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Second-order conditioning in Drosophila

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SECOND-ORDER CONDITIONING

IN *DROSOPHILA*

by

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ABSTRACT

Second-Order Conditioning in *Drosophila*

by

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Animals possess the ability to associate neutral stimuli in their environment with both rewards and punishment. A conditioned stimulus (CS1) such as a smell or sound, can become associated with an unconditioned stimulus (US), such as a food reward, to elicit what is known as the conditioned response (CR). This type of learning is commonly referred to as classical conditioning or first-order conditioning (FOC). Second-order conditioning (SOC) is an extension of this type of association wherein a novel stimulus is introduced (CS2) and associated with a previously conditioning first-order stimulus (CS1). As a result, the organism may show an attraction or avoidance towards the novel stimulus (CS2) even though it was never directly paired with the original unconditioned stimulus (US). In nature, there is a potential for SOC in almost any circumstance involving exposure to a sequence of learned events. For example, honeybees often memorize complex navigational pathways by associating landmarks with the presence of flowers. While a house or a tree may not reward the insect with nectar, it can be associated with a series of stimuli that eventually lead to a beneficial reward.

My work in this dissertation focuses on conclusively demonstrating SOC for the

first time in *Drosophila* along with utilizing genetic and molecular techniques to investigate the neuronal basis of this behavior. The fruit fly has numerous advantages underlying its usefulness as a model organism: its genome has been sequenced, it possesses a relatively short time of development, it can be easily subjected to genetic alterations, and it is studied by numerous laboratories around the world. Using an automated, computer-controlled olfactory-based learning paradigm, I will demonstrate the ability of *Drosophila* to form these complex, higher-order memories initially believed to be reserved only for the vertebrate learning model. In addition, I will show that *Drosophila* are also capable of conditioning in situations of complex odor presentations for both first- and second-order conditioning. Furthermore, through the use of a transgenic neuron silencing approach exclusive to the *Drosophila* animal model, I will examine whether previously studied neuronal circuits fulfill similar roles in both first- and second-order conditioning.

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CHAPTER 1

INTRODUCTION

1.1 Early Investigations of Second-Order Conditioning.

The landmark publication Conditioned Reflexes published by Ivan Pavlov in 1927 radically altered the scientific investigation of the phenomenon of learning and memory (Pavlov, 1927). In his book, Pavlov describes in detail many of the properties of what is now commonly known as classical conditioning or first-order conditioning (FOC). Classical conditioning is a form of learning wherein a neutral stimulus (CS, such as a tone or smell) is associated with an unconditioned stimulus (US, such as food or electric shock) to elicit a conditioned response (CR). This conditioned response exhibited by the animal is similar to the unconditioned response (UR) normally elicited by the presentation of the US alone. Once conditioned, an animal will respond to the presentation of the CS in a manner similar to the presentation of the US.

In addition to describing the various properties of classical conditioning, Pavlov also described a form of higher-order conditioning which would become known as second-order conditioning" (SOC). In his studies, Pavlov first conditioned a dog to salivate in the presence of an auditory cue by pairing this cue with the presentation of food (classical or first-order conditioning). After this initial pairing of sound and food, Pavlov would present a black square in the presence of the CS1 tone. After ten pairings, the animal would begin salivating at the sight of the black square, even though this was not presented with the original food reward (Pavlov, 1927). This is the earliest example of second-order conditioning in a laboratory setting and the

basis for my work in this dissertation (Figure 1.1).

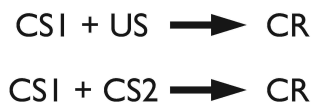

Figure 1.1: Summary of Second-Order Conditioning. A first round of training associated a conditioned stimulus (CS1) with an unconditioned stimulus (US), eliciting a conditioned response (CR). Following this first round of conditioning, a second training sessions associates a novel conditioned stimulus (CS2) with the previously conditioned stimulus (CS1, as opposed to a US) to elicit a similar conditioned response (CR) as the first-order conditioning.

Following its initial observation by Pavlov, SOC was less vigorously studied by scientists in the following 40 years as compared to other forms of learning such as operant and first-order conditioning. Notable exceptions include research on the honeybee *Apis mellifera* by the laboratories of Takeda (Takeda, 1961) and Grossman (Grossmann, 1970). Both researchers discovered clear evidence of SOC in insects using colors and odors with reward learning. These experiments were among the earliest examples of higher-order learning in insects.

1.2 Properties of Second-Order Conditioning.

In the early 1970's, the field of psychology experienced a renewed focus on second-order conditioning research following the publications of Robert A. Rescorla. His study in 1972 was the first to provide an in-depth examination of the relationships formed between stimuli during SOC. Rescorla demonstrated that extinction of the

initial first-order conditioned stimuli has no effect on the second-order conditioned response (Rizley and Rescorla, 1972). In these experiments, an animal is first trained with first- and second-order conditioning and then subsequently exposed to the FOC CS1 stimulus until it demonstrates a decrease in the conditioned response due to extinction. After this phase the animal is then tested for its response to a SOC stimulus. If the FOC and SOC stimuli are directly associated or linked in the brain of the animal, one might expect a decrease in the SOC response once the FOC response is abolished. However, this is not observed in Recorla's experiments (Rizley and Rescorla, 1972). Rats demonstrated the same SOC response both before and after extinction of the FOC stimulus.

These studies raised interesting and important questions regarding the associations formed during second-order conditioning. How does the brain of the animal interpret the layers of interactions between stimuli during SOC? What effect does extinction of the FOC stimulus or a devaluing of the original US have on the behavior of the animal? Does the extinction of the SOC stimuli have any effect on the animal's response to FOC stimuli? In the next four decades, numerous psychologists and neurobiologists have approached these problems with varying results. In the following pages I will summarize the results of their experiments and outline the reasoning for the use of *Drosophila* in SOC.

In second-order conditioning the original unconditioned stimulus is not used as a reinforcer for the second-order conditioned stimuli (Pavlov, 1927). Therefore, one might expect that any modulation of the US would result in little change in an animal's second-order conditioned response. Indeed, early work by Rescorla has demon-

strated that habituation to the original unconditioned stimulus after second-order conditioning has no effect on the response to an SOC stimulus (Rescorla, 1973; Holland and Rescorla, 1975a). In earlier experiments, rats were conditioned to a tone or light stimulus during first- and second-order conditioning. The unconditioned stimulus was presented as a startling noise during FOC. Gradual habituation towards this noise (presentation of the noise over repeated trials until the startle effect was significantly less pronounced) lowered the response of the rats to the first-order conditioned stimuli but not the second-order conditioned stimuli. In addition, it was also shown that an inflation of the US (where the strength of the US is raised, as opposed to lowered) affected the FOC response but not SOC response (Rescorla, 1974). These tasks were performed with an increasing US shock presentation after both first- and second-order training bouts. These results, in unison with the US habituation experiments, indicate that any associations formed between the original US and CS1 are independent of CS2.

A second topic of interest for researchers studying second-order conditioning is the effect of CS1 extinction on CS2 performance. The first study to examine the role of CS1 extinction after SOC was published by Recorla's group in 1972 (Rizley and Rescorla, 1972) using rats as a model organism. Unreinforced presentation of the original CS1 stimulus (light) eventually lead to a lower response towards this stimulus but had no effect on the animal's response to the CS2 (tone). This was the first demonstration in animals of CS1 extinction with preservation of the CS2 response. In addition, a similar finding was presented several years later by Holland and Rescorla through the use of appetitive conditioning also in rats (Holland and

Rescorla, 1975a). There have been numerous studies since the 1970's by other research groups in a variety of model organisms that have provided support for the view of CS1 CS2 separation during extinction (Amiro and Bitterman, 1980; Ross, 1986; Davey and Arulampalam, 1982).

However, there have also been several studies that point to an attenuation of CS2 response following extinction of the CS1. The first of such studies was presented in 1977 using second-order conditioning of the pigeon's key-peck response (Rashotte et al., 1977). In these experiments, pecking of a key-light (CS2) was diminished when the previous CS1 stimulus that also evoked pecking was extinguished. A similar finding was replicated by Rescorla's group two years later demonstrating that the use of a common sensory modality for both first- and second-order stimuli can lead to a diminished response towards the CS2 after CS1 extinction (Rescorla, 1979). This response appears to be restricted to a limited number of cases wherein autoshaping plays a major roll and stimuli are confined to a similar sensory input. However, even in these cases the CS2 is not extinguished at the same lower level of response as the CS1 (Rescorla, 1979). Furthermore, there is evidence that this extinction of CS2 with CS1 is due to a simultaneous training effect wherein the animal learns both CS1 and CS1-CS2 associations via a similar sensory modality at nearly the same time (Rescorla, 1982). When the CS1 and CS2 training bouts are presented in a sequential order, the extinction effect of CS1 on CS2 is lost (Rescorla, 1982).

These properties of SOC are important not only for understanding the differences between first- and second-order conditioning, but also in their practical application to therapy and psychological investigation. One of the most commonly studied illnesses

involving SOC is Post Traumatic Stress Disorder, or PTSD. PTSD is an common anxiety disorder effecting individuals who have encountered a traumatic experience such as physical abuse or military combat. It is estimated that as many as 39% of men who have experienced combat demonstrate some form of PTSD (Bisson and Andrew, 2007). The therapeutic difficultly in extinguishing associative events in PTSD share striking similarity to associations formed in second-order conditioning (Wessa and Flor, 2007). Therefore, understanding not only the associations formed during SOC but also how to disrupt and reverse those associations would be extremely useful for the treatment of PTSD.

With the advent of modern neurobiology, scientists are now beginning to examine the molecular components, neural networks, and brain structures responsible for the behavior observed in higher-order learning such as second-order conditioning. In the past 15 years, several research groups have attempted to examine specific regions of the brain and specific neurotransmitters that may play a role in both fear and appetitive SOC (Gewirtz and Davis, 2000). The basolateral amygdala (ABL) has been identified as an important brain structure for second-order conditioning in vertebrates (Hatfield et al., 1996; Nader and LeDoux, 1999). Lesions in this specific region of the brain prevent rats from forming SOC associations during higher-order training (Hatfield et al., 1996). The ABL is required only for the acquisition of second-order conditioned stimuli but not its maintenance or retrieval (Setlow et al., 2002a). Within the amygdala, NMDA receptors are required for the formation of SOC (Gewirtz and Davis, 1997). Blocking these receptors via chemical antagonists is sufficient to disrupt the formation of SOC in rats (Gewirtz and Davis, 1997). These

investigations represent the majority of work focused on deciphering the biological processes responsible for SOC on a neuronal or molecular level in vertebrates.

Research of second-order conditioning in invertebrates has centered mostly around the honeybee (*Apis mellifera*) with more recent pharmacological work utilizing crickets (*Gryllus bimaculatus*). The Takeda group was the first to demonstrate this higher-order learning with the honeybee in the 1960's (Takeda, 1961). This is also one of the earliest examples of the paired-paired protocol which is used by numerous researchers in both invertebrate and vertebrate models throughout the next several decades (Table 1.1).

Protocol	**Description**	**SOC?**
Paired-Paired (P-P)	CS1 + US paired, CS2 + CS1 paired	Yes
Unpaired-Paired (U-P)	CS1 + US unpaired, CS2 + CS1 paired	No
Paired-Unpaired (P-U)	CS1 + US paired, CS2 + CS1 unpaired	No

Table 1.1: Summary of the Paired-Paired Experimental Protocol. This approach is commonly used in the field of psychology to demonstrate and study SOC. Properly pairing the first- and second-order conditioning stimuli results in a conditioned response when testing for SOC. However, unpairing associations during either first- or second-order conditioning abolishes the final SOC response.

Demonstrations by Klaus Grossman recreated experiences observed by the honeybee in nature; insects could be trained with second-order conditioning using combinations of color, smell, and sugar reward cues (Grossmann, 1970). This experimental setup closely mimicked the flower visitations of forager bees and is an early example of SOC in a more natural environment. More recent work with honeybees has

demonstrated the ability to form backward SOC, similar to sensory-preconditioning with sequential, as opposed to simultaneous, presentation of two stimuli (Hussaini et al., 2007). Association of the first stimuli after backward conditioning can lead to the formation of a conditioned response to the second stimuli, even though the second stimuli was not paired with the same unconditioned stimulus. Before describing the most recent advances in insect neurobiology regarding second-order conditioning, neurotransmitters, and neuronal circuits, it is necessary to elaborate on the current model of learning and memory as it exists within the field of *Drosophila* research.

1.3 A Model of Insect Learning.

The mushroom body, a structure composed of 2500 neurons known as Kenyon cells, is the center of olfactory-based classical learning and memory in *Drosophila* (de Belle and Heisenberg, 1994; Heisenberg, 2003). The term itself is derived from the mushroom-like structure originally observed in the brains of honeybees (Dujardin, 1850). The mushroom body receives input from projection neurons that innervate the antennal lobes located at the central anterior region of the brain (Jefferis et al., 2001). These antennal lobes receive input directly from olfactory receptor neurons that travel from the antennae where they serve as detectors for a wide variety of odor molecules (Vosshall et al., 2000). When mushroom bodies are ablated through administration of the chemical hydroxyurea during larval development, flies are unable to perform learning tasks in an olfactory-based learning and memory paradigm (de Belle and Heisenberg, 1994).

The mushroom body has been topographically dissected into a number of lobes based on both visible morphology and experimental evidence of structure-function relationships (Tanaka et al., 2008). There are five main subsets of lobes: alpha, alpha prime, beta, beta prime, and gamma. These can be further dissected into 14 subcategories depending on the specific regions innervated by the neurons of the mushroom body (Tanaka et al., 2008). Synaptic transmission from alpha and beta lobes is required for memory retrieval (McGuire et al., 2001; Dubnau et al., 2001) while output from alpha prime and beta prime are required during acquisition and consolidation (Krashes et al., 2007). There has also been some evidence of the requirement for alpha and alpha prime lobes in long-term memory (Pascual and Preat, 2001).

Apart from the mushroom body, distinct subsets of aminergic neurons with predominantly single classes of neurotransmitters have been identified as serving specific roles for learning and memory in brain of *Drosophila* and other insects (Schroll et al., 2006). Perhaps the most intensively studied of these circuits are those consisting of dopaminergic and octopaminergic neurons (Honjo and Furukubo-Tokunaga, 2009; Vergoz et al., 2007; Hammer, 1997) . The extensively studied VUM neuron in honey bees is a well-known example of an octopaminergic neuron involved in appetitive learning (Hammer, 1993, 1997). Direct stimulation octopamine-specific circuits in the honey bee brain via injection of octopamine leads to associative olfactory learning (Hammer and Menzel, 1998). In addition, blocking the expression of octopamine receptors via RNA-interference prevents the formation of appetitive memory in honey bees (Farooqui et al., 2004). In fruit flies and crickets, octopamine has also been identified as a critical neurotransmitter for appetitive learning (Schwaerzel et al., 2003;

Mizunami and Matsumoto, 2009).

Studies in aversive learning in insects has also highlighted the importance of dopamine as a critical neurotransmitter for associative conditioning (Schwaerzel et al., 2003; Vergoz et al., 2007; Aso et al., 2010). Restriction of neurotransmission from dopamine neurons during aversive olfactory-based conditioning in *Drosophila* results in an abolishment of learning (Schwaerzel et al., 2003). Furthermore, direct light-induced activation of dopamine neurons in *Drosophila* larvae can be utilized to artificially create a learned avoidance behavior to a particular stimulus (Schroll et al., 2006). Studies involving direct imaging of dopamine neurons during associative conditioning have revealed a punishment prediction elicited by a presentation of a previously conditioned odor (Riemensperger et al., 2005). In other words, firing of dopamine neurons can be correlated with an prediction of an aversive event. In addition to studies in *Drosophila*, dopamine has been examined for its role in aversive conditioning in both honey bees (Vergoz et al., 2007) and crickets (Mizunami and Matsumoto, 2009). These studies, among others, demonstrate the importance of both dopamine and octopamine in insect associative learning.

While the field of Psychology has made great strides in understanding the complex behavior of animals in situations of higher-order learning, there still remains a disconnect in bridging what we observe with how the underlying neurobiology is generating these behaviors. Pioneering research by a handful of laboratories has highlighted the neurobiological mechanisms of SOC in vertebrate systems. For example, we are beginning to understand differing roles of the hippocampus and amygdala in fear conditioning (Phillips and LeDoux, 1992). More specifically, the basolateral

amygdala appears to be a focal point for SOC in the rat model (Hatfield et al., 1996; Setlow et al., 2002b,a; Lindgren et al., 2003). Manipulation of amygdala function both *in vivo* or *in vitro* have demonstrated a distinct different in first- and second-order conditioning neuronal networks in the mammalian system.

While SOC studies using the rat model system have begun to bridge the gap between the psychology and neurobiology of SOC, there remain many unanswered questions in deciphering the complete structure-function relationship. In order to further correlate specific changes in neuronal activity with behavior, there must exist a simple model organism that can be easily manipulated with modern genetic and neurobiology techniques, and yet still be capable of exhibiting complex higher-order learning. It is for this reason that I chose to focus on *Drosophila* as a model organism for examining second-order conditioning.

A survey of the literature reveals that only a handful of studies exist examining complex behavior in *Drosophila* and within those, only a single previous attempt at second-order conditioning in the fruit fly has been published (Brembs and Heisenberg, 2001). In 2001, experiments by Brembs and Heisenberg utilized visual learning to examine sensory-preconditioning and second-order conditioning in *Drosophila*. While they were ultimately successful in eliciting sensory-preconditioning, their experiments failed to decisively demonstrate second-order conditioning. Furthermore, there are currently no published efforts to attempt second-order conditioning in *Drosophila* using a purely olfactory-based approach.

I sought to utilize the T-maze paradigm first development by Tim Tully and Chip Quinn in 1985 (Tully and Quinn, 1985). This particular device has been widely

used by a number of laboratories worldwide for examining olfactory-based associative learning and memory in the fruit fly (Keene and Waddell, 2007). Therefore, developing a second-order protocol using this device may facilitate its adoption in other *Drosophila* learning and memory laboratories. In addition, the breadth of knowledge surrounding the roles of the mushroom body and aminergic neurons in fruit flies presents a unique opportunity for examining second-order conditioning. Almost all of the dozens of learning and memory studies involving fruit flies have utilized only first-order conditioning. My project begins with the construction and programming of an automated "training room" in which to elicit strong first-order responses from the fruit fly. After successfully achieving first-order conditioning using the T-maze paradigm, I then proceed to demonstrate second-order conditioning using an approach modified from earlier work in honey bees (Takeda, 1961). Finally, I demonstrate a novel heat-shocking method for examining the role of dopamine neurons and the mushroom body during different stages of SOC.

CHAPTER 2

MATERIALS AND METHODS

The ability to examine the behavior of *Drosophila melanogaster* in a controlled laboratory environment has been well documented since the pioneering work of Seymour Benzer (Benzer, 1967). Benzer's lifetime of work was focused on examining the relationship between genes and behavior, often utilizing the fruit fly as a model organism for his studies (Tanouye, 2008). It is from these studies, and the studies of other behavioral geneticists of the mid-twentieth century, that we can progress and develop useful laboratory paradigms for both quantifying and qualifying the behavior of *Drosophila melanogaster* and other model organisms.

In 1974, William Chip Quinn, William Harris, and Seymour Benzer published an influential paper describing the conditioning of *D. melanogaster* through the use of electric shock and odor pairing (Quinn et al., 1974). In their experiments they used semi-operant conditioning to train flies to avoid various odors that had been paired with an electric shock. The training device consisted of vertical acrylic tubes that were lined with a copper grid and covered with a thin layer of odor. Two distinct chemicals, 3-octanol (OCT) and 4-methylcyclohexanol (MCH), were used to train the flies. Administering an electric shock (90 VDC) while flies walked on the odor-covered grid would eventually lead to the formation of a conditioned response. When presented with a choice to run between a tube containing one of these odors odor and a blank tube, more flies would avoid the shock-associated odor versus a blank tube than the non-shock associated odor versus a blank tube. In other words, they learned to associate an electric shock with the trained odor. The avoidance of these

flies could be measure by counting the number of flies that avoided a shock-associated odor versus a non-shock associated odor. This performance index was then used as a measurement of the ability of a particular group of flies to form a conditioned response.

While this operant training paradigm was successful at demonstrating several well-known characteristics of classical learning, such as habituation and extinction, it was not without flaws. For example, the average performance index achieved for wild-type flies was around 0.34 or 34% (Tully, 1984). This was problematic as it did not allow for broad comparisons between groups of mutant or wild-type flies with such a low score as the highest performance index of the control group. In addition, by moving downward away from the copper grid, flies would enter a blank tube where they might smell the odor from the shock-tube but would not form an association as they lacked exposure to the shock itself. This ability to avoid conditioning within the paradigm may have lead to a lower overall performance index at the end of the training period.

To remedy the problems of the operant conditioning paradigm, Tim Tully and William Quinn devised a new method of classical conditioning to be used with *Drosophila melanogaster*. In 1985 they published a paper describing a device known as a "T-maze" to train fruit flies using a classical conditioning approach (Tully and Quinn, 1985). The T-maze allows for classical conditioning within a copper-wire lined acrylic training tube through which air and odors can flow (Figure 2.1). Flies placed within this acrylic chamber are forced to experience the training regime. This distinguishes the T-maze paradigm from the operant Benzer apparatus. The same

odors, OCT and MCH, were used to classically condition both wild-type and mutant *Drosophila* with a 90 VDC shock provided as the unconditioned stimulus. Following a training session in the top chamber, flies were transferred down through an elevator to a choice point where they were allowed to choose between two additional tubes, each containing either OCT or MCH. A performance index is calculated for these tests as the normalized avoidance of flies from a conditioned odor. Perfect avoidance, or zero flies in the conditioned odor tube, results in a score of 100. A 50:50 distribution of flies between the conditioned and unconditioned odor results in a score of 0 while all flies in the conditioned odor tube results in a score of -100.

For my second-order conditioning experiments, I used the T-maze apparatus to condition fruit flies. With slight modifications to the protocol and odor delivery system I was able to elicit a conditioned response to a second-order stimuli using an olfactory-based approach. In addition, I have written numerous programs to investigate the ability of *Drosophila* to discriminate between mixtures of various odors and to experimentally determine the optimal values for both first- and second-order conditioning. Using the T-maze paradigm has allowed me to build on the past research of Tully, Quinn, and Benzer, to create a system that is both robust and reliable for eliciting complex behavior from the fruit fly.

