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Dopaminergic and Activity-Dependent Modulation of Mechanosensory Responses in Drosophila Melanogaster Larvae

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DOPAMINERGIC AND ACTIVITY-DEPENDENT MODULATION OF MECHANOSENSORY RESPONSES IN DROSOPHILA MELANOGASTER LARVAE

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
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By
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Director: Dr. Robin Lewis Cooper, Associate Professor of Biology

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ABSTRACT OF DISSERTATION

DOPAMINERGIC AND ACTIVITY-DEPENDENT MODULATION OF MECHANOSENSORY RESPONSES IN *DROSOPHILA MELANOGASTER* LARVAE.

A central theme of this dissertation is nervous system plasticity. Activity-dependent plasticity and dopaminergic modulation are two processes by which neural circuits adapt their function to developmental and environmental changes. These processes are involved in basic cognitive functions and can contribute to neurological disorder. An important goal in modern neurobiology is understanding how genotypic variation influences plasticity, and leveraging the quantitative genetics resources in model organisms is a valuable component of this endeavor. To this end I investigated activity-dependent plasticity and dopaminergic modulation in *Drosophila melanogaster* larvae using neurobiological and genetic approaches.

Larval mechanosensory behavior is described in Chapter 2. The behavioral experiments in that chapter provide a system to study mechanisms of plasticity and decision-making, while the electrophysiological characterization shows that sensory-motor output depends on neural activity levels of the circuit. This system is used to investigate activity-dependent plasticity in Chapter 3, i.e., habituation to repetitive tactile stimuli. In Chapter 4, those assays are combined with pharmacological manipulations, genetic manipulations, and other experimental paradigms to investigate dopaminergic modulation. Bioinformatics analyses were used in Chapter 5 to characterize natural genetic variation and the influence of single nucleotide polymorphisms on dopamine-related gene expression. The impact and suggested future directions based on this work are discussed in Chapter 6.

Dopamine also modulates cardiomyocytes. Chapter 7 describes biochemical pathways that mediate dopaminergic modulation of heart rate. The final two chapters describe neurobiology research endeavors that are separate from my work on dopamine. Experiments that have helped characterize a role for *Serf*, a gene that codes for a small protein with previously unknown function, are described in Chapter 8. In the final chapter I describe optogenetic behavioral and electrophysiology preparations that are being integrated into high school classrooms and undergraduate physiology laboratories. Assessment of student motivation and learning outcomes in response to those experiments is also discussed.

KEYWORDS: Dopamine, neural circuit plasticity, mechanosensory habituation, electrophysiology

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CHAPTER ONE

Overview of mechanosensation and dopamine signaling in *Drosophila melanogaster*

Neural circuits and the big picture

The fundamental purpose of this research is to advance our understanding of how neural circuits work. Understanding neural circuit function is a goal of modern neuroscience because a working knowledge of neural circuits will ultimately lead to new ways to diagnose and treat cognitive disorders. Mental health disorders and neurodegenerative disease are devastating to patients and their families, and they occur in an alarmingly high percentage of the population. Neural disorders that are not completely debilitating, such as depression or addiction, also cause immeasurable suffering. Treatment options for neural disorders are limited because the biology of abnormal behavior is quite complex, undoubtedly influenced by large gene networks, large neural networks, and the environment. The limitation to a better understanding of mental health is knowing how these factors interact throughout an animal's life cycle.

By investigating the nervous system in animal models we control the environment, minimizing variability in the environmental factor. We are also increasingly adept at measuring the total molecular makeup of cells at the DNA, RNA, and protein levels, which enhances our control of the genetic network factor. Where we lack resolution and control is with the measurement of dynamic processes in the intact nervous system, i.e., the neural network factor. Neuronal populations are extremely dense and heterogeneous, both in terms of morphology and physiology. Not only do neurons vary in the expression of ion channels and neurotransmitter phenotypes, individual neurons also exhibit variation at the DNA sequence level by acquiring large

mutations (McConnell et al., 2013). These are only a few of the reasons why it has been difficult to fully comprehend how neural networks work, but with sophisticated neurogenetic tools, progress in this field seems imminent.

This is the point where the focus turns to *Drosophila melanogaster* neurobiology. *Mus musculus*, *Danio rario*, *Caenorhabditis elegans,* and other genetically tractable organisms afford many of the same experimental advantages as *D. melanogaster* to control and measure neural activity in defined populations of neurons. From the molecular level to the brain region level many functions have also been conserved throughout the evolution of insects and mammals (Strausfeld and Hirth, 2013). *D. melanogaster* was chosen because it offers a compromise in neural complexity between the relatively invariable *C. elegans* nervous system and the dauntingly complex vertebrate nervous systems. Ultimately, deciphering neural circuit function across different phyla is advantageous because it reveals features that are necessary to scale up the number of neurons and behavioral complexity.

