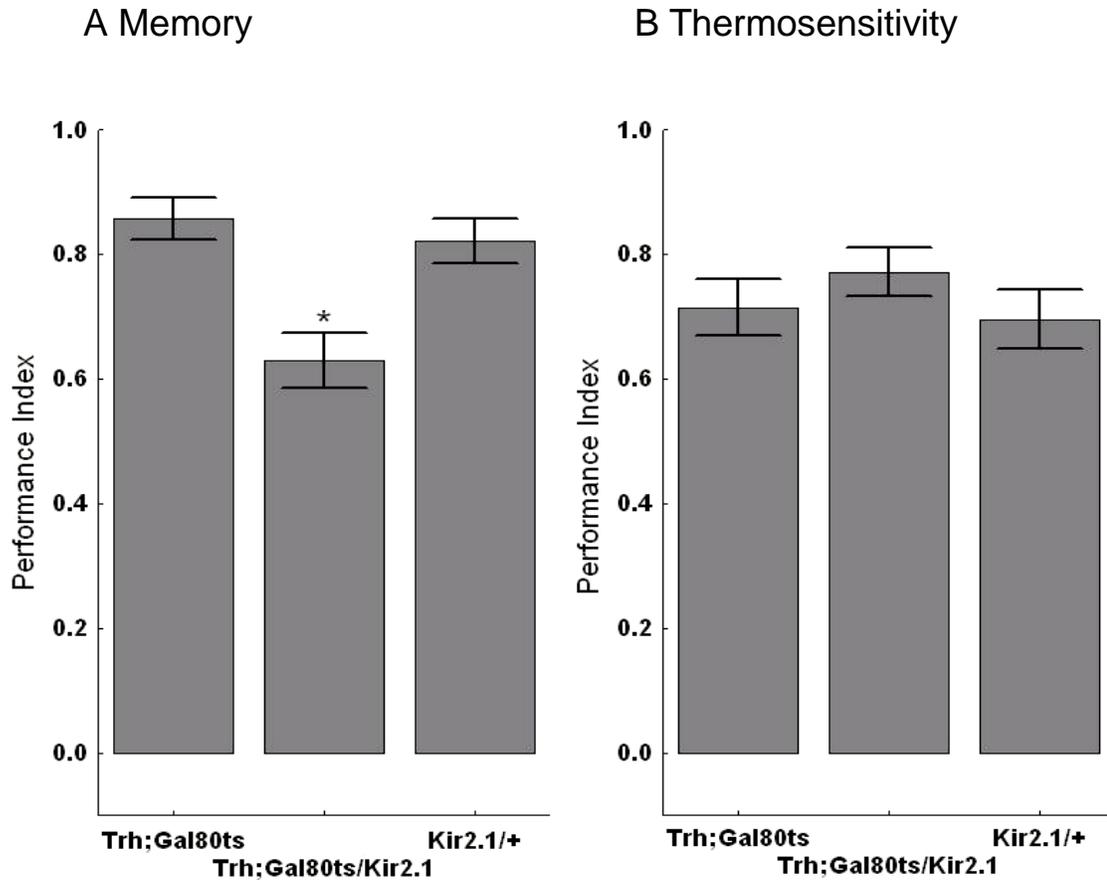


Figure 3-12. Electrical silencing of serotonergic neurons labeled by Trh-Gal4 impairs conditioning in the heat box. Further, decreasing the excitability of Trh positive neurons restricts memory performance. A) UAS-Kir2.1 was expressed in the Trh positive serotonergic neurons during adulthood using a temperature sensitive Gal80ts (Trh-Gal4; Gal80ts / Kir2.1). Trh-Gal4; Gal80ts / + and Kir2.1 / + represent the driver and transgene alone in a wild type background. Only flies expressing Kir2.1 driven by Trh-Gal4; Gal80ts driver had a deficit in conditioned memory performance, multiple comparisons indicate significant differences between Trh-GAL4; Gal80ts / UAS- Kir2.1 and both Trh-Gal4; Gal80ts / + and UAS- Kir2.1 / + [$H(2, N= 477) = 25.7$ $p = 0.000$]. B) Expression of Kir2.1 during adulthood does not alter thermosensitivity or avoidance of the flies to 41°C [$H(2, N= 307) = 4.1$, $p = 0.12$]. Values represented are means and error bars are SEMs marked significant as * $= p < 0.05$.

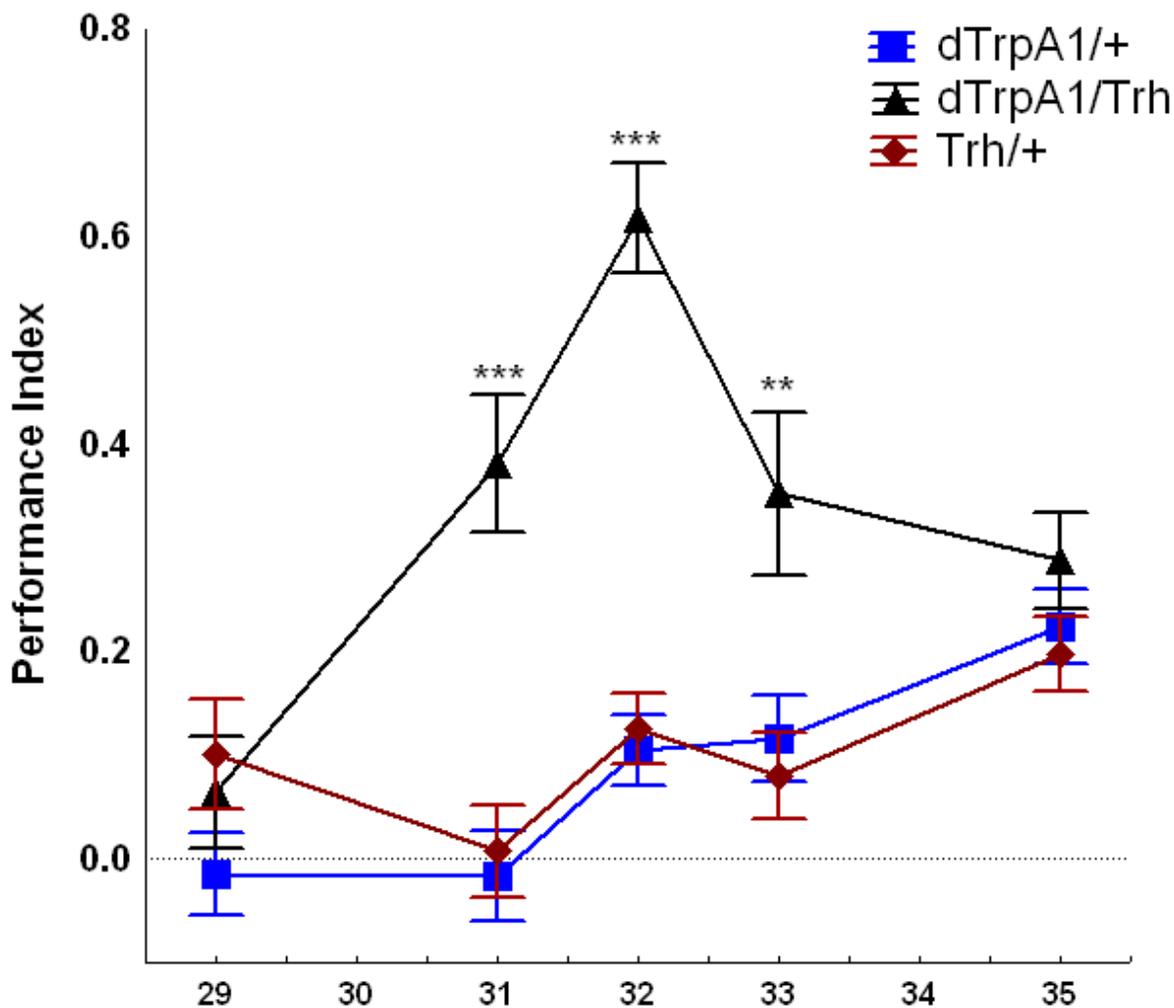


Figure 3-13. Activation of Trh positive serotonergic neurons mimics high temperature reinforcer. Trh-Gal4/dTrpA1, Trh-Gal4 /+ and dTrpA1/+ flies were conditioned using 29, 31, 32, 33 and 35°C, legend on top right corner indicates the tested genotypes. Multiple comparisons of mean ranks were carried out between genotypes for a particular temperature as they were tested in parallel. For 29°C, [H (2, N= 274) =5.14 p =.08], for 31°C, [H (2, N= 273) =27.22 p =0.000], for 32°C, [H (2, N= 419) =70.25 p =0.000], for 33°C, [H (2, N= 234) =12.84 p =.0016], for 35°C, [H (2, N= 574) =2.64 p =.27]. Values represent mean and SEMs, marked significant ** = p<0.01 and *** = p<0.001.

Table 3-2. Thermosensitivity of Trh/ dTrpA1 flies and their controls

Genotype	24/24	24/30	24/32	24/37	24/41	24/45	N
Trh/+	0.07± 0.05	0.15±0.05	0.22±0.04	0.38±0.05	0.64±0.05	0.82±0.03	80
Trh/dTrpA1	0.08± 0.05	0.10±0.03	0.23±0.05	0.33±0.06	0.59±0.06	0.64±0.06	77
dTrpA1/+	0.07± 0.03	0.09±0.04	0.18±0.04	0.36±0.03	0.63±0.03	0.77±0.05	76

Table 3-2. dTrpA1 expression in serotonergic neurons targeted by Trh-Gal4 does not alter the ability to sense and avoid temperatures. Flies expressing dTrpA1 in Trh positive neurons (Trh-Gal4 / dTrpA1) were tested for their thermosensitivity of temperatures 24-45°C with their genotypic controls Trh/+ and dTrpA1/+. Multiple comparisons of mean ranks shows H (2, N=233) for 24/24 =0.76, p=0.68, for 24/30 =3.96, p=0.14, for 24/33 =3.9, p=0.14, for 24/37 =2.5, p=0.29, for 24/41 =3.01, p=0.22, for 24/45 =3.67, p=0.16. Values represent means± SEMs.

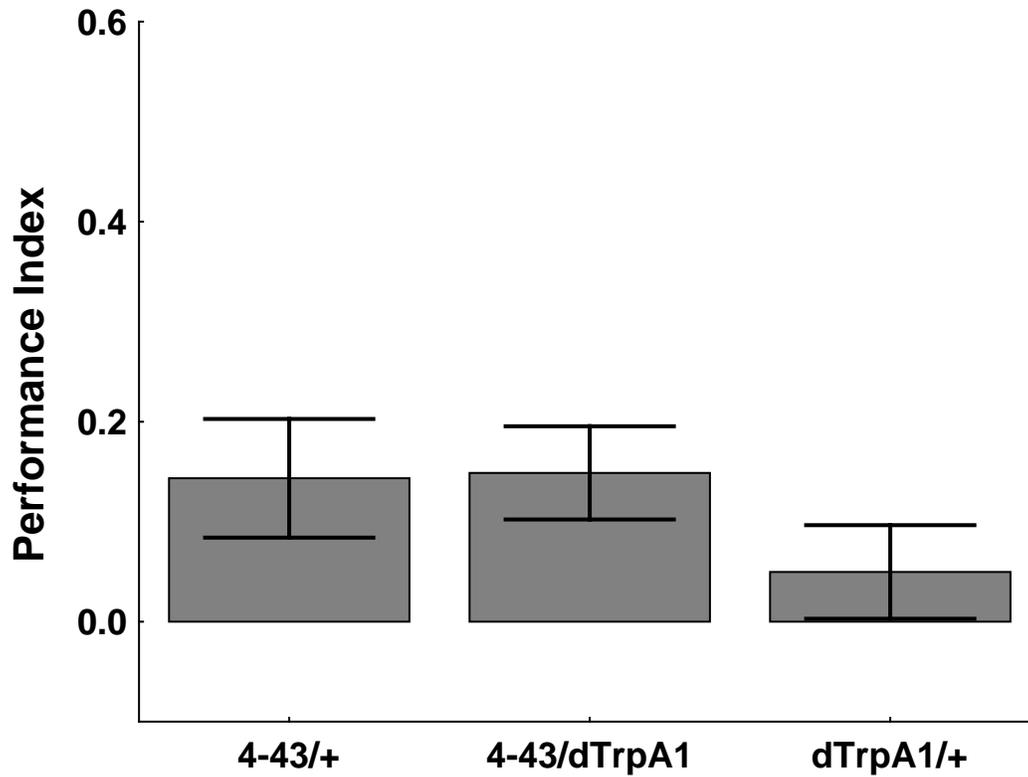


Figure 3-14. Activation of non- serotonergic neurons labeled by the 4-43 Gal4 by dTrpA1 does not increase avoidance in the heat box. 4-43 Gal4/ dTrpA1, dTrpA1/+ and 4-43 Gal3/+ flies were conditioned with 32° C. Multiple comparisons of mean ranks by Kruskal-Wallis show $H(2, N=238) = 4.21, p = 0.12$. Values represent mean and error bars are SEMs.

Summary and Conclusions

We extended the earlier behavioral analysis of the *white* gene and the likely molecular pathways dependent of *white* function. The White transporter belongs to the ABCG class of ABC transporter superfamily and its human homologue maps to 21q22.3. Some studies have shown that polymorphisms of this locus are associated with mood and anxiety disorders (Kirov et al., 2001). Indeed, a mutation in the ABCG1 gene has been identified in male patients with depression (Nakamura et al., 1999). Further, the expression of some of the ABCG transporters has been identified and localized specifically in mouse brain (Lein et al., 2007; Tachikawa et al., 2005). The *white* mutant flies have severely reduced memory performance and show severely decreased levels of both serotonin and dopamine. The mutant behavioral phenotype in *Drosophila* can be phenocopied by reducing *white* expression in the cells that make serotonin.

Independent of *white* function we also found that blocking synaptic transmission in the serotonergic cells reduces memory performance. Further, pharmacological reduction of serotonin reduces memory performance. Finally, we also carried out a comprehensive analysis of the localization and quantification of serotonergic neurons in the central nervous system which will be critical in screening new Gal4 and Gal80 lines that target specific subsets of serotonergic neurons. This will greatly aid identification of brain structures where serotonin acts to mediate the reinforcement information. To gain confidence that the serotonergic system mediates the reinforcement information we used two Gal4 lines targeting two different genes involved in serotonin synthesis. The additional use of Th-Gal80 with Ddc-Gal4 and alpha- methyltyrosine helps excludes a function for dopamine in place learning.

More importantly, altering the excitability of Trh-Gal4 positive serotonergic neurons by dTrpA1 and Kir2.1 in the positive and negative direction respectively is sufficient to modulate levels of place memory. Serotonin mediates the negative reinforcement in the heat box, but conclusions

about this system being a generally aversive signal cannot be made as there is no clear evidence for serotonin function in other learning paradigms in *Drosophila*.

Serotonin modulates a wide variety of innate and learned behaviors in both vertebrates and invertebrate animals. Serotonin has been implicated in associative learning in organisms ranging from *C. elegans*, leeches, and mollusks to mice and humans (Brunelli et al., 1976; Buhot et al., 2000; Farley and Wu, 1989; Zhang et al., 2005). However, the best understanding of the role of serotonin in mechanisms of neural plasticity underlying associative learning come from studies conducted in *Aplysia*. The gill- and siphon-withdrawal reflex of *Aplysia* can be classically conditioned by pairing a light tactile stimulus to the siphon (the conditioned stimulus-CS) with tail or mantle shock (the unconditioned stimulus-US). Siphon sensory neurons (SN) and their follower cells (Motor neurons-MN) are important for conditioned gill and siphon withdrawal in response to the light touch. The SN–MN synaptic plasticity depends on the release of the neurotransmitter serotonin during training (Glanzman et al., 1989; Marinesco and Carew, 2002). A single application of serotonin induces short-term synaptic facilitation, which is rapid and does not require protein synthesis (Brunelli et al., 1976). However, repeated application of serotonin causes long-term synaptic facilitation that lasts for more than a day, and requires transcriptional and translational changes (Dale et al., 1987). This long-term plasticity is correlated with the growth of new synaptic connections between the sensory and motor neurons (Glanzman et al., 1990). The serotonin induced long-term potentiation involves activity-dependent activation of the cAMP signaling pathway (Dash et al., 1990), cAMP-response element (CRE)-binding protein (CREB) and consequent transcriptional changes. Even though the role of serotonin is important for learning, it is not clear how serotonergic signaling is regulated to influence conditioning. Other than the place learning phenotype a clear definitive function of serotonin has not yet been reported in *Drosophila*. As long as the role of serotonin in olfactory learning remains controversial it appears that the biogenic amines that are necessary for classical olfactory learning and place learning are different (Blenau and Baumann, 2001; Tempel et al., 1984). This conclusion reflects

at least some specialization in the genetic bases of memory formation in the fly (Zars, T., under revision).

CHAPTER 4: OCTOPAMINE IN PLACE LEARNING

Introduction

Our experiments thus far indicate that serotonergic system is necessary and sufficient for spatial learning reinforced by high temperature. We have not found a function for dopamine in place learning. There could however be a second system important for reinforcing place conditioning. Conditioning in the heat box pairs a rising temperature to one of the chamber halves (e.g., the back half of the chamber associated with a maximum temperature of 41°C) and a falling temperature with the other chamber half (typically 24°C). That flies alter their back and forth walking behavior to avoid the chamber half associated with the rising temperature, and continue to do so when the temperature is kept at 24°C, is usually interpreted as an aversive memory. One might also imagine, however, that the chamber half associated with 24°C is used by a fly to form a reward memory for that place in the chamber. Thus, it is possible that what one measures after conditioning could be an aversive memory, a reward memory, or a mixed reward and aversive memory.

We examined whether octopamine might play a role in place learning. Because octopamine has a role in some rewarded memories (Hammer and Menzel, 1998; Schwaerzel et al., 2003; Unoki et al., 2005, 2006) and the falling temperature associated with a chamber-half might be rewarding to a fly, we reasoned that this biogenic amine could provide a critical component to place learning in the heat-box. This notion is further supported by thermotolerance mechanisms in the locust, in which a temperature-dependent physiological plasticity depends on octopamine function (Armstrong et al., 2006).

Octopamine and Tyramine are trace biogenic amines with several modulatory functions in invertebrates. Tyramine is synthesized from Tyrosine by the enzyme Tyrosine decarboxylase (Tdc) which is then converted to octopamine by Tyramine β -hydroxylase ($T\beta H$) (Figure 1-6). The role of tyramine in physiology and behavior are not very well understood. However, tyramine has been implicated in chloride permeability in the *Drosophila* malpighian tubule and behavioral responses to cocaine (Blumenthal, 2003; McClung and Hirsh, 1999). On the other hand octopamine is thought to be an arthropod ortholog of vertebrate norepinephrine given their similar molecular structure. Mutants of the *T β h* gene have severely reduced octopamine levels and have a characteristic defect in egg laying and this ovulation defect leads to sterility that can be rescued by feeding octopamine or ectopic expression of *T β h* (Cole et al., 2005; Monastirioti et al., 1996). More recently octopamine has been implicated in both innate behaviors like sleep, aggression, and courtship and learned behavior such as appetitive olfactory learning. Silencing of both octopaminergic and tyramineric neurons inhibits arousal, therefore, decreasing wakefulness in these flies (Crocker and Sehgal, 2008). As well, multiple lines of evidence show males lacking octopamine and tyramine have reduced lunge frequency used as a measure of aggression (Hoyer et al., 2008). More importantly, the role of octopamine has been studied in olfactory learning where mutant *T β H*^{nM18} flies have reduced appetitive learning while aversive learning is unaffected (Schwaerzel et al., 2003).

Results

Octopamine and Tyramine are not required for place learning

To address the possibility that octopamine or tyramine are required for place learning, we examined memory formation in mutant and transgenically manipulated flies. First, a loss of function mutation of the *T β H* gene, that inhibits synthesis of octopamine, was examined. Second, the regulatory regions for the Tyramine decarboxylase-2 (TDC2) gene have been fused to the

Gal4 coding sequence to provide a tool for manipulating the octopaminergic and tyraminerpic neurons (Busch et al., 2009; Certel et al., 2007; Cole et al., 2005; Sinakevitch and Strausfeld, 2006). We first sought to confirm the genotypic identity of the TDC2-Gal4 driver and the *TβH^{nM18}* mutant lines. For TDC2-Gal4, we examined the expression profile of UAS- GFP expression in the adult fly brain by confocal microscopy. The characteristic cell body location and innervation pattern of the TDC2-Gal4 driver was found (Figure 4-1, n=5) (Busch et al., 2009; Sinakevitch and Strausfeld, 2006). That is, TDC2-Gal4-positive neurons were identified in the VM, AL1, AL2, VL, ASM, PB, and PB2 cell clusters. The innervation patterns of these neurons were found in the characteristic regions of the sub-esophageal ganglion (sog), antennal lobes (al), mushroom bodies (mb), fan-shaped body (fb), protocerebral bridge (pb), and other regions of the brain.

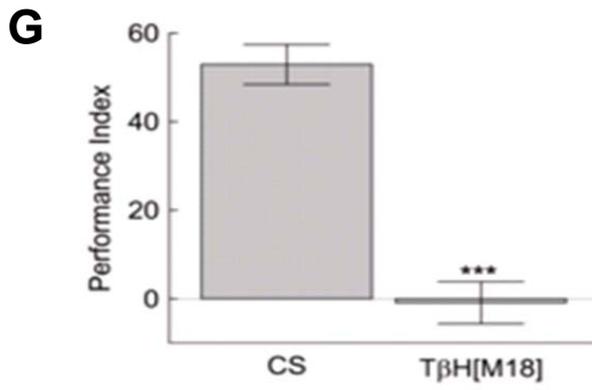
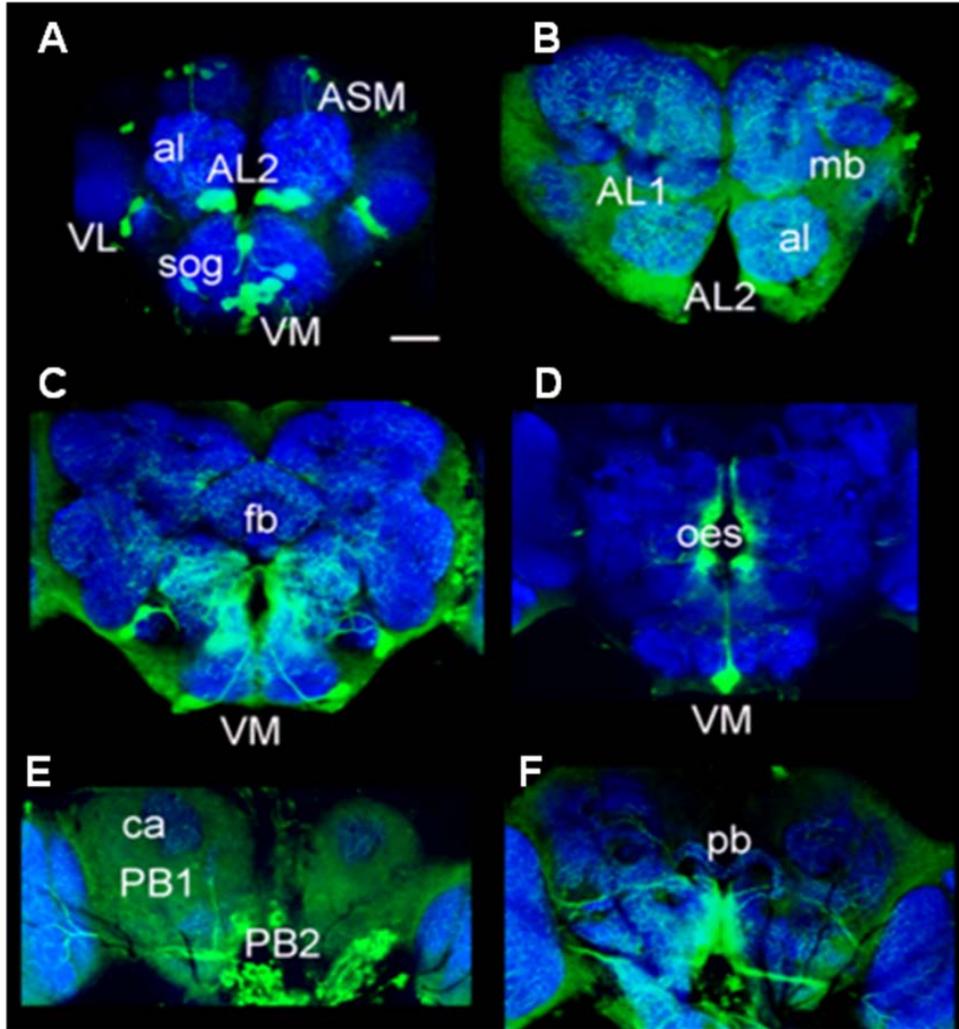


Figure 4-1. Expression pattern of the TDC2-Gal4 driver and behavioral analysis of *TβH^{nM18}* mutants to validate their genotypic identity. The expression pattern of the TDC2-GAL4 driver in the fly brain was examined using the mCD8-GFP marker (labeled as green), brains were co-challenged with the nc82 antibody to illuminate neuropil structure (labeled in blue). Selected sections from anterior (A) to posterior (F) are shown. Neurons in the Ventro-Lateral (VL), Ventro-Medial (VM), Antennal-Lobe 1 (AL1), Antennal-Lobe 2(AL2), Protocerebral-Bridge 1 and 2 (PB1 and PB2) neuron clusters were identified (Busch et al., 2009). Innervation of these octopaminergic neurons is evident in the antennal lobes (al), sub-esophageal ganglion (sog), mushroom bodies (mb), fan-shaped body (fb), the neuropil surrounding the esophagus (oes), the calyx of the mushroom bodies (ca), and the protocerebral bridge (pb). The scale bar represents 50 μm. G) The rewarded olfactory memory is strongly reduced in *TβH^{nM18}* mutant flies (Tbh [M18]) compared to wild-type CS flies ($F(1, 6) = 67.7, P < 0.001 = ***$). The values represent the means and error bars are SEMs.