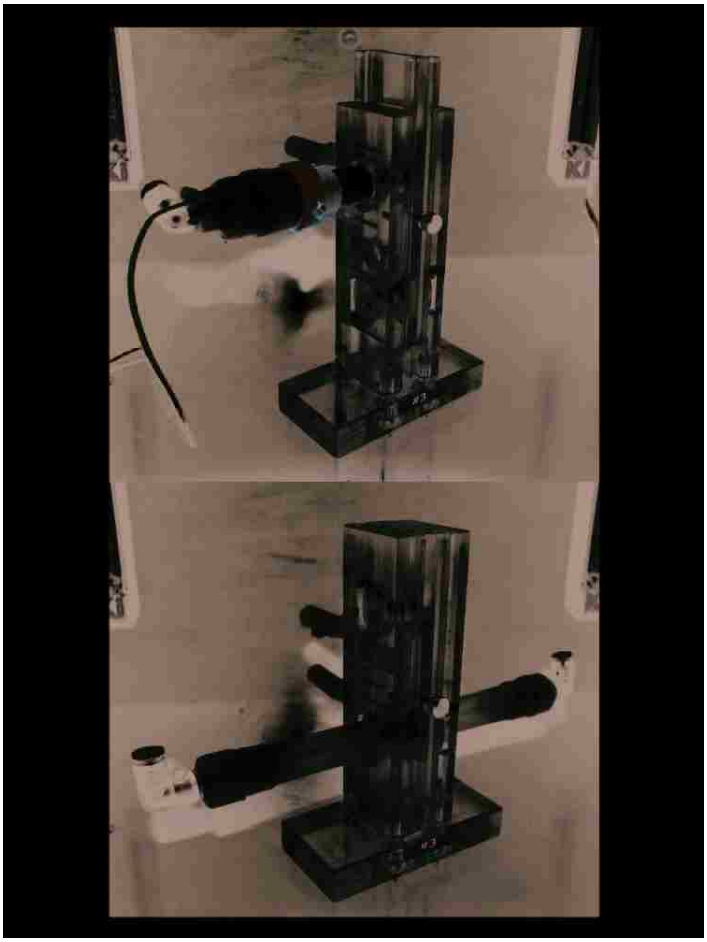

Figure 2.1: Tully & Quinn "T-Maze". The top portion of the photo demonstrates the maze in a training position. Approximately 75-100 flies are loaded into the copper-lined acrylic tube through which odors can pass while administering electric shock. The bottom half of the photograph demonstrates the choice position. Flies are free to move between either tube, each one contains an odor from the training portion of the test.

2.1 Training Room Equipment.

I used a semi-automatic odor and shock delivery system to condition flies in our T-maze apparatus (Figure 2.2). The setup consisted of three separate work stations allowing for concurrent training of up to three groups of flies for first-order conditioning or two groups of flies for compound and second-order conditioning. At the core of this conditioning apparatus is an odor delivery system developed by Analytical Research Systems and controlled by National Instruments FieldPoint relays via LabView software. In total, 82 different computer programs were written to control odor and shock delivery in 7 distinct paradigms (Table 2.1). LabView uses an object-based programming language involving distinct modules that can be chained together to create specific events. For our setup, each module was directly linked to a FieldPoint relay and could be positioned in either an on or off state to close or open a circuit. In this manner, any device in our training system that could be hardwired to the relay would be controlled by the LabView software. This allowed for automatic control of variables and stimuli such as airflow, odor presentation, and both electric and heat shock delivery.

Airflow was provided by an in-house vacuum system regulated at 8 different points (system-wide high and low airflow adjustments along with individual high and low airflow adjustments at each station). A electro-pneumatic switch (Festo), powered by an 18 VDC power supply (Circuit Specialists CSI12001X) and regulated by laboratory air, allowed relay-controlled alternation between high and low air airflow rates.

Figure 2.2: *Drosophila* Training Room. All experiments were carried out in a closed environmental chamber with 88 ± 5% humidity at 24 °C.

Protocol	**Number of Programs**
Classical Conditioning	8
Odor Balancing and Controls	13
Extinction	9
Compound Extinction	18
Compound Classical Conditioning	8
Long-Term Memory	4
Second-Order Conditioning	24

Table 2.1: Summary of Training Room Programs. 84 different programs were authored for both first- and second-order conditioning along with other forms of higher-order associations. Those used for sensory acuity tests, FOC, SOC, and compound conditioning are further examined in Chapter 2. The rest are listed in Appendix A.

Low airflow was set at 650 or 750 mL min^{-1} per machine, depending on experimental procedure, and high airflow was set to either 1300 or 1500 mL min^{-1}, respectively. An airflow reservoir was situated immediately before the laboratory vacuum connection to ensure a constant negative pressure, particularly in situations of higher air movement.

Electric shock was delivered to each training station via 18 gauge wire connected to an electric relay (Omron G9EB-1-B, max 250 VDC @ 25 A). Because the FieldPoint relays only support a maximum of 35 VDC @ 3 A, the Omron relays provide a means for delivering 90 to 120 VDC without damaging the LabView system. Three Omron relays provided shock currents from three power supplies (Circuit Specialists CSI12001X) to each training station. These relays were in turn controlled by the FieldPoint system on a separate circuit from the high voltage line. Closing the FieldPoint relay would close the Omron relay which would then provide the shock stimuli to the copper-wire inside of the acrylic training tubes. The voltage for this electric shock could be regulated between 1 to 130 VDC.

The odor delivery system was designed by Analytical Research Systems in collaboration with JS de Belle. Each unit consists of six 12 VDC powered solenoids connected to three glass bubblers (Figure 2.3). At every bubbler there are two entry points for tubes that connect to both the left and right set of solenoids. Airflow tubes extend from the back of each group of solenoids, thus allowing air to exit either the right or left side of each bubbler, travel down into the solenoids, and exit via the rear of the solenoid group. This configuration enables two airflow tubes from each odor delivery unit to each carry any combination of the odors from three bubblers. In a

typical setup, the blank odor bubbler was setup first and farthest from the exhaust in the solenoid row. This allowed for the blank air to help clear any residual air from solenoids closer to the rear exit tube.

For each bubbler, 5 mL of heavy mineral oil (Mallinckrodt) was used as the base solution. Odorants were added to this oil as a method of delivering stimuli to each training station. Varying dilutions were used for three odors depending on the experimental procedure. Three odors were used for all experiments: benzaldehyde (BEN), 4-methylcyclohexanol (MCH), and 3-octanol (OCT). After each full day of training room use, all bubblers were removed from their delivery system and cleaned with polished water + Alconox soap and thoroughly rinsed and dried for the next session of training. No residual odor remained in the bubblers after this cleaning.

All experiments were performed under strict environmental conditions and dim red light (Figure 2.4) in a closed climate-controlled chamber (RWSmith and Co.). Humidity and temperature were held at 88 ± 5% and 24 ± 0.5 °C, respectively. Dim red light was used throughout all experiments to restrict the visual capacity of *Drosophila*. All flies were allowed to acclimate to the room temperatures for fifteen minutes before trials. Humidified incubators, set at 35 ± 5% humidity and 24 ± 0.5 °C, were used to raise all *Drosophila* stocks on a 12:12 light:dark cycle. Flies were isolated at either 2 to 5 days or 3 to 6 days of age for training, depending on the experimental paradigm.

Figure 2.3: Automated Relays and Glass Bubblers. This system was constructed by Analytical Research Systems (ARS) based on designs by J. Steven de Belle. Each set of bubblers is linked to six individual 12 volt DC relays by two clear plastic tubes. This allows for odors from each bubbler to travel down either row of relays, giving three odors the possibility of traveling down two delivery tubes in any combination.

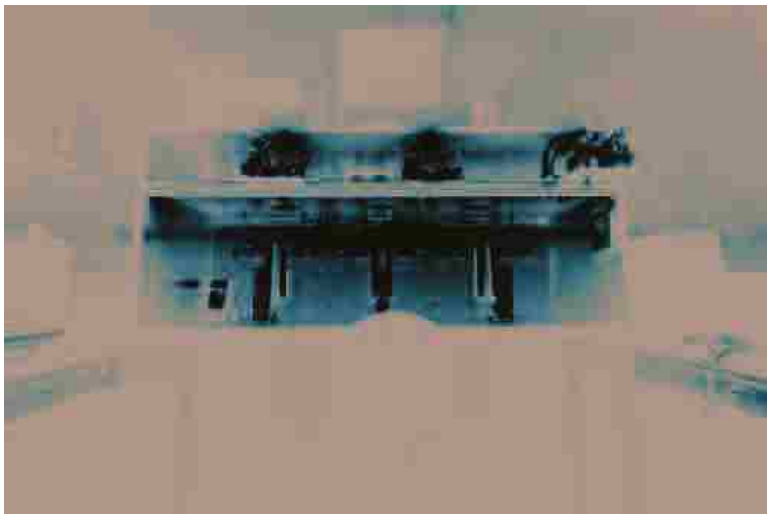

Figure 2.4: Training Room Red Light Conditions. All experiments were carried out under dim red light provided via light emitting diodes.

All stimuli presentation, airflow adjustments, and experimental timing was handled by the FieldPoint relays. Four eight-channel single-pole single-throw (SPST) relay modules (FP-RLY-420) were chained together using terminal bases (FP-TB-1) and connected to both a power supply (FP-PS-4) and network module (FP-1601). Each relay module consisted of 8 individual relays (max 35 VDC @ 3 A) for a total of 32 possible relay circuits. Network communication was established via a crossover cat-5 Ethernet cable connected to a Windows 2000 PC (built in-house).

2.2 Programming.

The classical conditioning protocols used for the training room were based largely on the research of Tully and Quinn (Tully and Quinn, 1985) with adjustments in timing to suit our experimental setup. The Tully and Quinn protocol was used for several reasons. First, it provided a means to examine the basic learning and memory abilities of multiple stocks, such as wild-type, white-eyed, or any transgenic lines that were used. Second, because this classical conditioning paradigm is widely studied throughout the field, it allowed for a comparison of the capability of the training room to elicit a strong conditioned response from our animals. Finally, I could use this program to examine the effect of changing variables, such as odor concentration or airflow rate, and note the difference in the performance of the flies. Designing a paradigm that results in a strong response for second-order conditioning often relies on a strong response from first-order conditioning (Bower, 1972) including the fine-tuning of every variable associated with associatively conditioning. The programs

described in this section allowed for precise trouble-shooting and adjustment of every variable for both first- and second-order conditioning.

All programs were authored in National Instruments LabView software on a custom in-house PC running Windows 2000 Professional. The FieldPoint relays are represented as colored boxes on the programming screen and each relay can be individually cycled on or off, depending on the commands of the program (see sample program layout, Figure 2.5). Also utilized were time-delays and custom inputs to allow the user to modify the program's variables on-the-fly (Figure 2.6).

2.2a Odor Balancing and Sensory Acuity Controls.

When comparing the behavior of flies with different genetic backgrounds, it is vital to ensure that all animals share a similar level of sensory acuity for both odor and shock perception. Furthermore, it is important, even in groups of genotypically identical flies, to ensure a positive avoidance behavior to all odors and electric shock used in these experiments. Adjustments of odor dilutions, shock intensity (including substitution of the shock tube itself), odor balance, and timing are all critically important variables in our behavioral paradigms.

To examine the response of flies to different stimuli I used a total of 13 different programs. The basic overview to an odor avoidance paradigm can be seen in Figure 2.7. Similar to odor avoidance, odor balancing programs were written to examine the odor avoidance behavior of naive (unconditioned) flies in a choice situation between two odors.

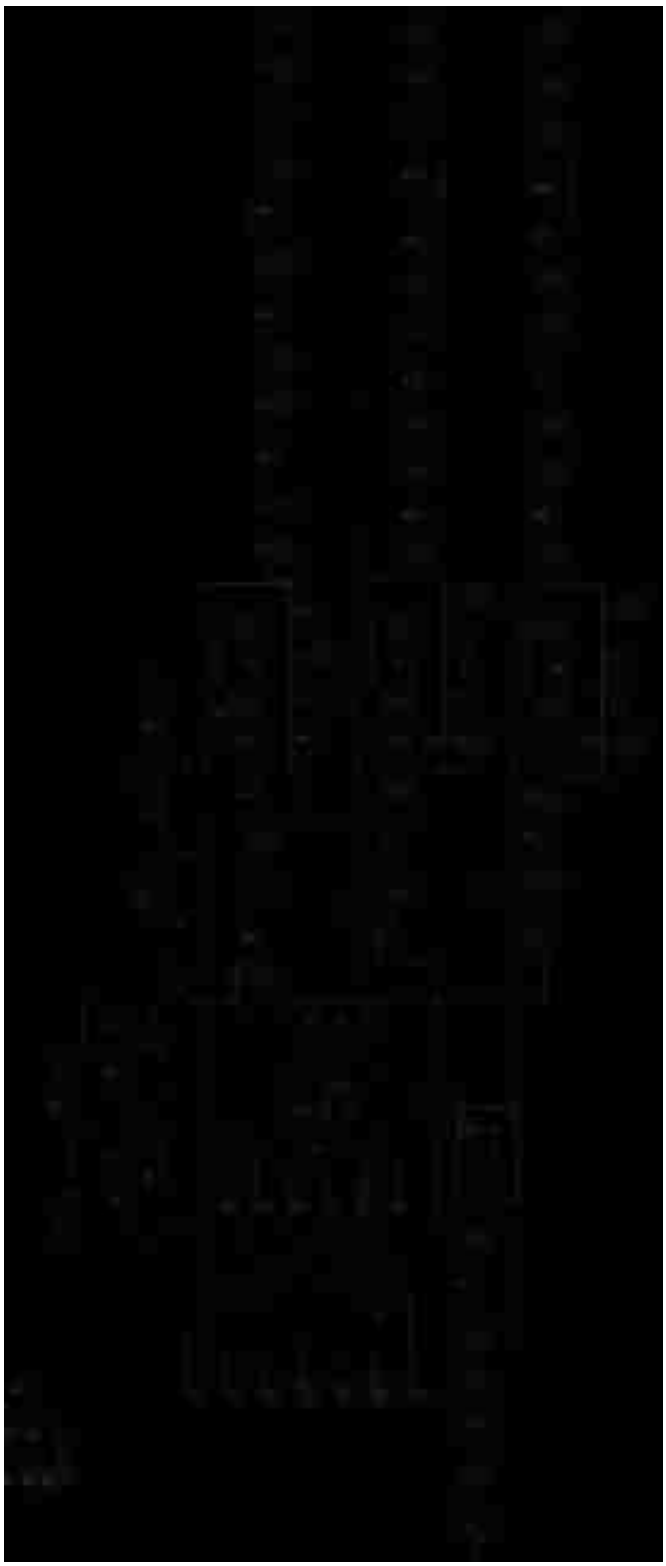

Figure 2.5: Sample LabView Workflow. Boxes represent relay open/close commands and time delays. Lines represent connections between variables within the program.

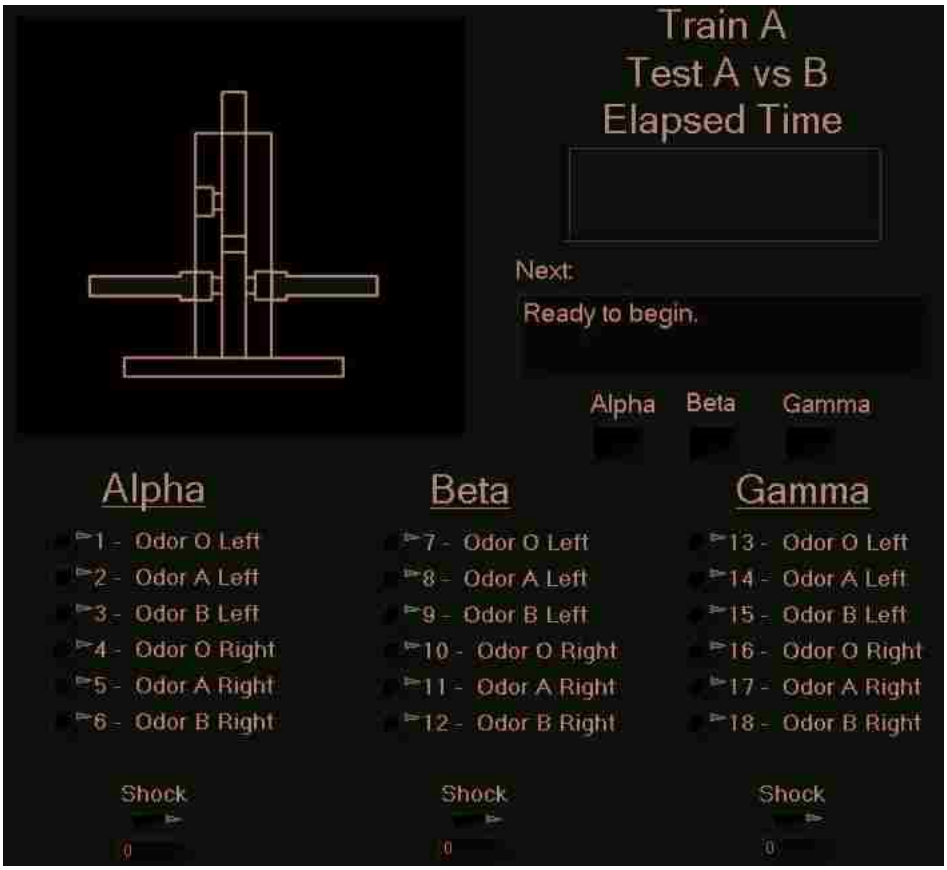

Figure 2.6: Graphical User Interface. Animations located at the top left corner indicate the next step as required by the researcher. Time elapsed is displayed at the top right of the interface with written instructions for each step immediately below. Lights across the bottom of the panel indicate the open or closed status of each relay (for both odor and shock).

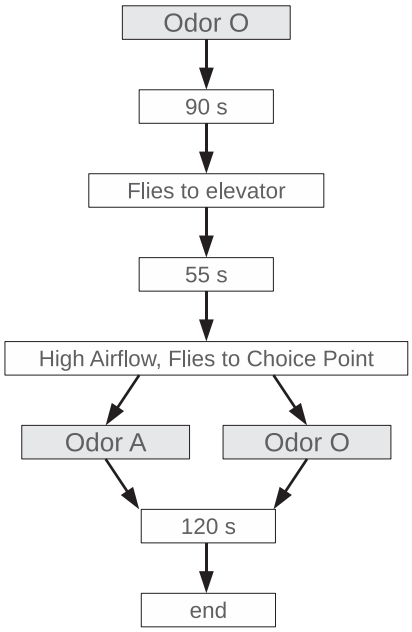

Figure 2.7: Program for Odor Avoidance. Shaded boxes indicate odor exposure, white boxes indicate time passed or events.

If one particular odor is too strongly aversive to a fly, the results of any conditioning trials will be skewed by this overwhelming innate avoidance of a stimuli. All odors dilutions were determined experimentally by utilizing these programs.

For odor avoidance, flies were first exposed to a blank odor (Odor O, mineral oil + air) for 90 s. This was followed by an immediate transfer into the T-maze elevator for 55 s. After transfer to the elevator, flies were lowered to the choice point and allowed two minutes to choose between a tube containing a single odor (e.g. Odor A) and a tube containing a blank odor (Odor O). After two minutes the test ended and flies were restricted to the tubes of their choosing. Our goal for these experiments was to demonstrate positive odor avoidance at concentration levels similar to those used for first- and second-order conditioning. Furthermore, these concentration levels would be utilized by other members of our laboratory as well as any outside collaborators visiting to conditioning flies in our training chamber (Figure 2.8).

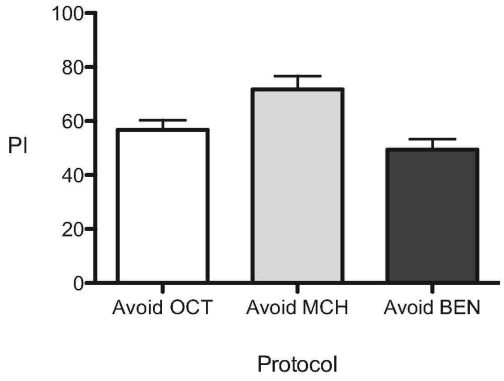

Figure 2.8: Odor Avoidance. Scores for odor avoidance using wild-type flies (2 to 5 d.o.) and dilutions of 2 x 10^{-3} (OCT), 1.4 x 10^{-3} (MCH), and 8 x 10^{-4} (BEN). Positive avoidance at a level greater than zero was observed for all odors at these dilutions (t-test, t[5] = 15.41, P $<$ 0.0001; t[5] = 14.34, P $<$ 0.0001; t[5] = 12.39, P $<$ 0.0001 for OCT, MCH, and BEN respectively), $n = 6$ for all groups

Electric shock avoidance was assayed in a similar manner (Figure 2.9). Flies were exposed to a blank odor for 90 s before being transferred to an elevator for 55 s. After the elevator, flies were lowered to a choice point containing two copper-wire lined acrylic tubes. Odor O was presented in both tubes. In addition, one of the copper-wire grids was electrified with a 90 VDC every 5 s for 1.25 s with 3.75 s intervals. This pulsing lasted for a total of 2 minutes, after which time the choice point was closed and the flies movement between tubes was restricted. We assayed the shock avoidance of wild-type flies at 90 VDC in all of our available shock tubes to determine the equipment best suited to deliver the US during our conditioning experiments (Figure 2.10). This level of analysis was necessary as our tubes were manufactured over two decades via different manufactures in both Germany and the United States.

Odor balancing followed the same initial odor presentation of Odor O for 90 s followed by 55 s in the T-maze elevator. After lowering to the choice point, flies were presented with a choice between two odors or a combination of odors (Figure 2.11). The goal of the odor balancing experiments was to equalize the avoidance to both odors so that flies distributed as close to 50:50 as possible. As previously mentioned, if one of the stimuli is too strong of a repellent for the flies, the scores for any learning tests that are performed will be artificially skewed in avoidance of that odor.

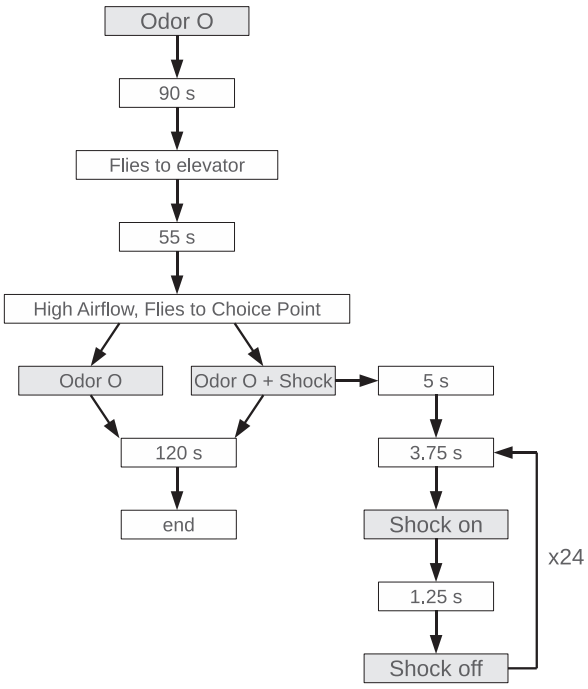

Figure 2.9: Program for Shock Avoidance. Shaded boxes indicate odor exposure, white boxes indicate time passed or events. The total time given for shock avoidance is 2 minutes with 24 individual shocks.