The specific aspect of neural circuit function that was addressed in this work is neural plasticity, which is the foundation of development, learning, and memory. How an animal will react to a stimulus depends on the physiological state of its nervous system, and plasticity allows the nervous to process the outcomes of those decisions. Progress in understanding neural networks is being made by determining the mechanisms that regulate input/output decisions for specific neural circuits, and then determining if the mechanisms are generally applicable to other circuits and in other organisms. Over time this approach should reveal how separate circuits interact and which mechanisms of plasticity are the most critical to specific behaviors and disease states. In this work, I characterized two different mechanisms for plasticity in a specific circuit. The circuit consists of the neurons involved in *D. melanogaster* larval mechanosensory behavior, and the mechanisms for plasticity are repetitive stimulation and dopaminergic

modulation. Both mechanisms of plasticity are relatively well characterized but neither have been investigated in this circuit, which is easily approachable on many levels. Over the next few sections I review the pertinent literature on mechanosensation and dopamine signaling, providing a background for chapters 2-5. Background specific to those experiments can also be found in the introductions of each chapter.

Larval mechanosensation as a model system for identifying molecular mechanisms of tactile sensory transduction and neural circuits for mechanosensory behavior

The field of larval mechanosensation was ignited by a forward genetic screen for mutants with tactile sensory defects (Kernan et al., 1994). This screen revealed molecular components of mechanotransduction, and just as importantly, it introduced an assay that would be used in future screens to identify additional molecular components of mechanotransduction and separate components with different thresholds. Wild type larvae responded to a gentle brush on the head by executing one of a limited number of stereotypical behaviors, and mechanosensory mutants were identified based on deficits in the response behavior. Genes from this screen were cloned and found to be expressed in a specific subtype of sensory neurons (Chung et al., 2001), those which are associated with sensilla (Hartenstein, 1988). Around that time the GAL4/UAS binary expression system was becoming common (Brand and Perrimon, 1993), and specific drivers were developed to drive the expression of transgenes in specific types of sensory neurons that innervate the larval epidermis (Gao et al., 1999). Classification of sensory neurons innervating the larval body wall were soon updated to differentiate external sensory organs, chordotonal organs, and multidendritic (MD) neurons (Bodmer and Jan, 1987). The multidendritic neurons were then further differentiated into 4 separate classes based on their distinct morphologies (Grueber et al., 2002).

The first functional separation of these neurons was achieved by applying painful stimuli (40°C hot iron or 40mN hard poke) to larvae with genetically silenced subsets of neurons (Tracey et al., 2003). In larvae generated by crossing an MD neuron-specific GAL4 line (GAL4109(2)80 (Gao et al., 1999)) with a line carrying UAS-TeTxLC (a tetanus toxin light chain transgene that blocks calcium-dependent synaptic vesicle release (Sweeney et al., 1995)), responses to the painful stimuli were almost completely eliminated without affecting response to a gentle brush stimulus (Tracey et al., 2003). Since then, several mechanotransduction ion channels have been identified that detect a range of thresholds (Ainsley et al., 2003, Zhong et al., 2010, Kim et al., 2012, Tsubouchi et al., 2012, Yan et al., 2013). At the neural circuit level, class-IV MD neurons are known to be necessary and sufficient for a defensive rolling behavior, whereas activation of other classes of MD neurons evokes an accordion-like behavior (Hwang et al., 2007). Multidendritic neurons and chordotonal neurons also provide sensory feedback during crawling (Caldwell et al., 2003, Song et al., 2007).

This system is interesting for two reasons, one is that the ion channels for mechanotransduction have human homologues, the other is that the behavior is ethologically relevant. Larvae use the nocifensive roll and other mechanosensory behaviors to escape parasitoid wasps (Hwang et al., 2007, Hodges et al., 2013, Robertson et al., 2013). Therefore the circuits underlying these behaviors have evolved to be efficient. What makes this is a powerful system to study neural circuits is that in addition to all that is known about mechanosensory neurons, the anatomy and function of motor units have also been exquisitely well characterized (Sink and Whitington, 1991, Kurdyak et al., 1994, Landgraf et al., 1997, Baines and Bate, 1998). There are also exceptional driver lines and well established protocols to investigate mechanosensory behavior and electrophysiology. Despite all that is known about larval mechanosensation, relatively little is known about how the information is integrated into

motor outputs. My aim was to leverage the tools and all of the data on inputs and outputs of the circuit to explore action selection. How is a specific behavior selected over others within its normal behavioral repertoire and what intrinsic neural circuit properties affect that decision (Chapter 2)? How is the decision affected by repetitive stimulations (Chapter 3), or the presence of neuromodulators (Chapter 4), or genetic variation (Chapter 5)? Answering those questions is an important first step in using this system to understand the logic of neural networks. In addition to the reasons mentioned above, I think that mechanosensory integration is an intriguing system to use for this purpose because the response is not a simple reflex or a single fixed action pattern, instead there are multiple fixed action patterns and they must be released within the context of rhythmic activity in central pattern generators. These are problems that likely occur in cognitive centers of the brain.