The $T\beta H^{nM18}$ flies were examined in sugar-rewarded appetitive olfactory memory. The $T\beta H^{nM18}$ flies had strongly reduced appetitive olfactory memory performance levels (Figure 4-1), as expected from the results from (Schwaerzel et al. 2003). Thus, the flies from the TDC2-Gal4 driver and $T\beta H^{nM18}$ lines are expressing the expected phenotypes (Figure 4-1) and indicate the genotypes of the flies are correct.

In addition to testing $T\beta H^{nM18}$ flies, we blocked evoked synaptic transmission in the octopaminergic and tyraminerpic neurons by expressing the tetanus toxin light chain (TNT) under the control of TDC2-Gal4 (Cole et al., 2005; Scholz et al., 2000; Sweeney et al., 1995). Because mutation of $T\beta H$ and blocking of TDC2-neurons both lead to reduced octopaminergic function, but increase or decrease tyraminerpic function, a similar phenotype in flies manipulated with both tools is used to suggest a role of octopamine in regulating a behavior. If only one of the manipulations alters a behavior, high or low tyramine is then implicated in modulating a behavior.

The hypothesis here is that flies use the falling temperatures as a reward in developing a preference for a part of the heat-box chamber, and if octopamine is important for all rewarded memories, that $T\beta H^{nM18}$ flies would show a memory deficit. Our tests show that memory performance levels are not different between $T\beta H^{nM18}$ and wild type CS flies (Figure 4-2). Again, we found no statistically significant difference in memory performance levels between the TDC2-Gal4 / TNT flies and genotypic controls (Figure 4-2). The UAS-TNT genotype was confirmed by test crosses with the elav-Gal4 driver, which killed all 1st instar larvae (not shown) (Sweeney et al., 1995). Altering the octopaminergic and tyraminerpic systems with these tools did not lead to alteration in place learning. Thus, neither octopamine nor tyramine appears to have a critical role in heat-box conditioning.

The octopaminergic and tyraminerpic system have also been shown to be important for locomotion and flight initiation/maintenance in *Drosophila* (Hardie et al., 2007; Saraswati et al.,

2004). Learning in the heat box is dependent on the flies' ability to determine the spatial positions associated with negative reinforcement. To test if these flies display a locomotor deficit in the heat box chamber and make certain that any place learning phenotype is not a result of its locomotor impairments a 30 second pre-test activity was measured in the heat box prior to conditioning. This period was chosen because the walking activity during the pre-test is not altered by a learning-dependent modulation of locomotor activity (Putz and Heisenberg, 2002). In this assay, we did not detect a difference in locomotor activity between wild-type CS and $T\beta H^{nM18}$ flies, or between TDC2-GAL4 / TNT flies and genetic control flies (Figure 4-2).

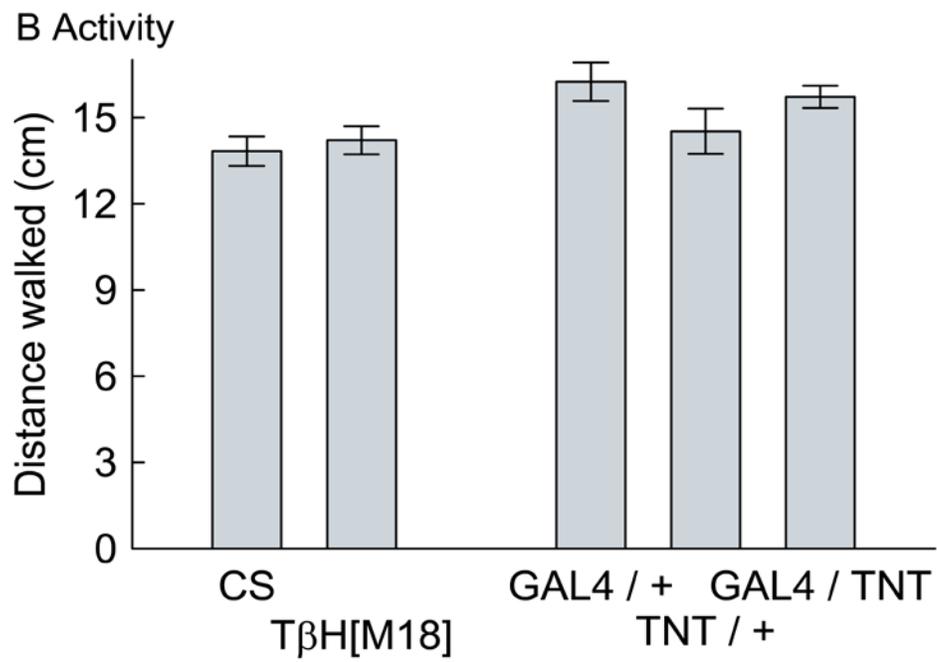
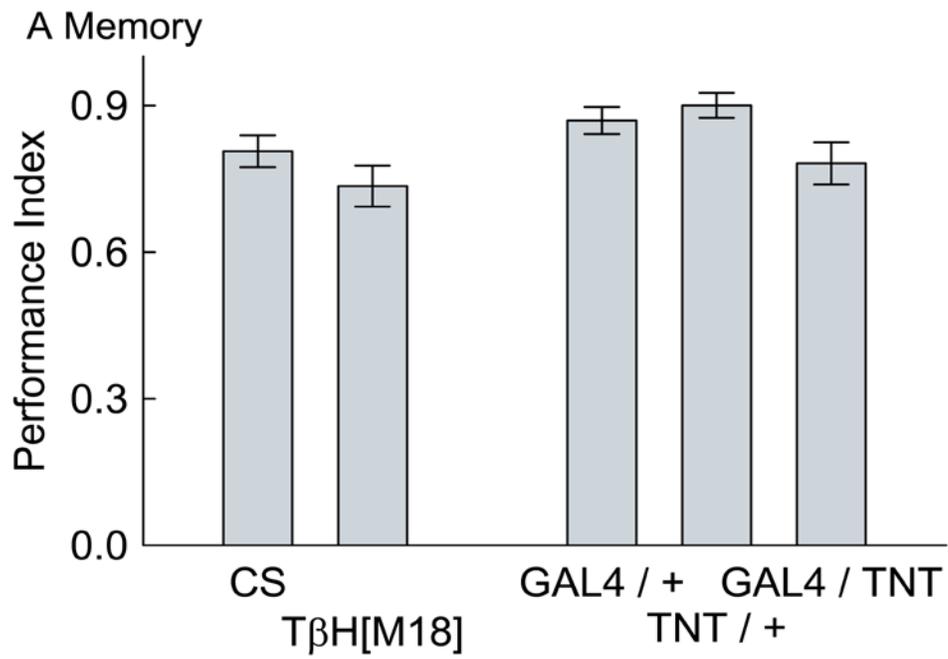


Figure 4-2. Altering octopamine and tyramine function does not alter place memory levels. Flies of control and experimental genotypes were trained for 20 min with 41 °C as negative reinforcement and tested for place memory (A) and their walking activity was determined (B).

A) The place memory performance was not significantly different between the wild-type CS flies and $T\beta H^{nM18}$ mutant flies ($H(1, N = 362) = 1.7, p = 0.2$). Similarly, blocking evoked synaptic transmission in the octopamine and tyramine neurons by expressing the tetanus toxin light chain (TNT) with the TDC2-Gal4 driver (GAL4) did not alter memory levels compared to flies of the control genotypes ($H(2, N = 414) = 6.1, p = 0.04$, no significant differences between the genotypes when examined with Multiple Comparisons). B) The walking activity of $T\beta H^{nM18}$ mutant flies during the pre-test phase of the conditioning experiments was compared to wild-type CS flies; there was no significant difference in the distance traveled between these genotypes ($U=39356.0, Z=0.23, P=0.8$). Similarly, there were no significant differences in distance walked in the pre-test phase in the TDC2-Gal4 / TNT flies and genetic controls ($H=0.76, P=0.7$). The values represent means and error bars are SEMs.

Pre-exposure effect is also independent of octopaminergic / tyraminerbic signaling

Octopamine also functions in some insects in conferring thermotolerance. In the locust, for example, octopamine is critical for the neuroprotective effects of a priming high-temperature exposure on neuronal function with a second high temperature challenge (Armstrong et al. 2006). This effect in the locust prompted our test of the octopaminergic system in establishing the pre-exposure effect. The pre-exposure effect is an enhancement of memory formation after the exposure of flies to unavoidable exposure to high temperatures (i.e., 41° C) (Sitaraman et al. 2007). We, therefore, exposed wild-type CS, TDC2-Gal4 / TNT, TDC2-Gal4 / + and TNT / + flies to 41 °C in three one-min sessions spaced over five min. The memory was tested 20 min later using 30 °C as negative reinforcement. In these experiments, the flies of these four genotypes performed at statistically indistinguishable levels (Figure 4-3). Thus, the pre-exposure effect on associative place memory is independent of normal octopamine and tyramine signaling. That we did not find an effect of octopamine signaling blockade on acquisition of the pre-exposure effect argues against a commonality in the thermotolerance and pre-exposure effects in learning.

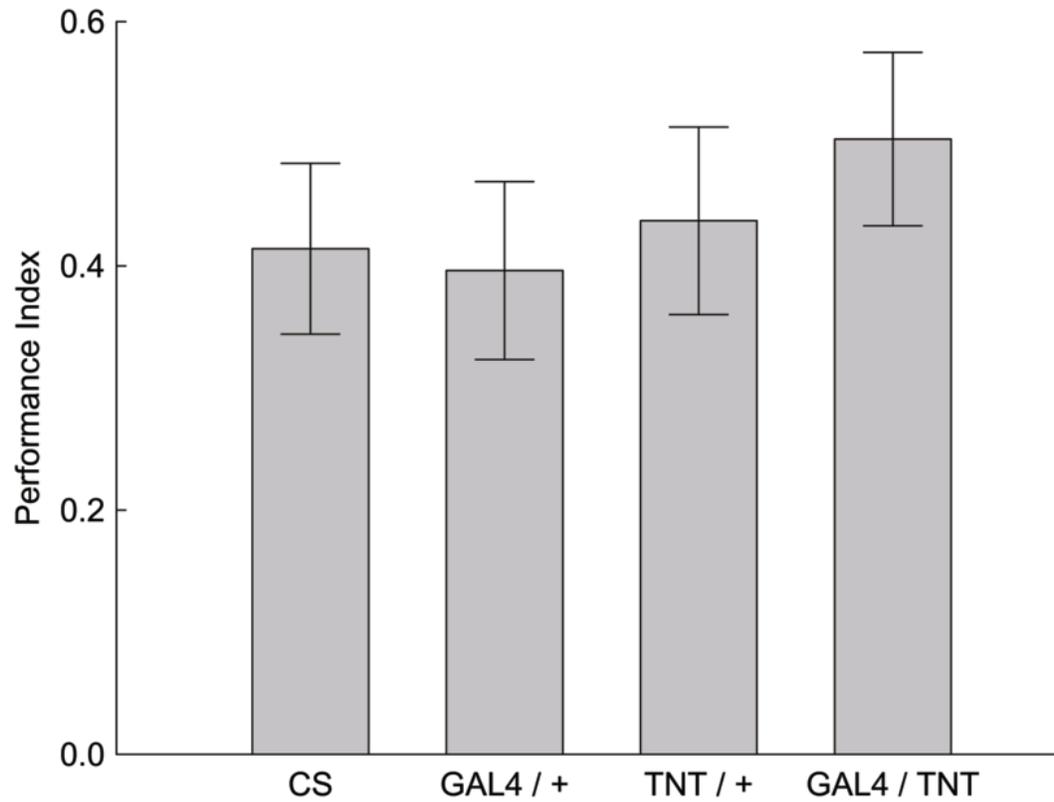


Figure 4-3. Reinforcement pre-exposure enhancement of place memory is independent of TDC2-GAL4-positive neurons function. Flies were exposed three times to a one-minute 41 °C high temperature trial and then tested for memory formation 20 min later using 30 °C as the negative reinforcing temperature. Flies in which the tetanus toxin light chain (TNT) was expressed in the TDC2-Gal4-positive neurons (GAL4) had similar memory performance as genetic control flies (CS, Gal4 / +, and TNT / +) ($H(3, N = 384) = 1.99, P = 0.57$). The values represent the means and error bars are SEMs.

Summary and conclusion

We hypothesized that if octopamine plays a general role in rewarded learning, then octopamine would be necessary for at least a component of a memory that depends on temperature and behavior / place associations (Sayeed and Benzer, 1996; Zars, 2001). That is, the strongly preferred temperature of 24 °C could be rewarding to flies as they approach and stay in parts of the chamber associated with this relatively cool temperature.

Two distinct genetic tools show that octopamine is not required for place learning. Further, that we did not find an effect of octopamine signaling blockade on acquisition of the pre-exposure effect argues against a commonality in the thermotolerance and pre-exposure effects in learning. Another octopamine-dependent behavior is the regulation of locomotion. *TβH^{nM18}* mutant larvae, with elevated tyramine levels and reduced octopamine levels, have a severe locomotion phenotype. *TβH^{nM18}* larvae spent much more time in pausing episodes than wild-type larvae and have reduced speed and linear translocation (Saraswati et al., 2004). However, in a different study the locomotor activity of adult *TβH^{nM18}* flies was found to be normal as compared to the wild type flies (Hardie et al., 2007). These differences could result from distinct locomotor assays in the two studies or differential neural regulation of the locomotion phenotypes in larvae and adult flies. In our experiments, when we examined locomotor activity in the pre-test of the conditioning experiments we did not find a difference between octopamine / tyramine altered and control flies. Presumably, differences in the behavioral assays account for these different results. For example, our tests of locomotion occur within the 30 sec that follow insertion into the heat-box chambers. This short time period falls within a 'reactive' period of locomotion in flies, and might mask locomotion changes that are evident with experiments that last longer and are less influenced by a fly's reaction to a new environment (Lebestky et al., 2009; Martin et al., 1999).

CHAPTER 5: PRE-EXPOSURE ENHANCES SPATIAL MEMORY FORMATION

Introduction

Learning can be influenced by apparently uncorrelated experience with rewards or punishments. That is, 'pre-exposure' to appetitive and aversive stimuli can strongly influence later learning in traditional operant or classical conditioning paradigms. In the spatial operant learning paradigm using the heat box, an organism can dwell on the side associated with high temperature or move away from this punished zone. However, in nature organisms don't always control their environment and are often subjected to unexpected good and bad events. How these exposures influence later conditioning with similar or different reinforcers is not well understood.

In many cases unpaired experiences can either obstruct or enhance later learning. Several phenomena have been identified that reduce later learning, including learned irrelevance and learned helplessness (Bennett et al., 1995; Seligman, 1972). An example of this effect was first identified in dogs and similar influences on associative learning have been seen in other vertebrates and invertebrates. Behaviorally, several conditioning experiments in rats show inhibited learning with CS-US pairing after pre-exposure of that US (Clafin and Buffington, 2006; de Brugada et al., 2004). Examples in humans also show that the presentation of an unexpected stimuli, reduces their ability to form associative memories later with those same stimuli (Myers et al., 2000). Learned helplessness has also been thought to be responsible for some symptoms of post traumatic stress disorder (Foa et al., 1992). This effect is however not restricted to vertebrate animals. In the honeybee, unpaired sugar or shock exposure retards later associative conditioning (Abramson and Bitterman, 1986; Sandoz et al., 2002). These suppressing effects are in contrast to those experiences that can enhance later conditioning. For example, fear conditioning in one context can be behaviorally sensitized when rats experience electric shock in a different context (Rau et al., 2005). Although tail shock in *Aplysia* can induce memory savings

evident in later sensitization experiments (Philips et al., 2006), there is no clear evidence that reinforcement pre-exposure can enhance later performance in associative conditioning in an invertebrate animal. We therefore sought to identify the influence of unexpected aversive events on conditioning in *Drosophila* using the heat box.

Results

Reinforcement pre-exposure enhances memory in the heat box

The heat box lends itself to behavioral, cellular and molecular analysis of a potential pre-exposure effect. We investigated here whether unpaired high-temperature exposure can have an effect on later conditioning in *Drosophila*. To do this, we examined the effect of high-temperature exposures on later conditioning using 41°C and 30°C reinforcement (Sitaraman et al., 2007). Flies pre-exposed to 1 minute of 41°C but not 30°C and 24°C had an enhancement with 30°C reinforcer in later conditioning (Figure 5-1). However, none of the above pre-exposures had any effect on later conditioning with 41°C reinforcer. We next wanted to test if changing the number of pre-exposure blocks to alter the duration of pre-exposure could influence later conditioning. To investigate the duration of the sensitizing effect of 41°C exposure, we exposed three groups of flies to either 0, 1, or 3 minutes of high temperature and tested conditioned memory after 1, 10, or 20 minutes (Figure 5-2). Under these conditions we found that three exposures had a somewhat larger enhancing effect on memory than one exposure, although this was not statistically significant. Both one and three exposures, however, had a large effect on memory performance compared to no exposures at 1, 10, and 20 minutes retention intervals (the time between the high temperature exposure and conditioning). Thus, the enhancing effect of unpaired high temperature exposure lasts at least 20 min. We, thus, provide the first example of this type of enhancement in an invertebrate animal.

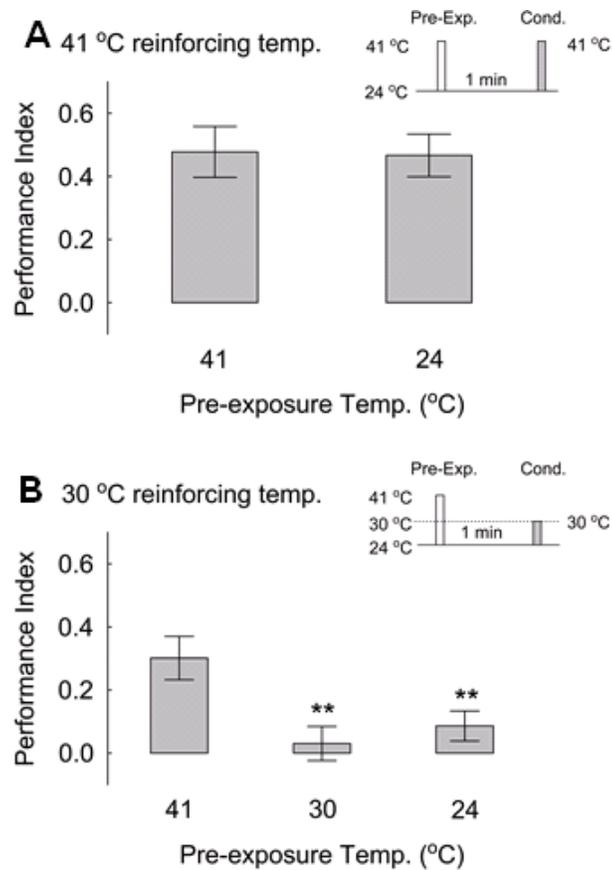


Figure 5-1. Unpaired high-temperature exposure enhances warm temperature reinforced memory. Flies were exposed for 1 min to 24, 30, or 41°C and then conditioned with a 30 or 41°C reinforcing temperature (schematized in upper right panels). A. There was no measurable effect of unpaired high-temperature exposure on 41°C reinforced conditioned memory ($H(1, N = 144) = 0.55, p = 0.5$). In contrast in B flies that had been exposed to 41°C had significantly enhanced memory performance compared to flies exposed to 24 or 30°C ($H(2, N = 234) = 15.6, p = 0.0004$; multiple comparison tests reveal significant differences between the 41°C group and both 30 and 24°C groups, $** = p < 0.01$). Flies were exposed for 1 min to 24, 30, or 41°C and then conditioned with a 30 or 41°C reinforcing temperature (schematized in upper right panels). The bars represent the mean values, error bars are SEMs.

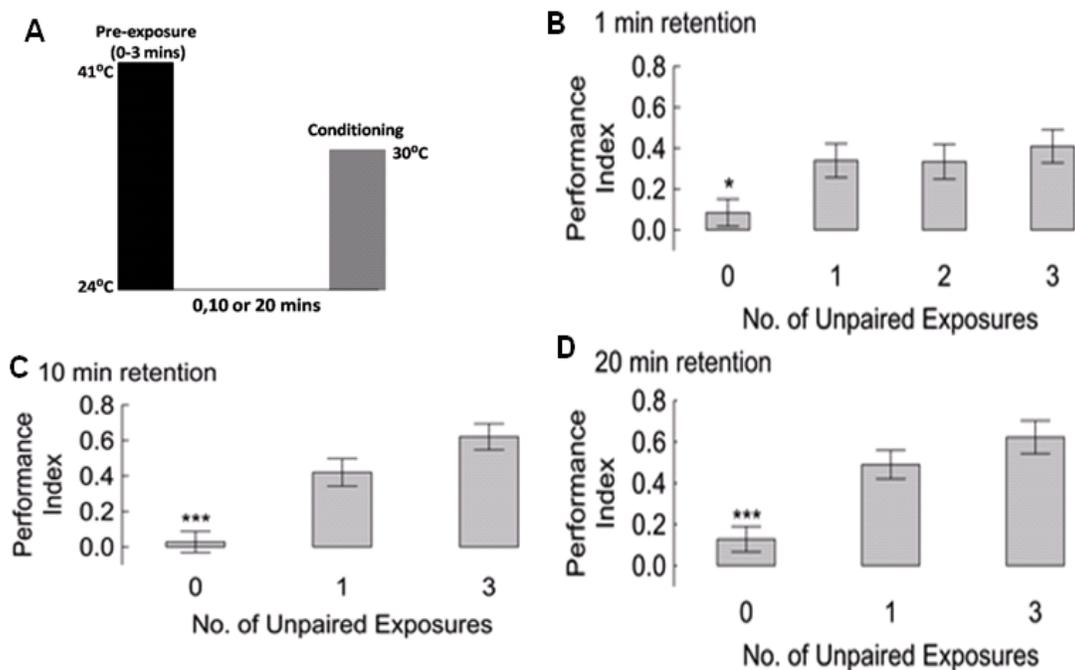


Figure 5-2. Unpaired high-temperature enhancement of memory formation lasts at least 20 minutes. When tested 1 min after exposure, 1, 2, or 3 exposures enhanced memory formation. A. Experimental protocol schematized black bar represents pre-exposure while the grey bar represents conditioning 0, 10 and 20 minutes after pre-exposure. B. Flies tested after 1 min of pre-exposure were compared and we found [$H(3, N = 333) = 13.0, p = 0.005$]. There was no significant effect of increased number of exposures on memory formation for both the 10 and 20 min retention tests. C and D. 10 min retention [$H(2, N = 216) = 34.6, p = 0.0000$] and 20 min retention [$H(2, N = 243) = 30.1, p = 0.0000$]. Although three exposures increased later memory performance compared to one exposure, this was not significant in either the 10 or 20 min retention tests (multiple comparison tests reveal significant differences only between non-exposed groups and the other groups, $* = p < 0.05$, $*** = p < 0.001$). The bars represent the mean values, error bars are SEMs.

High temperature exposure does not change unconditioned avoidance behavior and is independent of the antennal thermosensor

The ability of the flies to enhance their memory performance after reinforcement pre-exposure could be a result of altered thermosensitivity. To address this possibility flies were pre-exposed to 41°C under conditions identical to those used in the above conditioning experiments followed by testing of their avoidance for 30°C and 41°C. In the thermosensitivity assay, flies are presented with a chamber half held at an elevated temperature (30 or 41°C) while the other chamber half is 24°C. Flies normally avoid the chamber half that is higher (or lower) than 24°C, and measures the ability of a fly to both sense and avoid a high temperature source. In all conditions, pre-exposure to 41°C did not have a significant effect on avoidance behavior of either 30 or 41°C (Figure 5-3). Thus, the pre-exposure effect on conditioned behavior is independent of changes in high temperature avoidance behavior.

Interestingly, temperatures above and below 30°C appear to be sensed by different mechanisms. Removal of the antennae abolishes flies' ability to sense and avoid 30°C or less (Sayeed and Benzer, 1996; Zars, 2001). Furthermore, temperatures above 30°C are typically required for place memory formation (Zars and Zars, 2006; Zars, 2001). When the antennae are removed no conditioning has been detected using this temperature (Zars, 2001). We, therefore, tested whether the antennal thermosensor was necessary for the pre-exposure effects on memory conditioned with 30°C. To do this, flies with or without antennae were presented with one or three 41°C exposures and tested 20 min later for conditioned place memory using the short protocol and 30°C reinforcement. In both cases, we found that removal of the antennae had no effect on the memory enhancing effect of unpaired high temperature pre-exposure (Figure 5-4). Therefore, 41°C exposure recruits the high-temperature thermosensor into responding to 30°C and place memory can be reinforced.