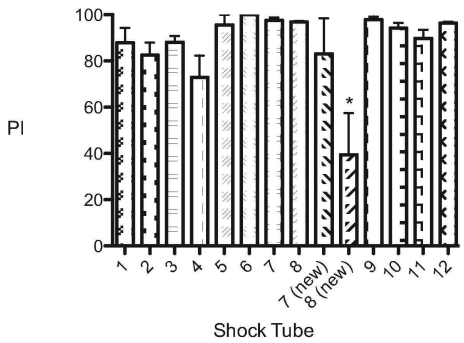

Figure 2.10: Shock Tube Performance Indices. All 14 tubes were tested at 90 v DC using wild-type flies (2 to 5 d.o.). Only one, "8 new" showed statistical difference to other shock tubes (ANOVA, $F[13,27] = 4.384$, $P < 0.0001$; Tukey, $P \leq 0.05$;), $n = 3$ for all groups except tube 5, $n = 2$

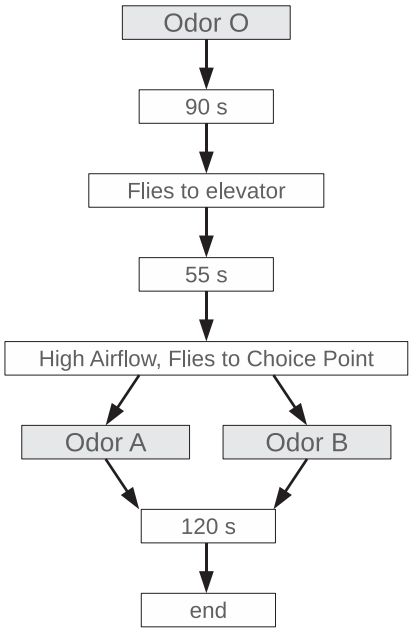

Figure 2.11: Program for Odor Balance. Shaded boxes indicate odor or shock exposure, white boxes indicate time passed or events.

In other words, avoiding that odor will be easier during the testing phase of an experiment because of the potency of the odor. Lowering the concentration of the more repellent odor remedies this problem. However, it is also important to have a high enough concentration of odors so that flies can still avoid them at a reasonable level (statistically greater than zero) during the normal odor avoidance paradigm (any Odor vs Odor O). Following the construction of our training room, initial odor dilutions for both balancing and avoidance needed to be determined *de novo* for our unique automated setup and odor delivery system. While we were able to achieve balancing for odorants at dilutions of 1.0 x 10^{-2} (OCT) and 2.6 x 10^{-3} (MCH) (Figure 2.14), final odor dilutions were balanced at a much lower level (around 5.0 x 10^{-4} (OCT) and 6.0 x 10^{-4} (MCH), Figure 2.15) to avoid "overwhelming" our training apparatus with a strong odor scent between trials and possibly skewing test results due to lingering odorants.

2.2b Classical Conditioning Learning Paradigm.

The classical conditioning learning paradigm (Tully and Quinn, 1985) is the focus of all our first-order experiments and the basis for developing second-order conditioning (Figure 2.16). In this paradigm, flies were first exposed to a blank odor (Odor O, mineral oil + air) for 90 s. Next, an odor was presented to the flies (Odor A) for 65 s with an electric shock applied every 5 s for 1.25 s (90 VDC) beginning after 8.75 s of odor exposure.

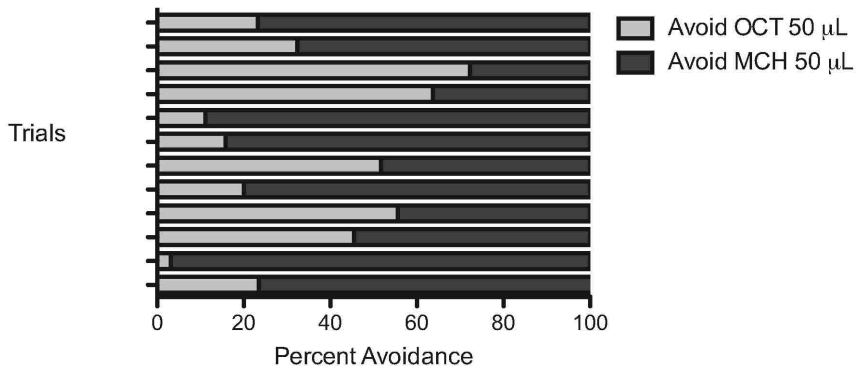

Figure 2.12: Odor Balancing for 50 μL of OCT vs 50 μL MCH suspended in 5 mL of mineral oil giving final concentration of 1.0 x 10^{-2}. Early odor balance experiments to determine odor dilutions for octanol (OCT) and methylcyclohexanol (MCH) using wild-type flies (2 to 5 d.o.). Each horizontal bar presents an individual trial. Both odors were significantly different from a 50:50 distribution (t-test, t[11] = 2.36, P $<$ 0.05), $n = 12$.

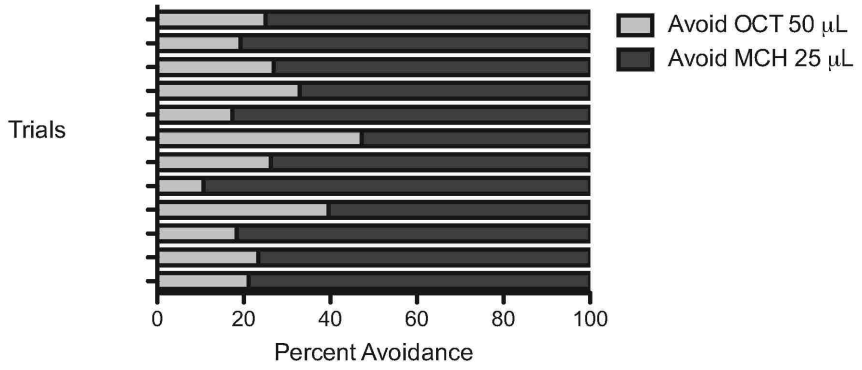

Figure 2.13: Odor Balancing for 50 μL of OCT vs 25 μL MCH suspended in 5 mL of mineral oil giving final dilutions of 1.0 x 10^{-2} (OCT) and 5.0 x 10^{-3} (MCH). Early odor balance experiments to determine odor dilutions for octanol (OCT) and methylcyclohexanol (MCH) using wild-type flies (2 to 5 d.o.). Each horizontal bar presents an individual trial. Both odors were significantly different from a 50:50 distribution (t-test, t[11] = 8.321, P $<$ 0.0001), $n = 12$.

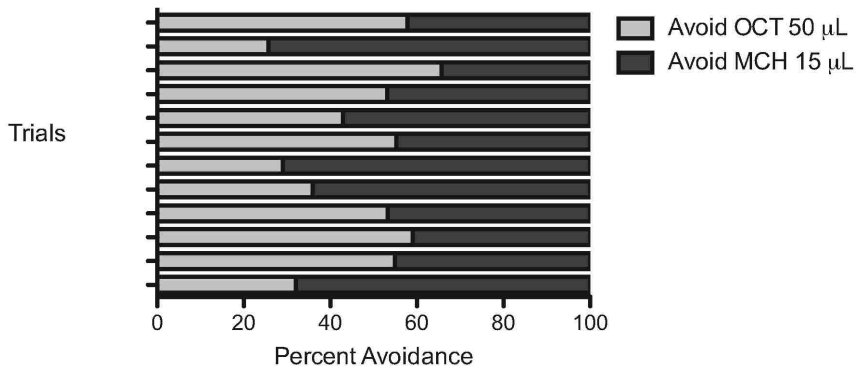

Figure 2.14: Odor Balancing for 50 μL of OCT vs 13 μL MCH suspended in 5 mL of mineral oil giving final dilutions of 1.0 x 10^{-2} (OCT) and 2.6 x 10^{-3} (MCH). Early odor balance experiments to determine odor dilutions for octanol (OCT) and methylcyclohexanol (MCH) using wild-type flies (2 to 5 d.o.). Each horizontal bar presents an individual trial. Both odors were not significantly different from a 50:50 distribution (t-test, t[11] = 0.733, P = 0.4558), $n = 12$.

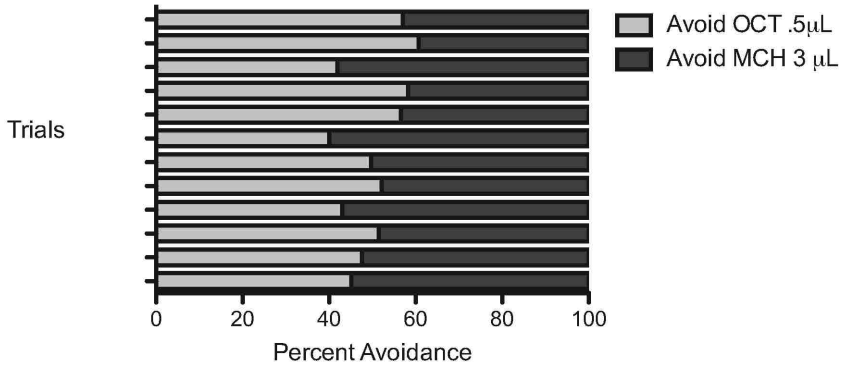

Figure 2.15: Odor Balancing for 0.5 μL of OCT vs 3.0 μL MCH suspended in 5 mL of mineral oil giving final dilutions of 5.0 x 10^{-4} (OCT) and 6.0 x 10^{-4} (MCH). Early odor balance experiments to determine odor dilutions for octanol (OCT) and methylcyclohexanol (MCH) using wild-type flies (2 to 5 d.o.). Each horizontal bar presents an individual trial. Both odors were not significantly different from a 50:50 distribution (t-test, t[11] = 0.1366, P = 0.8938), $n = 12$.

A blank odor was then presented for 45 s followed by a 65 s exposure to a second odor (Odor B). During odor B presentation, no electric shock was given. After an additional 55 s presentation of a blank odor, flies were moved to a choice point in the bottom of the T-maze where they were given two minutes to choose between one tube containing Odor A and a second containing Odor B. After this two minute period, the test ended and the tubes were blocked to prevent flies from moving between stimuli.

After optimization of our training machines for shock, odor balancing and avoidance, airflow, and timing of stimuli presentation, several tests were run in order to determine the level of score obtained from simple first-order conditioning (Figure 2.17 through Figure 2.19). The average scores of these tests were similar to those previously published by Tully & Quinn (Tully and Quinn, 1985), indicating that our experimental setup could successfully recreate the performance indices of his laboratory.

2.2d Compound Conditioning.

Several programs were written to assess the ability of *Drosophila* to undergo conditioning with compound odor mixtures. Our SOC paradigm utilizes the same sensory modality for the presentation of CS1 + CS2 (in an overlapping manner), therefore flies must be able to form associations in situations of compound odor presentation (described in detail in Chapter 3). While the timing of these programs are identical to the classical conditioning programs, multiple simultaneous odors are presented during the training and/or testing procedure.

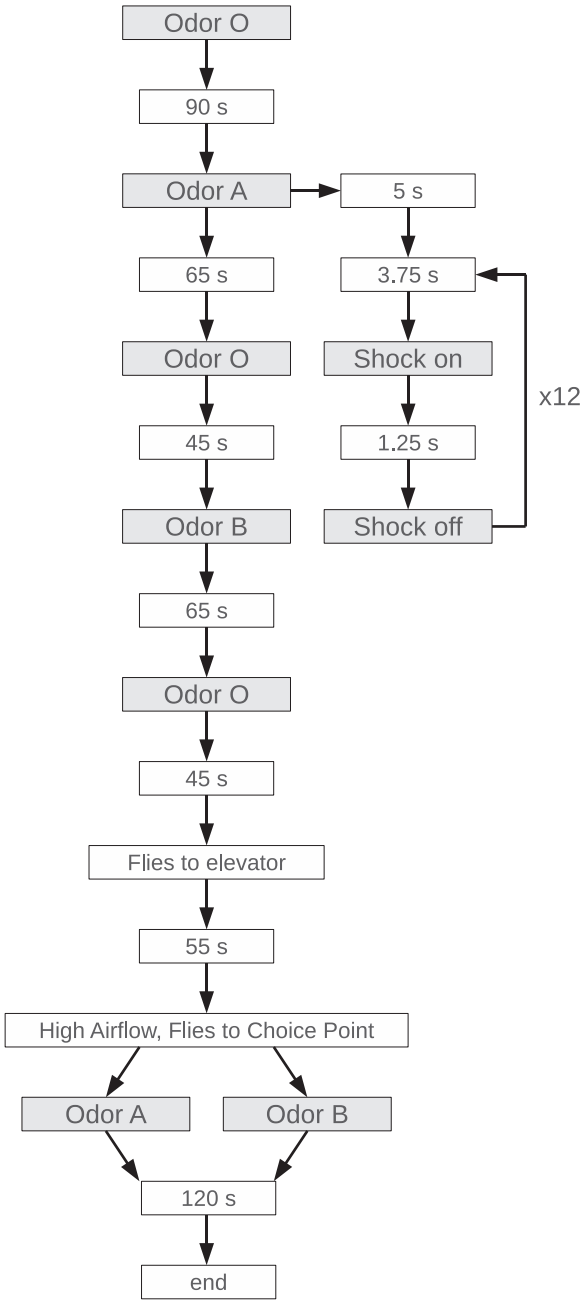

Figure 2.16: Program for Associative Conditioning. Timing was derived from earlier studies by Tully & Quinn (Tully and Quinn, 1985) along with previous experiments performed by J. Steven de Belle. Shaded boxes indicate odor or shock exposure, white boxes indicate time passed or events.

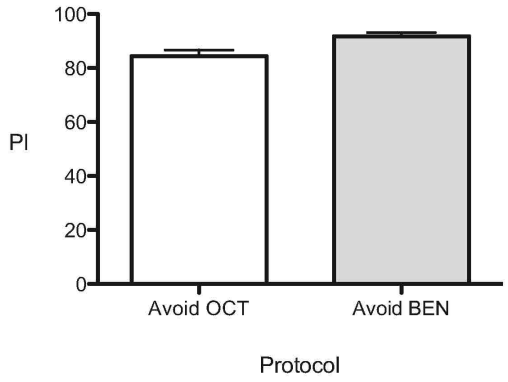

Figure 2.17: Simple First-Order Learning with OCT/BEN. Tests were conducted with 2-3 d.o. wild-type flies using 6.0 x 10^{-4} OCT and 1.0 x 10^{-3} MCH with 90 VDC shock reinforcement, $n = 12$.

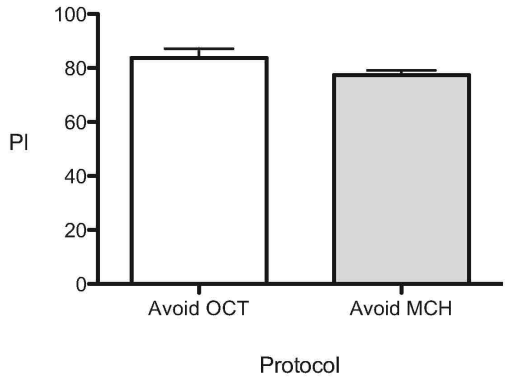

Figure 2.18: Simple First-Order Learning with OCT/MCH. Tests were conducted with 2-3 d.o. wild-type flies using 1.0 x 10^{-4} OCT and 6.0 x 10^{-4} MCH with 90 VDC shock reinforcement, $n = 12$.

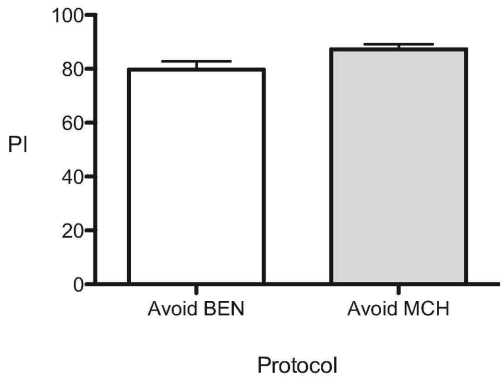

Figure 2.19: Simple First-Order Learning with BEN/MCH. Tests were conducted with 3-5 d.o. wild-type flies using 8.0 x 10^{-4} BEN and 1.4 x 10^{-3} MCH with 90 VDC shock reinforcement, $n = 12$.

In addition, there were only two T-maze machines employed during these conditioning trials, as opposed to the three that are concurrently used for classical conditioning. Four different versions of the compound conditioning programs were were written. The first variant (Figure 2.20) uses a compound stimulus only during the shock-associated conditioning procedure. The animals are tasked with identifying the a component of the compound mixture during the testing phase. A second version of this protocol exchanges the compound stimulus (A+B) with that of the CS- (C), thereby requiring the animal to distinguish the CS- during the testing phase (Figure 2.21). A third variant conditions the animal to a single stimulus during training but requires the fruit fly to distinguish this trained odor from a mixture during the test (Figure 2.22). Lastly, a fourth protocol uses a compound CS- during the testing phase while only training the flies using single odors (Figure 2.23).

2.2f Second-Order Conditioning.

The design of a proper protocol for second-order conditioning was paramount to successfully eliciting a higher-order response from *Drosophila*. To achieve this goal, I modified a protocol first examined in second-order conditioning of the honey-bee (Takeda, 1961) and commonly employed in numerous other studies of higher-order learning (de Belle and Heisenberg, 1994; Hussaini et al., 2007). This protocol is often referred to as the paired-paired approach because of the temporal pairing between first- and second-order stimuli.

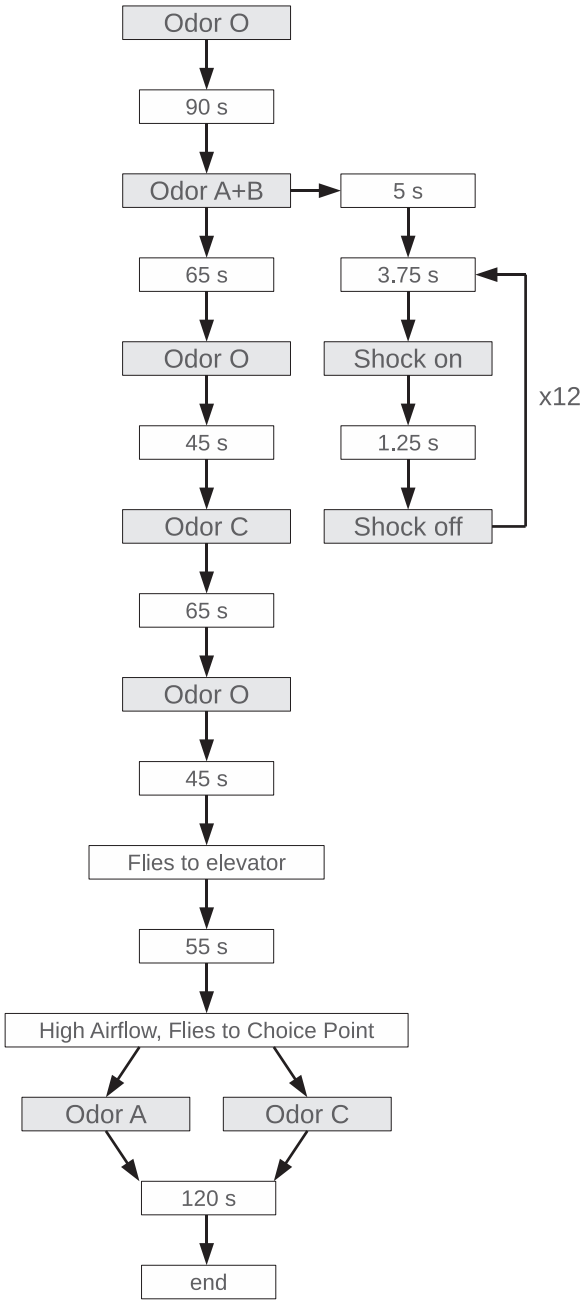

Figure 2.20: Program for Compound Conditioning Protocol 1. Flies were trained to avoid a two odor mixture (A + B) while being tested on their performance to only one component of the mixture (A). Shaded boxes indicate odor or shock exposure, white boxes indicate time passed or events.

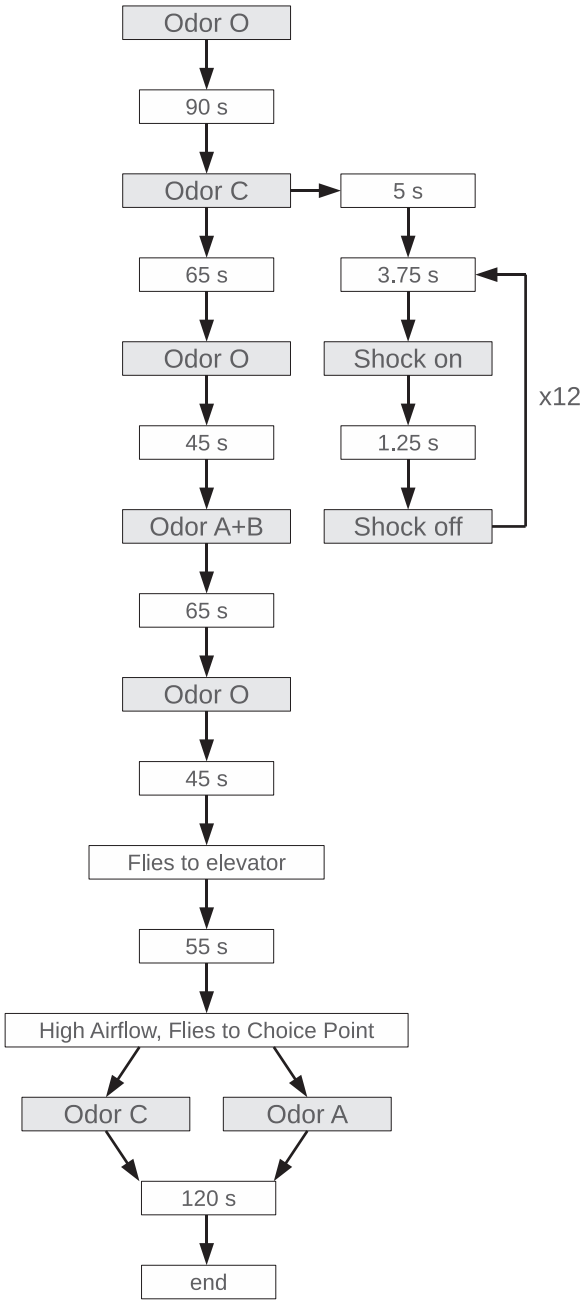

Figure 2.21: Program for Compound Conditioning Protocol 2. In a reciprocal test to Protocol 1, flies were training to avoid a single odor (C) while the unreinforced stimuli was presented as an odor mixture (A + B). Shaded boxes indicate odor or shock exposure, white boxes indicate time passed or events.