Anatomical and functional organization of the fruit fly central nervous system

The fruit fly nervous system consists of a brain, segmented nerve cord, and peripheral nervous system. Reiterative sensorimotor processing for body wall segments (or limbs and wings in adults) occurs in the thoracic and abdominal regions of the nerve cord. In the brain there are various segregated nuclei dedicated to taste, vision, olfaction, learning and memory, much like one would find in vertebrate brains. These regions are defined as neuropils separated by glial compartments (Younossi-Hartenstein et al., 2003). The insect midbrain and vertebrate midbrain possess several commonalities, especially within the vertebrate basal ganglia and the insect central complex. The following structures were considered to be homologous within the protocerebra: superior protocerebrum:frontal cortex, mushroom body:hippocampus, inferior medial protocerebrum:somatosensory cortex, inferior medial protocerbrum:thalamus, fan-shaped body and protocerebral bridge:striatum, ellipsoid

body:globus pallidus, and the insect ventral lateral protocerebrum corresponds to the mammalian motor cortex (Strausfeld and Hirth, 2013). Dopaminergic modulation of these ganglia has also been maintained through 1 million years of divergent evolution. Just as the substantia nigra sends dopaminergic processes to the striatum, the PPL1 cluster of dopaminergic neurons projects to the fan-shaped body and ellipsoid body.

Anatomical and functional organization of dopaminergic neurons in the fruit fly central nervous system

Dopaminergic neurons in *D. melanogaster* are specified near the end of embryonic development (Neckameyer and White, 1993). In the larval ventral ganglion there are dorsal segmented pairs and unpaired cell bodies whose axons terminate ventrally into wide arborizations along the anterior-posterior tracts. In the brain there are also paired and unpaired cell bodies that send projections to almost every neuropil. Anatomy of the larval dopamine system is highly stereotyped with almost no variation between animals.

There are approximately 90 dopaminergic neurons in the larval nervous system (Budnik et al., 1986, Selcho et al., 2009). Thirty cell bodies are seen in the brain and 60 in the ventral nerve cord. Generally the cell bodies are located peripherally with their axons projecting into brain neuropils and fascicles. All four regions of the protocerebrum and the sub esophageal ganglion receive extensive dopaminergic innervations.

In adult flies it has been suggested that there is variation in the number of dopaminergic cells (Sykes et al., 2004). Dopaminergic neurons in the adult brain are typically classified in terms of separate clusters: paired posterior lateral 1 and 2 (PPL1 and PPL2), paired posterior medial 1 and 2 which are typically grouped together (PPM1/2), PPM3, paired anterior lateral (PAL) and paired anterior medial (PAM) (White et al., 2010). The anterior cells and the PPM1/2 cells innervate the protocerebrum. The

PPL cells innervate the mushroom bodies, and the PPM3 cells innervate the central complex and protocerebrum. This stereotypical innervation pattern to specific brain regions with well characterized functions supports the accumulating evidence for involvement of dopamine in various neurobiological processes. In the adult ventral nerve cord, dopaminergic neurons do not have an organized distribution pattern that is observed in larvae. Relatively little is known about the functional organization of dopaminergic cells in the ventral nerve cord in adults or larvae. In both systems they project into regions where sensorimotor circuitry is located and there is evidence that dopamine is involved in those behaviors.

Functions of dopamine in the fruit fly

New dopaminergic functions in the *D. melanogaster* nervous system are constantly being established. Aggressive behavior (Alekseyenko et al., 2013) and response to sugar (Marella et al., 2012) have been associated with individual dopaminergic neurons and receptor subtypes. Appetitive olfaction (Wang et al., 2013), aversive olfaction (Aso et al., 2012), reward signaling (Burke et al., 2012, Liu et al., 2012), learning (Berry et al., 2012), sleep and arousal (Ueno et al., 2012), hormone signaling (Karpova et al., 2012, Keleman et al., 2012), motor behavior (Hirsh, 1998, Riemensperger et al., 2011), courtship behavior (Neckameyer, 1998, Liu et al., 2009), and neuronal morphology (Neckameyer and Bhatt, 2012). Note that many of these functions are also modulated by dopamine in vertebrates. These are also mostly adult nervous system functions. Dopamine modulates the activity of nerves that innervate muscles acting on the mandible as well as circuitry that runs between segmental nerves.