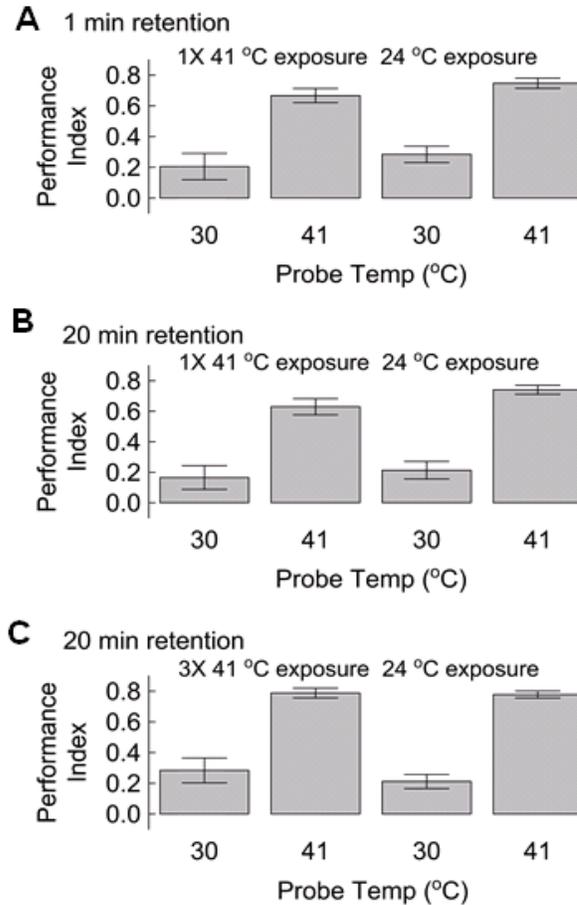


Figure 5-3. Pre-exposure to high temperature does not alter high-temperature avoidance behavior. With a 1 min retention interval, flies exposed to either 41 or 24 °C showed similar avoidance of 30 and 41°C probe temperatures. Statistical comparisons of mean ranks by Kruskal Wallis test reveals for probe temperature 30°C, $H(1, N = 207) = 0.067$, $p = 0.79$; probe temperature 41°C, $H(1, N = 207) = 1.21$, $p = 0.27$. Similarly, after a 20 min retention interval, neither one or three 41°C exposures had an effect on avoidance of 30 or 41°C probe temperatures (Single exposure: probe temp 30 °C, $H(1, N = 176) = 0.002$, $p = 0.97$; probe temp 41°C, $H(1, N = 176) = 1.88$, $p = 0.17$. Three exposures: probe temp 30°C, $H(1, N = 173) = 2.53$, $p = 0.11$; probe temp 41°C, $H(1, N = 173) = 1.07$, $p = 0.30$). The bars represent the mean values, error bars are SEMs.

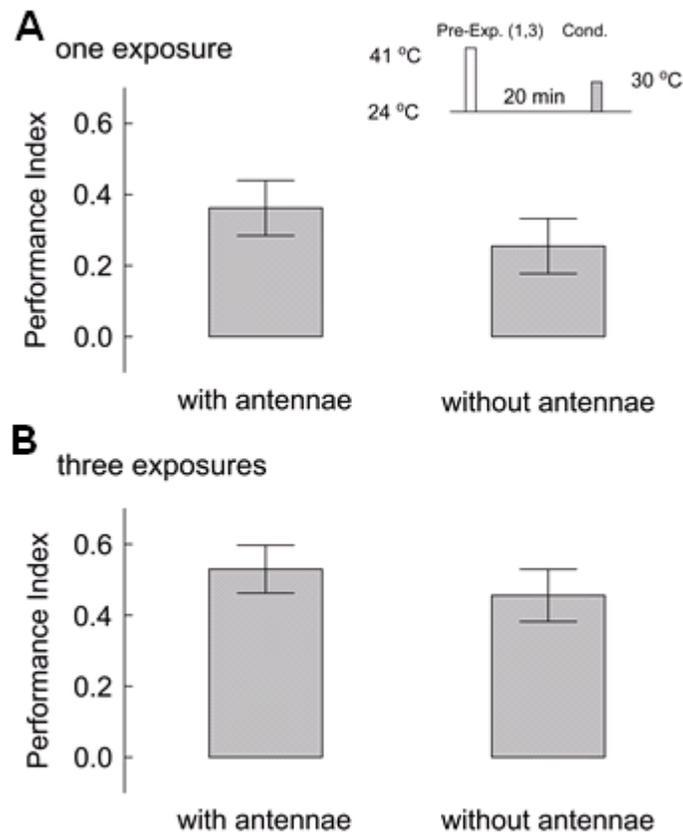


Figure 5-4. A high-temperature thermosensor is sufficient for reinforcement pre-exposure enhancement of memory formation. Although flies without antennae performed slightly lower than flies with antennae after either one or three exposures (experiment schematized in upper right panel), they did not perform significantly worse in either case A. one exposure $H(1, N = 180) = 0.45, p = 0.50$, or B. three exposures $H(1, N = 175) = 0.29, p = 0.59$. In all groups, memory performance was significantly greater than zero (one exposure with antennae $Z = 3.27, p = 0.001$; one exposure without antennae $Z = 2.2, p = 0.02$; three exposures with antennae $Z = 4.7, p < 0.001$; three exposures without antennae $Z = 3.9, p < 0.001$). The bars represent the mean values, error bars are SEMs.

Pre-exposure influences reinforcement processing

Training wild types flies for varying time periods in the range of 4 to 20 minutes shows that flies can make an association between spatial position and negative reinforcement in as little as 4 minutes. As expected, increased training leads to increases in memory levels reaching an asymptote at 10 minutes of training (Diegelmann et al., 2006). The short (4 minutes) and long training (20 minutes) allows for identification of acquisition and reinforcement processing deficits. We tested wild-types flies pre-exposed to 3 blocks of 41°C and conditioned using 4 and 20 minute training. The pre-exposure induced enhancement on later conditioning with 4 minute training is consistent with previous results (Sitaraman et al., 2007). A significant enhancement was observed with 20 minutes training (Figure 5-5). This suggests that the pre-exposure influences reinforcement processing mechanisms. We next sought to investigate the role of serotonin in the pre-exposure effect since this system is required for reinforcement processing in place learning (Sitaraman et al., 2008).

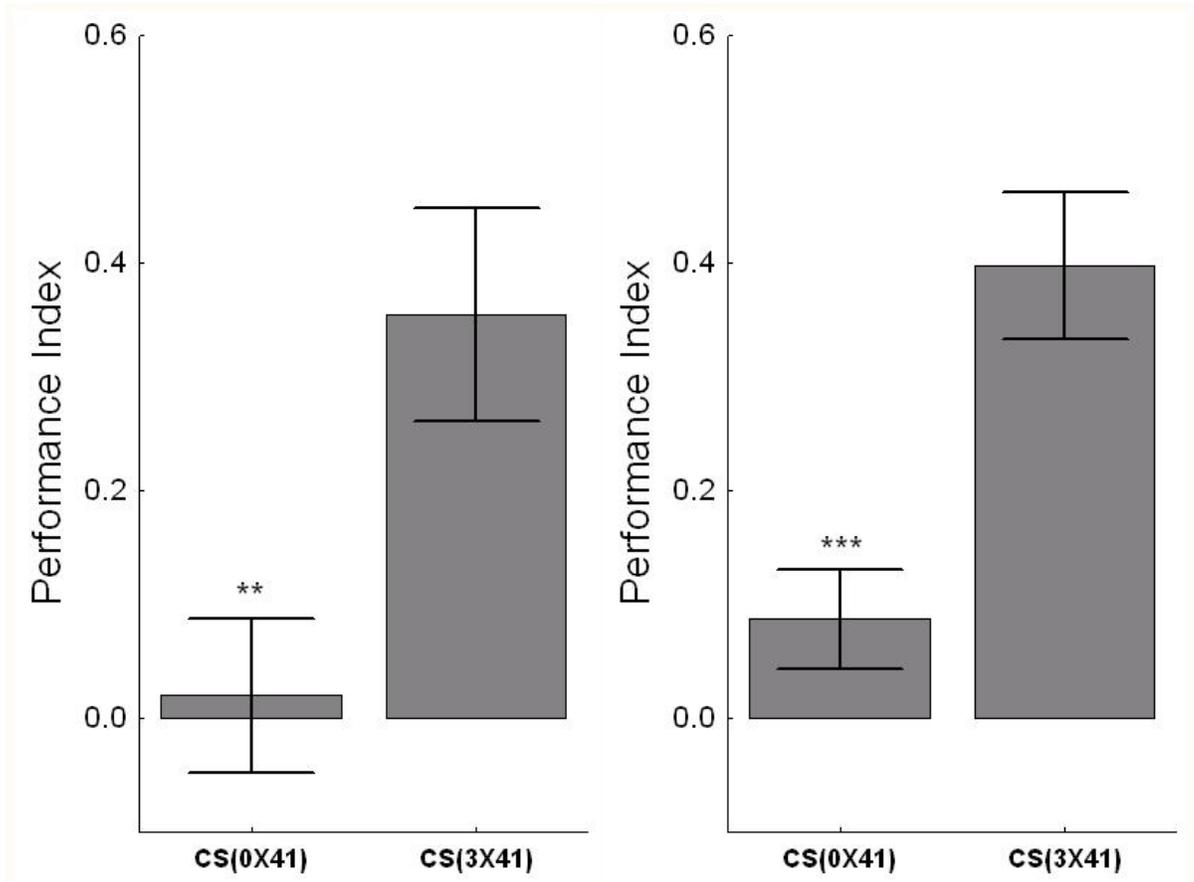
A. 4 minutes training**B. 20 minutes training**

Figure 5-5. Pre-exposure effect induces enhancement of short (4 minutes) and long (20 minutes) conditioned memory in the heat box. Wild type CS flies were pre-exposed to 24° C (0X41) or 41° C (3X41) and conditioned with 4 min training in A, and 20 minute training in B. Statistical comparisons between the (0X41) and (3X41) groups for A and B were made by Mann-Whitney U test: for A, $U=1462$, $z=-2.8$, $p=0.005$, $N=128$. With 20 minute training in B, $U=4880$, $z=-4.14$, $p=0.0004$, $N=238$. The time gap between pre-exposure and training was 10 minutes for both A and B. The bars represent the mean values, error bars are SEMs marked significant as ** = $p<0.01$ and *** = $p<0.001$.

Reinforcement processing mechanisms are important for the pre-exposure effect

Wild type flies can increase or decrease their memory levels indicated by performance index in as little as 2 minutes in response to an increase or decrease in the reinforcement intensity (Zars and Zars, 2009). Based on memory matching results from spatial learning we hypothesize that the flies inflate the high temperature reinforcing value of 30°C after pre-exposure leading to high performance indices. We know that the high temperature reinforcement information is mediated by serotonin (Sitaraman et al., 2008). Since memory levels with 20 minute training can be enhanced with pre-exposure we hypothesized that the serotonergic system would also be important for the pre-exposure effect. To test this directly we genetically blocked synaptic output from serotonergic neurons targeted by DdcGal4;Th-GAL80 by expressing tetanus toxin light chain (Sweeney et al., 1995). These flies were then tested for their ability to form memories 10 minutes after pre-exposure with 3 minutes of 41°C.

To determine that the phenotype observed from these experiments was a result of pre-exposure effect and not of impaired 30°C conditioning; flies with pre-exposure to 3 minutes of 24°C under the same chamber environment were used as controls (Figure 5-6). Additionally, flies were conditioned with 4, 6, 8, 10, and 20 minutes of 30°C reinforcement to see if the serotonergic system is required for reinforcement processing of the pre-exposure effect. The tested genotypes were also tested for their ability to sense and avoid temperatures used for later conditioning after 0X41 and 3X41 exposures (Table 5-1). As seen with direct conditioning experiments, memory levels during later conditioning increases with increased training, reaching an asymptotic level at 10 minutes. Therefore, we can conclude that pre-exposure, modulates the serotonin dependent reinforcement processing mechanisms in place learning.

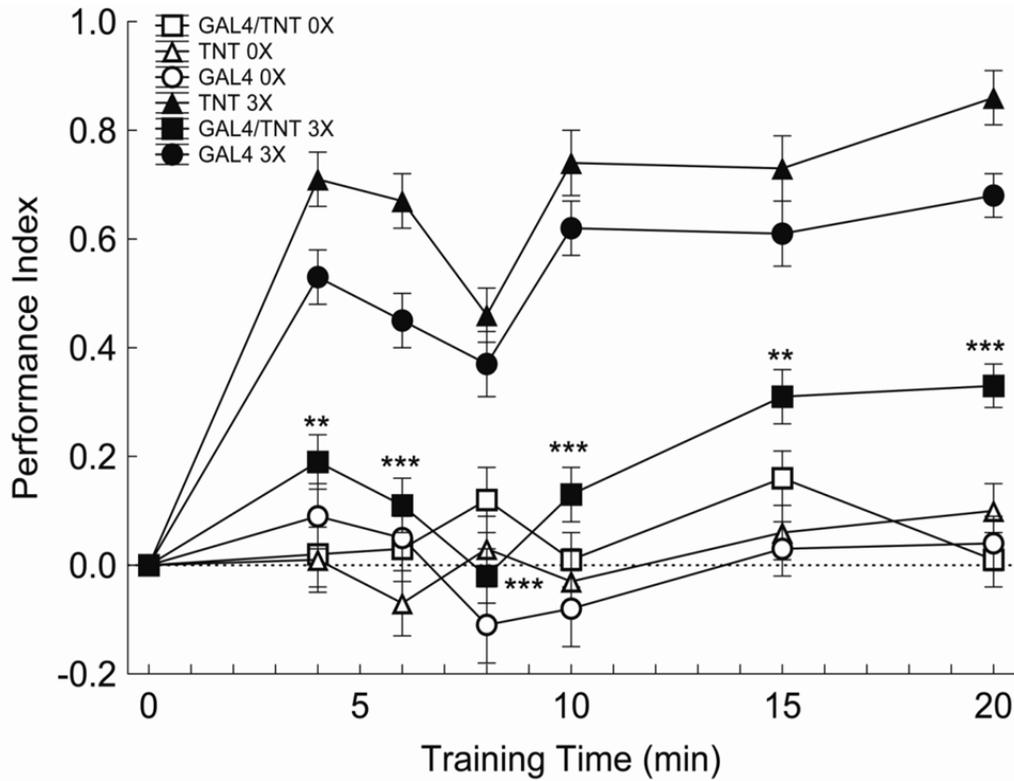


Figure 5-6. Ddc-Gal4; Th-Gal80 neurons are required for acquisition and maintenance of the pre-exposure effect. Tetanus toxin light chain (TNT) was expressed in Ddc; Thgal80 positive serotonergic neurons and conditioned for 4, 6, 8, 10, 15, and 20 minutes at 30°C after pre-exposure. Legend on left corner of the figure shows the three genotypes tested 10 minutes after pre-exposure with either 3 minutes of 41°C (3X) or 24°C (0X). Open symbols indicate 0X and shaded symbols indicated 3X exposures. Statistical analysis by multiple comparisons of mean ranks of the 6 tested groups at different time points indicate for 4 minutes H (5, N=553)=132.25, p=0.000, for 6 minutes H (5, N=486)=124.05, p=0.000, for 8 minutes H (5, N=428)= 57.85, p=0.000, for 10 minutes H (5,N=433)=125.41, p=0.000, for 15 minutes H (5, N=477)= 103.74, p=0.000, for 20 minutes H (5, N=615)=212.66, p=0.000. The points represent the mean values, error bars are SEMs, marked significant as ** = p<0.01 and *** = p<0.0001.

Table 5-1. Thermosensitivity of Ddc-Gal4;Th-Gal80/TNT flies after pre-exposure

A. (3X41) (Mean PI \pm SEM)

Type	24/24	24/30	24/41	N
Ddc;Th-Gal80/+	-0.11 \pm 0.08	0.14 \pm 0.08	0.71 \pm 0.03	70
Ddc;Th-Gal80/TNT	-0.02 \pm 0.08	0.13 \pm 0.08	0.71 \pm 0.05	74
TNT/+	0.15 \pm 0.05	0.16 \pm 0.06	0.70 \pm 0.06	68

B. (0X41) (Mean PI \pm SEM)

Type	24/24	24/30	24/41	N
Ddc;Th-Gal80/+	0.04 \pm 0.05	0.16 \pm 0.05	0.70 \pm 0.03	71
Ddc;Th-Gal80/TNT	0.10 \pm 0.03	0.15 \pm 0.04	0.62 \pm 0.03	79
TNT/+	0.10 \pm 0.03	0.19 \pm 0.03	0.64 \pm 0.03	71

Table 5-1. Blocking synaptic output from serotonergic neurons labeled by Ddc; Th-Gal80 does not alter the ability to sense and avoid temperatures after pre-exposure. Flies were pre-exposed to either A. 3 minutes of 41°C (3X41) or B. 3 minutes of 24°C (0X41) and tested for thermosensitivity 10 minutes after pre-exposure. Flies expressing tetanus toxin light in serotonergic neurons (Ddc-Gal4; Th-Gal80/TNT) were compared with their genotypic controls (Ddc-Gal4; Th-Gal80/+ and TNT/+). Multiple comparisons by mean ranks show for A: H (2, N= 208) for 24/24 = 3.53, p=0. 17, for 24/30 = 0.66, p= 0.72, for 24/41 =2.43, p=0.29. Statistical comparisons in B: H (2, N= 221) for 24/24 = 2.64, p=0. 27, for 24/30 = 1.97, p= 0.37, for and for 24/41= 3.7, p=0.15.

Serotonergic neurons targeted by Trh-Gal4 are necessary for the pre-exposure effect

Since Ddc-Gal4;Th-Gal80 system also labels the dopaminergic PAM neurons (Friggi-Grelin et al., 2003; Mao and Davis, 2009) and a cluster of non serotonergic neurons (Sitaraman et al., 2008) additional experiments were carried out using other tools available to gain confidence in the role of serotonergic system in pre-exposure. To this end we used a second serotonergic driver Trh-Gal4 targets a different gene required for serotonin synthesis. We hypothesize that blocking synaptic output of Trh-Gal4 positive neurons would restrict the pre-exposure induced enhancement. An effect of a second Gal4 driver would help gain confidence in the role of serotonergic system in pre-exposure. To this end flies expressing tetanus toxin light chain (TNT) in the Trh-Gal4 positive cells were subjected to 3X41 and 0X41 and later conditioned with the 30° C long training protocol was used to identify a processing phenotype. We find that the Trh-Gal4/ TNT flies showed lower memory performance levels during later conditioning compared to the genotypic control levels. However, the memory levels after 0X41 were not significantly different between tested groups (Figure 5-7).

The ability of the tested genotypes after 3X41 and 0X41 to sense and avoid temperatures relevant to the later conditioning and pre-exposure (24, 30, and 41° C) were tested using the thermosensitivity assay (Table 5-2). The performance index of the three groups for 3X and 0X were not significantly different.

A Pre-exposed with 0X41

B Pre-exposed with 3X41

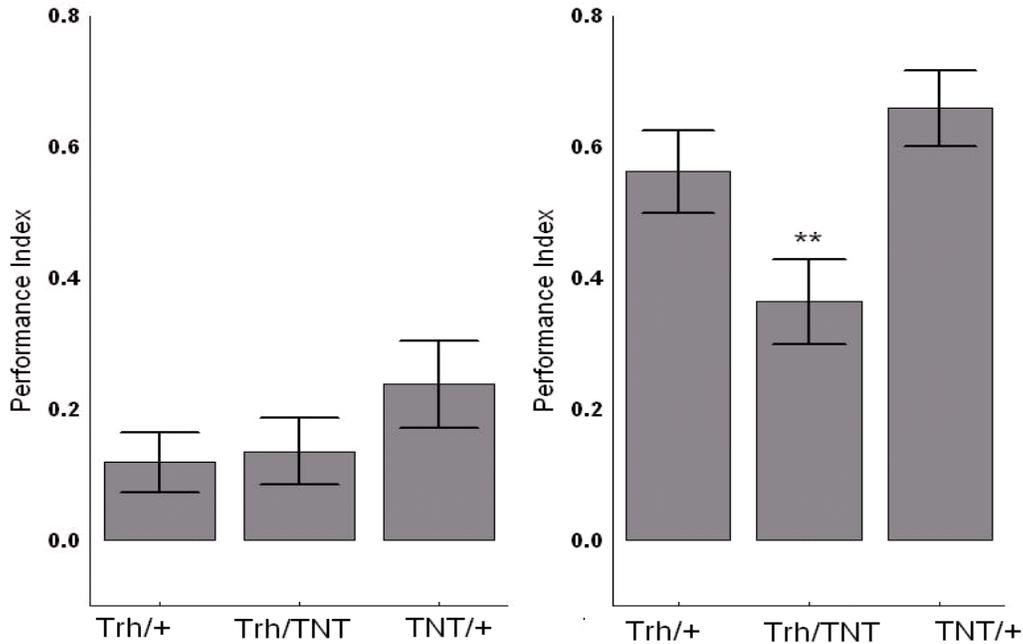


Figure 5-7. Pre-exposure induced enhancement is inhibited by blocking output from Trh-Gal4 positive serotonergic neurons. Flies expressing tetanus toxin light chain (TNT) in Trh positive serotonergic neurons (Trh-Gal4 / TNT) were compared to their genotypic controls (Trh-Gal4 / + and TNT / +) for conditioning. Experimental regimen for pre-exposure was 3 minutes of 24°C (0X41) in A, and 3 minutes of 41°C pre-exposure (3X41) in B. Multiple comparisons of mean ranks for A. $H(2, N=327) = 2.84, p=0.24$ and for B. $H(2, N=407) = 25.44, p=0.0000$.

Performance index values of Trh-Gal4 / TNT were significantly different from Trh-Gal4 / + and TNT / + in B. The values represent the mean values, error bars are SEMs, marked significant as ** at $p < 0.01$.

Table 5-2. A. Thermosensitivity of Trh-Gal4/TNT and control flies

A. 3X41

Type	24/24	24/30	24/41	N
Trh/(3X41)	0.04±0.07	0.14±0.07	0.60±0.06	87
Trh/TNT(3X41)	-0.05±0.07	0.11±0.08	0.54±0.06	81
TNT/(3X41)	0.05±0.07	0.19±0.07	0.58±0.06	81

B. 0X41

Trh/(0X41)	0.07±0.05	0.16±0.05	0.67±0.06	80
Trh/TNT(0X41)	0.09±0.02	0.08±0.04	0.60±0.04	87
TNT/(0X41)	0.04±0.03	0.14±0.05	0.64±0.05	78

Table 5-2. Blocking synaptic output from serotonergic neurons targeted by Trh-Gal4 pre-exposure induced memory enhancement but doesn't affect thermosensitivity. Flies expressing tetanus toxin light chain in Trh-Gal4 positive neurons (Trh-Gal4 / TNT) were pre-exposed to either 3 minutes of 41°C (3X41) or 3 minutes of 24°C (0X41) and tested for thermosensitivity along with the genotypic controls Trh-Gal4 / + and TNT/+. Multiple comparisons of mean ranks by Kruskal-Wallis test for A: $H(2, N=249)$, for temperature 24°C =0.49, $p=0.78$ for 30°C = 0.81, $p=0.6$ and for 41°C =2.75, $p=0.25$. Statistical comparisons with B: $H(2, N=245)$, show for temperature 24°C =2.2, $p=0.33$, for 30°C = 2.3, $p=0.31$, for 41°C =5.4, $p=0.07$. Values represent mean± SEMs.

Pharmacological inhibition of serotonin synthesis severely restricts pre-exposure induced memory enhancement

To test the possibility of a developmental role for serotonin and complement the genetic analysis of the pre-exposure effect we used pharmacology to inhibit serotonin synthesis in wild type adult flies. Wild type CS flies were fed serotonin synthesis inhibitor, α -methyltryptophan (am-W) or water (sham control) for 2 days after which the drug fed flies and sham controls were tested for the pre-exposure effect with 20 minutes training in later conditioning (Figure 5-8). The flies with reduced serotonin levels with the 3X41 pre-exposure did not show a memory enhancement as compared to the sham controls. Both am-W and sham fed flies performed at similar levels with 0X41 pre-exposure indicating that the inhibition of serotonin synthesis itself does not impair conditioning with the low temperature 30°C reinforcer. Combined with the genetic evidence from Trh-Gal4 and Ddc-Gal4; Th-Gal80 we find that the impairment in conditioning after pre-exposure does not result from deficits to process this low temperature reinforcement. All the three independent methods of manipulating the serotonergic system impaired processing of the pre-exposure effect.

Finally, we tested the ability of α -methyltryptophan (am-W) and sham fed flies to sense and avoid temperatures used in conditioning to make sure that the inability to enhance memory does not result from altered thermosensory ability (Table 5-3). The sham and am-W fed flies did not show a significant different in avoidance of 30 and 41 °C showing that pre-exposure does not alter thermosensory ability .