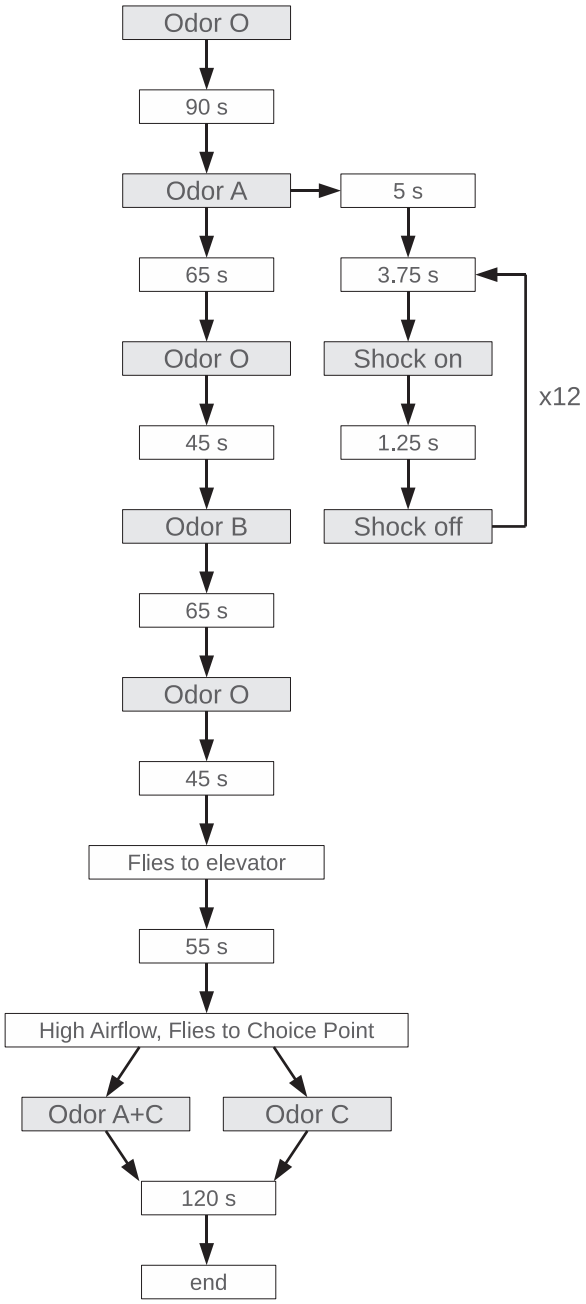

Figure 2.22: Program for Compound Conditioning Protocol 3. Flies were trained to avoid a single odor (A) but tasked with avoiding an odor mixture during the test (A + B). Shaded boxes indicate odor or shock exposure, white boxes indicate time passed or events.

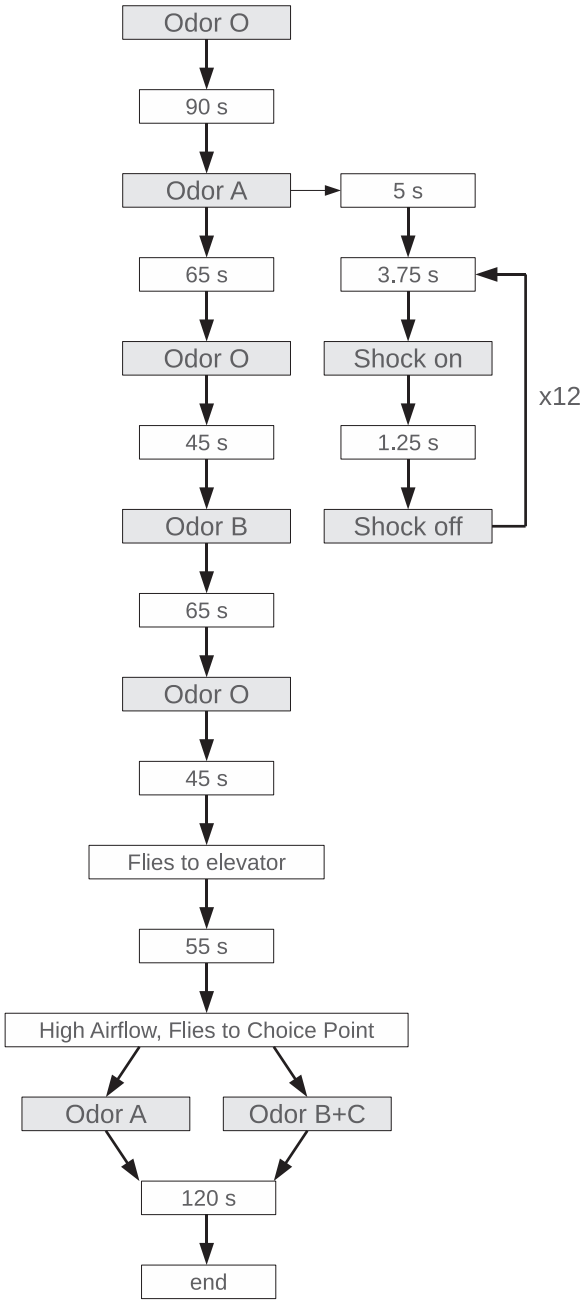

Figure 2.23: Program for Compound Conditioning Protocol 4. In a reciprocal test to Protocol 3, flies were trained to avoid a single odor (A) and exposed to a single unreinforced odor (C). During testing they were tasked with avoiding the trained odor and choosing an odor mixture as the unreinforced stimuli (B + C). Shaded boxes indicate odor or shock exposure, white boxes indicate time passed or events.

The test involves six separate experiments focusing on examining both first- and second-order responses to stimuli (Figure 2.24). The paired-paired (P-P) protocol associated the first-order and second-order stimuli in a temporal framework that serves to transfer the learned response from one stimulus to the next. In other words, the fly is first trained to avoid a first-order stimuli and then subsequently presented with a second-order stimuli in close proximity to this original first-order stimuli. If the transfer of learned avoidance behavior is successful, the fly will response to the second-order stimuli in a similar manner to the first-order stimuli.

The exact method of concurrently presenting the first and second stimuli was derived from earlier experiments by Tully and Quinn (Tully and Quinn, 1985). In optimizing the conditioning of *Drosophila* during first-order conditioning with shock and odor presentation, Tully found that pulsing 90 VDC twelve times over the course of a minute (one 3.75 second pulse every 5 seconds), the strongest possible response of the animal could be achieved. For this reason, I attempted to recreate this environment when associating SOC + FOC. Instead of pulsing an electric shock during the training phase of SOC, I presented the second-order odor and pulsed the previous first-order odor with which the fly had been conditioned to avoid. In this manner, the training protocol was similar to Tully and Quinn's technique for eliciting a strong behavioral response, but it involved the pulsing of an odor as opposed to the pulsing of an electric shock.

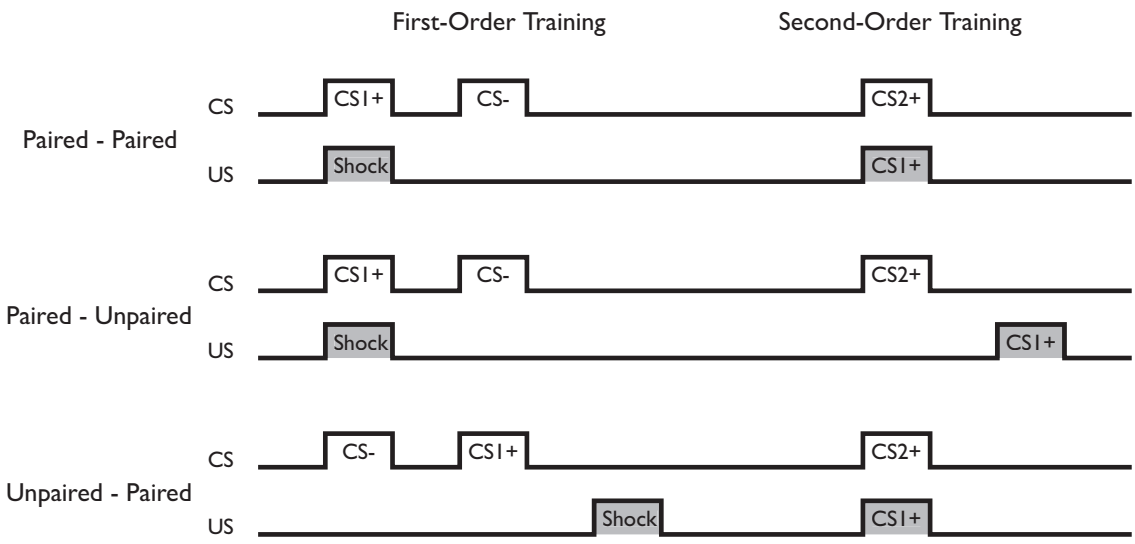

Figure 2.24: Pairing Protocol of Second-Order Conditioning. For all second-order conditioning experiments involving wild-type flies, I used an adaptation of earlier work pioneered by *Apis mellifera* research (Takeda, 1961). LabView coding for this protocol is shown in Figures 2.27 through 2.29.

The programming overview for "paired-paired" is shown in Figure 2.27. LabView controls all aspects of odor and shock delivery using the previously described solenoids. Extra time (65 s) is added to the end of first- and second-order conditioning to adjust the overall length of the test to match the unpaired-paired and paired-unpaired protocols. Since these two additional protocols (P-U, Figure 2.28 and U-P, Figure 2.29) require extra time for the unpairing of an conditioned and unconditioned stimulus, this delay must also be included in the paired-paired program. For both U-P and P-U protocols, the gap inserted between respective stimuli is 45 s. This is a sufficient gap to disrupt association according to earlier investigations of FOC (Tully and Quinn, 1985). However, there is still evidence of a slight trace conditioning (see Chapter 3, U-P is statistically greater than zero when testing for FOC) in the U-P protocol, indicating that flies are capable of forming a weak association between CS1 + US, even with a 45 s space in stimuli presentation. Trace conditioning is a form of conditioning involving a "gap" in the presentation of the CS and US during FOC (**?**). Animals are still capable of forming an association between these two stimuli, so long as the gap is not extensive, with the belief that a "trace" of the CS exists in the brain of the animal (even after removal of the physical CS stimulus) and is later associated with the US. In *Drosophila*, trace conditioning has not been extensively studied and we do not believe it has any effect on our observations of SOC.

We also investigated extinction within our SOC paradigm by exposing the flies to a modified version of the P-P protocol (further elaborated in Chapter 3). An initial P-P protocol presented FOC/SOC in the exact manner as previously described, but tested for FOC response. A second, modified protocol presented FOC/SOC without the

presence of the CS1 during SOC. While extinction is known to be a learning process distinct from FOC and SOC (Quirk and Mueller, 2007), we still needed to investigate whether any "minor" extinction may have influenced the learning scores of *Drosophila* during SOC. Previous studies in fruit flies using an extinction protocol involving twenty rounds of CS1 exposure noted a reduction of half the final performance index (Tully and Quinn, 1985) for FOC. Therefore, we did not expect to observe notable extinction during our SOC trials with only 3 rounds of unreinforced CS1 presentation.

2.3 Heat Shock Application

In all experiments requiring presentation of a heat shock (Chapter 4), acrylic tubes were wrapped with commercial heating tape (Omega, HTWAT051-010 & HTWAT051-008) permitted rapid heat shocking of flies during both first- or second-order stages of the experiment (Figure 2.25). A curve of temperature vs time can be seen in Figure 2.26. This graph represents a mean of $n = 5$ individual heat shocks as applied during a typical pre-training period for associative conditioning (1 heat shock per trial, the graph represents the mean of 5 individual trials). The dotted line indicates the beginning of a training regime after a ten minute pre-trial heat shock interval. The temperature spikes to an average of $34.60 \pm 0.5\,^{\circ}\mathrm{C}$ at 6 minutes before returning to $31.06 \pm 0.4\,^{\circ}\mathrm{C}$ at the beginning of training (10 minute mark).

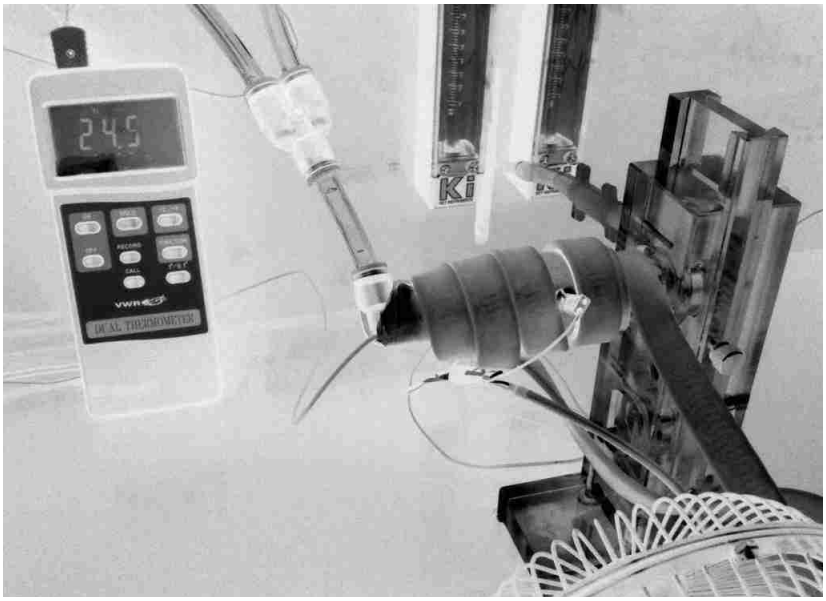

Figure 2.25: Delivering Heat Shock Via Heating Tape. Omega, HTWAT051-010 & HTWAT051-008 model heating tape was wrapped around the training tubes to deliver rapid heat shocks to fruit flies during conditioning.

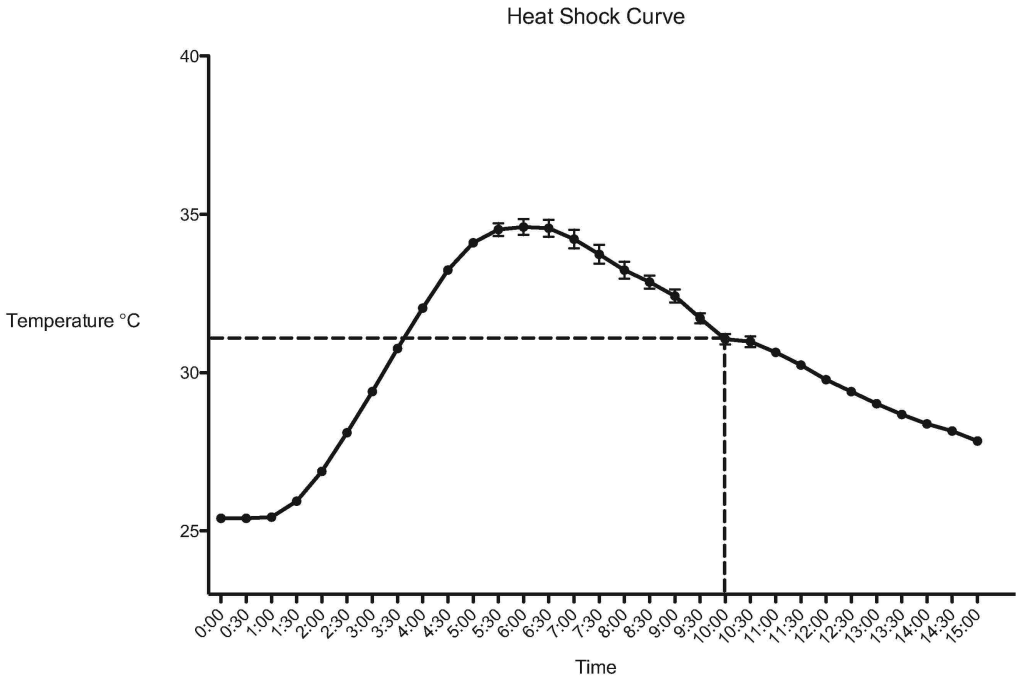

Figure 2.26: Heating Tape Heat Shock Temperature Curve. Five individual heat shock trials were average to produce a curve of temperature inside the training tube to confirm the function of the heating tape. Each heat shock application during first- and second-order conditioning was monitored via a digital thermometer and temperatures were kept within $\pm$ 0.5 °C of these average values.

For experiments requiring heat shock application during the testing phase (as opposed to the training phase) of conditioning, acrylic tubes were incubated at 42± 0.5 °C in a VWR mini shaker (https://www.vwrsp.com/catalog/product/index.cgi?catalog_number=12620-938). In contrast with heat shock during training, no heating tape was wrapped around these tubes after their removal from the incubator and connection to the T-maze. This was to avoid inconsistent application of heat shock by accidentally wrapping one side of the collection tubes differently from the other. In this manner, the only heat source for the heat shock of these collection tubes was provided prior to testing using the VWR incubator. This may have resulted in a quicker dissipation of heat as compared to the heating tape method.

2.4 *Drosophila* Stocks.

Wild-type Canton Special (*CS*) were used for all experiments not involving transgenic flies (Chapters 2 and 3). The transgenic dominant negative *P{UAS-shits1.K}* (http://flybase.org/reports/FBtp0013545.html) containing two insertions, one on the X chromosome and one on the III chromosome, was a gift from Joanna Young (Kitamoto, 2000). The dopamine neuron-specific *GAL4* driver *P{TH-GAL4.F}3* (http://flybase.org/reports/FBrf0188651.html) was a gift from Troy Zars (Friggi-Grelin et al., 2003). The *c739-GAL4* (http://flybase.org/reports/FBti0002926.html) driver (*P{GawB}Hr39^{c739}*) (Yaoyang, 1995) and *y[1] w[*] ; Pin[Yt]/CyO ; P{w[+mC] =UAS-mCD8::GFP.L}LL6* reporter (http://flybase.org/reports/FBtp0002652.html) were obtained from the laboratory stocks of J. Steven de Belle. The *c739-GAL4*

stock was previously out-crossed to our laboratory's *w1118 Canton Special* stock for 7 generations. All other fly genotypes were not out-crossed (for lack of time); but all controls (heterozygous drivers and responders) were crossed to the same original Canton Special *CS* line for every FOC/SOC test. In addition, all stocks were raised in identical conditions at 24.0 °C with 35 ± 5% humidity under a 12 hour light:dark cycle on Bloomington Stock Center Food (Table 2.2).

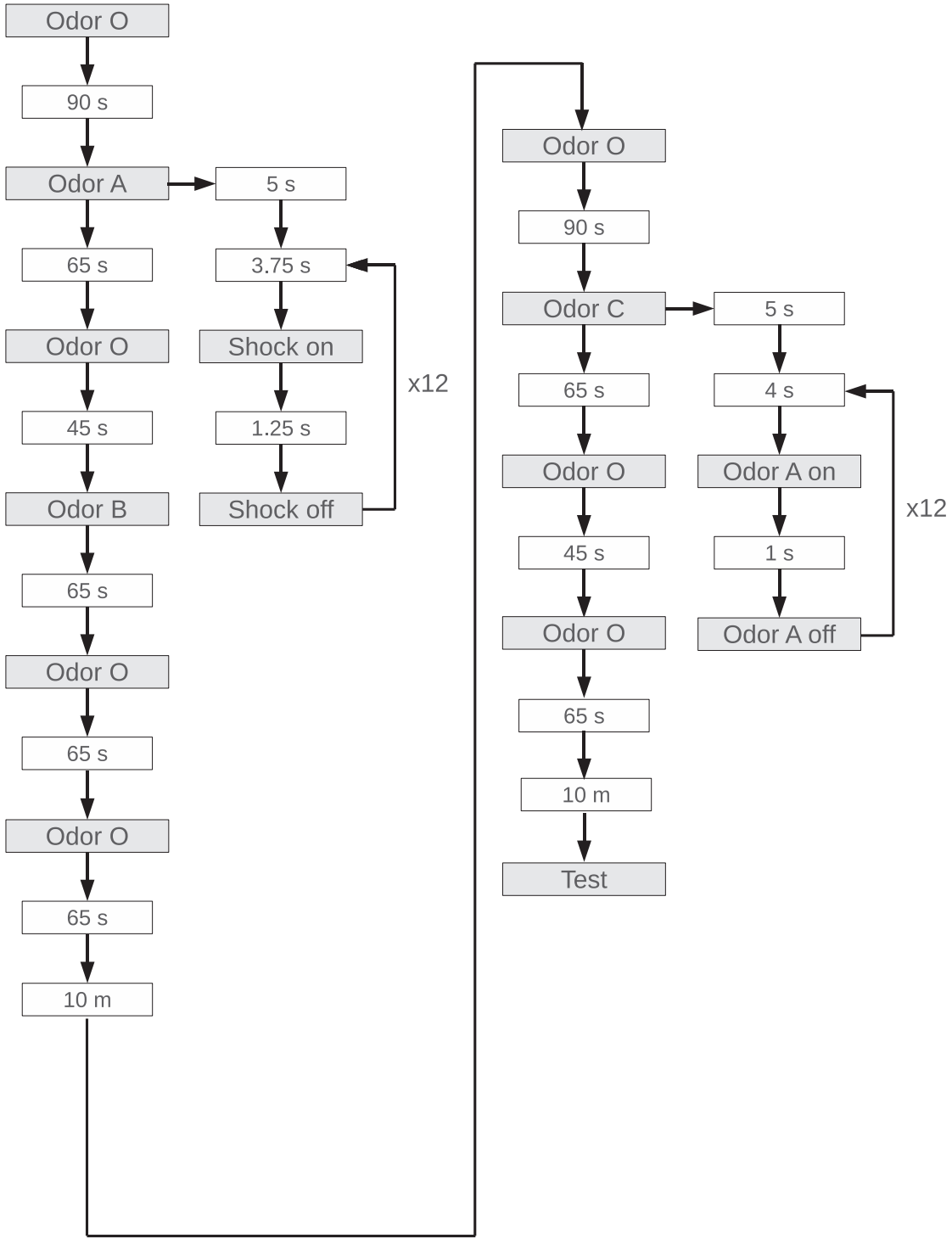

Figure 2.27: Program for Paired-Paired SOC. Shaded boxes indicate odor or shock exposure, white boxes indicate time passed or events.