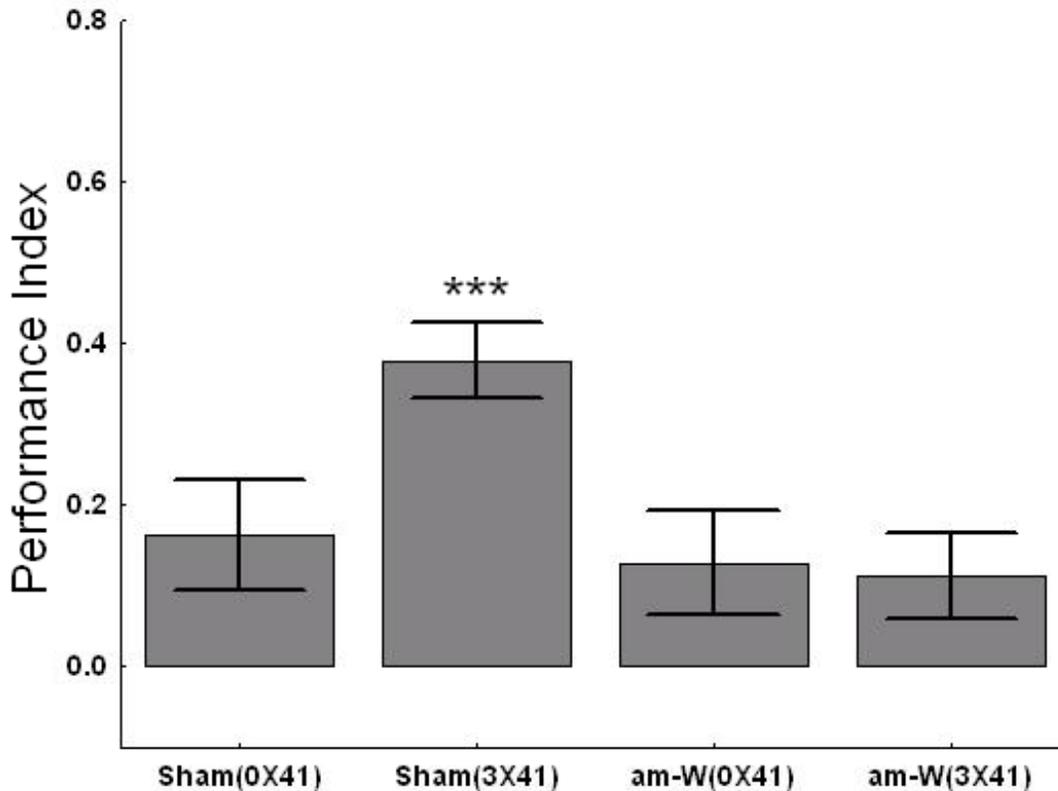


Figure 5-8. Inhibition of serotonin synthesis impairs pre-exposure induced memory enhancement. Sham (water fed) and α -methyltryptophan or am-W (drug fed) flies were pre-exposed to either 3 minutes of 41°C (3X41) or 3 minutes of 24°C (0X41) and conditioned at 30°C, 10minutes after pre-exposure. Pharmacological blocking of serotonin synthesis lead to impairment in ability of wild type flies to enhance conditioned memory after pre-exposure. Multiple comparisons of mean ranks, Kruskal-Wallis test: $H(3, N= 566) = 19.16$ $p = 0.000$. No significant differences were found between the sham (0X41) and am-W (0X41) flies. The values represent the mean values, error bars are SEMs marked significant as ***= $p < 0.001$.

Table 5-3. Thermosensitivity of am-W and sham flies after pre-exposure

Type	24/24	24/30	24/41	N
Sham(0X41)	0.08±0.07	0.40±0.06	0.66±0.06	90
am-W(0X41)	0.09±0.06	0.36±0.06	0.72±0.04	92
Sham(3X41)	0.02±0.05	0.30±0.05	0.70±0.04	101
am-W(3X41)	-0.01±0.08	0.26±0.08	0.50±0.07	101

Table 5-3. Inhibition of serotonin synthesis impairs pre-exposure induced memory enhancement but does not affect thermosensitivity. Sham (water fed) and am-W (drug fed) flies were pre-exposed to either 3 minutes of 41°C (3X41) or 3 minutes of 24°C (0X41) and tested for thermosensitivity 10 minutes after pre-exposure. Inhibition of serotonin synthesis did not lead to impairment in sensing and avoiding temperatures (30 and 41°C). Multiple comparisons of mean ranks by Kruskal-Wallis test H (3, N=384), for temperature 24°C =1.33, p=0.72, for 30°C =4.91, p= 0.18, and for 41°C =5.75, p=0.12. Values represent means± SEMs.

The Pre-exposure effect survives context interruption

Our experiments with Ddc; Th-Gal80, Trh-Gal4 and α -methyltryptophan thus indicate that the serotonergic system is required for the pre-exposure effect. The inhibition of serotonergic output and synthesis only produce a partial reduction in pre-exposure induced enhancement, which may indicate that other mechanisms are also important. A second component could result from a stress response. We next sought to test the possibility of a pre-exposure induced stress response in memory enhancement.

In *Drosophila*, a mixture of volatile compounds is thought to be released in response to stressors such as electrical and mechanical shock. Even though the exact composition of this stress odorant mixture is not known an important component of the dSO (*Drosophila* stress odorant) is carbon dioxide (Suh et al., 2004). The emission of dSO or carbon dioxide alone is an aversive cue to the flies and flies also show a dosage dependant avoidance behavior in response to CO₂ through specific neurons (Gr21) in the glomeruli (Suh et al., 2007; Suh et al., 2004). Pre-exposure to an aversive reinforcer could induce the release of volatile compound which primes the flies to perform at higher levels and exhibit an exaggerated avoidance response to 30°C during conditioning.

To address this possibility we pre-exposed the flies and instead of leaving them in the chamber for 10 minutes we placed them in a fresh empty vial for the period between pre-exposure and conditioning. Additionally the chambers were aired out to remove a potential odorant or volatile compound. The flies were then loaded in the chambers and conditioned using 30°C. Interestingly, the flies still exhibited an enhanced conditioned behavior (Figure 5-9). Combined with earlier experiments where flies were pre-exposed to both 0X and 3X41 in the same chambers and handled in identical fashion for later conditioning we have no evidence that odor deposits in the chamber itself influences conditioning. Thus, it is highly unlikely that a possible stress odorant

release resulting from the 3 minutes of 41°C exposure enhances conditioned behavior.
Furthermore, the effect of pre-exposure on conditioning survives context interruption.

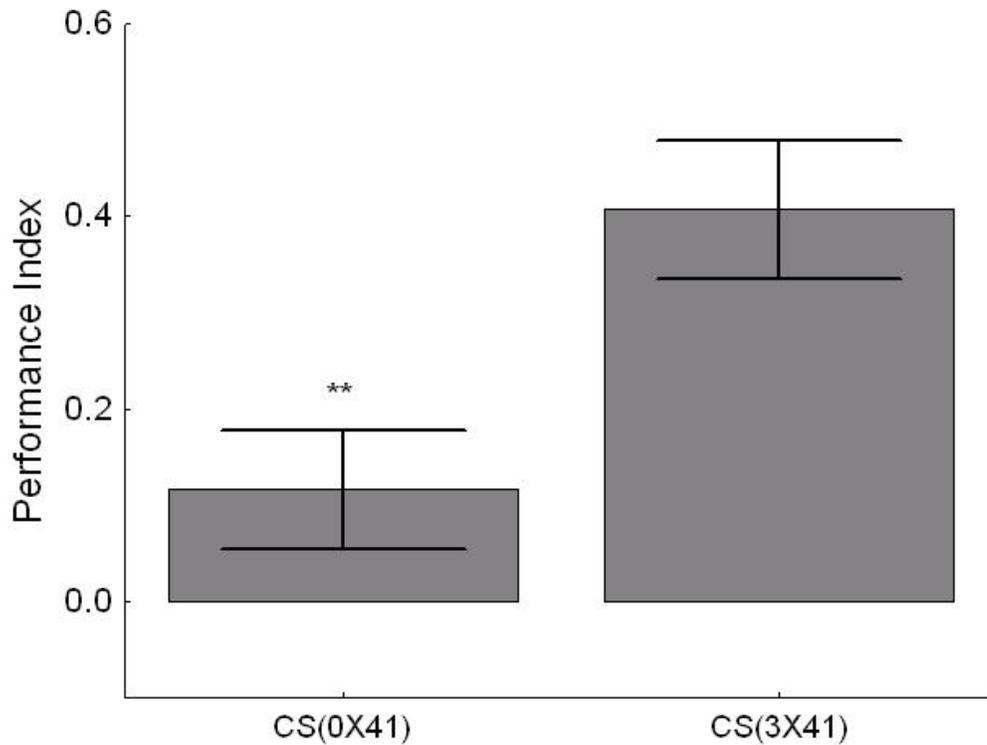


Figure 5-9. Pre-exposure effect does not depend on a pre-exposure induced stress odorant and survives context interruption. Wild types CS flies were pre-exposed to 41(3X41) or 24 (0X41) and removed from the heat box chambers during the gap between pre-exposure and conditioning. Performance index represents the average 3 minute memory score after training. Statistical analysis using Mann-Whitney U test where $U=1935$ $z=-3.05$, $p=0.002$, $N=148$. Values represent mean and error bars SEMs, marked significant as $**=p<0.01$.

Activation of Trh neurons during pre-exposure is sufficient to induce later memory enhancement

The recruitment of the serotonergic neurons supports the notion that the serotonergic reinforcement circuit mediating 41°C is also required for the pre-exposure effect. We wanted to test for the sufficiency of serotonergic neurons in inducing this effect. We expressed dTrpA1 channels in Trh-Gal4 positive serotonergic neurons. Activation of serotonergic neurons around 31-33°C leads to increased memory scores with optimal activation at 32°C (Figure 3-13). This indicates that the activation of serotonergic neurons alone can mimic the high temperature reinforcement. We wanted to test if increasing excitability of Trh positive serotonergic neurons could substitute for pre-exposure.

Flies expressing dTrpA1 in Trh-Gal4 positive serotonergic neurons were exposed to 30-32°C for 30 minutes (20 minutes in a water bath at 30°C and 10 minutes in the heat box chamber at 32°C) followed by a 10 minute gap in which the flies were in the chambers maintained at 24°C. This long time period was chosen to potentially activate the serotonergic neurons strongly during pre-exposure. The 10 minute gap was followed by conditioning with 30°C (Figure 5-10). Trh-Gal4/dTrpA1 flies had a significant enhancement in conditioning as compared to the genotypic controls. To make certain that an activation event leads to memory enhancement the experimental and control flies were exposed to 24°C instead of 30-32°C during pre-exposure period. We found that only Trh-Gal4 / dTrpA1 flies pre-exposed to 30-32°C showed memory enhancement as compared to the controls. Trh-Gal4 / dTrpA1 flies pre-exposed to 24°C under identical chamber conditions did not show this phenotype. These results also show that the memory enhancement induced is a result of specific activation during pre-exposure period and not because of activation during later conditioning with 30°C. This conclusion is supported by the memory levels of the 24°C pre-exposed Trh-Gal4 / dTrpA1 flies (not significantly different from their genotypic controls) that were subjected to the same conditioning environment.

Since dTrpA1 is a temperature sensitive channel the phenotypes could also result from altered temperature avoidance. To test for this possibility we carried out a thermosensitivity assay to test for the ability of the flies to sense and avoid temperatures used in conditioning and pre-exposure (Table 5-4). And the results show that expression and activation of dTrpA1 in serotonergic neurons does not impair or enhance thermosensory acuity. No significant differences were found between performance indices of the tested genotypes for avoidance of 30 and 41°C.

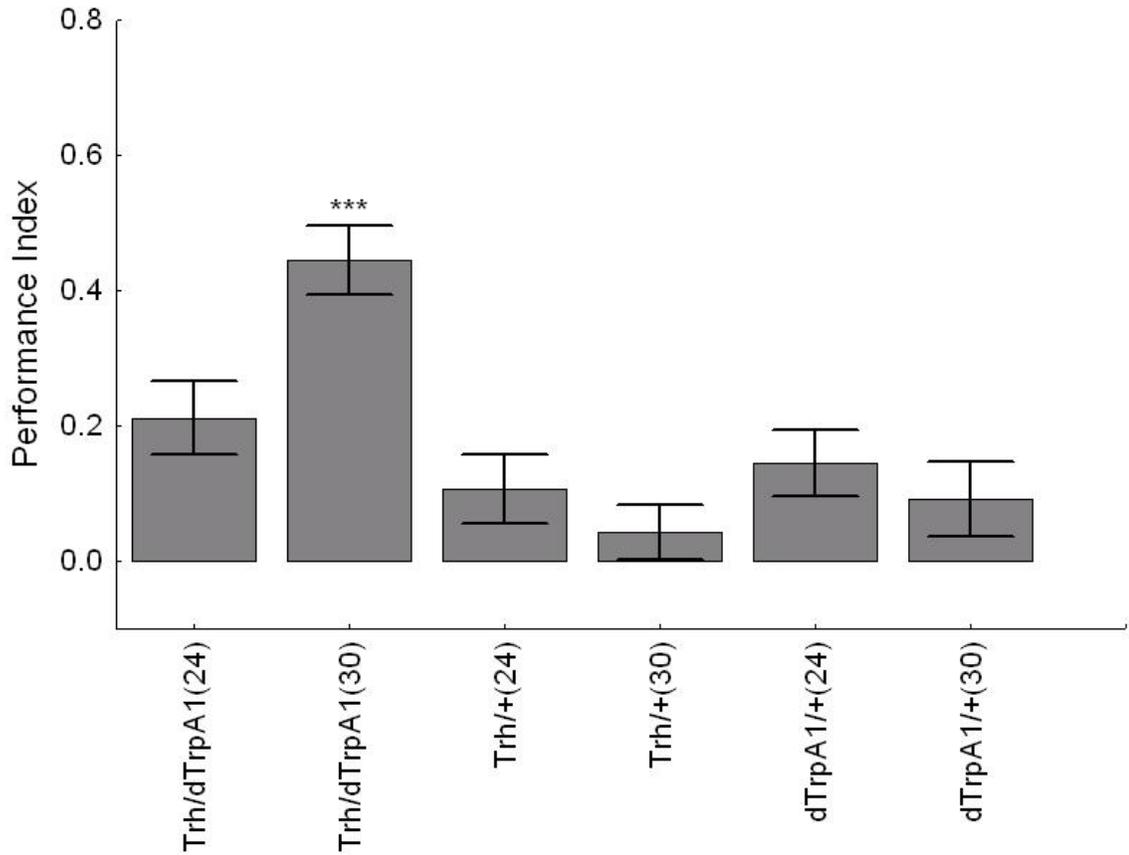


Figure 5-10. Activation of Trh-Gal4 targeted neurons induces a pre-exposure effect 10 minutes post activation. Trh-Gal4/ dTrpA1 flies that were incubated to 30-32°C during pre-exposure had a significantly higher memory score when tested with 30°C conditioning as compared to all genotypes tested. Multiple Comparisons of mean ranks by Kruskal-Wallis test: $H(5, N=737) = 31.85$ $p=0.000$. Values represent mean and standard error SEMs, marked significant as *** = $p<0.0001$.

Table 5-4. Thermosensitivity of Trh-Gal4/dTrpA1 flies and controls after pre-exposure

Type	24/24	24/30	24/41	N
Trh/+ (24)	0.18±0.04	0.26±0.05	0.75±0.04	83
Trh/dTrpA1(24)	0.02±0.04	0.18±0.04	0.59±0.06	85
dTrpA1/(24)	0.07±0.04	0.16±0.04	0.70±0.04	83
Trh/+ (30)	0.05±0.04	0.22±0.05	0.72±0.02	88
Trh/dTrpA1(30)	0.06±0.04	0.14±0.05	0.66±0.05	84
dTrpA1/(30)	0.04±0.04	0.20±0.04	0.71±0.04	90

Table 5-4. The ability of the Trh-Gal4 / dTrpA1 flies to sense and avoid temperatures after pre-exposure to 24 or 30°C was compared to the genotypic controls. Both 30°C and 24 °C pre-exposed flies were tested for their avoidance of temperatures 30 and 41 °C and were not significantly different. Multiple comparisons of mean ranks indicates H (5, N=513) for 24/24, =9.47 p=0.1, for 24/30, = 5.38, p=0.37, and for 24/41 =6.24, p=0.28. Values represent mean± SEMs.

Lack of behavioral predictor induces the pre-exposure effect

In a typical pre-exposure experiment the flies are exposed to 41°C for three minutes. The pre-exposure effect could be the consequence of the difference in exposure time to 41°C reinforcer or the memory enhancement could result from the unpredictability of the pre-exposure stimulus. To address this possibility we carried out yoking experiments. In these experiments flies in odd numbered chambers had control of the environment. Such that every time they crossed the midline to venture into the punished zone they received a heat strike as the temperature rose from 24 to 41°C (identical to direct conditioning experiments). The flies in the odd numbered chambers controlled the environment based on their spatial position and are called master flies (M). In the even numbered chambers the flies received the same number, intensity and sequence of high temperature stimuli independent of their position in the chambers. These yoked flies have no control of their environment, and thus cannot develop a prediction about the reinforcement stimuli.

Yoking experiments allow memory formation in master flies but not yoked flies.

Yoking experiments in the heat box clearly show that the master learns and forms a robust memory. The yoked flies do not form a memory as they cannot make accurate associations (Putz and Heisenberg, 2002). A schematic of the experimental chambers of master (control) and yoked (no control) is shown in Figure 5-11.

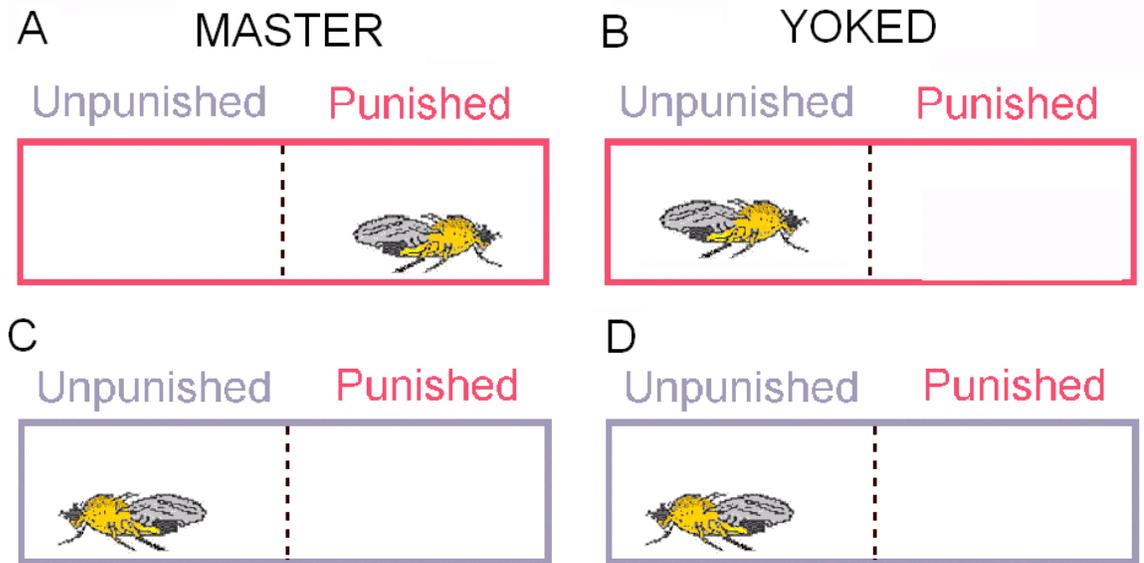


Figure 5-11. A schematic of the master and yoked flies in the heat box chambers. A The Master flies are punished (chamber temperature rose to 41°C from 24° C, shown as a red outline) when they cross the midline. B. The yoked chamber heats up like the master chamber (controlled by the master fly) irrespective of the position of the fly in the chamber. C When the master fly returns to the unpunished zone chamber temperature cools down from 41° C to 24° C (shown as a blue chamber outline). D. The yoked chamber also cools down when the fly is in the unpunished zone. A Master fly has a predictor for punishment while the yoked fly does not.

We next used the master/ yoked principles of dependence on prediction to understand the pre-exposure effect. Master flies were trained with 5 training blocks each lasting 6 minutes and the time spent by the flies experiencing 41°C was calculated (Table 5-6). 5 training blocks were chosen because initial experiments showed that in each training block master flies spend 45-52 seconds experiencing 41°C. So, to make the pre-exposure time close to 3 minutes we chose 5 training blocks. To ensure that the flies experience ~ 50 seconds of 41°C in each training session reversal conditioning was carried out. After the 5 training blocks the flies were held in the chamber at 24°C for 10 minutes followed by 4 minutes of training with 30°C.

To see if the master and yoked flies have predictors during training indicative of operant learning we looked at the performance indices during each of the 5 training sessions (Tr1-Tr5) (Figure 5-12 A, B, C, D and E). Training performance index of the master is ~ 0.8 which shows that the master flies avoid 41°C side. Yoked flies in contrast have performance close to 0 indicating they are unable to make an association between punishment and spatial position. We next calculated the exposure time of the master and yoked flies to 41°C and 24°C in each training block (Figure 5-13). Yoking during pre-exposure was followed by normal conditioning of the master and yoked flies with 30°C reinforcement. Like previous experiments the gap between pre-exposure phase and conditioning was 10 minutes (Figure 5-14). We found that the master and yoked flies were pre-exposed to 3.3 ± 0.4 minutes of high temperature (Table 5-6). During later conditioning yoked flies exhibited higher memory levels as compared to the master flies (Figure 5-14).

Flies from both master and yoked groups experience the same temperature exposure. Therefore, based on memory levels from later conditioning performance there would be two possibilities. First, that yoked group would have memory enhancement indicating that lack of prediction or uncertainty is important for memory enhancement. A second possible result could be no enhancement of memory levels in both yoked and master flies as the high temperature exposure is distributed more broadly over time than the 3 minutes of sudden unpleasant high temperature

exposure that causes the previous measured enhancement. We find that with 5 blocks of training flies without predictor (yoked) show a robust memory enhancement as compared to the master flies (Figure 5-14). Therefore, we can conclude the lack of predictor function is necessary to induce pre-exposure effect. Further, we hypothesise that when a fly is in control of its environment as is the case with master flies the reinforcement information readily enters and exits the reinforcement buffer, leading to rapid matching of memory to reinforcer levels

Additionally, to see if the yoked and master flies sense and avoid temperatures to the same extent a thermosensitivity assay was carried out 10 minutes after yoked pre-exposure training (Table 5-5). We found no significant differences between the master and yoked flies. Therefore, pre-exposure with and without predictor does not influence the ability to sense and avoid temperatures used in pre-exposure and conditioning.

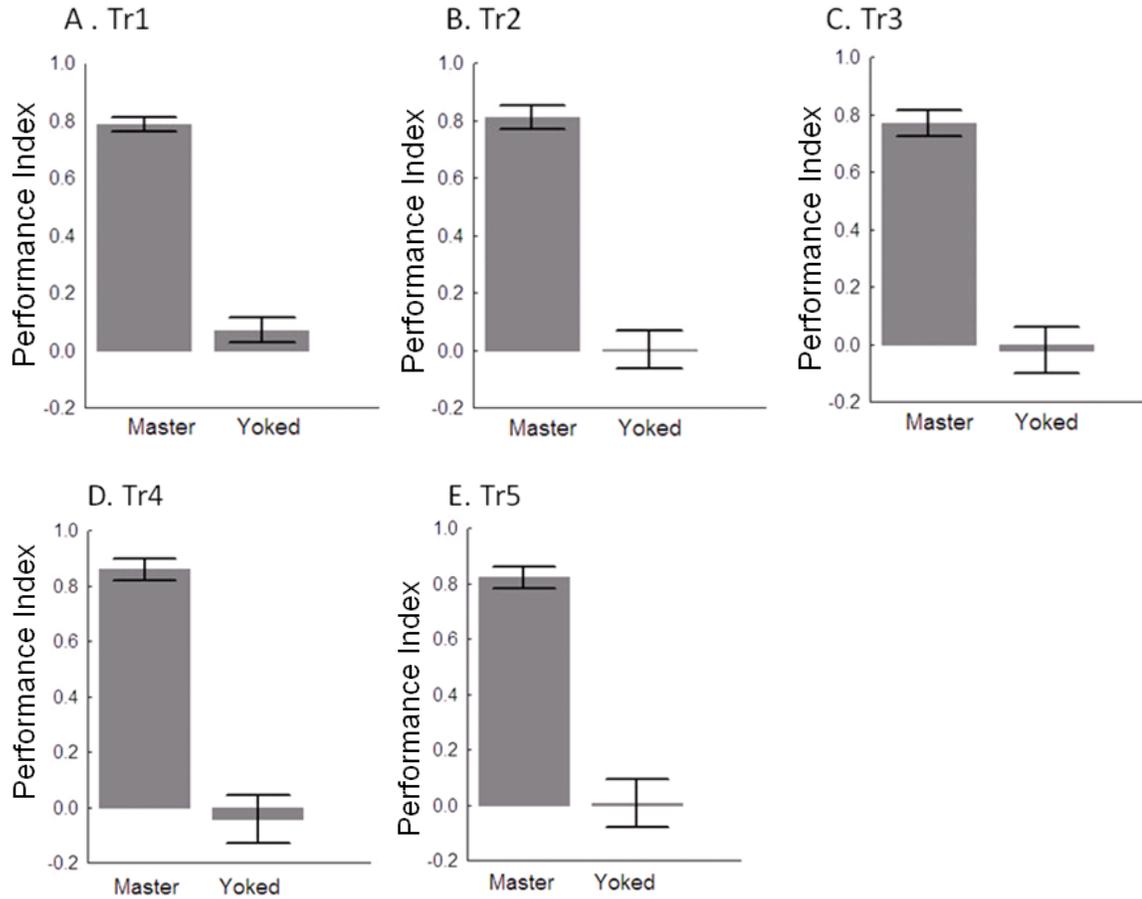


Figure 5-12. Performance index of each training block for master and yoked flies represent operant learning in the heat box for the master but not the yoked flies. The master flies show high training performance indicative of avoidance of high temperature associated side while yoked flies show low performance. The master flies have a predictor for punishment while yoked flies do not. A, B, C, D and E represent the learning score of trainings session 1-5. Each training session included a 30 second pretest followed by 6 min training with 41°C. Total number of master and yoked flies for each training session = 236. Values represent means and standard error SEMs.

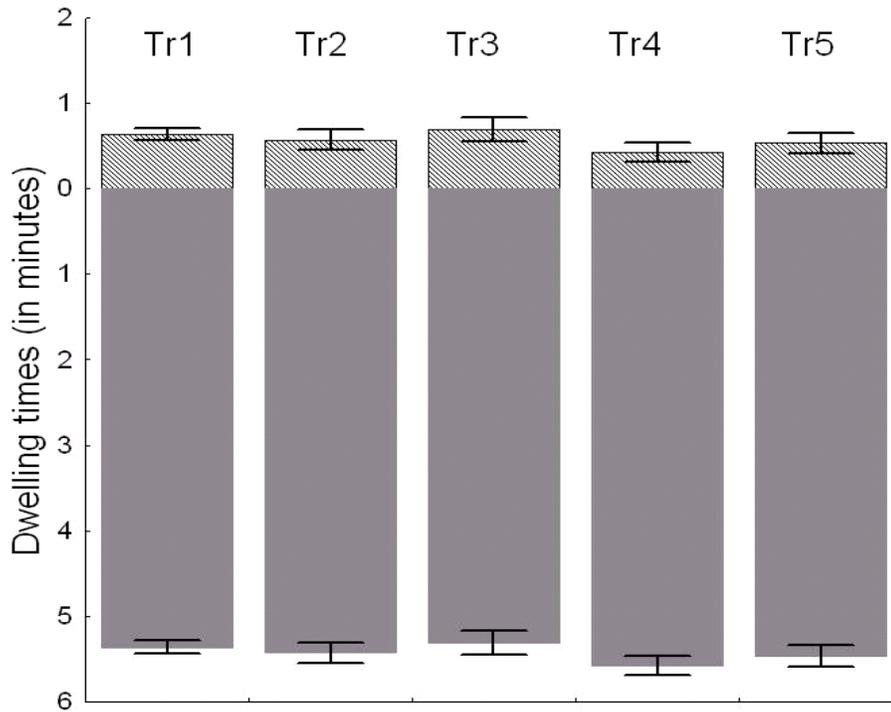


Figure 5-13. Dwelling time of master flies in punished and unpunished zone. Flies with a pre-exposure predictor are referred to as master and flies without a predictor are termed yoked. Exposure time of master and yoked flies in unpunished (associated with 24°C) and punished zone (associated with 41°C) over 5 training blocks. Each bar represents a training block of 6 minutes. The hatched bars indicate punished and solid bars the unpunished side dwellings. We find that the master and yoked flies are exposed to 41°C for a total of 3.3 ± 0.4 minutes during the 5 training blocks.

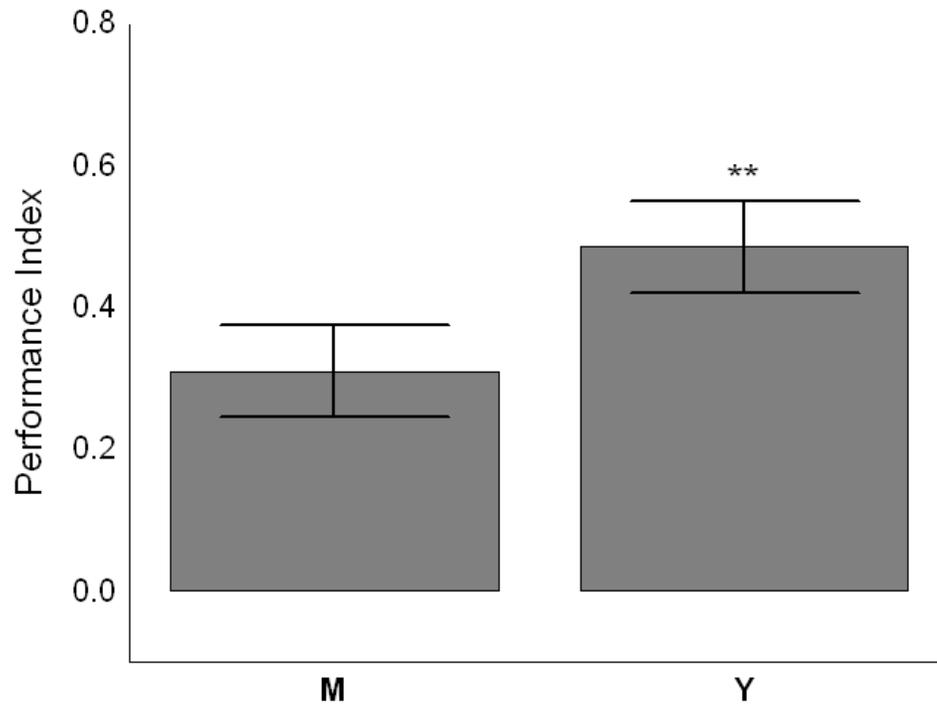


Figure 5-14. Flies without predictor exhibit robust enhancement. Conditioning after yoked pre-exposure results in memory enhancement of the yoked but not the master flies. Master and yoked flies were trained with 41°C using 5 training blocks (6 minutes each) and conditioned at 30°C. Statistical comparisons made by Mann-Whitney U Test where $U=5629.0$, $z=-2.5$, $p=0.007$ and $N= 236$. Values represent mean and error bars SEMs marked significant ** = $p<0.01$.

Table 5-5. Thermosensitivity of master and yoked flies.

Type	24/24	24/30	24/41	N
Master	0.0±0.06	0.38±0.06	0.60±0.06	102
Yoked	-0.1±0.07	0.27±0.07	0.56±0.06	95

Table 5-5. The ability of wild type flies to sense and avoid temperatures after yoked training was measured in the heat box. Both master and yoked flies were tested for their avoidance of temperatures 24-45°C. The master and yoked flies showed similar quantitative avoidance to high temperatures. No significant differences were found in any of the temperatures tested. Mann-Whitney U test: For 24°C: U=4415, z=1.07, p=0.28; 30°C: U=4524, z=0.80, p=0.42; 41 °C: U=4642, z=-0.51, p=61. Values represent Mean PI ± SEM.

Amount of pre-exposure is critical for memory enhancement of yoked flies.

The pre-exposure effect is sizeable when the timing of pre-exposure is in the range of 1-3 minutes (Sitaraman et al., 2007). We asked if flies exposure time in yoking experiments had similar temporal properties. Yoking experiments can be conducted with decreasing or increasing the number of training blocks (to alter exposure time). By altering the number of trainings the master and yoked flies can be exposed to varying pre-exposure time periods. The dwelling times of the flies in the 41°C side was calculated from the training performance indices of each fly in a single training block using reversal conditioning. Flies were trained using 1, 2, 4, and 7 training blocks to cover a pre-exposure time period of 0.5 to 3.6 minutes (Table 5-6).

The master and yoked flies were held in the chamber at 24°C after these training blocks and thereafter conditioned using 30°C. Interestingly, we found that the yoked flies pre-exposed with 2, 4, and 7 training blocks (Tr) showed a memory enhancement and were significantly different from the master flies (Figure 5-15). On the other hand both master and yoked flies that received one training block did not show a memory enhancement, pointing to a threshold requirement of pre-exposure for later enhancement. Therefore, similar to our earlier results, acquisition of the pre-exposure effect requires more than one minute of 41°C exposure. The yoked flies that were subjected to 1 training block spent 0.52 minutes experiencing 41°C side while the yoked flies with 2, 4, 5, and 7 training blocks experienced 41°C in the range of 1-4 minutes (Table 5-6). The yoked flies subjected to 2, 4, and 7 Training blocks during pre-exposure without predictor performed at significantly higher levels as compared to the master flies. Thus, we can conclude that it is lack of prediction that induces the pre-exposure effect, not simply the experience with high temperature.

Table 5-6. Training blocks and exposure time to high temperatures

No. of training blocks	Time spent by the fly in the high temperature side (minutes)	N
1	0.5 ± 0.04	96
2	1.0 ± 0.1	112
4	1.7 ± 0.1	120
5	3.3 ± 0.4	118
7	3.6 ± 0.6	80

Table 5-6. The training blocks ranging from 1-7, altering the exposure time of master and yoked flies to the high temperature. N indicates the number of master flies in each experiment that control the chamber environment. For training blocks 1-7 flies spent 0.5-3.6 minutes in the high temperature side. Values represent mean ± SEM.

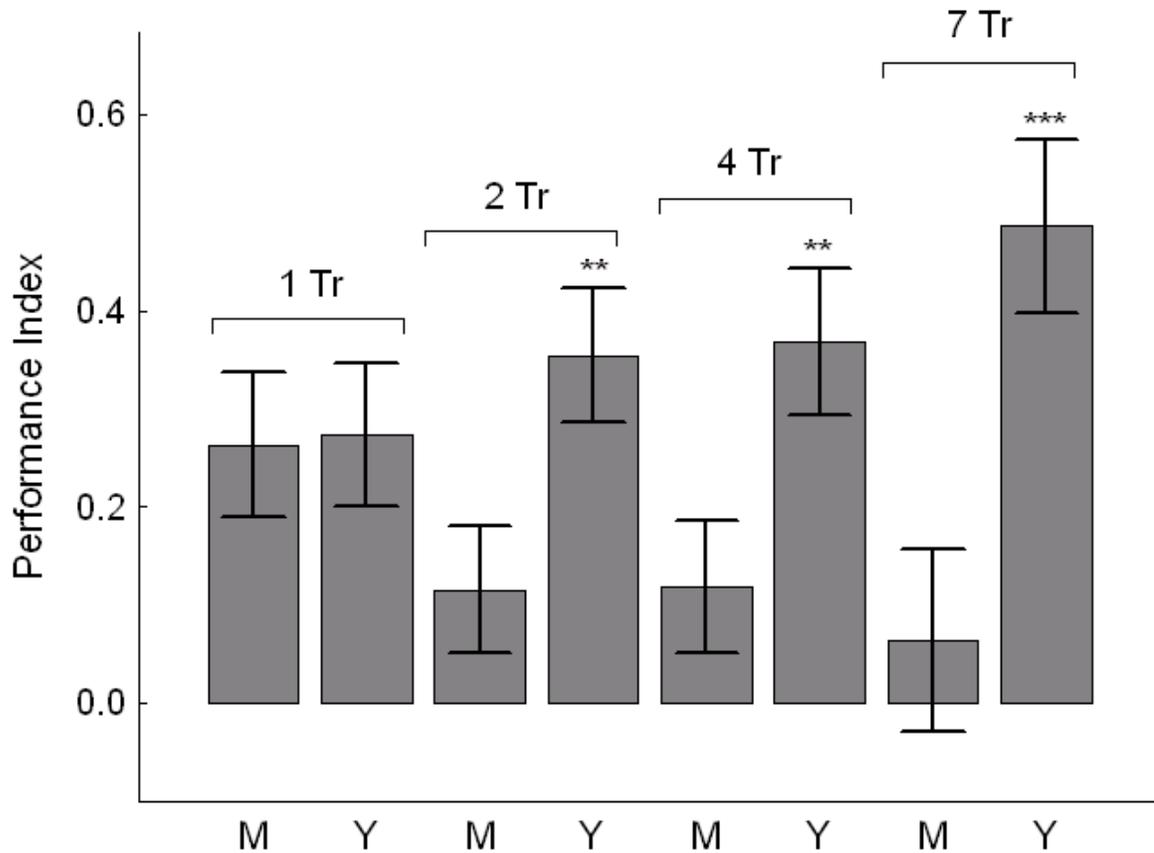


Figure 5-15. Conditioning performance indices of Master (M) and yoked (Y) after 1 Tr, 2 Tr, 4 Tr, and 7 Tr blocks. The flies trained for two, four and seven, 6 minute training blocks (2, 4, and 7 Tr) show enhancement in yoked but not master flies. The master and yoked flies perform at the same level after 1 Tr. Statistical analysis by Mann-Whitney U test for master and yoked flies with 1 training block (1 Tr): $U=2800.5$, $z=-0.184$, $p=0.85$, $N=150$; 2 training (2 Tr): $U=3675.5$, $z=-2.83$, $p=0.004$, $N=196$; 4 training blocks (4 Tr): $U=3729.0$, $z=-2.7$, $p=0.006$, $N=196$; 7 training blocks (7 Tr): $U=2550.5$, $z=-3.1$, $p=0.0010$, $N=168$. Values represent mean and error bars SEMs, marked significant ** = $p<0.01$ and *** = $p<0.001$.

Unrelated reinforcement experiences also induces a pre-exposure effect

Pre-exposure to unpaired high temperature in the heat box leads to a memory enhancement that is dependent on the serotonergic system and occurs only in the absence of a predictor. Next we asked if the memory enhancement is specific to unexpected high temperature pre-exposure or can be induced by other unpaired and unrelated reinforcers. The other reinforcers that have been used extensively in *Drosophila* for associative learning are electric shock as an aversive stimulus and concentrated sucrose as an appetitive reinforcer (Schwaerzel et al., 2003; Tully and Quinn, 1985). To induce aversive olfactory memory, 12 pulses of 100V of electric shock are usually delivered in one minute to flies in a shock tube. We exposed wild type flies to this aversive shock and then placed them in the heat box chambers at 24°C for 10 minutes, followed by conditioning with 30°C. Flies placed in a shock tube that did not receive the 100V shock were used as a control. Shocked and unshocked flies were tested in parallel in odd and even numbered chambers, respectively.

Interestingly, the shocked flies showed a memory enhancement (Figure 5-16). Additionally the flies from these two groups sensed and avoided temperature normally (Table 5-7). The ability of a distinct aversive reinforcer to influence place memory indicates that reinforcement signaling recruited in shock are also important for place learning. Electric shock reinforcement is mediated by dopamine in olfactory learning and whether dopamine plays a role in pre-exposure effect is not known and under investigation.

We also tested the influence of positive reinforcers on place learning. Flies were starved for 16-20 hours, pre-exposed to 1M sucrose, and then placed in the heat box for 10 minutes at 24°C, followed by conditioning at 30°C. The control group was pre-exposed to water in parallel before testing in the heat box. Both the groups exposed to sugar and water performed at the same levels, suggesting that this protocol, which is used to condition flies in olfactory learning does not induce memory enhancement (Figure 5-16). This may mean that appetitive pre-exposures are not

capable of inducing an enhancement, or that appetitive reinforcement pre-exposure on aversive conditioning does not interact. The flies pre-exposed with sugar and water performs at lower memory levels as compared to unshocked group. This could be a result of 16-20 hours of starvation.

Finally, the shock related pre-exposure effect could be dependent on the serotonergic system. To test for this possibility Ddc-Gal4; Th-Gal80/TNT flies were pre-exposed to 100V shock as before and conditioned in the heat box. Interestingly, we found that the flies with impaired neurotransmission in serotonergic neurons targeted by Ddc-Gal4; Th-Gal80 were able to show normal memory enhancement in response to shock (Figure 5-17). This indicates that shock induced pre-exposure is independent of the serotonergic signaling that mediates the high temperature pre-exposure effect and reinforcement information. This shows that shock recruits a separate system. Evidence from the short term memory in olfactory paradigm indicates that electric shock is mediated by the dopaminergic system and required for aversive learning (Claridge-Chang et al., 2009; Schwaerzel et al., 2003). At the level of downstream effectors both aversive (in specific regions of the brain) and appetitive olfactory learning are dependent on the adenylyl cyclase (Schwaerzel et al., 2003; Zars et al., 2000b). Therefore it is not uncommon in associative learning in *Drosophila* for different reinforcers to be mediated by distinct upstream signaling processes and common downstream effectors.

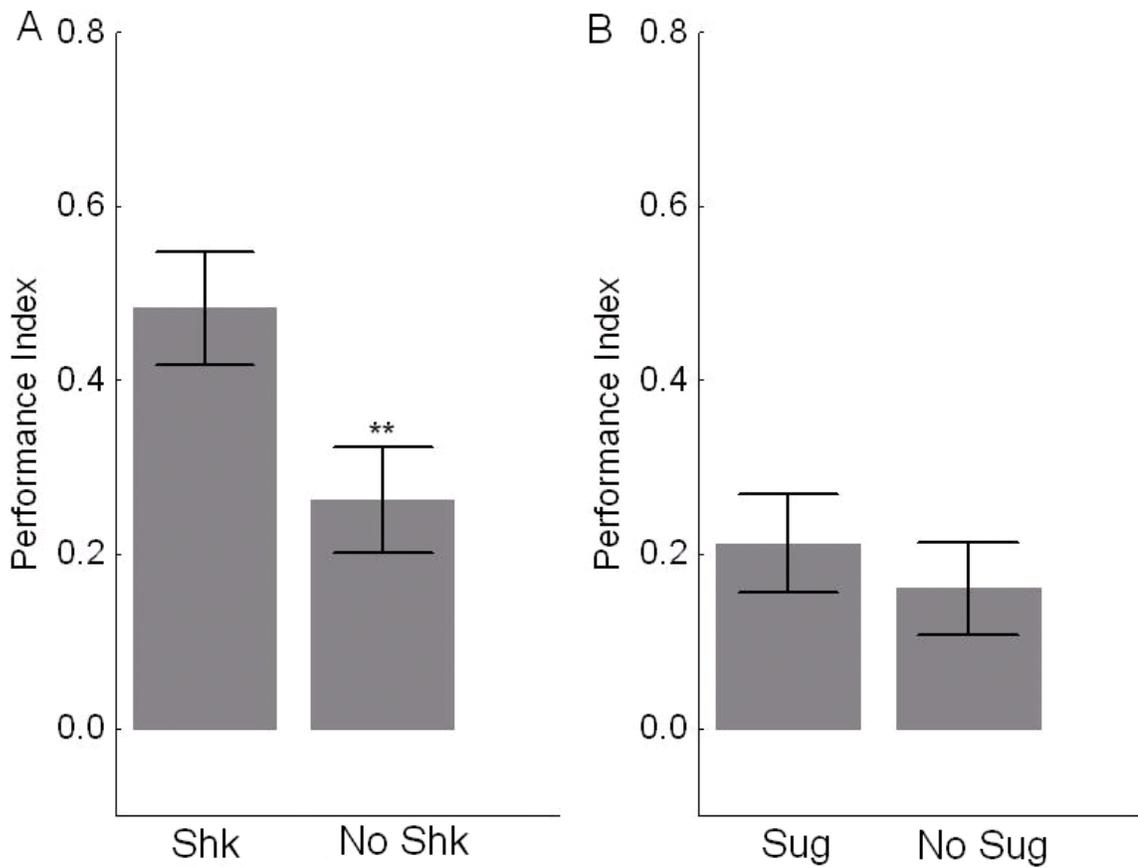


Figure 5-16. Aversive shock but not appetitive sugar pre-exposure induces memory enhancement. Wild type flies were pre-exposed to shock (Shk) and tested in the heat box 10 minutes post-shock with 30°C. Unshocked flies (No Shk) tested in parallel were used as controls. The shocked group showed memory enhancement as compared to the unshocked group. B. Instead of shock, flies were pre-exposed to 1M sucrose (sug) or Water (No sug) and then conditioned in the heat box. Comparisons by Mann-Whitney U test for shocked and non shocked group show $U=4995.5$, $z=2.64$, $p=0.008$ and $N=223$. For sugar and no sugar group $U=4805.5$, $z=0.71$, $p=0.47$ and $N=202$. Values represent mean and error bars SEMs, marked significant at ** = $p<0.01$ and * = $p<0.05$.

Table 5-7. Thermosensitivity of shocked and unshocked flies

Type	24/24	24/30	24/41	N
Shk	0.08±0.1	0.25±0.1	0.63±0.07	64
NoShk	0.08±0.0	0.26±0.09	0.75±0.05	64

Table 5-7. The ability of the shocked and unshocked wild type flies to sense and avoid temperatures was measured in the heat box. Both shocked and unshocked flies were tested for their avoidance of temperatures 30 and 41°C relevant to the pre-exposure effect. PI close to 0 means no net avoidance while positive PI score indicates a strong avoidance. The shocked and unshocked flies showed similar quantitative avoidance to high temperatures. No significant differences between shocked and unshocked groups were found in any of the temperatures tested. Statistical analysis was made using Mann-Whitney U test for temperature avoidance (24-45°C). For temperatures 24°C: U=1996.5, z=-0.25, p=0.81; 30°C: U=2009.5, z=0.18, p=0.85; 41 °C: U=1708.5, z=-1.62, p=0.11. Values represent mean±sems.

A. DdcGal4; Th-Gal80/+

B. DdcGal4; Th-Gal80/TNT

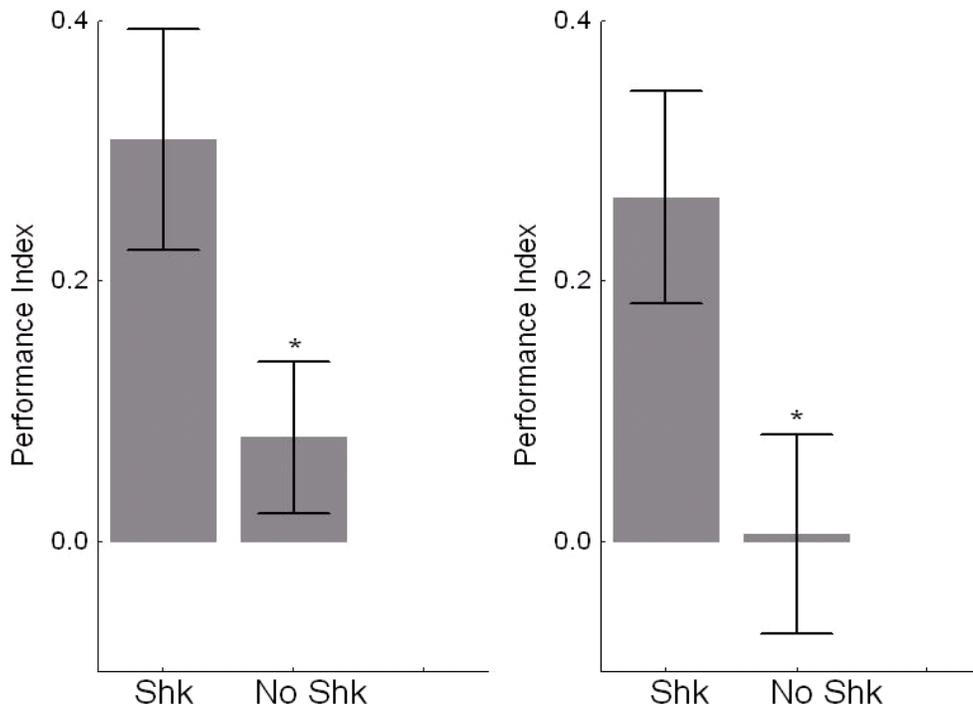


Figure 5-17. Aversive shock induced memory enhancement is independent of the serotonergic system. In A and B Ddc-Gal4;Th-Gal80/TNT and Ddc-Gal4;Th-Gal80/+ flies were pre-exposed to shock (Shk) and tested in the heat box 10 minutes post shock with 30°C. Unshocked flies (No Shk) of the same genotypes were tested in parallel were used as controls. The shocked group showed memory enhancement as compared to the unshocked group. Multiple Comparisons of mean ranks by Mann-Whitney U test for Ddc-Gal4; ThGal80/TNT: U=990, z=2.2, p= 0.03, N=103 and for Ddc-Gal4; Th-Gal80/+: U=957.5, z=2.0, p= 0.04, N=102. Values represent mean and error bars SEMs, marked significant * = p<0.05.

Summary and Conclusion

In an attempt to understand the influence of unexpected punishments and rewards in place learning we find that pre-exposure of aversive reinforcers such as high temperature and electric shock can induce an enhanced memory in later place learning. The high temperature mediated memory enhancement is independent of the antennal thermosensor required for sensing and avoiding temperatures in the range of 30-32° C. Also, the pre-exposure does not alter thermosensitivity. Wild types flies can increase or decrease their memory levels indicated by performance index in as little as 2 minutes in response to an increase or decrease in the reinforcement intensity (Zars and Zars, 2009). This switch in conditioned behavior in response to changing reinforcement intensity seems to be a conserved property of learning circuit (Herrnstein, 1997; Sugrue et al., 2004). Since memory matching is dynamic, long training would be expected to switch the enhanced memory levels to lower performance indices during conditioning if the pre-exposure effect had the same dynamics as conditioning. Our results show that the pre-exposure effect persists and flies do not match their memory to 30°C reinforcer even after 20 minutes of training.