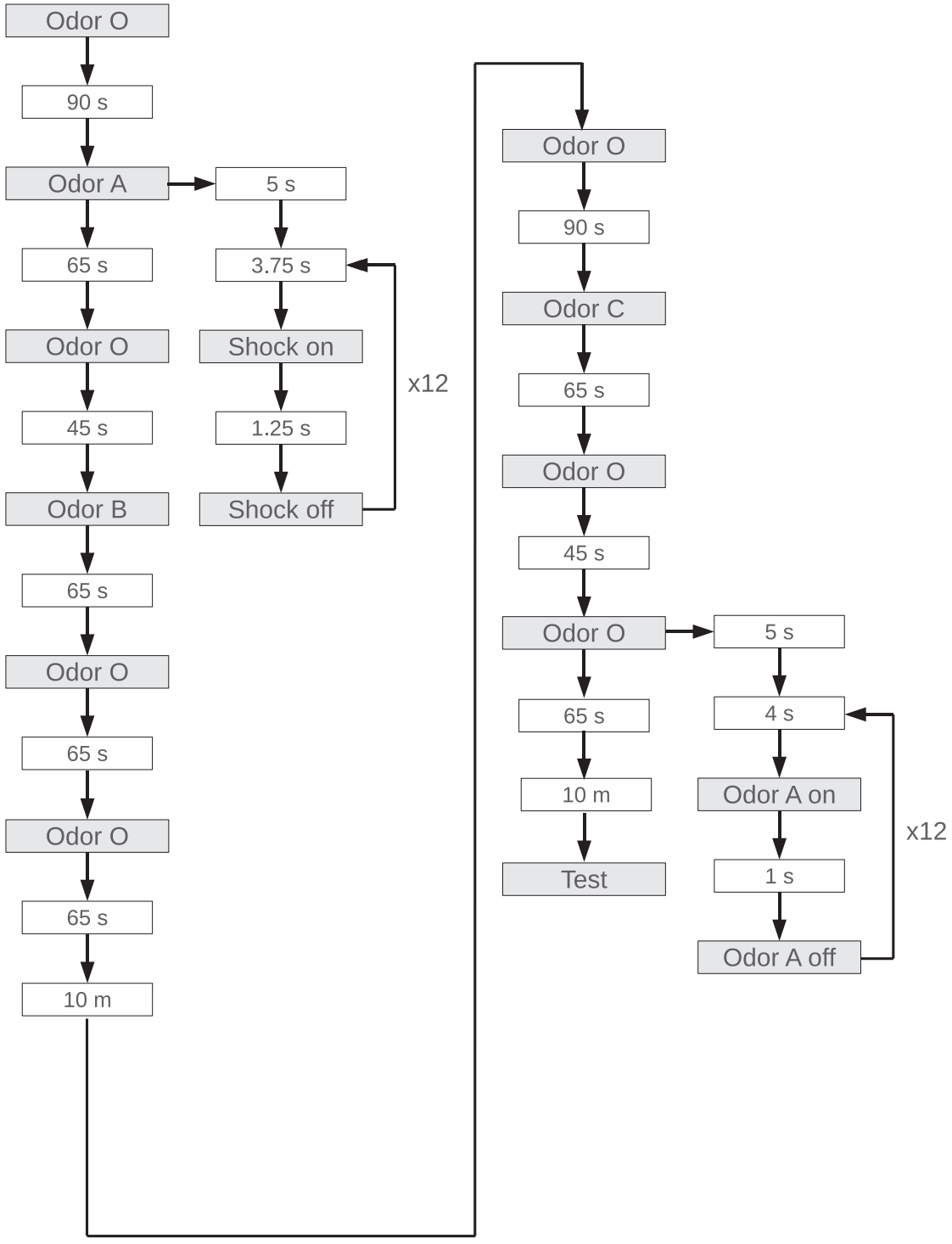

Figure 2.28: Program for Paired-Unpaired SOC. Shaded boxes indicate odor or shock exposure, white boxes indicate time passed or events.

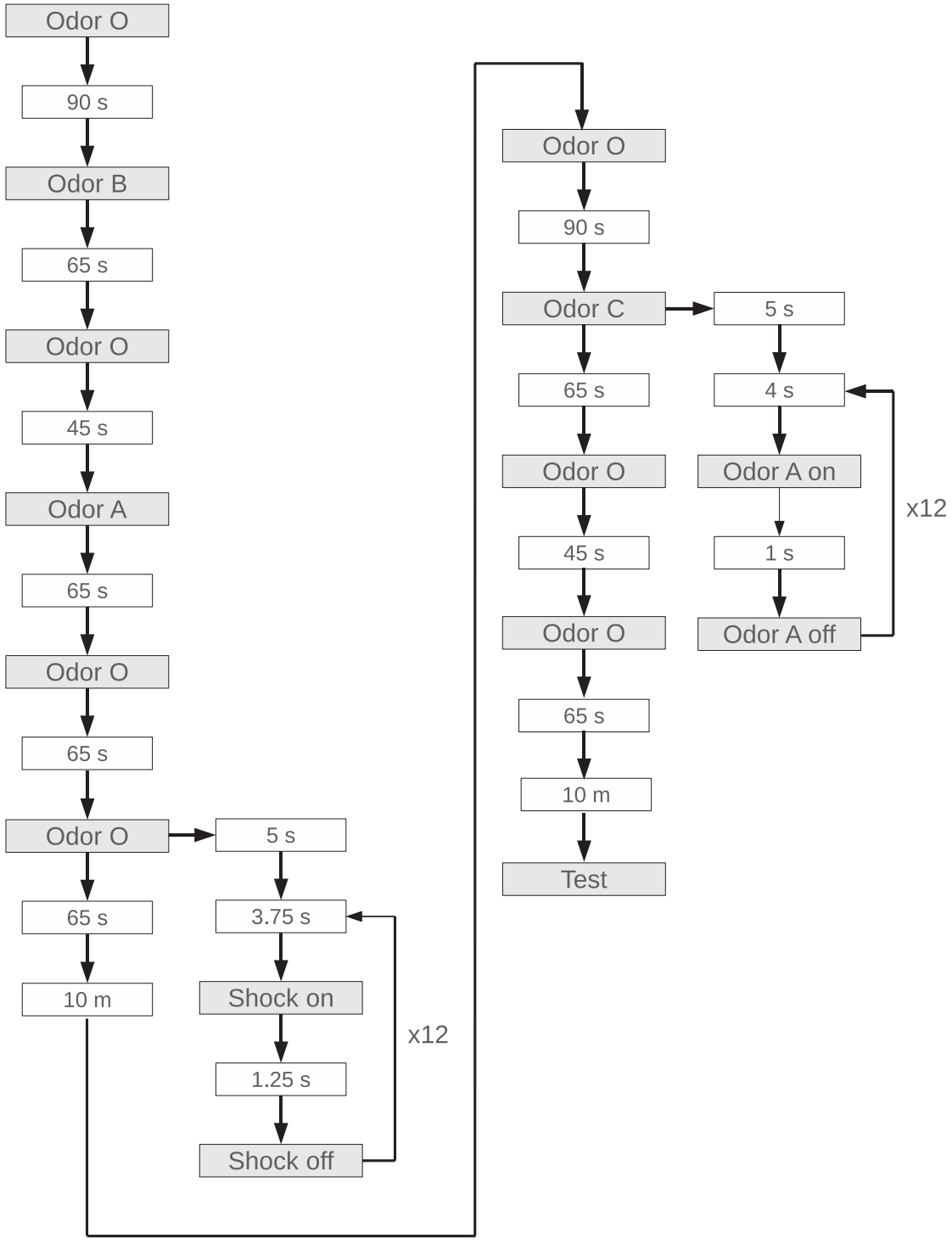

Figure 2.29: Program for Unpaired-Paired SOC. Shaded boxes indicate odor or shock exposure, white boxes indicate time passed or events.

Ingredient	Quantity
Water	39 liters
Yeast	675 grams
Soy flour	390 grams
Yellow cornmeal	2,850 grams
Agar	225 grams
Light corn syrup	3 liters
Propionic acid	188 milliliters

Table 2.2: Bloomington Stock Center Food. This is the standard recipe used for all fly stocks during behavioral tests to avoid any change in behavior that may result from poor nutrition (Guo et al., 1996).

2.4 Confocal Imaging.

All transgenic flies with CNS-specific *GAL4* drivers were checked for expression patterns by visualizing GFP and comparing to previously published work from other laboratories. Female *y[1] w[*] ; Pin[Yt]/CyO ; Pw[+mC]=UAS-mCD8::GFP.LLL6* virgins were crossed to each *GAL4* driver stock. Brains from adult flies no less than 2 days post-eclosion were dissected and imaged (without fixing) using a Zeiss LSM 510 confocal microscope.

CHAPTER 3

SECOND-ORDER CONDITIONING IN *DROSOPHILA*

The following chapter was accepted for publication in the journal Learning and Memory with the following author list: Christopher J. Tabone and J. Steven de Belle. My contribution to this work is as follows: Designed and executed all experiments, analyzed all statistics, wrote the publication. J. Steven de Belle advised on the direction of experiments and helped edit and improve the manuscript.

3.1 Introduction.

Second-order conditioning is a form of higher-order associative learning wherein a previously conditioned stimulus (CS1) can associate with a second conditioned stimulus (CS2) to elicit a conditioned response. Initial training involves pairing of CS1 with an unconditioned stimulus (US) during a first-order conditioning (FOC) session followed by a second-order conditioning (SOC) session in which CS1 is paired subsequently with a novel stimulus, CS2. If successful, an animal will demonstrate a conditioned response to CS2 similar to CS1, even though it has not been exposed to the original US during CS2 + CS1 association. Although SOC was originally described by Pavlov (1927) and has been thoroughly studied by psychologists for nearly four decades (Rizley and Rescorla, 1972), modern neuroscience has only recently turned to this paradigm as a means for understanding the mechanisms of learning and memory (Gewirtz and Davis, 2000). SOC presents a unique opportunity to investigate the internal transfer of information from one conditioned stimulus to another (CS1

CS2), leading to a conditioned response.

The ability to form higher order associations is prevalent in nature. SOC studies have spanned numerous animal models, including sea slugs (Hawkins et al., 1998), honeybees (Takeda, 1961; Grossmann, 1970; Bitterman et al., 1983; Hussaini et al., 2007), crickets (Mizunami and Matsumoto, 2009), pigeons (Rashotte et al., 1977; Rescorla, 1979), rats (Rizley and Rescorla, 1972; Holland and Rescorla, 1975b; Debiec et al., 2006), and humans Jara et al. (2006); Karazinov and Boakes (2007). While investigations of learning with flies have demonstrated complex behavior such as sensory preconditioning, the results of previous vision-based SOC studies were only significant when results from two different experiments were pooled together (Brembs and Heisenberg, 2001). This finding encouraged our investigation of SOC in *Drosophila* using a robust olfactory-based conditioning paradigm. Flies have served as a model for many types of learning and memory studies (Pitman et al. 2009) and are arguably the best understood animal genetics model as well. Their ease of manipulation using transgenic technologies and conditional expression of neuron-silencing transgenes (Keene and Waddell, 2007) along with the availability of numerous learning and memory-associated mutations (McGuire et al., 2005) makes the fly an extremely powerful system for examining neural mechanisms of behavioral plasticity.

3.2 Results.

We used the Pavlovian olfactory conditioning T-maze paradigm (Tully and Quinn, 1985) for all experiments. Flies were Canton special raised at 24 ± 0.5 °C and 35 ±

5% humidity on standard Bloomington *Drosophila* medium with a 12:12 h light:dark cycle. Experiments were performed at 88 ± 5% humidity and 24 ± 0.5 °C under dim red light. For training, groups of approximately 100 2- to 5-day-old adults were aspirated into an acrylic tube lined with an embedded double-wound copper coil to deliver electric foot shock (90 V dc; US). Current was provided by a dc-regulated power supply (Circuit Specialists). Three odorants, benzaldehyde (BEN; Aldrich), 4-methylcyclohexanol (MCH; Aldrich), and 3-octanol (OCT; Aldrich) were suspended in heavy mineral oil (Mallinckrodt) at dilutions of 8 x 10-4, 1.4 x 10-3, and 2 x 10-3 respectively. Room air was bubbled through the odorants in a custom-built automated delivery system (Analytical Research Systems, Figure 3.1) at a flow rate of 650 mL min-1. Presentation of all stimuli was controlled by FieldPoint relays using LabVIEW software (National Instruments).

Our assessment of SOC in *Drosophila* used three different protocols involving alternated pairing and unpairing of conditioned stimuli (Figure 3.2), adapted from earlier studies by Rescorla (1972) and Takeda (1961). The paired-paired (P-P) protocol was designed with both CS1 and CS2 temporally paired with the US and CS1, respectively. The paired-unpaired (P-U) protocol retained pairing of CS1 and the US but delayed association of CS2 with CS1 by 45 s. Alternatively, the unpaired-paired (U-P) protocol delayed association of CS1 with the US by 45 s while CS2 and CS1 remained paired. After training, flies were transferred to a choice point between converging air currents and tested for their responses to either first- or second-order-conditioned stimuli. A performance index (PI) was calculated for each test as the normalized percent of flies that avoided a previously conditioned stimulus (CS1 or

CS2, depending on the protocol) versus an unreinforced stimulus (CS). A PI of 100 indicates 100 avoidance of a previously conditioned stimulus while a PI of 0 represents a 50:50 distribution (i.e. no learning). We calculated a full PI as the average of two tests using separate groups of flies. CS1 (FOC) or CS2 (SOC) and CS odors in the first test were reversed in the second. In this way we accounted for a possible odor bias among different populations of flies (Tully and Quinn, 1985).

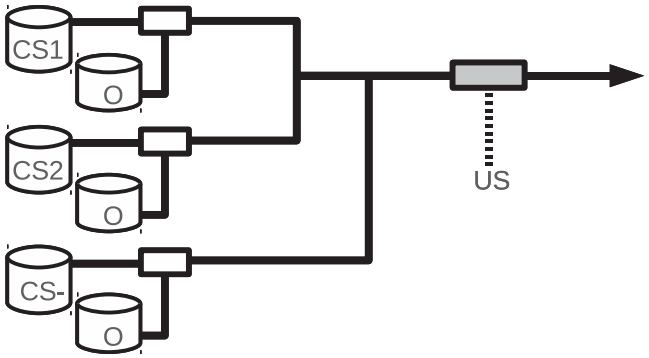

Figure 3.1: Schematic representation of our automated odor and shock delivery system. Bubblers contained odorants suspended in mineral oil [BEN (8 x 10^{-4}), MCH (1.4 x 10^{-3}) and OCT (2 x 10^{-3}); CS1, CS2 and CS] or mineral oil alone (O) and drew ambient room air using an in-house vacuum system (650 mL min-1). Solenoids (Analytical Research Systems; white rectangles) directed airflow by opening or closing in response to computer-controlled relays. Air flowed through telfon-coated tubing (Tygon SE-200; solid lines, arrow indicates direction) from bubblers to solenoids, then into acrylic copper coil-lined training tubes (gray rectangle). We presented mixed odors by opening two solenoids simultaneously. Electric shock (90 V dc; dotted line) was delivered from a dc-regulated power supply (Circuit Specialists) directly to the training tubes.

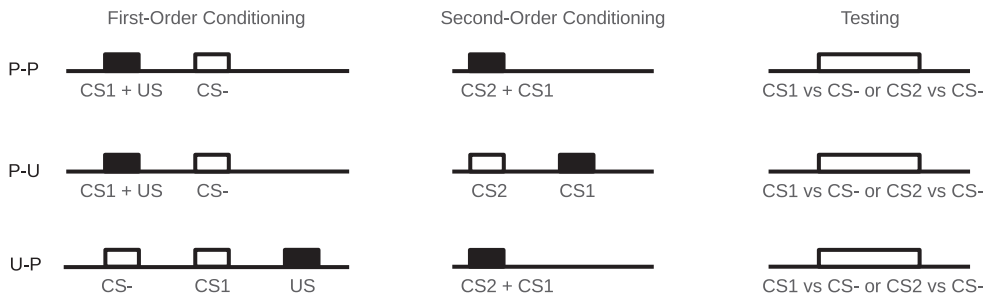

Figure 3.2: Timeline representations of training and testing. Squares represent stimuli; solid indicate reinforcement, open indicate the absence of reinforcement. All flies received both FOC and SOC and were tested for their responses to either CS1 (3.3) or CS2 (3.4) versus the CS. In the paired-paired protocol (P-P) both CS1 and CS2 were reinforced. In the paired-unpaired protocol (P-U) CS2 preceded CS1 by 45 s, while in the unpaired-paired protocol (U-P) CS1 preceded the US by 45 s. During the SOC phase of the P-P and U-P protocols, CS2 was presented alone for 7 s followed by simultaneous presentation of both CS1 and CS2

In our study, three odorants (BEN, MCH, and OCT) deployed in three different protocols (P-P, P-U, U-P) with reciprocal tests to account for odor bias, and tests for both FOC and SOC, give a total of 36 possible unique experimental combinations. For expedience, we focused on a subset of odor pairings. When measuring FOC responses, MCH and OCT were exchanged as CS1 and the CS while BEN remained the CS2 odor. Similarly, when we measured SOC responses, BEN and MCH alternated as CS2 and the CS while OCT remained the CS1 odor. Tests involving alternative combinations of CS1, CS2, and CS odors yielded results similar to those reported here (data not shown).

In FOC, flies were exposed to a blank odor (air bubbled through mineral oil) for 90 s, followed by a 60 s exposure to the CS1 odor paired with twelve 1.25 s pulses of 90 V dc shock delivered every 5 s. The training tube was flushed for 45 s with the blank odor followed by a presentation of an unreinforced CS odor for an additional 60 s. First-order conditioning was repeated three times with 10 min rest intervals of no odor or airflow.

SOC began with exposure to the blank odor for 90 s followed by presentation of a novel CS2 alone for 7 s. The previously conditioned CS1 was then presented in twelve 4 s pulses alternating with a blank odor every 5 s for 60 s, paired with a constant flow of CS2 odor. Pulsing of the CS1 odor generated stronger second order conditioned responses than constant exposure (data not shown). Paired odors converged in the airflow before entering the training tube. The tube was then flushed with blank odor for 45 s. CS was not presented to the flies during the second-order training phase of the experiment to minimize the time between conditioning and testing. Absence of the

CS was shown to have only minimal influence on performance following FOC (Masek and Heisenberg, 2008). After repeating second-order conditioning three times with 10 min rest intervals, flies were tested for their avoidance of either first- or second-order-conditioned stimuli.

When CS1 and the US were explicitly unpaired during FOC, we observed a significant decline in avoidance of the first-order-conditioned stimulus (ANOVA, $F[2,21] = 152.0$, $P < 0.0001$; Tukey, $P \leq 0.05$; Figure 3.3). As previously observed in FOC experiments, inserting a 45 s gap between shock and odor presentation severely reduces avoidance of a conditioned odor (Tully and Quinn, 1985). Furthermore, the lack of a significant difference between the paired-unpaired (P-U) and paired-paired (P-P) groups indicates that unpairing second-order stimuli has no distinguishable effect on FOC.

During SOC tests, flies demonstrated a significant avoidance of second-order stimuli compared to either unpaired first-order or second-order controls (ANOVA, $F[2,21] = 14.68$, $P < 0.0001$; Tukey, $P \leq 0.05$; Figure 3.4). Coincident exposure to both first-order (CS1 + US) and second-order (CS2 + CS1) stimuli is required to form a conditioned response to the second-order stimulus. Explicit unpairing during either first- or second-order conditioning, with a 45 s gap in stimulus presentation, was sufficient to disrupt this association.

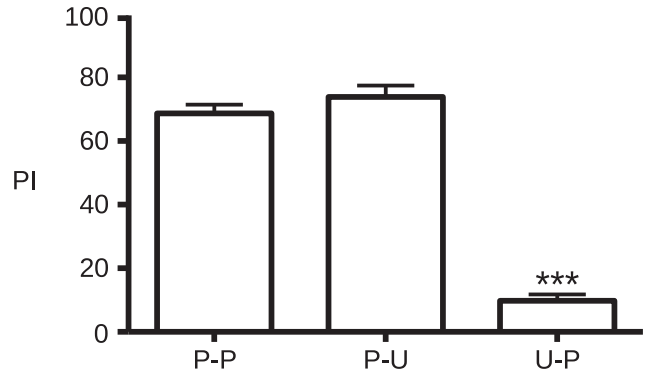

Figure 3.3: Pairing stimuli during FOC was required to generate a conditioned response to CS1 versus the CS (ANOVA, $F[2,21] = 152.0$, $P < 0.0001$; Tukey, $P \leq 0.05$). Note that unpairing of stimuli during SOC did not reduce the first-order-conditioned responses of flies using the P-U protocol. Bars indicate mean ± SEM; n = 8/bar.

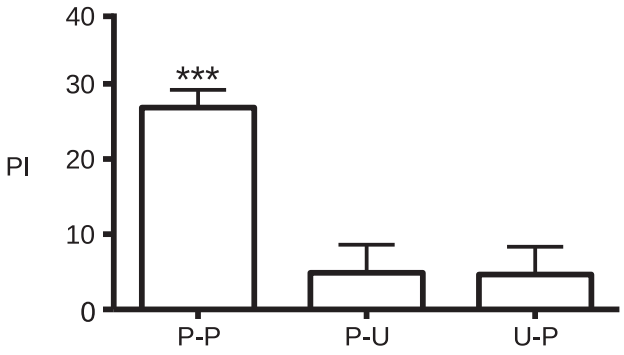

Figure 3.4: Pairing of stimuli during both FOC and SOC was required to generate a conditioned response to CS2 versus the CS (ANOVA, $F[2,21] = 14.68$, $P < 0.0001$; Tukey, $P \leq 0.05$). Bars indicate mean ± SEM; n = 8/bar.

In our SOC paradigm, both CS1 and CS2 use the same sensory modality and are presented simultaneously during training. Therefore, flies must be able to distinguish both CS1 and CS2 components. Processing of odor mixtures has been previously investigated in *Drosophila* (Silbering and Galizia, 2007), but not in the context of associative conditioning in adults. We conducted a series of four shock-associated FOC experiments to examine the flies capacity to discriminate components of odor mixtures both during training and testing (Figure 3.5). In our first protocol flies were conditioned to avoid an odor mixture (OCT + BEN) and tested for their avoidance of one component of this mixture (OCT). Protocol 2 is the inverse of protocol 1, with one component (OCT) of an unreinforced mixture (OCT + BEN) presented during the test. In protocol 3, flies were conditioned to avoid a single odor (OCT) and tested for their avoidance of the same odor in a mixture (OCT + BEN). Protocol 4 is the inverse of protocol 3, with an unreinforced odor (OCT) presented in a mixture (OCT + BEN) during the test. The PI for all four protocols was significantly greater than zero (t-test, $t[13] = 8.64$, $P < 0.0001$; $t[13] = 9.13$, $P < 0.0001$; $t[13] = 11.50$, $P < 0.0001$ and $t[13] = 14.24$, $P < 0.0001$; Figure 3.6). These results clearly demonstrate that flies possess the capacity to discriminate odors from odor compounds, and also illustrates why one sensory modality can be used for concurrent presentation of both CS1 and CS2 during SOC. Furthermore, we observed no significant differences between any of the four protocols (ANOVA, $F[3,52] = 1.779$, $P = 0.1626$), indicating an equivalence of this capacity during both training and testing.

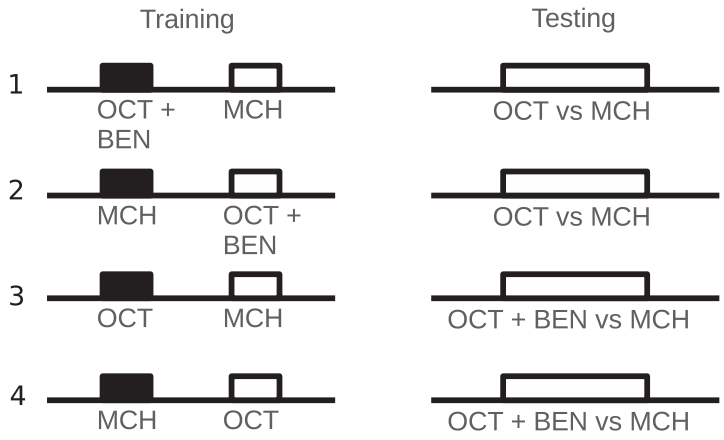

Figure 3.5: Timeline representations of training and testing. Squares represent odor; solid indicate electric shock (90 V dc) reinforcement, open indicate the absence of reinforcement. We assessed discrimination of odors from odor mixtures during both training (protocols 1 and 2) and testing (protocols 3 and 4).

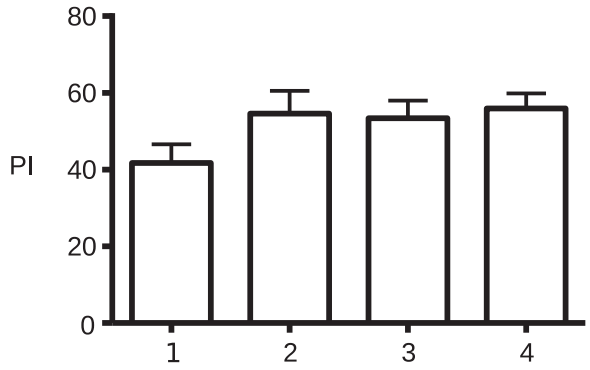

Figure 3.6: Flies demonstrated significant avoidance of all conditioned stimuli (t-test, $t[13] = 8.64$, $P < 0.0001$; $t[13] = 9.13$, $P < 0.0001$; $t[13] = 11.50$, $P < 0.0001$ and $t[13] = 14.24$, $P < 0.0001$). Differences between groups were not significant (ANOVA, $F[3,52] = 1.779$, $P = 0.1626$). Bars indicate mean ± SEM; n = 14/bar.

Exposing flies to a previously conditioned stimulus in the absence of a US can lead to extinction during FOC (Tully and Quinn, 1985). During SOC, flies were exposed to the CS1 in the presence of CS2 without the original US. This exposure throughout all three cycles of SOC may have lead to the decreased salience of CS1 as a reinforcer of CS2. To test for the possibility of such extinction during SOC, we assayed avoidance of CS1 using two training protocols (Figure 3.7). In one, flies received identical training to the P-P protocol described earlier and were exposed to CS2 + CS1 during SOC. The second group received identical FOC but was not exposed to CS1 during SOC. We observed no significant difference between the two groups when testing for FOC response (t-test, $t[10] = 1.083$, $P = 0.3042$; Figure 3.8), indicating that unreinforced exposure to CS1 did not accelerate the extinction of this stimulus during SOC. A similar result was observed in studies with Apis mellifera, in which the P-P group still demonstrated a first-order response even after numerous spaced SOC trials (Bitterman et al. 1983).

3.3 Discussion.

These experiments clearly demonstrate SOC in D. melanogaster using an olfactory conditioning paradigm. Three FOC cycles followed by three SOC cycles are sufficient to produce an avoidance response to CS2. We also confirmed that flies are capable of discriminating odors from odor mixtures during associative conditioning; a necessary prerequisite for our olfactory-based SOC paradigm.

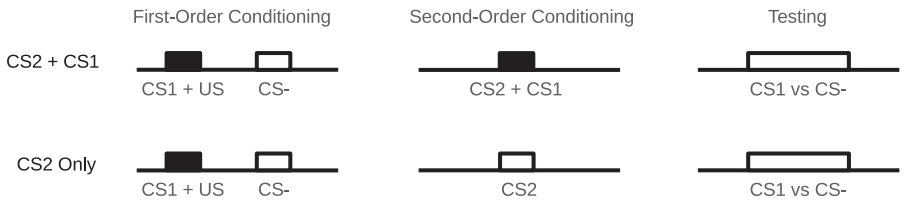

Figure 3.7: Timeline representations of training and testing. Squares represent odor presentation; solid indicate reinforcement, open indicate the absence of reinforcement. We assessed the effect of CS1 extinction during SOC (protocol 1), relative to the control group (protocol 2).

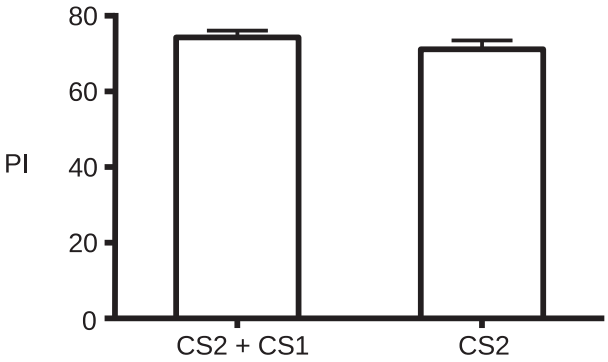

Figure 3.8: Presenting CS1 in the absence of the US did not accelerate extinction of the conditioned response (t-test, t[10] = 1.083, P = 0.3042). Bars indicate mean ± SEM; n = 6/bar.

Furthermore, we did not observe extinction in this study, indicating that our SOC training regime effectively elicits a conditioned response without a significant loss of CS1 salience.

There remain several properties of SOC open for investigation using our protocol. For example, a test of sensory preconditioning would be another, more robust assessment of odor discrimination in the context of our SOC experiment. This would involve presentation of a CS2 + CS1 odor mixture prior to pairing of a CS1 component + US reinforcement. Flies would then be tested for their avoidance of the unreinforced CS2 component of the mixture. Although the phenomenon has been demonstrated in *Drosophila* using a visual-based paradigm (Brembs and Heisenberg, 2001), no example of a successful olfactory-based approach yet exists.

Our SOC paradigm may be useful for studying neuronal and molecular mechanisms of extinction in flies (Qin and Dubnau, 2010). For example, extinction of CS1 after several rounds of SOC was previously shown to have no effect on the conditioned response to CS2 in rats (Rizley and Rescorla, 1972). Combining our robust approach together with *Drosophila* molecular-genetic tools should lead to a better understanding of the biological processes underlying Rizley and Rescorla's observations on extinction and SOC.

SOC also presents an opportunity to revisit the roles of well-studied learning and memory genes and the neuronal circuits in which they operate. In this regard, we are interested to know whether or not FOC mutants *rutabaga* (calcium-sensitive adenylyl cyclase) and *dunce* (cAMP-specific phosphodiesterase) have SOC phenotypes. *Drosophila* NMDA receptors *dNR1* and *dNR2* have also been implicated in

Pavlovian learning (Xia et al., 2005). Interestingly, blocking NMDA receptor activity in the amygdala prevents second-order fear conditioning in mice (Gewirtz and Davis, 1997). Our SOC paradigm will help to determine whether these evolutionarily conserved receptors are required for higher-order learning in flies as well.

Several neurotransmitter-specific circuits necessary for associative learning have been identified in insects. For instance, dopaminergic neuron activity has been shown to provide aversive US reinforcement during FOC in *Drosophila*Schwaerzel et al. (2003); Riemensperger et al. (2005), honey bees (Vergoz et al., 2007) and crickets (Unoki et al., 2005). In SOC, a previously conditioned stimulus (CS1) provides reinforcement of CS2, rather than a US. Recent work by Mizunami *et al.* (2009) has demonstrated that a second-order conditioned association in crickets can be blocked by administering a dopamine antagonist prior to SOC, indicating that neurotransmission from these neurons is required at this stage of learning. We are currently using a transgenic approach with our SOC paradigm to examine whether dopaminergic neurons function in a similar manner in *Drosophila*.

While *Drosophila* behavioral neuroscience has provided some understanding of the mechanisms underlying first-order learning and memory, much less emphasis has been placed on studies of more complex and ecologically relevant behavior. The work presented here demonstrates the flies' capacity for higher-order associative learning and offers a simple and reliable method for investigating the neurobiology of this phenomenon.

CHAPTER 4

INVESTIGATING THE NEURONAL MECHANISMS OF SOC

4.1 Introduction.

Studies in *Drosophila* learning and memory have predominantly focused on two major neurotransmitters, dopamine and octopamine, both known as important central nervous system signaling molecules in invertebrate neurobiology (Roeder, 1999, 2005).

The role of octopamine in reward learning was first elucidated from research of the honey bee VUMmx1 neuron and its octopaminergic control of reinforcement (Hammer, 1993, 1997). Inhibiting the firing of this neuron or preventing the synthesis of octopamine receptors prevents appetitive conditioning (Farooqui et al., 2004). In *Drosophila*, octopamine has been shown to be necessary for appetitive sugar-based reward learning (Schwaerzel et al., 2003). Restricting neurotransmission from these neurons via *GAL4* specific expression of the *UAS-Shi^{ts}* transgene during conditioning prevents the formation of appetitive memories (Schwaerzel et al., 2003). Furthermore, direct activation of these neurons via targeted expression of the Channelrhodopsin-2 protein, a light-activated ion channel derived from algae, results in appetitive conditioning in *Drosophila* larvae even in the absence of a positive reward (Schroll et al., 2006). Additionally, octopamine is known to be required for appetitive conditioning in the cricket *Gryllus bimaculatus*, pharmacologically inhibiting this neurotransmitter during learning blocks reward-based associations *in vivo* (Mizunami and Matsumoto, 2009).

In addition to octopamine, dopamine and its role in aversive conditioning has been extensively studied in *Drosophila* first-order conditioning (Waddell, 2010). In a manner similar to restricting octopaminergic neurons for appetitive learning, blocking neurotransmission from dopaminergic neurons via targeted expression of *UAS-Shi^{ts}* prevents the formation of aversive memory during electric-shock based associative conditioning (Schwaerzel et al., 2003). Light-activation of dopamine neurons using Channelrhodopsin-2 results in the formation of aversive memories in *Drosophila* larvae (Schroll et al., 2006) in a similar manner to octopamine light-activated reinforcement. Recent work has identified small subsets of dopamine neurons (distinct subpopulations of those originally examined by (Schwaerzel et al., 2003)) in the fly brain that are necessary and sufficient for aversive odor and shock-based conditioning (Aso et al., 2010).

In this chapter I will demonstrate the successful replication of data concerning the restriction of dopamine signaling in first-order conditioning. I will also outline an approach for investigating the role of dopamine in a second-order conditioning paradigm and discuss the results of studies restricting neurotransmission during SOC.

Apart from neurotransmitters, the role of the mushroom body in *Drosophila* learning and memory has been extensively investigated with paradigms of first-order conditioning (Heisenberg, 2003). Output from the mushroom body is shown to be required for retrieval of memory after conditioning (McGuire et al., 2001; Dubnau et al., 2001; Yu et al., 2006; Akalal et al., 2010) and specific lobes of the mushroom body have been implicated in various stages of memory acquisition and consolidation (Krashes et al., 2007). In addition to examining the role of dopamine in SOC, I will also describe

experiments to investigate the role of the mushroom body during SOC.

All experiments restricting neurotransmission from neurons in the *Drosophila* brain utilized the dominant-negative transgene Shi^{ts} under *GAL4/UAS* control. At 29.0 °C and above this transgene undergoes a conformational change that results in a dominant negative inhibition of synaptic vesicle recycling (Kitamoto, 2000). Environmental chamber temperature was fixed at 24.0 °C for all experiments with heat shock applied via "heating tape" as described in the next section.

4.2 Results.

Previous studies examining the role of dopamine and mushroom body neurons and utilizing the dominant-negative transgene *UAS-Shi^{ts}* spanned several hours to allow for acclimation of the environmental temperature to different degrees (either permissive or restrictive temperatures). For example, in the first publication demonstrating mushroom body restriction via *UAS-Shi^{ts}*, the time between training and testing was adjusted to either 165 or 180 minutes, depending on the particular experiment (McGuire et al., 2001). Furthermore, in studies investigating the role of dopamine in first-order conditioning, the time between training and testing was 60 minutes (Schwaerzel et al., 2003). However, although the time between training and testing was often $>$ 1 hour, a brief period of only 15 minutes is sufficient to induce silencing of neurotransmission via expression of *UAS-Shi^{ts}* (Thum et al., 2006). In my experiments, the total time of both first- and second-order conditioning is 1 hour and 37 minutes with only 10 minute intervals between periods of training (Figure 4.1).

Therefore, I was not able to allow for long time delays between the training of first- or second-order conditioning as well as the final test.

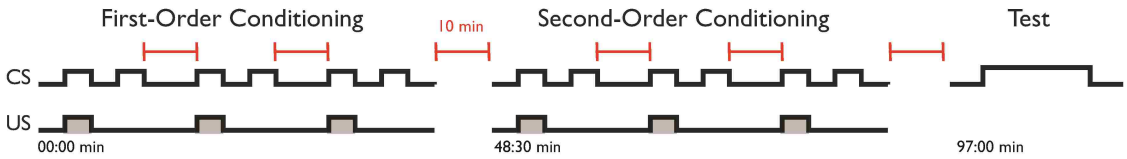

Figure 4.1: Timing Intervals of SOC.

To remedy this problem, I devised a novel method of applying heat shock to the flies during training and testing that would both not interrupt the current staging of the SOC experiments and allow for sufficient recovery time between training first- and second-order conditioning and testing (see Chapter 2, section 2.3). In all cases, the temperature drops below 29.0 °C (the restrictive temperature) by the 14 minute mark (reaching 28.38 ± 0.22 °C on average). At the point of dropping below the restrictive temperature (14 minute mark) there are 34:30 minutes remaining until the start of the next training or testing phase. This is sufficient recovery time for the *UAS-Shi^{ts}* transgene according to previous *Drosophila* behavioral studies of learning and memory (Dubnau et al., 2001). Therefore, we can attribute any defect in learning from these heat shock restrictions to the particular phase in which the heat shock occurred (either FOC, SOC, or the final test).

In order to confirm the efficacy of this approach, I attempted to recreate data from earlier dopamine restriction studies performed during first-order conditioning in

Drosophila (Schwaerzel et al., 2003). Flies were exposed to a 10 minute HS treatment before undergoing single first-order conditioning trials with a 10 minute rest interval before testing. The results are shown in Figure 4.2. After heat shock, *THGAL4 / UAS-Shi^{ts}* flies demonstrated a significantly reduced performance index compared to controls (ANOVA, F[3,13] = 34.65, P $<$ 0.0001; Tukey, P $\leq$ 0.05). This result indicates that my heat tape heating method is capable of recreating the same dopaminergic neurotransmission restriction experiments previously reported by other laboratories (Schwaerzel et al., 2003; Riemensperger et al., 2005). Flies from this driver stock were also crossed to *UAS-mCD8::GFP* and imaged to confirm the expression pattern of *THGAL4* (Figure 4.3).

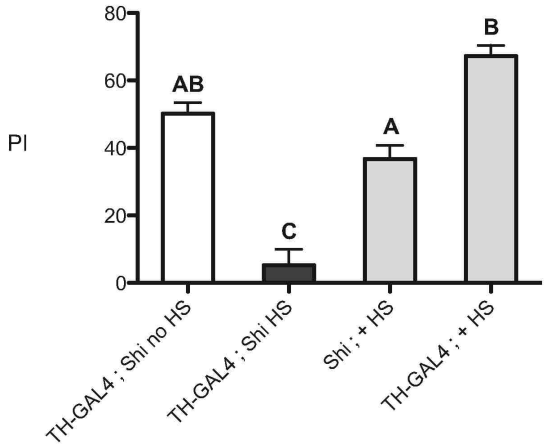

Figure 4.2: Restricting Dopaminergic Neurons during FOC Training. Flies were subjected to a 10 minute pre-training heat shock exposure followed by a single first-order conditioning session. After 10 minutes of recovery flies were tested for their avoidance to the conditioned first-order odor. Heat shocked *THGAL4 / UAS-Shi^{ts}* flies demonstrated a decreased performance index as compared to their respective controls (ANOVA, F[3,13] = 34.65, P $<$ 0.0001; Tukey, P $\leq$ 0.05). Letters indicate statistical difference. Sample size (left to right) is *n = 6,6,3,2.*

Figure 4.3: *THGAL4* CNS Expression Pattern. To confirm expression of the *THGAL4* driver, *THGAL4 / UAS-mCD8::GFP* flies were examined using confocal microscopy. Top panel shows GFP fluorescence, bottom panel is a computer-generated 3D model of the same exposure.

Next, I sought to apply this same heat shocking technique in an attempt to elucidate the role of dopaminergic neurons during SOC. I applied two heat shocks to two different experimental groups of flies, one group received heat shocks during FOC training and a second during SOC training. Theoretically, disrupting FOC by silencing dopaminergic neuron neurotransmission should prevent or lessen the ability of the fly to form a SOC memory, (i.e., lowering the saliency of FOC gives a weaker basis for SOC). The second group of experimental flies received a heat shock during SOC training. According to previous research in crickets, restriction of dopaminergic neurotransmission during SOC should prevent the formation of an SOC memory (Mizunami and Matsumoto, 2009). This may also be the expected result since dopaminergic neurons are known to signal during the testing phase of FOC (Riemensperger et al., 2005); this time point would correlate with the training period of SOC. An overview for these heat shocks is shown in Figure (4.4).

Subjecting *THGAL4/UAS-Shi^{ts}* flies to a heat shock during FOC and SOC results in a reduction of performance index when testing for SOC score (Figure 4.5). However, heat shocking the control line *UAS-Shi^{ts}*/+ during SOC also results in a decreased learning score. This "leaky" nature of *UAS-Shi^{ts}* has been observed in previous *Drosophila* learning experiments in FOC (McGuire et al., 2001) and unfortunately prevents me from making a strong conclusion on whether dopaminergic neurons are required during SOC.

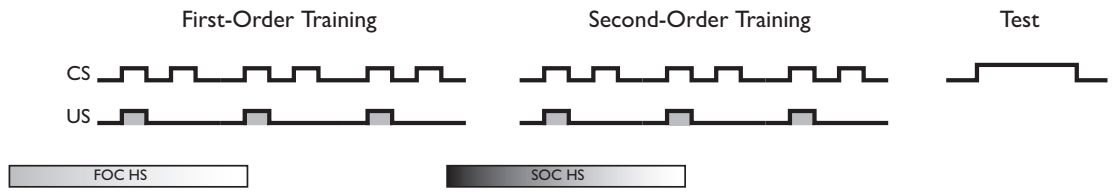

Figure 4.4: Heat Shocks Applied During *THGAL4 / UAS-Shi^{ts}* Conditioning. Light grey bars indicate a heat shock given during FOC, dark grey bars indicate heat shock given during SOC.

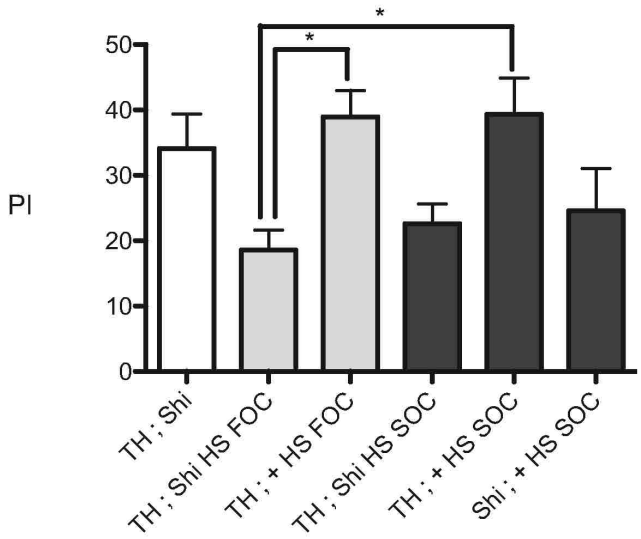

Figure 4.5: Performance Indicies for *THGAL4 / UAS-Shi^{ts}* During FOC/SOC. Significant differences were observed only between *THGAL4 / UAS-Shi^{ts}* and respective *TH/+* controls for FOC/SOC (ANOVA, $F[5,37] = 3.722$, $P < 0.001$; Tukey, $P \leq 0.05$).

Therefore the only statistical differences are seen between the *THGAL4/UAS-Shi^{ts}* flies during FOC with heat shock and their respective driver control *TH/+* for FOC/SOC (ANOVA, $F[5,37] = 3.722$, $P < 0.001$; Tukey, $P \leq 0.05$). Despite my best efforts to vary environmental chamber temperature, odor concentration, and shock level, I was unable to raise the score of the *UAS-Shi^{ts}/+* line to a level where it became statistically significant from the experimental group.