Furthermore, the high temperature mediated enhancement is dependent on serotonergic signaling. Recruitment of this system points to the plasticity of the reinforcement circuitry that underlies the pre-exposure effect. The mismatch between the expected and actual reinforcement evident during later conditioning as a result of pre-exposure is dependent on serotonin. Additionally, the flies with a pre-exposure predictor form memories at lower levels as compared to the yoked flies, this ability to influence later conditioning supports the notion that the unpredictability or uncertainty of the aversive reinforcer is required for memory enhancement. Based on the yoking experiments we can make a conclusion that reinforcement information is buffered, with accurate prediction as is the case with master flies there is a rapid draw-down in reinforcement value. Since pre-exposure dependant on uncertainty and biases later conditioning

we also refer to this behavior as the uncertainty bias. The neural circuitry supporting this uncertainty bias behavior involves the serotonergic system but how the prediction component influences the downstream effectors is unknown. Finally, pre-exposure effect can also be induced by aversive reinforcements unrelated to temperature, and that this effect of sudden unexpected punishments operates across modalities.

CHAPTER 6: SEROTONIN IN AVERSIVE OLFACTORY LEARNING

Introduction

In *Drosophila* olfactory learning, when electric shocks overlap odorant presentation a mixed appetitive / aversive memory is formed (Diegelmann et al., 2006; Tanimoto et al., 2004; Yarali et al., 2009). That is, flies largely avoid an odor associated with electric shock and approach the odor not associated with shock. However, when the timing of the electric shocks is altered in such a way that the odorant is present when the shock is turned off, flies will approach that odorant rather than avoid it (Tanimoto et al., 2004; Yarali et al., 2009). Furthermore, mutation of the *white* ABC transporter leads to higher than expected aversive memories, as if a positive component of a mixed aversive / appetitive memory is missing. The serotonin reduction in *white* mutants has provided important insights into the reinforcement signaling in place learning. So we wanted to investigate if the *white* phenotype in olfactory learning has provided a clue to the role of serotonin in olfactory learning.

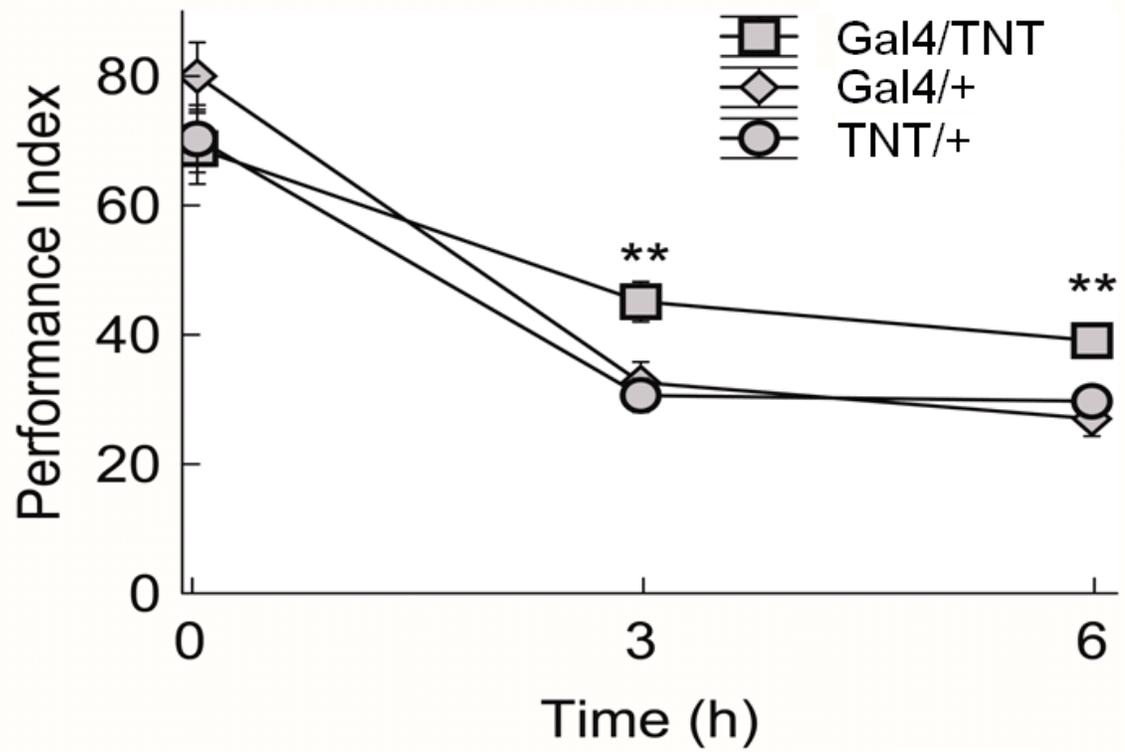
The role of serotonin in *Drosophila* aversive short-term memory is not well understood. If one were to identify a role of serotonin in either aversive or appetitive olfactory short-term memory, it would indicate multiple modulators can be important for the acquisition of a given memory type. Serotonin plays a critical role in short-term aversive place memories (Sitaraman et al., 2008). Place and olfactory memory seem to recruit different sets of neurons and signaling molecules. In spite of these specializations, one might predict that serotonin plays an essential role in aversive short-term olfactory memories, which would be consistent with a more general role of serotonin in aversively reinforced memories.

Results

To test the role of the serotonergic neurons in aversive olfactory memory, we expressed the tetanus toxin light chain (TNT) with either the Ddc-Gal4; ThGal80 driver combination or Trh-GAL4. We found that blocking synaptic output from serotonergic neurons does not influence aversive olfactory memory with 100V tested directly after training (Figure 6-1). We next looked at memory decay in Ddc-Gal4; Th-Gal80/TNT 3 and 6 hours after training. A higher than expected olfactory memory was found in the DdcGAL4; THGAL80 / TNT flies compared to genetic control flies. This indicates that normal serotonergic output is required for normal memory decay. Inhibition of this component leads to persistence of this memory.

The Ddc-Gal4; Th-Gal80 also labels the PAM dopaminergic cluster and dopaminergic signaling has been shown to be sufficient to mediate the negative reinforcement information in olfactory learning (Claridge-Chang et al., 2009). To ascertain a serotonergic function in memory decay we used another serotonergic driver, Trh-Gal4 and tested memory after 3 hours of training. Blocking synaptic output from Trh-Gal4 neurons had the same phenotype as with Ddc-Gal4; Th-Gal80. The 3 hr memory was higher than control while the short term memory was unaffected. In both these treatments the acquisition was normal but only the decay kinetics was altered. Since all the tested genotypes acquire short term memory at the same levels sensory acuity of shock and odors cannot explain the later enhanced 3 and 6 hr memory. To further address that possibility, however, assays to measure the shock and odor avoidances of the tested genotypes are underway. The lack of difference between the tested genotypes upon impairing serotonergic neurotransmission could be a ceiling effect. Since, serotonin plays an important role in matching reinforcement intensity and memory levels in place learning we hypothesized a similar role for serotonin in aversive olfactory learning. To this end we used 20 V a lower intensity reinforcer and tested short term olfactory learning. We found that Ddc-Gal4; Th-Gal80/ TNT flies performed at the same level as the genotypic control with the low intensity reinforcer (Figure 6-2).

A DdcGal4; Th-Gal80



B Trh-Gal4

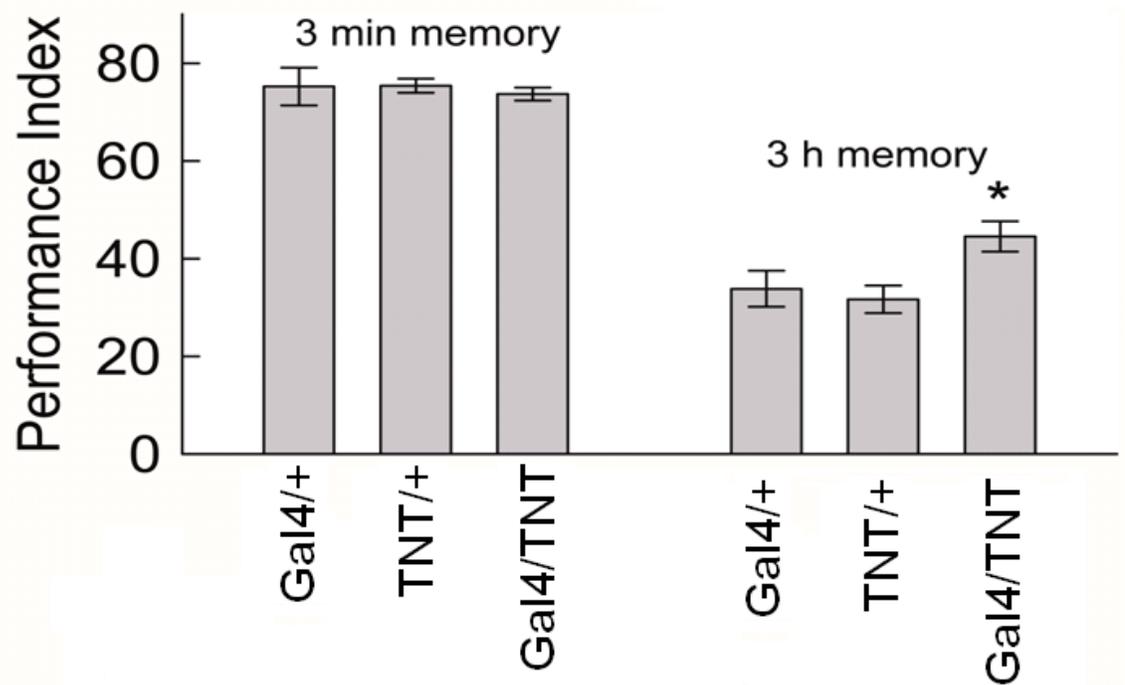


Figure 6-1. The serotonergic neurons are required for a positive component of the electric-shock-associated olfactory memory. Evoked synaptic transmission was blocked by expressing the tetanus toxin light chain (TNT) in serotonergic neurons with either the DdcGAL4; THGAL80 driver combination (A) or the TrhGAL4 driver (B). Olfactory memories of experimental and genetic control flies were tested at 3 minutes, 3, and 6 hours post-training. Significant differences appeared at 3 and 6 hours (DdcGAL4; THGAL80 only) post-training between the experimental and control genotypes (DdcGAL4; THGAL80 / TNT compared to DdcGAL4; THGAL80 / + and TNT / + at 3 min: $F(2,15)=2.6$, $p=0.11$; 3 h: $F(2,36)=6.9$, $p=0.003$; 6 h: $F(2,42)=6.2$, $p=0.004$. TrhGAL4 / TNT compared to TrhGAL4 / + and TNT / + at 3 min: $F(2,12)=0.14$, $p=0.9$; 3 h: $F(2,24)=4.5$, $p=0.02$; significant differences after Newman-Keuls post-hoc tests are shown: * = $p < 0.05$, ** = $p < 0.01$). The values represent means and error bars are SEMs.

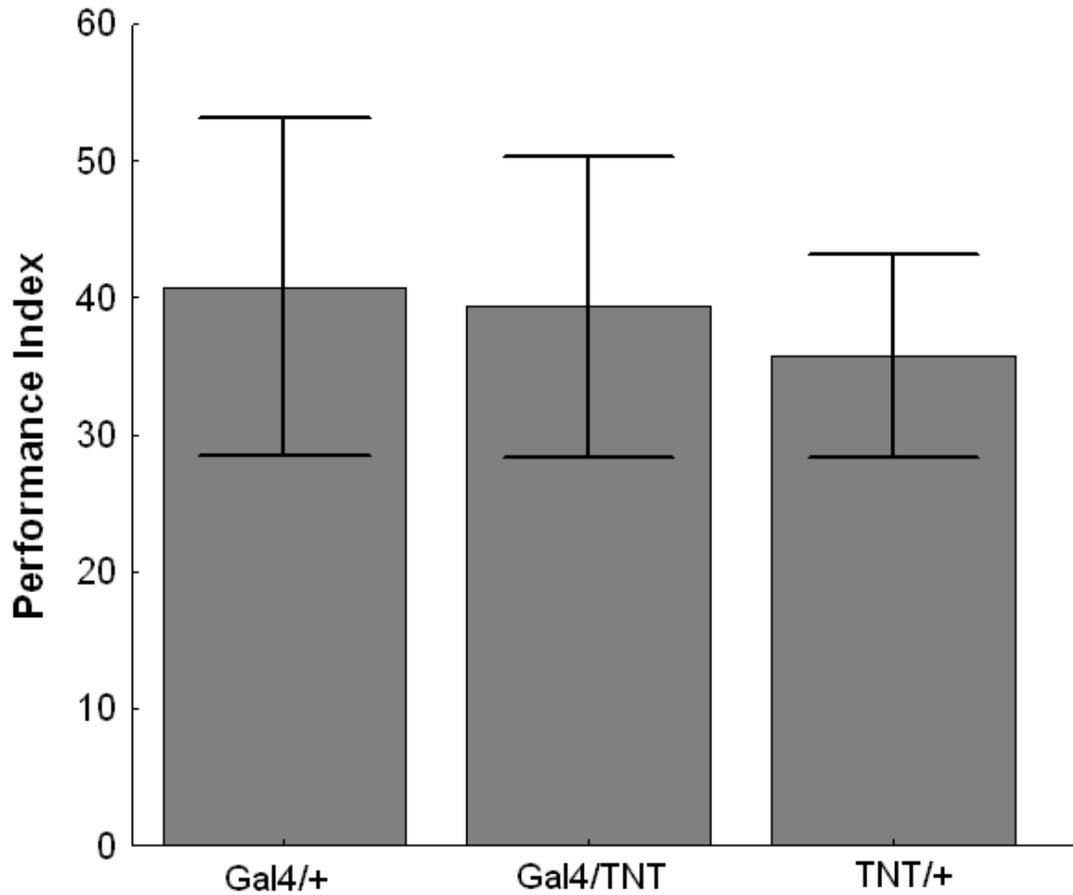


Figure 6-2. Inhibition of serotonergic output does not lead to increased 20V olfactory short term memory. Ddc-Gal4; Th-Gal80/TNT (Gal4/TNT) flies were trained and tested along with the genotypic controls Ddc; Th-Gal80/+ (Gal4/+) and TNT/+. No significant differences were found between tested genotypes $F(2, 15) = 0.06$, $p = 0.94$. Statistical comparisons were made by Neumann-Keuls post hoc tests. Values represent mean and error bars SEMs.

Summary and Conclusion

Our results with two different serotonin drivers reveal a role of serotonin in the establishment of a delayed positive component in an otherwise largely aversive olfactory memory. Serotonin is not required for acquisition of aversive olfactory learning. Therefore, the increased memory performance at later time points indicate that serotonin plays an important role in memory decay process. This inhibitory function of serotonin in memory decay is the first clear evidence of serotonin function in aversive olfactory learning in *Drosophila*. Given the role of mushroom bodies in acquisition and consolidation of aversive olfactory memory this function could be mediated by serotonergic neurons that innervate this structure (de Belle and Heisenberg, 1994; Dubnau et al., 2001; Heisenberg et al., 1985). Further, neurotransmission from MB $\alpha\beta$ neurons is only required for memory retrieval (Krashes et al., 2007). Finally, the role of serotonin in appetitive olfactory learning is unknown and under active investigation.

CHAPTER 7: DISCUSSION

Signaling processes that mediate reinforcement information are critical to learning. One of the goals of this dissertation was to identify the reinforcement signaling mechanisms in a spatial operant learning paradigm, the heat box. The discussion is addressed in six parts. First, the Wolf-Heisenberg model of operant learning will be examined in the context of serotonin. Second, the function of the *white* gene in place learning will be discussed. Third, the function of serotonin in reinforcement processing is argued. Fourth, we will explore a potential appetitive component of place learning by addressing the role of octopamine. In the last two parts we will identify and characterize the pre-exposure effect on place learning and discuss results from olfactory tests.

Wolf-Heisenberg model for operant learning

The Wolf-Heisenberg model for operant learning based on work with the flight simulator in *Drosophila* provides a framework for our studies in place learning. At the core of this model is that animals have a goal or desired state (Figure 7-1). To reach a desired state an animal initiates and executes one of several motor programs. The model posits the existence of a coincidence detector that pairs an efference copy of the motor program with the difference between the current state and desired state. The efference copy is a representation of a motor program or command (Sperry, 1950). The probability of using a given motor program increases when there is a significant coincidence between a decreasing difference between the current and desired state and the efference copy. The behavior persists as long as the sensory information can be controlled by the conditioned behavior (Wolf and Heisenberg, 1991). Therefore, select one of

several motor programs to decrease the difference between current and desired state (Wolf and Heisenberg, 1991).

Applying the Wolf-Heisenberg model to place learning, the desired state or goal is an environment maintained at 24°C. Interestingly, the presence of the coincidence detector alone does not explain how flies can modify their conditioned memory levels based on the intensity of the reinforcer. This leads to another component of the operant learning circuit called the comparator (Diegelmann et al., 2006). The comparator most probably acts at the level of the current versus desired state and feeds it into the coincidence detector. For low temperature reinforcers close to the desired state of 24°C, the comparator provides a low output to the coincidence detector which matches it to one of several motor programs. The coincidence detection then selects a motor behavior (dwelling at different positions in the chamber, turning in the chamber, etc) that decreases the difference between current and desired state. The comparator is critical for determining the deviation from the desired state and provides input to the coincidence detector.

The learning circuit would not be complete without a feedback loop between the behavior initiator and the coincidence detector via the efferent and after-effect pathways (Figure 7-1) (Diegelmann et al., 2006). Molecularly, the comparator probably depends on the proper functioning of the *white*-ABC transporter as these flies perform at low levels (as expected from low temperature reinforcers) even when the punishing temperature is high and aversive. The comparator in these flies does not give an accurate representation of the difference between actual and desired state to the coincidence detector. The desired state in *white* flies is probably the same as wild-type flies indicated by their preference for 24°C over higher temperatures (Diegelmann et al., 2006). Further analysis of the *white* gene and the molecular systems dependent on it will be important in identifying signaling within the comparator. Since reduced memory performance in *white* is dependent on serotonergic signaling, the serotonergic system is most likely acting at the level of the comparator.

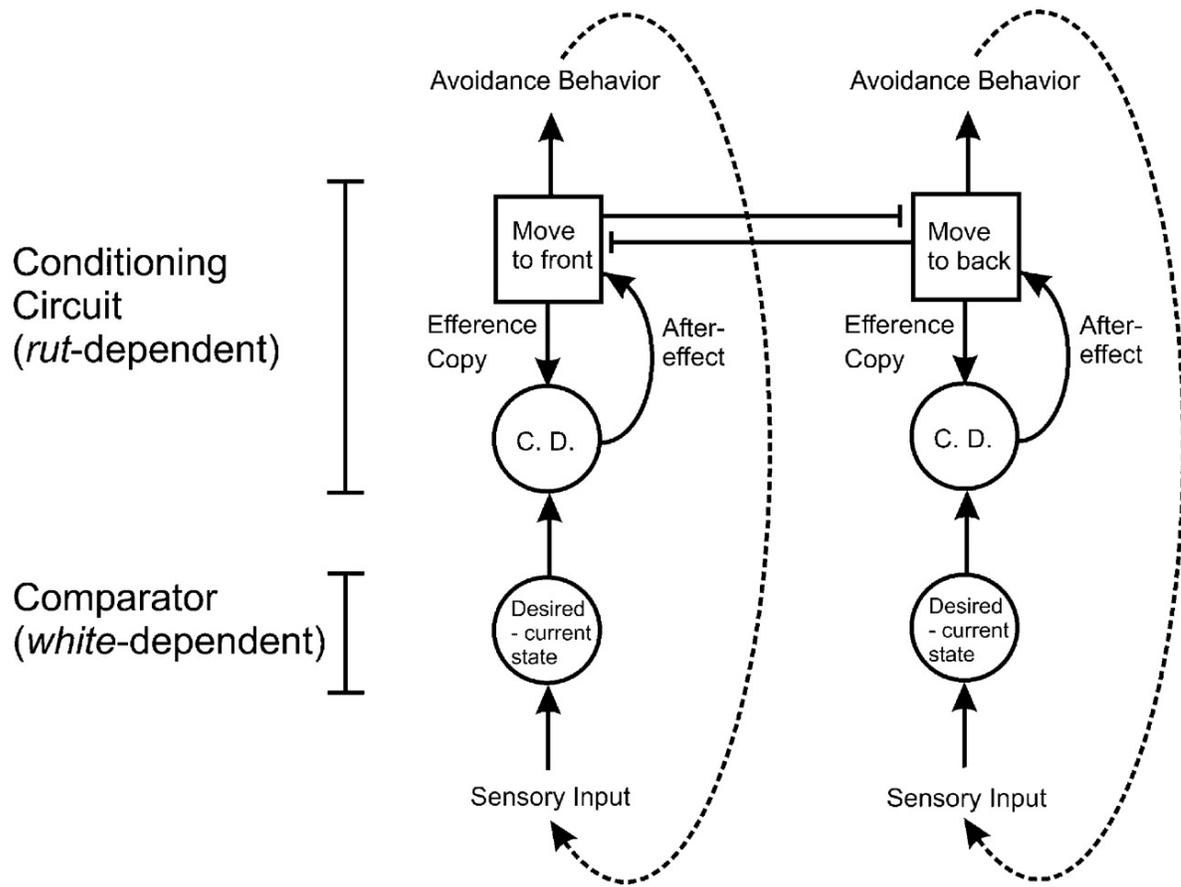


Figure 7-1. The Wolf-Heisenberg model of operant conditioning. The comparator receives information from the environment and is compared with a desired state. This difference is fed into the conditioning circuit, where behavioral motor programs are initiated. The initiated behaviors, some of which are mutually exclusive of other behaviors, can influence sensory input. With successful reduction in the difference between the desired and current state, the conditioning circuit is reinforced and has a higher probability of being implemented. In this model, the *white* mutation affects the comparator (Diegelmann et al., 2006).

white mutant flies have drastically reduced memory performance and show severely decreased levels of both serotonin and dopamine (Borycz et al., 2008; Diegelmann et al., 2006; Sitaraman et al., 2008). We have identified that serotonin is necessary and sufficient for the aversive high temperature reinforcement in *Drosophila* spatial learning. Thus we assign serotonin a function within the comparator, providing input to the coincidence detector. This eventually leads to initiation and sustenance of motor program that mediate avoidance of the zone associated with punishment or serotonergic activation.

The *white* gene and place learning

Multiple mutant *white* alleles have low place memory phenotypes. A reduction in serotonin and the effect of RNAi expression in serotonergic neurons has an effect place memory levels. Thus, it is probable that deficits of *white* mutants results from lack of neuromodulation by serotonin. Keeping with the role of amines in reinforcement systems the deficit in *white* we hypothesized that this deficit could possibly be rescued by increasing serotonin and/ or dopamine levels.

However, the attempts to rescue the learning deficits in *white* flies with feeding of serotonin and dopamine synthesis precursors were not successful (Appendix, Figure 8-2). Effects of pharmacological manipulations are often hard to predict in *Drosophila* for many reasons.

Injections into specific regions of the nervous system routinely carried out in vertebrates and larger insects such as honeybees, crickets and locusts are often difficult in *Drosophila*. The drugs fed may not be consumed in sufficient quantities by all the flies tested to produce a behavioral deficit or rescue. Additionally, the drugs may not be very stable in fly food making chronic feeding protocols difficult to execute. Besides, these technical issues the drugs may not be accessible to candidate structures required for a learning task. Other groups have also had problems with drug

feeding as supplying amines ectopically does not guarantee transport to tissues where they can be metabolized and used to modulate a certain behavior (Hardie et al., 2007; Hoyer et al., 2008).

Serotonergic function in reinforcement processing

We next characterized the serotonergic system in *Drosophila* central brain. There are 38-40 neuronal cell bodies per hemisphere which innervate most of the central brain. Many of these neurons can be manipulated with two different Gal4 approaches; Ddc-Gal4; Th-Gal80 and Trh-Gal4. We addressed serotonin in reinforcement processing by multiple transgenes ranging from those that block neurotransmission to the ones that alter excitability of target neurons. All these manipulations of the serotonergic system combined with pharmacological evidence alter conditioning in the heat box. The ability to modify behavioral output by activation and inhibition of serotonergic system suggests that either this system acts alone at the comparator level or is the central control of a reinforcement circuit. Further, analysis of this system will be imperative in understanding the specific serotonergic neurons that act as the source of serotonin and its precise postsynaptic targets that modulate spatial conditioning in the heat box.

Octopamine in place learning

Place learning could have a rewarded component. High temperature is associated with one half of the chamber, and a cool, strongly preferred temperature (defining a desired state or goal) with the other half of the chamber. The cool-temperature-associated chamber half could provide a rewarding stimulus to a fly, and thus the performance indices represent aversive, rewarded or memory resulting from the mixture of the two reinforcers. Evidence from learning experiments in

insects shows that the appetitive or rewarding stimulus is octopamine dependent (Schwaerzel et al., 2003; Unoki et al., 2005, 2006; Vergoz et al., 2007). Therefore, a molecular approach of identifying a potential appetitive component in spatial learning is to identify the role of positive reinforcing signal (octopamine) in the heat box. Using a mutation in the *tyramine beta hydroxylase* and blocking of evoked synaptic transmission in the octopamine (and tyramine) neurons labeled with a *tyramine decarboxylase-2* (TDC2) gene regulatory elements we found that reinforcement of place memories is independent of normal octopamine signaling. One might conclude, then, that a system other than octopamine might be important for a rewarding effect of falling temperatures, there might be redundant systems that support place learning in the absence of a rewarding octopamine signal, or falling temperatures as a reward has no role in reinforcing place learning. Future experiments will be required to differentiate between these possibilities.