I also examined the role of *c739-GAL4* neurons during SOC. The *c739-GAL4* driver expressed GAL4 protein within the Kenyon cells that constitute the mushroom body. Expression of *UAS-Shi^{ts}* via *c739-GAL4* has been shown to result in an inhibition of memory recall, but not learning or consolidation, in studies of FOC in *Drosophila* (McGuire et al., 2001). My goal for this portion of the experiment was to examine whether these same c739 neurons were required during SOC training or testing. I followed the same heat shock protocol as previously described for the *TH-GAL4/UAS-Shi^{ts}* experiments. During the testing phase, flies are moved to the lower part of the "T-maze" apparatus where they must move between acrylic tubes and choose an odor to avoid. Since wrapping these parts of the T-maze might result in uneven heating on one tube compared to the other, I incubated these collection tubes in a small VWR bacterial incubator until their internal temperature reached approximately 34 °C. However, there was no method to reliably heat the tubes during the testing period without disturbing the flies during their choice-making and possibly affecting the outcome of the test. Therefore, the heat shock during the testing phase was less intense as compared to the heat shock given by wrapping the training tube

with heating tape. However, when heat shocking flies for 10 minutes after a single FOC session and testing them in the T-maze using this approach, I was still able to observe a drop in *c739-GAL4 / UAS-Shi^{ts}* fly performance similar to the results of earlier studies in *Drosophila* retrieval (Figure 4.6). Similar to the *THGAL4* fly stocks, images of *c739-GAL4* crossed to *UAS-mCD8::GFP* were taken to confirm mushroom body expression (Figure 4.7).

Next, I moved to restrict neurotransmission from *c739-GAL4* neurons during SOC. Heat shock was applied during both SOC training and testing phases (Figure 4.8). Unfortunately, no statistical difference was observed between any groups for both HS and non-HS flies when testing for SOC score (ANOVA, $F[6,29] = 0.7568$, $P = 0.6094$). It is possible that the heat shock supplied during the testing phase was too weak as compared to the heat shock provided with the heating tape. However, this would only explain lower scores for the "SOC test" group. The "SOC train" group was heat shocked during the SOC training period using the same heating tape protocol as previously described. However, I did not observe any statistically significant differences for this group when testing for SOC performance.

While the initial dopamine restriction experiments involving single FOC tests revealed promising results through the use of this novel heat shock technique, ultimately I was unable to elucidate the roles of both dopamine and mushroom body neurons during SOC. If the scores of the SOC paradigm were closer to those observed for FOC, it might be possible to statistically separate the control and experimental group scores during heat shock experiments.

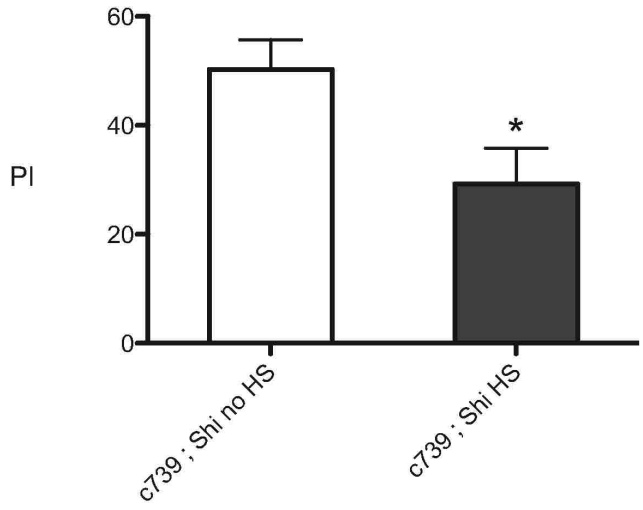

Figure 4.6: Restricting Mushroom Body Neurons during FOC Tests. Flies were subjected to a 10 minute post-training heat shock exposure followed by a choice between the conditioned and unconditioned FOC odors. Heat shocked *c739-GAL4 / UAS-Shi^{ts}* flies demonstrated a decreased performance index when subjected to a heat shock (t-test, $t[11] = 2.484$, $P < 0.05$), *n = 6 & 5*.

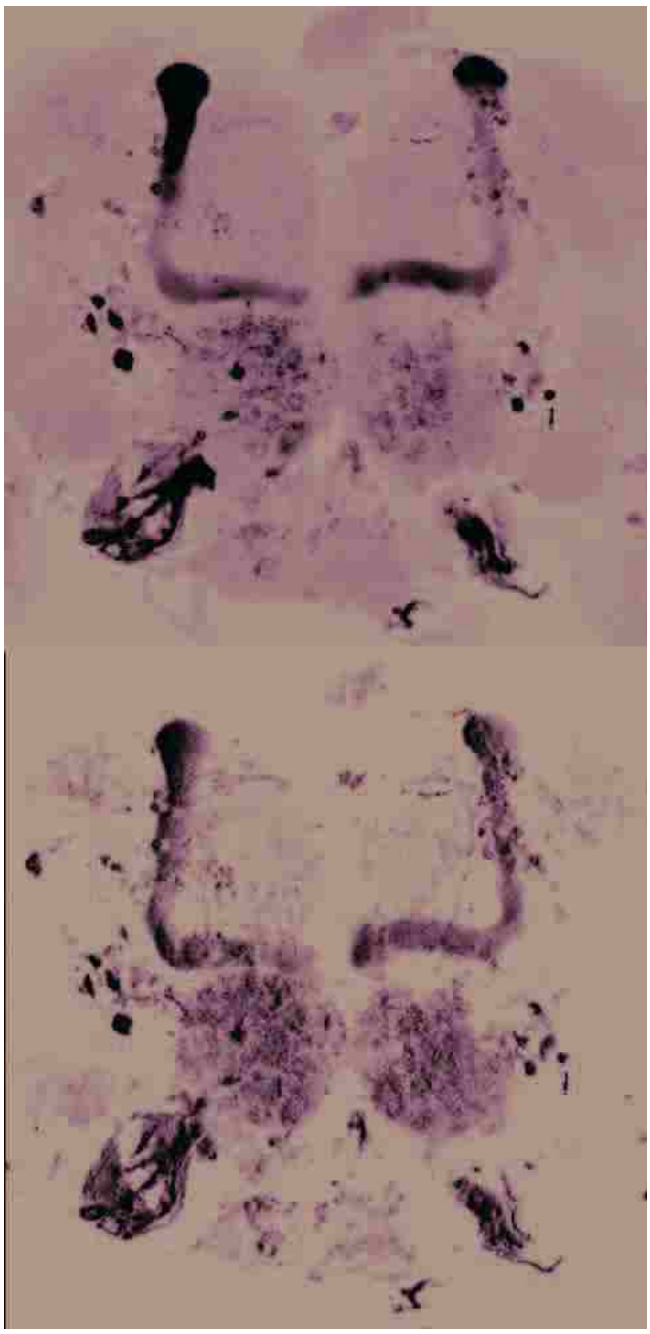

Figure 4.7: *c739-GAL4* CNS Expression Pattern. To confirm expression of the *c739-GAL4* driver, *c739-GAL4 / UAS-mCD8::GFP* flies were examined using confocal microscopy. Top panel shows GFP fluorescence, bottom panel is a computer-generated 3D model of the same exposure.

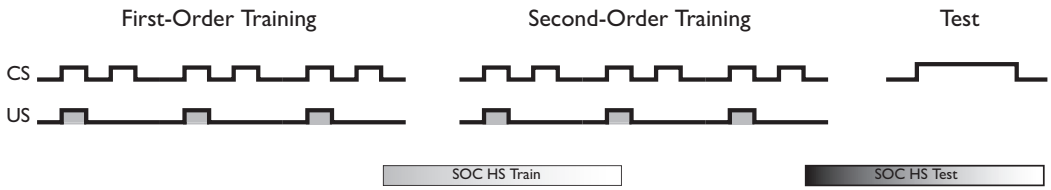

Figure 4.8: Heat Shocks Applied During *c739-GAL4 / UAS-Shi^{ts}* Conditioning. Light grey bars indicate a heat shock given during SOC training, dark grey bars indicate heat shock given during the SOC test.

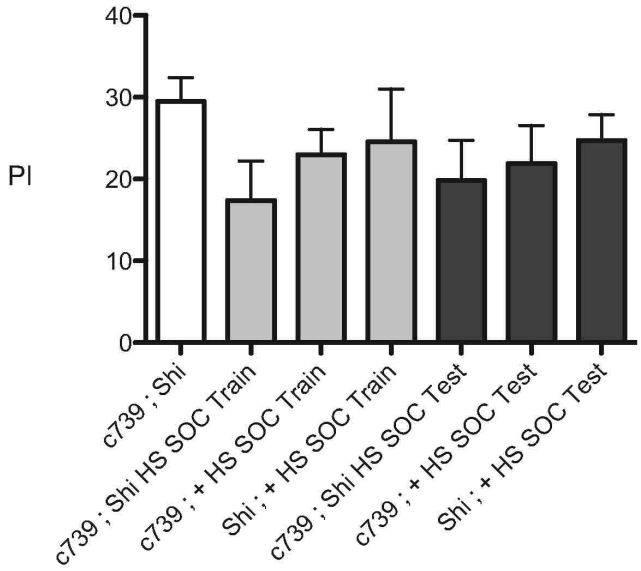

Figure 4.9: Performance Indicies for *c739-GAL4 / UAS-Shi^{ts}* During SOC Training & Test. No statistical difference was observed between any groups for both HS and non-HS flies when testing for SOC score (ANOVA, $F[6,29] = 0.7568$, $P = 0.6094$).

However, it would most likely require a new paradigm or odor delivery system to be implemented as I have been unable to increase scores over a PI of 40 using our current laboratory setup. Regardless, I remain optimistic that this paradigm can be further improved with new technology and hopefully the roles of dopamine and mushroom body neurons in SOC will be revealed in the near future.

CHAPTER 5

CONCLUDING REMARKS

This research successfully demonstrates that *Drosophila melanogaster* is capable of second-order conditioning and complex, compound learning with multiple odors. The fruit fly has routinely shown its flexibility as a model organisms for the past 100 years and this latest approach highlights its expanding role in the field of behavioral neuroscience. By utilizing an adaptation of a widely available learning paradigm (the "T-maze" paradigm) we have made this approach feasible for the growing number of laboratories around the world that study learning and memory in *Drosophila*.

Furthermore, we have constructed an semi-automatic approach to training the fruit fly for first- and second-order conditioning. Our LabView FieldPoint system minimizes any human error during the training process and enables higher-order learning to be precisely executed in a controlled environment. Our system is expandable to a large number of relays and additional paradigms with the simple addition of more programs and electronically controlled devices. With the large array of equipment available from National Instruments, this system can scale to include additional features such as temperature and pressure sensing probes along with various behavior feedback mechanisms (including accessories such as real-time video tracking for behavioral paradigms).

While this work has demonstrated several properties of SOC, namely the ability for flies to undergo SOC using the same sensory modality, and the lack of extinction in our paradigm, and the compound learning ability of *Drosophila*, there are still additional areas of research that will now be open for investigation. For example, sensory

preconditioning is one particular animal behavior, closely related to SOC, that has only scarcely been examined in *Drosophila* (Brembs and Heisenberg, 2001). A small modification in the order of shock and odor presentation should be sufficient to create a sensory preconditioning paradigm in which to study the behavior of *Drosophila*.

Although it is unfortunate that the experiments involving *UAS-Shi^{ts}* flies did not provide clear results for the roles of the mushroom body and dopamine neurons during SOC, the novel heat shock method presented here can serve as a useful tool for future neurotransmission-silencing transgenes. There are a already a growing number of heat-shock activated transgenic tools available for *Drosophila* (Thum et al., 2006) and it is reasonable to expect improvements to the *UAS-Shi^{ts}* allele along with the generation of novel silencing tools. These may help to elucidate the neurobiology of SOC sometime in the near future. In addition, it may be possible to administer dopamine antagonists during SOC in a manner similar to research in crickets (Mizunami and Matsumoto, 2009); provided the paradigm can be adapted for the analysis of individual fruit flies. We are hopeful that these improvements may one day enable *Drosophila* researchers to better understand the mechanisms of second-order conditioning to the same extent as first-order conditioning.

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APPENDIX A

COMPLETE LIST OF LABVIEW PROGRAMS

Protocol	**CS1+**	**CS-**	**Comments**
Classical Conditioning	A	B	test A vs B
	B	A	test A vs B
	A	B	test only, no training
	B	A	test only, no training
	A	B	training only, no test
	B	A	training only, no test
	A	B	with adjustable variables
	B	A	with adjustable variables

Table A.1: Classical Conditioning Programs. All variants are included in this table.

Protocol	CS1+	CS-	Comments
Balancing & Controls	A	O	odor avoidance
	B	O	odor avoidance
	O	A	odor avoidance
	O	B	odor avoidance
	S	O	shock avoidance
	O	S	shock avoidance
	A	B	odor balancing
	B	A	odor balancing
	AB	C	comp. odor balancing
	AC	B	comp. odor balancing
	BC	A	comp. odor balancing
	A	BO	comp. odor balancing
	A	CO	comp. odor balancing
	B	CO	comp. odor balancing

Table A.2: Balancing & Odor Control Programs. All variants are included in this table.

Protocol	**CS1+**	**CS-**	**Comments**
Compound Training	AB	C	test AB vs C
	C	AB	test C vs AB
	AB	C	test A vs C
	AB	C	test B vs C
	CA	B	test A vs B
	CA	B	test C vs B
	CB	B	test B vs A
	CB	C	test C vs A

Table A.3: Compound Training Programs. All variants are included in this table.

Protocol	CS1+	CS-	Comments
Compound Extinction	AB	C	extinguish A
	AB	C	extinguish B
	AB	C	extinguish C
	AC	B	extinguish A
	AC	B	extinguish B
	AC	B	extinguish C
	BC	A	extinguish A
	BC	A	extinguish B
	BC	A	extinguish C
	A	B	extinguish AB
	A	B	extinguish AC
	A	B	extinguish BC
	A	C	extinguish AB
	A	C	extinguish AC
	A	C	extinguish BC
	B	A	extinguish AB
	B	A	extinguish AC
	B	A	extinguish BC
	B	C	extinguish AB
	B	C	extinguish AC
	B	C	extinguish BC
	C	A	extinguish AB
	C	A	extinguish AC
	C	A	extinguish BC
	C	B	extinguish AB
	C	B	extinguish AC
	C	B	extinguish BC

Table A.4: Compound Extinction Programs. All variants are included in this table.

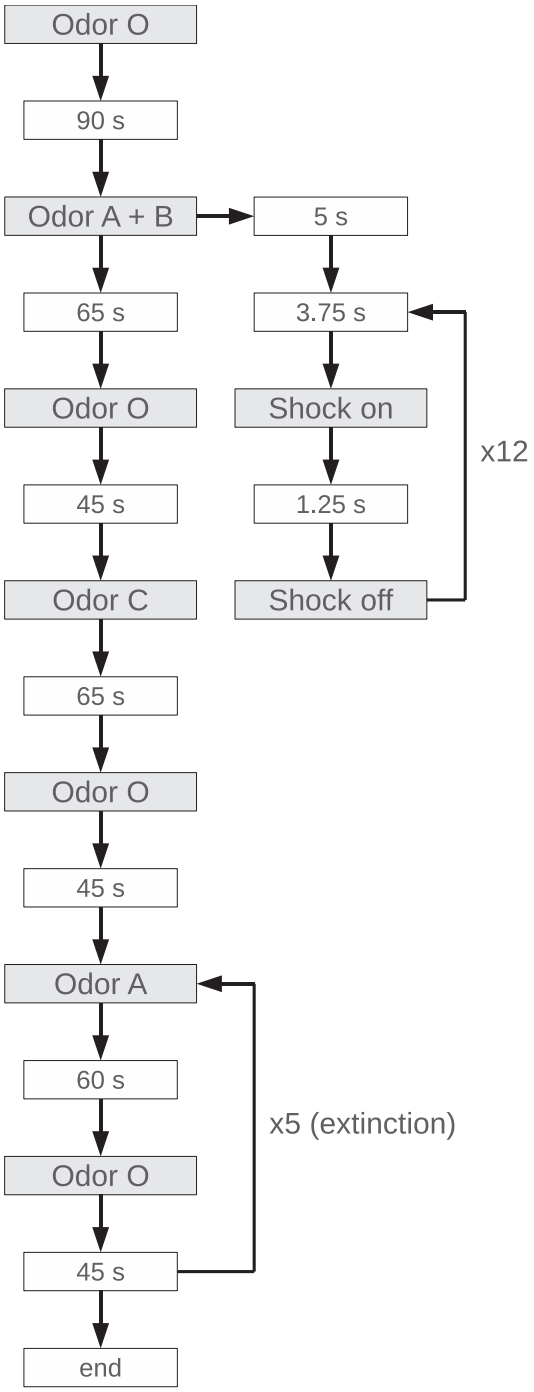

Figure A.1: Program for Compound Extinction.

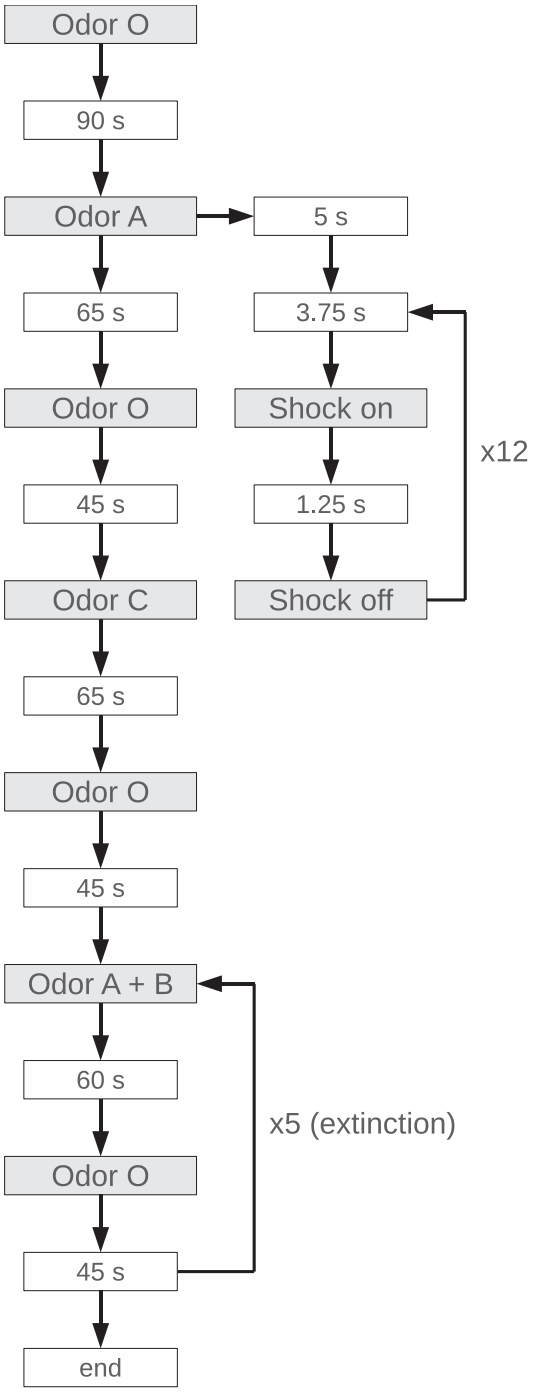

Figure A.2: Program for Compound Extinction 2.

Protocol	CS1+	CS-	CS2+	Comments
Second-Order Conditioning	A	B	C	paired-paired test 1st order
	B	A	C	paired-paired test 1st order
	A	B	C	unpaired-paired test 1st order
	B	A	C	unpaired-paired test 1st order
	A	B	C	paired-unpaired test 1st order
	B	A	C	paired-unpaired test 1st order
	A	B	C	paired-paired test 2nd order
	A	C	B	paired-paired test 2nd order
	A	B	C	unpaired-paired test 2nd order
	A	C	B	unpaired-paired test 2nd order
	A	B	C	paired-unpaired test 2nd order
	A	C	B	paired-unpaired test 2nd order

Table A.5: Second-Order Conditioning Programs. All variants are included in this table.

Protocol	CS1+	CS-	Comments
Long Term Memory	A	B	Spaced
	B	A	Spaced
	A	B	Massed
	B	A	Massed

Table A.6: Long Term Memory Programs. All variants are included in this table.

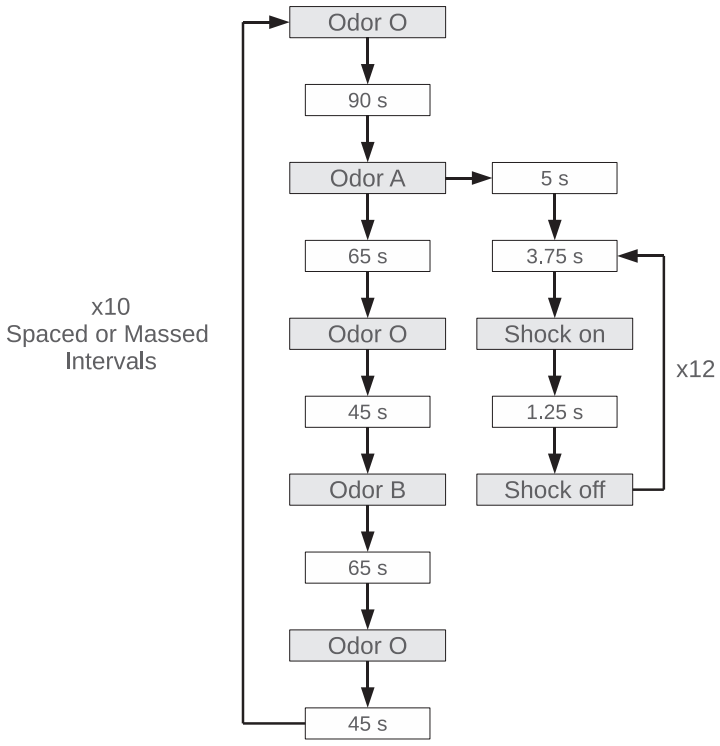

Figure A.3: Program for Long-Term Memory. Using the olfactory-based T-maze paradigm, it is possible to elicit long-term memory (¿24 hrs) in the fruit fly (Tully et al., 1994). There are two main methods for training fruit flies using long-term memory conditioning procedures. Spaced training uses classical conditioning procedures with 10 or 15 minute break intervals between training bouts whereas massed training removes these spacing periods and condenses all training into one continuous exposure (Tully et al., 1994). It has also been demonstrated that while spaced training is protein synthesis dependent and can be blocked by the administration of translation inhibitors such as cycloheximide (CXM), massed training is protein synthesis independent and unaffected by the same inhibitor (Tully et al., 1994). The program for training long-term memory is identical in procedure to the classical conditioning paradigm described earlier with the addition (or lack of) breaks between 10 repeated training sessions. During the spaced training sessions I used an ASCO valve (Red-Hat II 312190-T) controlled via relay to completely disrupt airflow in order to keep the flies free from any stimuli. The program was also easily adjustable to allow for either 10 or 15 minute breaks between training sessions, although it is preferable to use 15 minute breaks as this normally improves the learning performance of the flies (Tully et al., 1994). After 24 hours in a climate-controlled incubator, flies were tested using the Test A vs B program that does not include any additional training sessions.

APPENDIX B

COLLABORATION DATA

The following appendix includes data from 4 of the 5 collaborations undertaken during my graduate program at UNLV. Results obtained from a collaboration with the laboratory of David Krantz are not included in this section as the data is currently being processed by his graduate students and postdocs. Our automated training room setup provided consistent results for both odor avoidance and learning and memory assays - its use was often requested by our collaborators (Table B.1) and we provided behavioral data for numerous mutants and transgenic fly crosses.

Collaborator	**University**	**Notes**
Lee Fradkin	Leids University, Netherlands	*Manuscript in prep
David Krantz	University of California, Los Angeles	*Manuscript submitted
Mani Ramiswami	Trinity College, Ireland	Preliminary results
Mark Tanouye	University of California, Berkeley	Preliminary results
Jerry Yin	University of Wisconsin, Madison	Preliminary results

Table B.1: List of Collaborations undertaken during graduate study at UNLV.

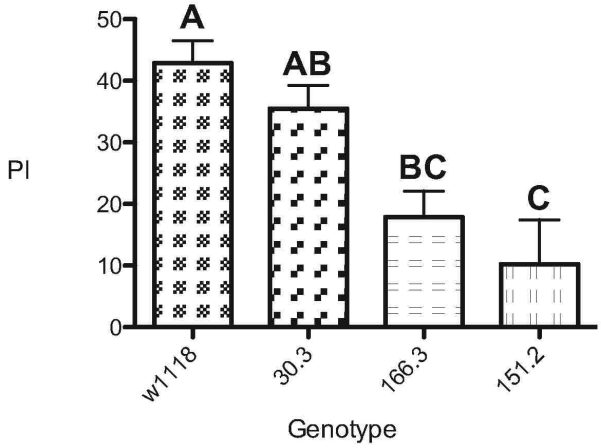

Figure B.1: The gene *dystrophin* has been extensively studied in humans for its role in Duchenne and Becker muscular dystrophies, a disease involving gradual deterioration of muscle tissue over time (Muntoni et al., 2003). In *Drosophila*, dystrophin is required as a retrograde neurotransmitter in specific neuropiles of the central nervous system (Fradkin et al., 2008). Our work with Lee Fradkin focused on examining the role of *dystrophin* as a retrograde (and possibly anterograde) neurotransmitter in the olfactory nervous system. Avoidance of benzaldehyde was examined in several fly genotypes mutant for *dystrophin* and a rescue was attempted at both the pre- and post-synaptic terminals of the olfactory receptor / antennal lobe CNS junction.
Dystrophin Avoidance, 1.2 x 10^{-3} BEN. Flies lacking wildtype expression of the gene *dystrophin* were tested for their avoidance to benzaldehyde at a concentration of 1.2 x 10^{-3}. Mutant alleles include 166.3, 30.3, (both protein null imprecise excision) and 151.2 (imprecise excision). Differences were observed in odor avoidance levels between wildtype and mutant flies (ANOVA, $F[3,32] = 10.72$, $P < 0.0001$; Tukey, $P \leq 0.05$; Letters indicate statistical significance between groups). All flies were raised at 24 $\pm$ 0.5 °C and 35 $\pm$ 5% humidity on standard Bloomington Drosophila medium with a 12:12 h light:dark cycle and isolated at 2 to 7 d.o. for each experiment. Bars indicate mean $\pm$ SEM; $n = 12$ for w1118, $n = 8$ for 30.3, 166.3, 151.2. Work performed in collaboration with the laboratory of Lee Fradkin.

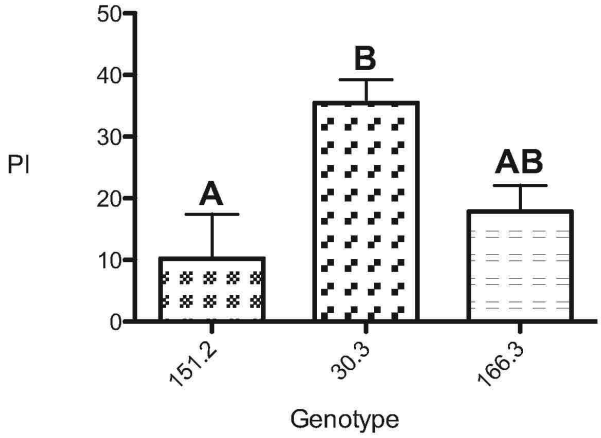

Figure B.2: *Dystrophin* Avoidance, 6 x 10^{-4} Benzaldehyde. Flies lacking wildtype expression of the gene *dystrophin* were tested for their avoidance to benzaldehyde at a concentration of 6 x 10^{-4}. Mutant alleles include 166.3, 30.3, (both protein null imprecise excision) and 151.2 (imprecise excision). Differences were observed in odor avoidance levels between mutant alleles 151.2 and 30.3 (ANOVA, $F[2,21] = 5.985$, $P < 0.001$; Tukey, $P \leq 0.05$; Letters indicate statistical significance between groups). All flies were raised at 24 ± 0.5 °C and 35 ± 5% humidity on standard Bloomington Drosophila medium with a 12:12 h light:dark cycle and isolated at 2 to 7 d.o. for each experiment. Bars indicate mean ± SEM; $n = 8$/bar. Work performed in collaboration with the laboratory of Lee Fradkin.

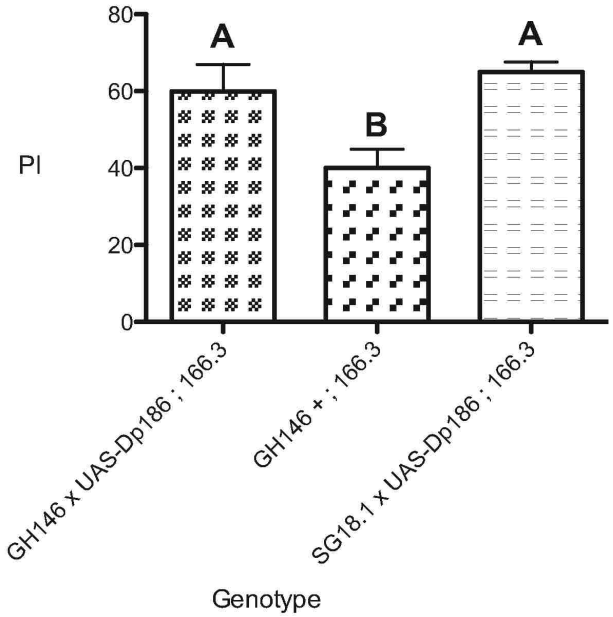

Figure B.3: *Dystrophin* Avoidance Rescue, 6 x 10^{-4} Benzaldehyde. Expression of wildtype *dystrophin* (*UAS-Dp186*) at either the pre-(*SG18.1*) or post-(*GH146*)synpatic terminals of antennal lobe / olfactory receptor lobe neurons is sufficient to rescue avoidance to benzaldehyde in a 166.3 mutant background. Differences were observed in odor avoidance levels between the rescue lines and *GH146 Gal4* driver in a mutant background (ANOVA, $F[2,21] = 6.533$, $P < 0.001$; Tukey, $P \leq 0.05$; Letters indicate statistical significance between groups). All flies were raised at 24 ± 0.5 °C and $35 \pm 5\%$ humidity on standard Bloomington Drosophila medium with a 12:12 h light:dark cycle and isolated at 2 to 7 d.o. for each experiment. Bars indicate mean ± SEM; $n = 8$/bar. Work performed in collaboration with the laboratory of Lee Fradkin.

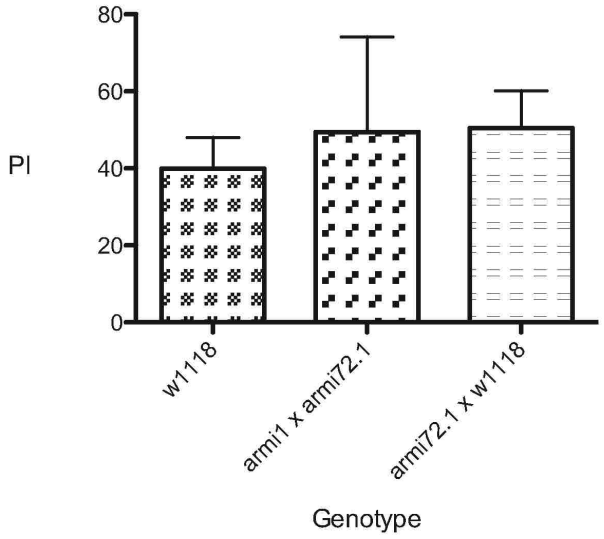

Figure B.4: *Armitage* is an RNA helicase required for the proper function of RISC complex in the RNAi silencing pathway of *Drosophila* (Tomari et al., 2004). In addition, disruption of this protein has resulted in learning deficits for protein synthesis dependent long-term memory in *Drosophila* (Ashraf et al., 2006). Preliminary work was performed with odor avoidance for *armitage* mutants as a starting point for learning and memory studies, however, further investigations were abandoned due to time constraints and commitments to other experiments / investigations.
Trans-heteroallelic *Armitage* Odor Avoidance at 3 x 10^{-4} Benzaldehyde. Preliminary data demonstrating equal benzaldehyde odor avoidance for w1118, armi1 x armi72.1, and armi72.1 x w1118 flies (ANOVA, $F[2,10] = 0.2437$, $P = 0.7882$). All flies were raised at 24 ± 0.5 °C and 35 ± 5% humidity on standard Bloomington Drosophila medium with a 12:12 h light:dark cycle and isolated at 2 to 7 d.o. for each experiment. Bars indicate mean ± SEM; $n = 6$ for w1118, $n = 3$ for armi1 x armi72.1, and $n = 4$ for armi72.1 x w1118. Work performed in collaboration with the laboratory of Mani Ramaswami.

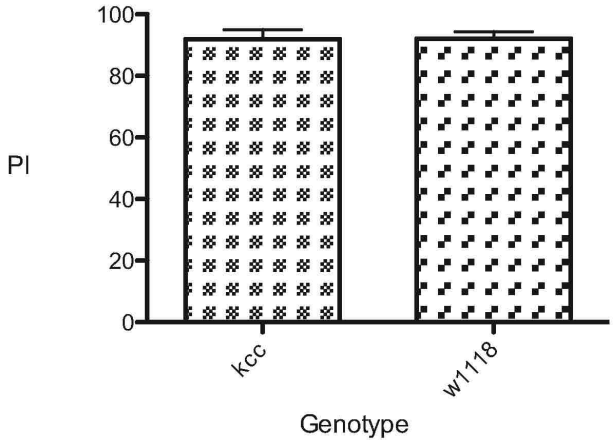

Figure B.5: *Kazachoc* is a K^+/Cl^- cotransporter whose mutantion results in increased seizure susceptibility in *Drosophila* (Hekmat-Scafe et al., 2006). Preliminary work was performed with the kcc mutant to investigate any behavioral changes in learning and/or memory. Unfortunately, due to issues with shock avoidance, the work was put on hold pending the outcrossing of kcc mutants to wildtype *Drosophila* backgrounds.
Kazachoc Shock Avoidance at 90 VDC. Preliminary data demonstrating equal shock avoidance for flies mutant for the *kazachoc* gene versus w1118 (t-test, t[10] =0.03334, P = 0.9741). All flies were raised at 24 ± 0.5 °C and 35 ± 5% humidity on standard Bloomington Drosophila medium with a 12:12 h light:dark cycle and isolated at 2 to 7 d.o. for each experiment. Bars indicate mean ± SEM; $n = 6$/bar. Work performed in collaboration with the laboratory of Mark Tanouye.

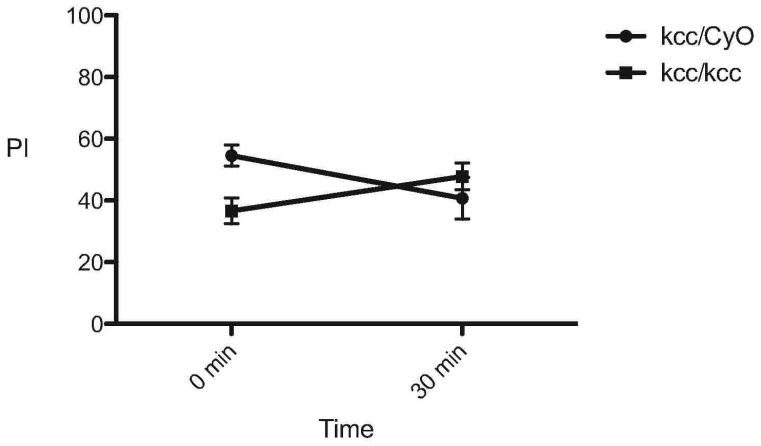

Figure B.6: *Kazachoc* Learning and STM. Preliminary data demonstrating a performance index for learning (0 min) and short-term memory (30 min) for kcc/CyO and kcc/kcc mutant flies. Shock voltage was 90 VDC, exact odor concentrations were recorded by postdocs working in the lab of Mark Tanouye. All flies were raised at 24 $\pm$ 0.5 °C and 35 $\pm$ 5% humidity on standard Bloomington Drosophila medium with a 12:12 h light:dark cycle and isolated at 2 to 7 d.o. for each experiment. Bars indicate mean $\pm$ SEM; $n = 12$ for 0 min, $n = 8$ for 30 min. Work performed in collaboration with the laboratory of Mark Tanouye.

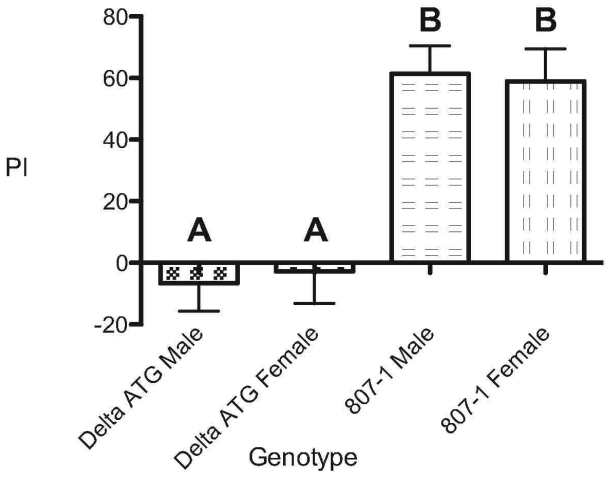

Figure B.7: *CREB*, or cAMP responsive element binding protein, is a transcription factor studied extensively in the field of learning and memory (Silva et al., 1998). In *Drosophila*, *CREB* has been directly implicated in learning and memory through investigations of both mutants and conditional expression of a dominant negative transcript (Yin et al., 1994; Yin and Tully, 1996). The following figures demonstrate odor and shock avoidance for two allelic variants of *CREB* in the fruit fly (with the ultimate goal of recreating previously published *CREB* research). Because odor avoidance differed between these two stocks, further learning and memory studies could not be pursued.
CREB Odor Avoidance, 1.4 x 10^{-3} MCH. Preliminary data demonstrating odor avoidance for *Creb* mutated (Delta ATG) versus *CREB* 807-1 transcript overexpression flies. Significant differences were observed between mutant and overexpression stocks in their avoidance to methylcyclohexanol (ANOVA, $F[3,20] = 14.53$, $P < 0.0001$; Tukey, $P \leq 0.05$; Letters indicate statistical significance between groups). All flies were raised at 24 ± 0.5 °C and $35 \pm 5\%$ humidity on standard Bloomington Drosophila medium with a 12:12 h light:dark cycle and isolated at 2 to 7 d.o. for each experiment. Bars indicate mean ± SEM; $n = 6$/bar. Work performed in collaboration with the laboratory of Jerry Yin.

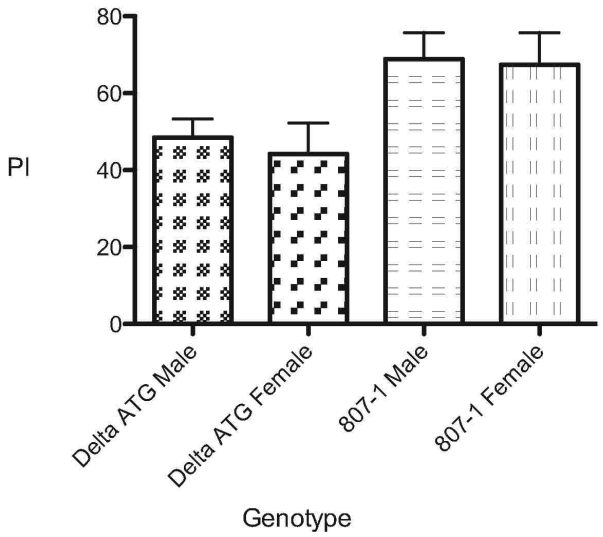

Figure B.8: *CREB* Odor Avoidance, 2 x 10^{-3} OCT. Preliminary data demonstrating odor avoidance for *Creb* mutated (Delta ATG) versus *Creb* 807-1 transcript overexpression flies. Means were calculated to be significantly different (ANOVA), however no statistical differences were observed when calculating a Tukey post-hoc test between all groups of mutant and overexpression stocks (ANOVA, $F[3,20] = 3.133$, $P < 0.05$). All flies were raised at 24 ± 0.5 °C and 35 ± 5% humidity on standard Bloomington Drosophila medium with a 12:12 h light:dark cycle and isolated at 2 to 7 d.o. for each experiment. Bars indicate mean ± SEM; $n = 6$/bar. Work performed in collaboration with the laboratory of Jerry Yin.

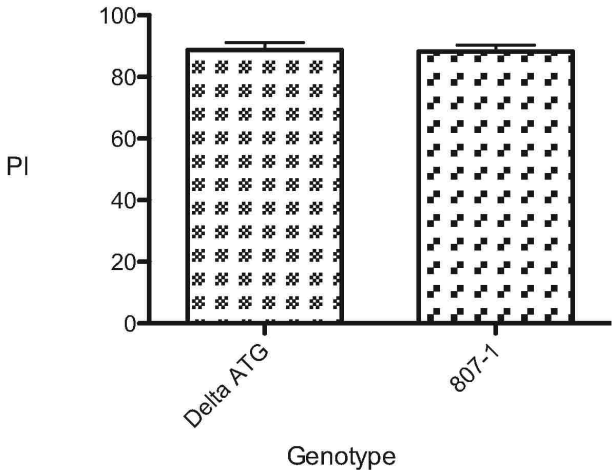

Figure B.9: *CREB* Shock Avoidance, 90 VDC. Preliminary data demonstrating shock avoidance for *Creb* mutated (Delta ATG) versus *Creb* 807-1 transcript overexpression flies. No significant differences were observed between mutant and overexpression lines . All flies were raised at 24 ± 0.5 °C and 35 ± 5% humidity on standard Bloomington Drosophila medium with a 12:12 h light:dark cycle and isolated at 2 to 7 d.o. for each experiment. Bars indicate mean ± SEM; $n = 6$/bar. Work performed in collaboration with the laboratory of Jerry Yin.

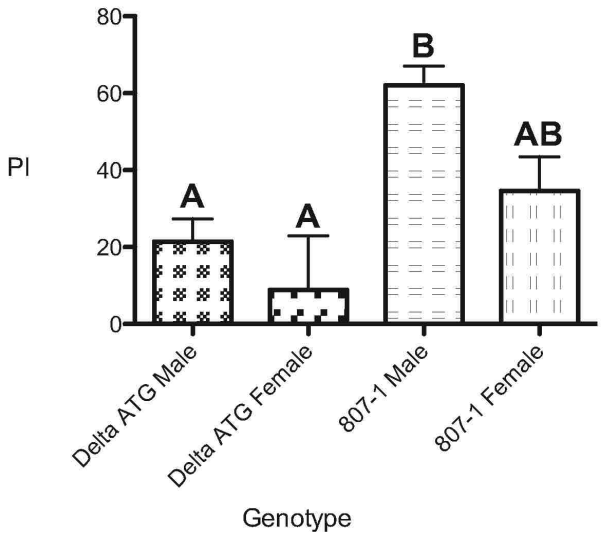

Figure B.10: *CREB* Odor Avoidance, 2.8 x 10^{-3} MCH. Preliminary data demonstrating odor avoidance for *Creb* mutated (Delta ATG) versus *Creb* 807-1 transcript overexpression flies. Significant differences were observed between mutant and overexpression stocks in their avoidance to methylcyclohexanol (ANOVA, $F[3,20] = 6.128$, $P < 0.01$; Tukey, $P \leq 0.05$; Letters indicate statistical significance between groups). All flies were raised at 24 ± 0.5 °C and 35 ± 5% humidity on standard Bloomington Drosophila medium with a 12:12 h light:dark cycle and isolated at 2 to 7 d.o. for each experiment. Bars indicate mean ± SEM; $n = 6$/bar. Work performed in collaboration with the laboratory of Jerry Yin.

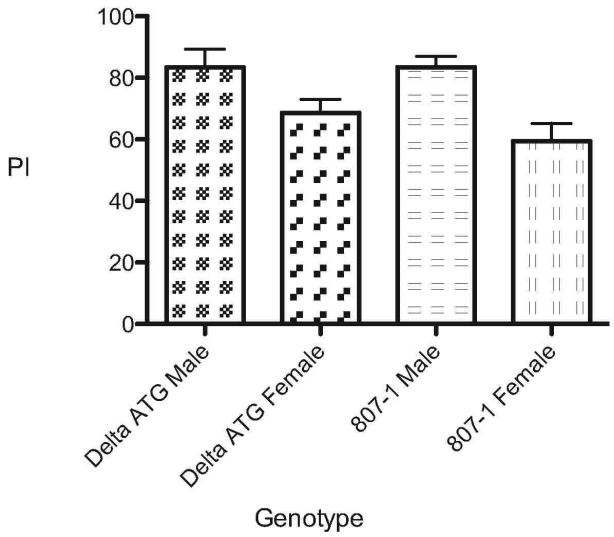

Figure B.11: *CREB* Odor Avoidance, 4.2 x 10^{-3} MCH. Preliminary data demonstrating odor avoidance for *Creb* mutated (Delta ATG) versus *Creb* 807-1 transcript overexpression flies. Significant differences were observed between mutant and overexpression stocks in their avoidance to methylcyclohexanol (ANOVA, $F[3,20] = 5.469$, $P < 0.01$; Tukey, $P \leq 0.05$; Letters indicate statistical significance between groups). All flies were raised at 24 ± 0.5 °C and 35 ± 5% humidity on standard Bloomington Drosophila medium with a 12:12 h light:dark cycle and isolated at 2 to 7 d.o. for each experiment. Bars indicate mean ± SEM; $n = 6$/bar. Work performed in collaboration with the laboratory of Jerry Yin.

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