The pre-exposure effect

What are the effects of unexpected reinforcers on learning. In the associative learning literature, it has been shown that non-reinforced exposure to a stimulus that is to serve in later Pavlovian conditioning can retard subsequent learning (Claffin and Buffington, 2006; Rickert and Lorden, 1989). This is thought to be a result of a decline in the associability of the stimulus produced by pre-exposure (McLaren and Mackintosh, 2002). On the other hand there are also a few examples where the pre-exposure facilitates later learning. In *Drosophila*, we find that the pre-exposure to a aversive reinforcer potentiates later associative learning using a reinforcer of lower intensity. This enhancement of associative learning could be in principle accomplished by increased sensitivity of the peripheral sensory afferent pathway, inflation of high-temperature reinforcing value, or activation of another system (Sitaraman et al., 2007). We find that the pre-exposure does not alter thermosensitivity. We find that pre-exposure influences the reinforcement processing

mechanisms. Using insights from the Wolf-Heisenberg model we hypothesize that the pre-exposure also influences or biases the comparator to represent larger deviations between current and desired states than expected.

The molecular mechanisms underlying pre-exposure induced enhancement is dependent on serotonergic signaling. As a result of the pre-exposure, flies most likely raise the reinforcing value of 30°C during conditioning, thereby increasing their memory levels. Since, high temperature reinforcement learning is mediated by serotonergic signaling it's not surprising that reducing output from serotonergic neurons impairs the pre-exposure effect. However, most strikingly dTrpA1 activation of serotonergic system during pre-exposure alone induces memory enhancement. Activation of serotonin neurons like pre-exposure, acts at the level of comparator and misrepresents the difference between desired and current state. However the pre-exposure or serotonergic activation itself does not alter the desired state since these flies still have a preference for 24°C similar to control levels in thermosensitivity assays. Thus, mismatch between actual and expected punishments lead to a larger output from the comparator to the coincidence circuit so that only certain motor behaviors are maintained that increase avoidance of 30°C associated spatial positions.

We further explored the role of a predictor in the pre-exposure effect by carrying out yoking experiments. The effect of not being operant during pre-exposure influences and potentially modifies the learning circuits. The plasticity of the reinforcement signaling in the heat box shows clearly that learning pathways in invertebrates possess complexities matching those of vertebrate systems. The role of predictor as such has not been explored in invertebrates so far. In vertebrates the role of control or predictor has been explored in some tasks. Experiments in mice and human have shown that lack of control of aversive reinforcements is not preferred by an organism. In mice no control is perceived aversive leading to activation of amygdala, a brain region in vertebrates associated with a fear response (Herry et al., 2007; Whalen, 2007).

Experiments with humans have also shown that when individuals can control, or even just perceive that they can control (illusory control) the duration of aversive air puffs administered to them, they show lower arousal, presumably by lowering of stress response (Glass et al., 1973). Moreover, unpredictable aversive stimuli induces a higher degree of anxiety compared to predictable stimuli in experiments with human subjects (Grillon et al., 2004). The flies in the yoked condition as compared to the master flies probably tend to be more sensitive to their environment leading to enhanced avoidance while the master flies with their ability to control begin to have reduced sensitivity to changes in their environment. However, the thermosensory abilities of both groups are not different. Therefore, this arousal does not occur at the levels of sensory system. We have not yet found an effective way of identifying the cellular / molecular nature of this predictor in spatial learning, but it is expected to influence the serotonergic system.

Serotonergic system in aversive olfactory learning

Finally, we also looked at the effect of impaired serotonergic signaling on aversive olfactory learning. Blocking output from serotonergic neurons did not restrict or enhance 20 V and 100 V short term memories. This indicates that serotonin is not required for acquisition of aversive olfactory learning. The memory levels of wild-type CS flies reduce after 3 minutes, followed by a slower decline in memory levels from 3 - 6 hours. When serotonergic output is inhibited the flies have increased memory performance levels as compared to their genotypic controls at both 3 and 6 hours. Like memory formation, memory decay could also be a complex process composed of excitatory and inhibitory components to regulate this process. We have identified serotonin as the inhibitory component of memory decay such that inhibition of this system leads to slower or reduced memory decay. Serotonergic inhibition affects both the fast (0-3 hour) and slow (3-6 hour) kinetics of decay. Serotonergic inhibition does not influence acquisition, therefore, providing an example of a system specifically required for memory decay. Memory decay could be a

dynamic process helping an organism differentiate between memory traces and selectively store or decrease decay of important memories. To conclude, mechanisms of reinforcement in place learning were sought. We found that serotonin, but not dopamine or octopamine are important. Pre-exposure leads to an enhancement of learning, and likely does so through the serotonergic reinforcement circuit.

CHAPTER 8: APPENDIX

Appendix: A

Localization of the White protein in fly head

In addition to genetic characterization of *white* function in the fly brain (Chapter 3, figure 3-3) we also tried to localize the White ABC transporter in the fly head. In an initial attempt to localize the White protein in *Drosophila* brain and heads we used a commercially available rabbit anti mouse ABCG4 antibody on a western blot. We probed CS and w^{1118} heads using the anti-ABCG4 that targets a 15 amino acid peptide in the highly conserved extracellular C-terminus of the mouse ABCG4 gene. CS and w^{1118} immunoblots had a single band in the region between 50 and 100 Kd. The molecular weight of the White protein is a predicted 76 Kd (687aa). A clear reduction in White-ABC transporter level is evident in w^{1118} flies which shows lighter band between the 100 and 50 KD bands of the molecular weight ladder.

This result indicates that anti-ABCG4 labels White in the fly head. Even though there are differences in the CS and w^{1118} lane the presence of a light band suggests other conserved ABCG transporters with a similar molecular weight to White, such as the Scarlet protein could be recognized by this antibody (Figure 8-1). Therefore, this antibody was not used for immunohistochemistry in the fly brains as it might label other ABCG transporters. Interestingly, *white* transcripts have been detected in fly heads that do not contain visual tissue using RT-PCR (Campbell and Nash, 2001). However, other than our immunoblot results White-ABC transporter itself hasn't been clearly identified in fly brains. Additionally, some of the detected White-ABC transporter levels in the immunoblots could be arising from the visual system.

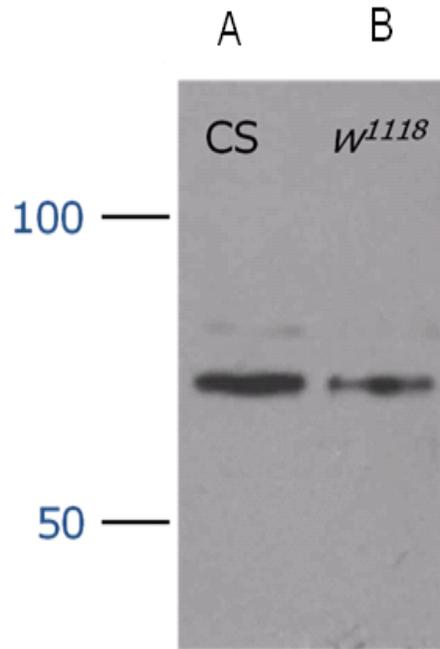


Figure 8-1. Immunoblot of CS and *w*¹¹¹⁸ heads probed with anti-ABCG4. Homogenate from 10 fly heads of wild-type CS and *w*¹¹¹⁸ was loaded in lanes A and B respectively. The lines on the left indicate the position of 100 KD and 50 KD bands of the protein ladder.

Pharmacological rescue attempt to feed serotonin and dopamine synthesis precursors does not ameliorate *white* induced place learning deficit.

To follow up on the conditioning and *white* dependant serotonin and dopamine reduction in place memory we asked if supplementing amines in *white* mutant flies could rescue the place memory deficit (chapter 3, Figure 3-1). To test that conditioning deficits in *white* flies directly result from reduced serotonin and/or dopamine, *w¹¹¹⁸* and CS flies were fed with 5- hydroxytryptophan (5HTP) and L-Dopa. 5-hydroxytryptophan (5HTP) is shown to increase the levels of serotonin within few hours of administration in mice (Fickbohm and Katz, 2000). HPLC results also show an increase in serotonin levels after overnight feeding in flies (Dierick and Greenspan, 2007). Similarly, L-Dopa can enhance dopamine levels in mice within a few hours of administration and widely used for symptomatic relief in patients suffering from Parkinsons disease. L-dopa has also been shown to elevate dopamine levels in *Drosophila* within few hours of administration (Liu et al., 2008). CS and *w¹¹¹⁸* flies were fed 5HTP and L-Dopa overnight and tested in the heat box with 4 minutes of training and 41°C as the reinforcing temperature (Figure 8-2). To address the possibility of requirement of both these neurotransmitters in conditioning CS and *w¹¹¹⁸* flies were fed a mixture of 5HTP and L-Dopa (sucrose-agarose media). Flies were put on new food (cornmeal-agar) before testing to reduce handling of drug food during experiments. The memory phenotype of *w¹¹¹⁸* flies administrated biogenic amine precursors does not lead to a rescue as the drug and sham fed *w¹¹¹⁸* perform at similar levels (Figure 8-2). This could result from insufficient time of exposure, concentration and the feeding step so we concluded that this many variables would be difficult to address and concentrated on other experiments.

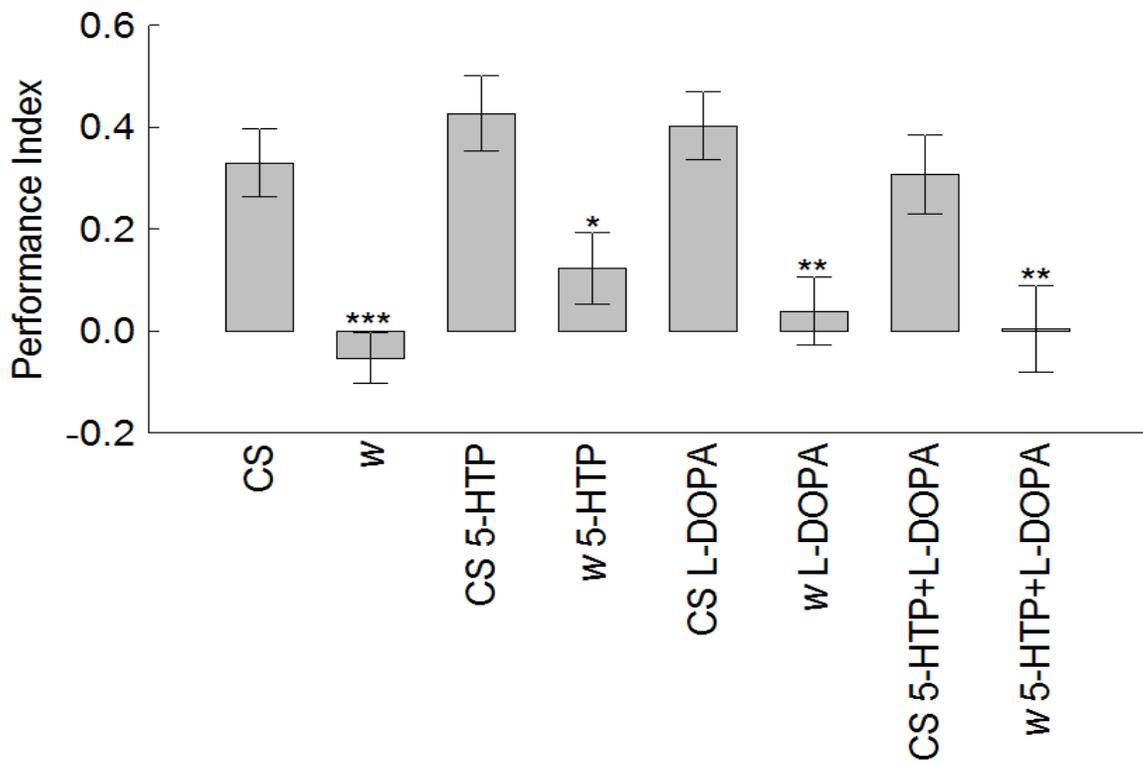


Figure 8-2. Pharmacological enhancement of the serotonin and dopamine levels in w^{1118} does not rescue the memory deficit after 4 minutes (short) training. CS and $w^{1118 \text{ flies}}$ were fed 5HTP (5mg/ml), L-DOPA (5mg/ml) and 5HTP+L-Dopa (5mg/ml each) for 12-16 hours and tested using 4 minute training. Multiple comparisons of mean ranks p values (2-tailed) Kruskal-Wallis test: $H(8, N= 846) = 78.46, p = .0000$. Significant difference indicated by * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. The bars represent mean and SEMs.

Acute pharmacological treatment with serotonin and dopamine synthesis inhibitors impairs acquisition but not reinforcement processing in place learning.

Inhibitors of biogenic amine metabolism have been used to effectively study physiology and behavior compared to supplementing amines (Andretic et al., 2005; Marican et al., 2004; Neckameyer, 1998a). To test this in memory formation we fed flies with the drug 4-Chloro-DL-phenylalanine (PCPA) to inhibit tryptophan hydroxylase activity and block the synthesis of serotonin (Banerjee et al., 2004). Alternatively, flies were fed 3-iodotyrosine (Marican et al., 2004) to inhibit tyrosine hydroxylase and dopamine biosynthesis (Neckameyer, 1996). When flies were fed either (or both, not shown) of these drugs for two or sixteen hours, place memory after long (20 min) training, no memory defects were found (Figure 8-3). To determine whether shorter training sessions might reveal an effect of PCPA and 3IY feeding, we trained flies for four minutes and tested their place memory. In this case, memory performance was strongly reduced in flies fed either drug (Figure 8-3). Neither drug had an effect on temperature avoidance behavior (Figure 8-3). These results indicate that both serotonin and dopamine are necessary for place memory formation after short training, but these treatments do not alter memory after long training.

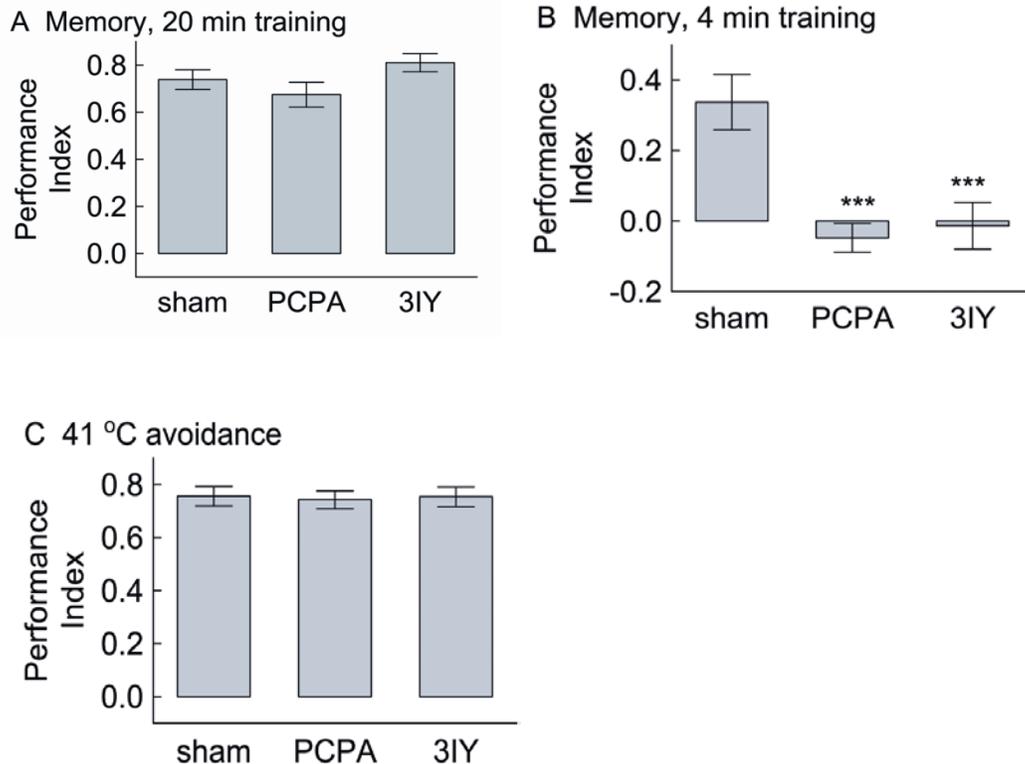


Figure 8-3. Pharmacological manipulation of the serotonergic and dopaminergic levels identifies a necessary function for both systems in memory performance after 4 minutes of training but spares memory after 20 minutes of training. A) Flies fed PCPA (5mg/ml) or 3IY (5mg/ml), reducing serotonin and dopamine levels respectively, did not have significant differences in memory performance after 20 minutes of training ($H(2, N = 470) = 7.4, p = 0.02$; no significant differences with multiple comparisons). B) Memory after four min of training in flies fed either PCPA or 3IY had strongly reduced memory performance compared to sham treated control flies ($H(2, N = 271) = 23.6, p = 0.0000$; multiple comparisons indicate significant differences between both PCPA and 3IY treated flies and Sham treated flies ($p < 0.001 = ***$)). C) Tests for the ability to sense and avoid a 41 °C high temperature source in the thermosensitivity assay found no significant differences between the treatment groups ($H(2, N = 159) = 0.30, p = 0.86$). The values represent means and error bars are SEMs.

Even though 3IY has been shown in the literature to reduce dopamine levels effectively in fly brain and head samples the efficacy of PCPA has not been shown clearly (Andretic et al., 2005; Neckameyer, 1998b; Yuan et al., 2005). To test whether PCPA decreases 5HT levels upon administration we measured 5HT in Sham, 3IY and PCPA fed flies. Interestingly, we find that 5HT levels were reduced in both 3IY and PCPA fed flies when compared to sham (Table 8-1). Since, serotonin and dopamine synthesis depends on a common enzyme dopamine decarboxylase this decrease could arise from compensatory down regulation resulting from inhibition of tyrosine hydroxylase by 3IY. This is, however, difficult to test without measuring actual levels of these enzymes. Furthermore, these decreases might not be drastic enough to lead to a conditioning phenotype and a role for these amines in conditioning after 20 minutes of training cannot be ruled out. An additional issue also arises from these acute pharmacological treatments since all biogenic amine inhibitor used so far act as competitive inhibitors and their absence in the environment before experiments and during training in the heat box can bring back the amine levels to normal. Even as pharmacology has proven to be a useful tool in assaying behavioral phenotypes by transgenic approaches and chronic drug treatments provide strong complementary approaches.

Table 8-1. Dopamine and Serotonin levels after drug treatment			
Treatment	Dopamine level (pg/head)	Serotonin level(pg/head)	N
Sham	423.7 ± 64.6	169.8 ± 10.2	5
3IY	20.24 ± 2.12 (***)	128.5 ± 1.23 (*)	5
PCPA	184.2 ± 23.7 (**)	59.1 ± 2.45(***)	5

Table 8-1. Treatment of CS flies with 3IY and PCPA reduces both serotonin and dopamine in the head tissue. Biogenic amine levels from homogenate of 10 fly heads were tested using HPLC and Enzyme immunoassay for serotonin and dopamine, respectively. Statistical comparisons made by multiple comparisons of mean ranks by Kruskal Wallis test dopamine levels $H(2, N=15) = 9.83, p=0.000$ and serotonin levels $H(2, N=15) = 15.87719, p=0.0012$. *, ** and *** indicates $p < 0.05, p < 0.01$ and $P < 0.001$ respectively.

Expressing a dn-ATPase in serotonergic neurons with Trh-Gal4 does not affect place learning using 32 °C reinforcer

TrpA1 activation of serotonergic neurons mimics the high temperature reinforcers and induces place memory levels atypical of those expected from 32°C (Figure 3-13). We also tested a second method of altering serotonin neurons by expressing a UAS-dnATPase in Trh positive neurons. Flies expressing dnATPase in Trh positive neurons were conditioned with 32°C as the negative reinforcer. These flies did not have an increased PI as compared to the genotypic controls (Figure 8-4). This could be a result of a developmental compensation of increased activation of Trh neurons resulting in no drastic increase of neurotransmitter at the synapse or ineffective transgene expression. One could manipulate expression levels by altering copy numbers of Gal4 drivers and effector transgenes to address these possibilities.

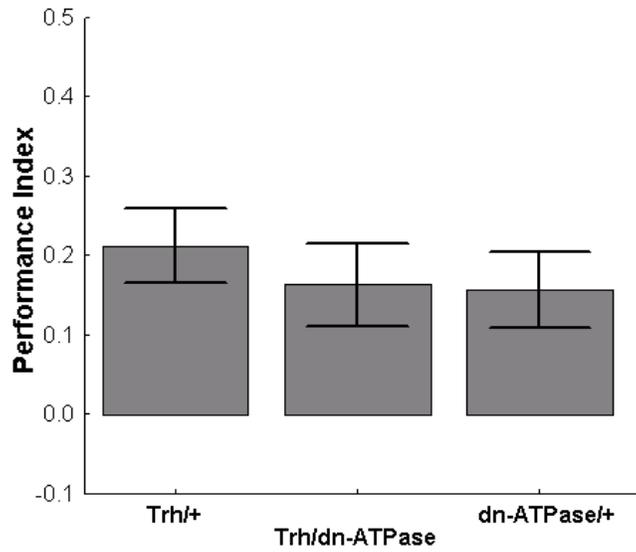


Figure 8-4. Expression of dn-ATPase in Trh positive serotonergic neurons does not lead to an enhancement in conditioned memory using 32°C as the negative reinforcer. Multiple comparisons of mean ranks Kruskal Wallis test $H(2, N=324) = 1.26, p=0.53$. Values represent mean and error bars SEMs.

Serotonin receptors in place learning

In our initial attempts to investigate the role of serotonin receptors and downstream events following serotonin release we tested the role of 5HT1b in the heat box. Of the four identified serotonin receptors in *Drosophila* a clear behavioral role has only been identified for the 5HT1a and 1b receptor (Yuan et al., 2006; Yuan et al., 2005). 5HT1b expresses in some clock neurons and seems to regulate a key kinase (GSK3b) required for stability of a circadian protein called TIM (timeless)(Yuan et al., 2005). 5HT1b receptor was a good starting point to unravel the downstream mechanisms of place learning because of the availability of tools and information about their expression patterns in the CNS. We first probed the expression pattern of 5HT1b-gal4 in the *Drosophila* brain and co-labeled with anti-5HT (Figure 8-5). 5HT1b had a few cell bodies with extensive innervations in mushroom body, central complex and the sub-esophageal ganglion (SG).

To test the role of 5HT1B receptor and role of candidate structures labeled by the 5HT1B-Gal4 in place learning we expressed tetanus toxin light chain in these neurons. Flies were then tested in the heat box using the long (20min) training protocol. We found that blocking output from 5HT1b-Gal4 positive neurons restricts memory performance as compared to the genotypic controls (Figure 8-6).

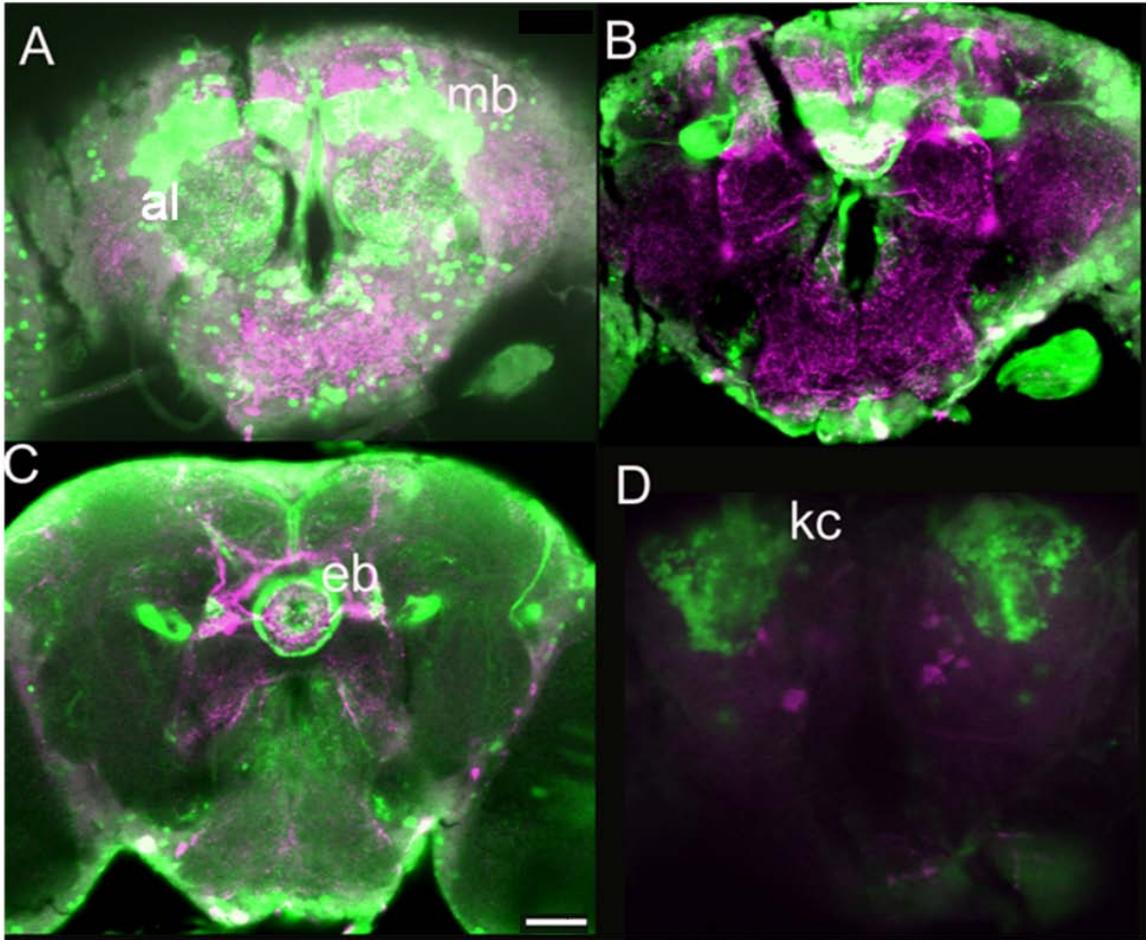


Figure 8-5. Whole mount brain sections of 5HT1bGal4-nlsGFP brains (n=5) stained with anti-GFP (green) and anti-5HT (magenta). A-D) Anterior to posterior sections showing Gal4 expression. Strong expression was seen in mb (mushroom bodies horizontal lobes), al (antennal lobes) in the anterior sections, eb (ellipsoid body) and KC (Kenyon cells) in middle and posterior sections. Each image is a projection of 3-10 μm slices of a whole mount Z-stack. Scale bar in A: 50 μm .

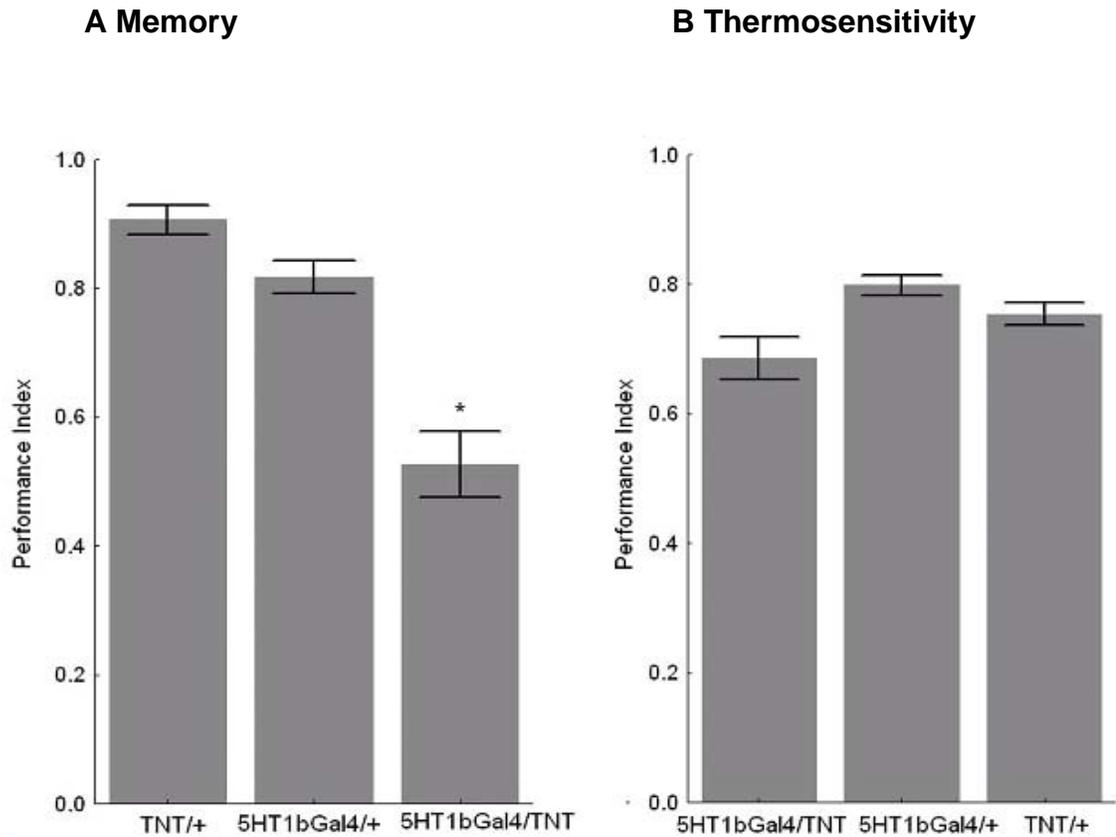


Figure 8-6. Blocking output from 5HT1B expressing neurons using d5HT1b-Gal4 suppress memory formation. A) 5HT1bGal4 / TNT flies performed at lower levels as compared to the genotypic controls CS/TNT and CS/5HT1b-Gal4. Statistical comparisons by Kruskal-Wallis test $H(2, N= 708)= 37.2, p=0.000$. B) 41°C avoidance although a bit lower for 5HT1bGal4/TNT flies was not significantly different from both the controls. Kruskal-Wallis test: $H(2, N=401)= 5.2, p=0.07$. Values represent means and standard errors SEMs, marked significant as * = $p<0.05$.

To complement the above results we tested a 5HT1b hypomorphic allele. A putative hypomorphic allele for *5HT1b* (*5-HT1bMB05181*) has a minos element inserted in the 2nd intron of the *5HT1b* gene (Bellen et al., 2004). This line is homozygous viable, fertile and has normal locomotor activity. Homozygous *5HT1bMB05181* flies in a w+ background was tested with wild-type CS flies as a control and did not show a memory phenotype as compared to the wild-type (n= 282, p=0.59 data not shown). This lack of a memory phenotype could either result from ineffective decrease in 5HT1b levels or redundancy of receptor function. Another line of evidence supporting the ineffective decrease in 5HT1b levels as a potential problem in identifying receptor function comes from no observable memory phenotype seen in flies expressing a 5HT1b-RNAi specifically in 5HT1bGal4 positive neurons (Figure 8-8). To address the redundancy problem and effectively identify receptor function we generated lines which were trans-heterozygous for 2nd chromosome carrying a *5HT1bMB05181* allele and a deletion of the locus that includes *5HT1a* and *5HT1b* gene (Df (2R) Exel6068, P {XP-U} Exel6068, 7550). These flies have reduced memory levels compared to the genetic controls (Figure 8-7).

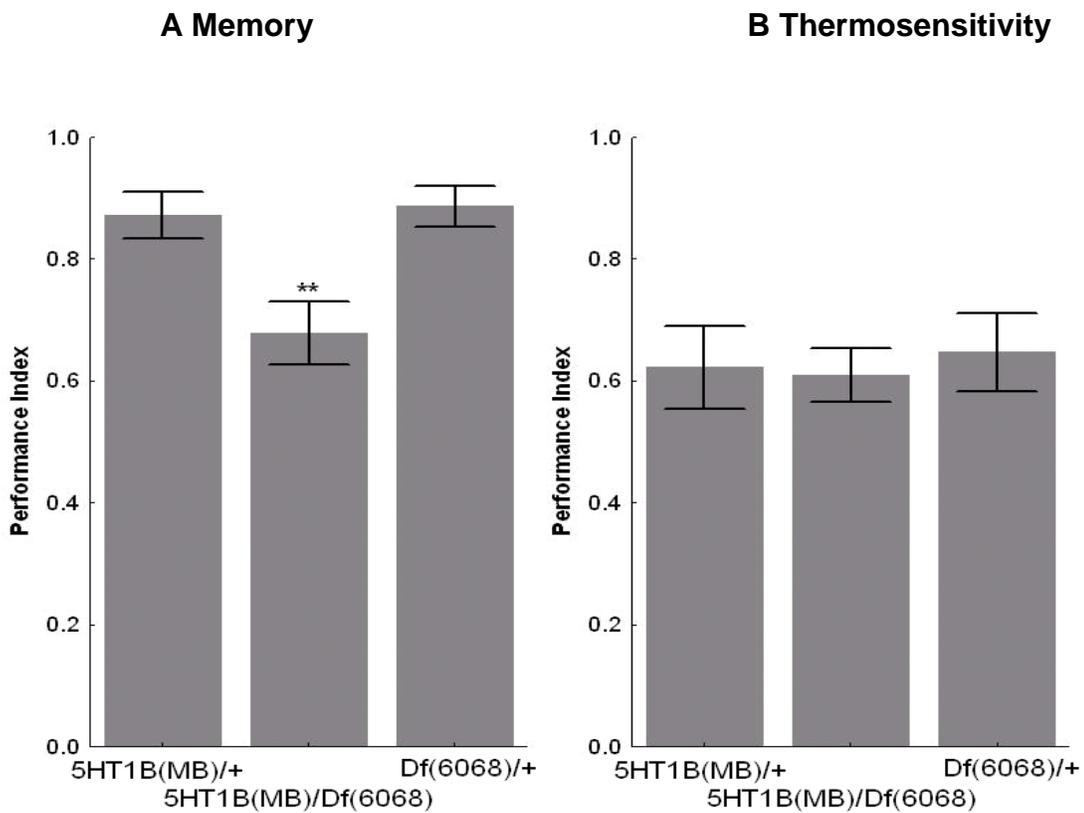


Figure 8-7. Trans-heterozygous flies with reduced 5HT1b receptor restrict memory performance without altering avoidance to 41° C. A) Flies carrying a deficiency over the 5HT1b hypomorph allele (Df (2R) Exel6068/ 5-HT1BMB05181) have lower performance index as compared to the flies of genotypic controls (Df(2R)Exel6068/+) and minus insertion (5-HT1BMB05181/+). Multiple comparisons of mean ranks, Kruskal-Wallis test: $H(2, N=324) = 26.92, p = .0000$. Marked as significantly different ** = $p < 0.01$. B) All genotypes sense and avoid 41° C to the same extent. No significant differences were found ($n=190$). Multiple Comparisons of mean ranks by Kruskal-Wallis test: $H(2, N=166) = 3.09, p = 0.21$. Values represented are means and error bars SEMs.

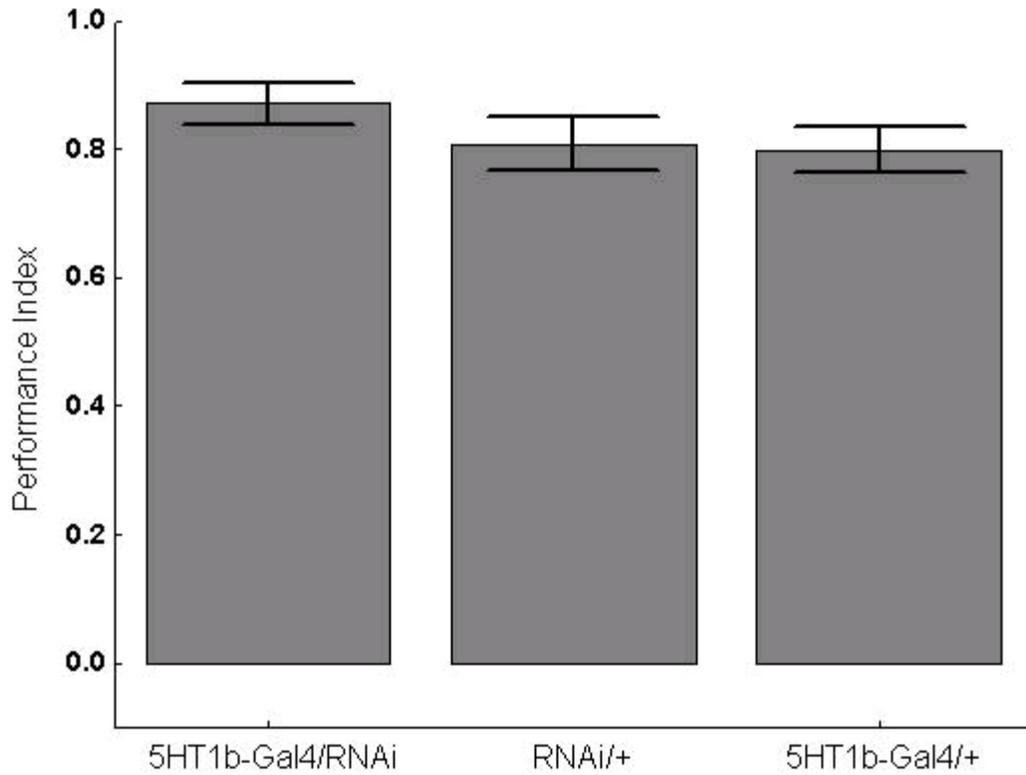


Figure 8-8. Expression of UAS-5HT1B-RNAi in cells targeted by 5HT1b-Gal4 does not reduce memory performance in the heat box. 5HT1b-Gal4/RNAi flies had normal reinforcement processing and memory performance with 41°C reinforce as compared to the genotypic controls RNAi/+ and 5HT1b-Gal4/+. Multiple comparisons of mean ranks indicates $H(2, N=427) = 2.8$, $p=0.2$. Values and error bars represent mean and SEMs.

Attempted activation of serotonergic neurons by ChR2 does not lead to memory enhancement

To activate the serotonergic neurons during pre-exposure we first used a blue light activated cation channel, Channelrhodopsin-2 (ChR2) in the Trh positive neurons. Neurons expressing ChR2 depolarize and show firing patterns as spikes when exposed to blue light. ChR2 activation requires the cofactor all-trans retinal (ATR) to function and flies do not synthesize their own all-trans retinal. Thus, to activate neurons using ChR2, all-transretinal (ATR) has to be provided in the fly food. Adult flies were fed with 10mM ATR which would be most likely be in excess and accessible to the neural structures targeted by Trh-Gal4 as experiments with larval locomotion and olfactory learning are fed ATR in the range of 100-500 μ m (Schroll et al., 2006). For the pre-exposure experiments the experimental group Trh/UAS-ChR2 and the controls Trh/+ , UAS-ChR2/+ were exposed to a strong blue led for 10-30 minutes in a glass vial followed by conditioning with 30°C.

The putative activation of Trh neurons by ChR2 using the above method did not lead to a memory enhancement (not shown). Increasing the feeding time and concentration of atr from 48 to 72 hours and 10mM to 100mM respectively also did not lead to memory enhancement. Additionally, a positive control did not produce the paralysis of adult flies induced by activation or inhibition of cholinergic neurons (Kitamoto, 2001b). The inability of these flies to produce a memory enhancement by activation of serotonergic neurons during pre-exposure could result from two major caveats. Firstly, the blue light might not penetrate the cuticle effectively or second that the ATR is not accessible to the Trh positive brain structures leading to either no or insufficient activation. A second conclusion could be that the Trh neurons are necessary but not sufficient for the pre-exposure effect. Another effector dTrpA1 argues against this possibility. Trp (transient receptor potential) channel misexpression system provides an effective alternative tool to study adult behavior in *Drosophila* (Krashes et al., 2009; Peabody et al., 2009).

Appendix: B

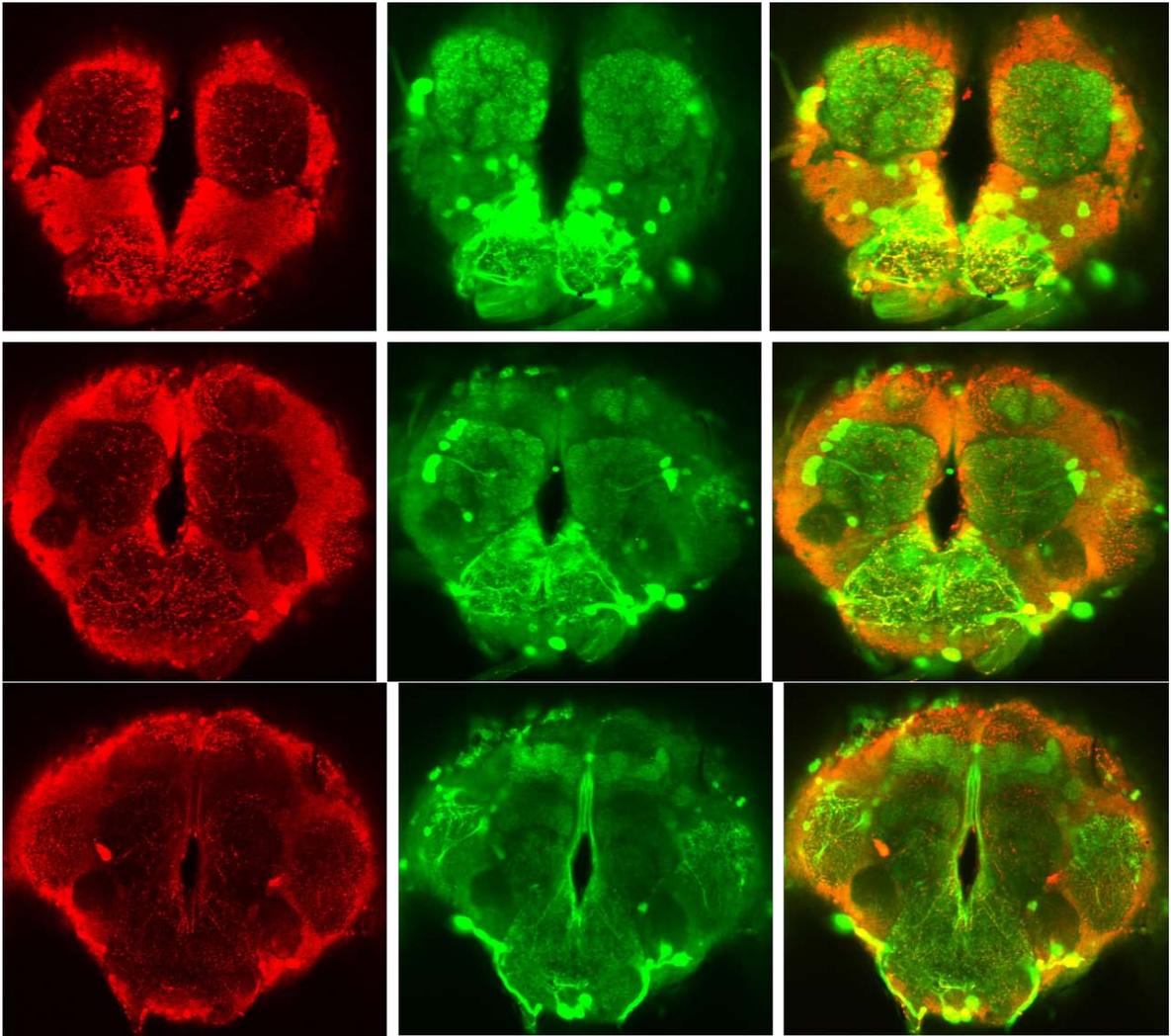
Identification and characterization of Trh positive serotonergic neurons

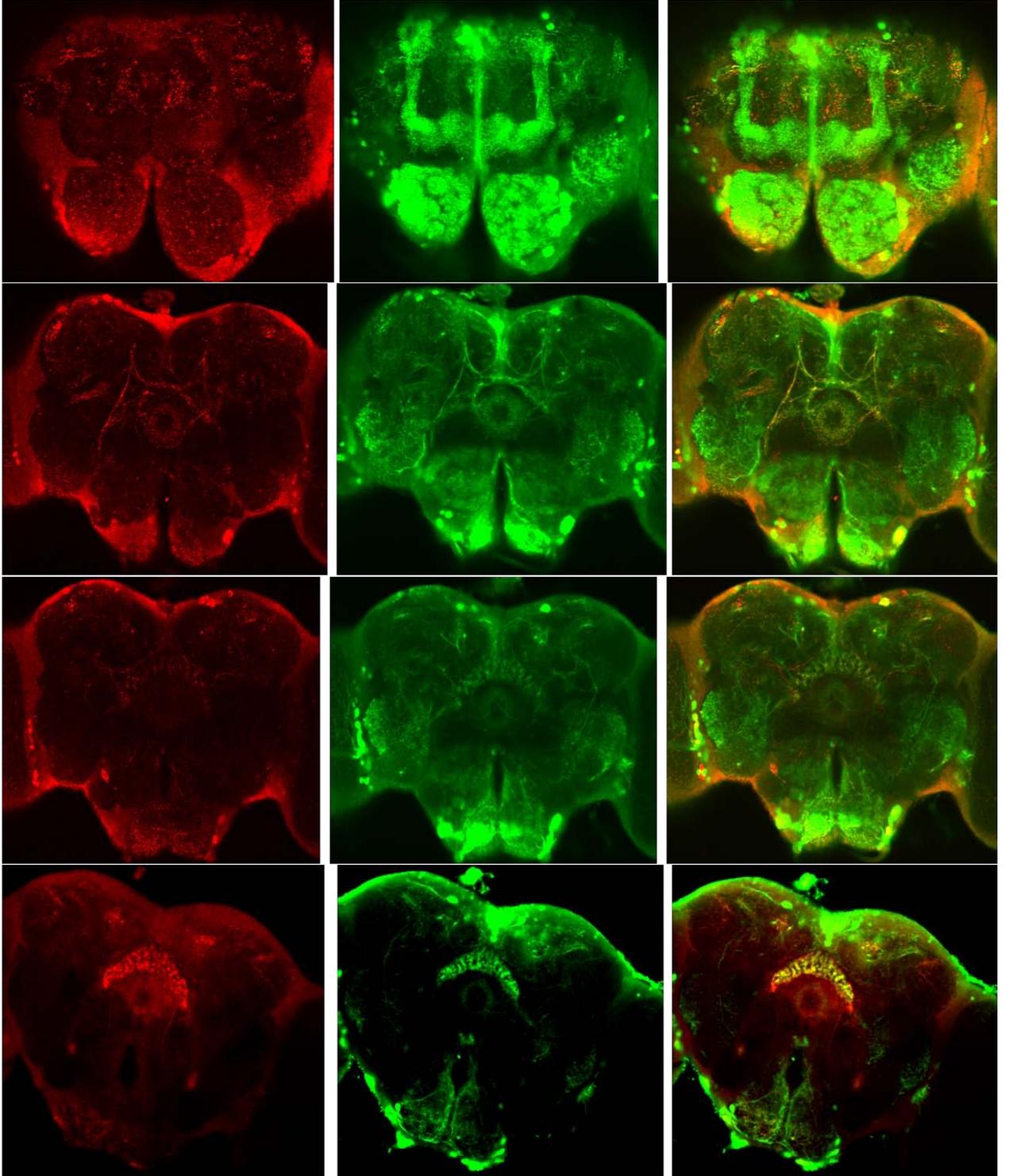
In an attempt to identify and characterize Trh-Gal4 as a serotonergic driver for behavioral experiments we probed Trh-Gal4; UAS-GFP fly brains. Whole mounts of 14 fly brains were probed with anti-GFP and anti-serotonin using confocal microscopy to observe possible co-localization. Co-localizations were found in several serotonergic clusters (Figure 3-11) and innervations patterns were observed in the mushroom body lobes, central complex and sub-esophageal ganglion. The anti-GFP, anti-serotonin labeling is shown in green and red respectively in Figure 8-9, the merge images show co-localization in yellow.

Anti- serotonin

Anti- GFP

Merge





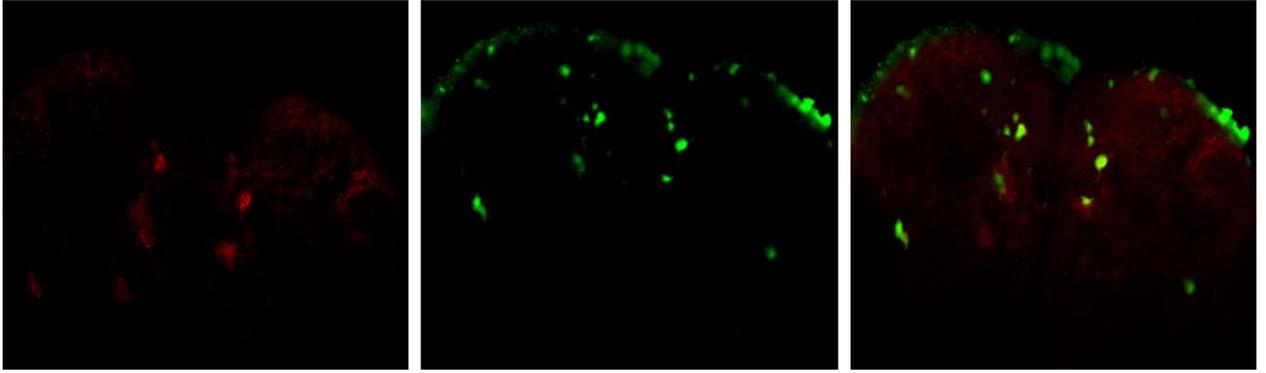


Figure 8-9. Trh-Gal4; UAS-GFP brains showing GFP and serotonin expression. Trh-Gal4; UAS-GFP fly brains were probed with anti-GFP and anti-serotonin to identify the co-localization patterns of cell bodies and innervations. The left panel shows anti-5HT immunoreactivity in red while the middle panel shows the anti-GFP signal (green). The right panel shows a merge of these images to identify potential colocalization (yellow).

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VITA

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