Because dopamine is involved in scleritization of the cuticle (Wright, 1987) it is vital for proper development. Tyrosine hydroxylase null mutants are unable to hatch from the embryo, and if aided to hatch, do not survive metamorphosis. Dopamine also

circulates in the hemolymph where it has renal-like function (analogous to vertebrate proximal tubule) in modulation of malphigian tubules (Barone, 1999, Draper et al., 2007). Lastly, our lab and others have shown that dopamine modulates heart rate in *D. melanogaster* larvae (Titlow et al., 2013).

Pharmacological and genetic approaches have been used to manipulate dopamine levels in *Drosophila* and investigate the resulting effects on nervous system development and function. Studies with alpha-methyl-tyrosine (AMT) and 3-iodo-tyrosine (3IY), competitive inhibitors of tyrosine hydroxylase (TH), show that inhibiting dopamine synthesis in first and second instar larvae slows down development and severely reduces the number of flies that eclose into adults (Noguchi et al., 1995, Neckameyer, 1996). Feeding 3IY to third instars does not affect eclosion or sensorimotor behavior when the flies are larva, but adult males who were fed the drug as larvae exhibited impaired copulatory behavior (Noguchi et al., 1995). Dopamine synthesis has also been inhibited in transgenic flies using RNAi to knockdown TH function (Neckameyer and Bhatt, 2012). That study showed that dopamine acts as a trophic factor for the development of serotonergic gut neurons. Dopamine's effect on serotonergic fibers was specific to type-2 dopamine receptors (Neckameyer and Bhatt, 2012). In decapitated adult flies, exogenous application of dopamine induces locomotor activity (Venken et al., 2011). Depleting the neural isoform of tyrosine hydroxylase decreases activity levels and impairs aversive olfactory learning (Riemensperger et al., 2011).

Dopaminergic neurons and the four identified subtypes of *Drosophila* dopamine receptors (DARs) are distributed throughout the CNS (Noguchi et al., 1995, Feng et al., 1996, Han et al., 1996, Kohsaka et al., 2012). The type-1 DAR is sensitive to SKF 38393 (vertebrate agonist) but SCH 23390 (vertebrate antagonist) does not bind with high affinity. S(+)-butaclamol and *cis*(Z)-flupentixol are more effective antagonists (for review see (Blenau and Baumann, 2001) (Gotzes et al., 1994). Neurons cultured from fly

embryos have been used to assess the pharmacological properties of dopaminergic modulation. Dopamine decreases the frequency of spontaneous cholinergic activity in cultured preparations and SKF 38393 mimics that effect (Keller et al., 2002).

Dopaminergic pathways in the human CNS

Soma of the dopaminergic cells are aggregated in various clusters that send projections to nearly all corners of the brain and spinal cord. From the brainstem the three major dopaminergic ganglia are the substantia nigra, retrorubral field, and ventral tegmental area. The ventral tegmental area innervates limbic structures in the forebrain and temporal/frontal cortices (mesolimbic and mesocortical pathways). Regions innervated by the substantia nigra and retrorubral field include the anterior cingulate/prefrontal/pyriform/ and entorhinal cortices, amygdala, nucleus accumbens, hippocampus, caudate and putamen. Dopaminergic neurons in the dorsal hypothalamus innervate the lower brain stem and spinal cord. The thalamus is innervated by dopaminergic neurons located along the wall of the third ventricle. Neurons from this region innervate the median eminence and pituitary (tuberoinfundibular pathway). Lastly, there are dopaminergic neurons in the olfactory tubercle, olfactory bulb, and retina (Kandel et al., 2000).

Dopamine is a critical signaling molecule in animals that have a central nervous system. In animals that possess the capacity for memory, sex, and aggression, dopamine signaling has evolved to regulate the cellular networks that drive these and other complex behaviors. In humans, defects in dopamine signaling cause schizophrenia and Parkinson's disease, possibly addiction, ADHD, and depression. An ultimate goal of dopamine neurobiology research is to generate therapies for these debilitating disorders.

Abnormal behaviors that are highly correlated with dopamine typically involve other neurotransmitter systems (Lau et al., 2013), but to determine how these signaling

pathways and different brain regions interact, a thorough working knowledge of dopamine's influence on behavior is necessary. Determining biological factors related to dopamine that influence behavior is how this dissertation research contributes to the field of neuroscience. This work describes mechanisms of dopaminergic modulation at the cellular level, the molecular level, during development, and in non-neuronal tissues.

Abnormal behavior is difficult to characterize because there are numerous abiotic and biological factors, and the biological factors are not completely understood. Many disorders are typically diagnosed based on behavioral criteria listed in the Diagnostic and Statistical handbook (American Psychiatric Association-DSM-V). Some behaviors are quantifiable and can be measurably impacted by treatment, but for other behaviors a more empirical quantification is desirable. Chemical biomarkers, (Asor and Ben-Shachar, 2012), functional imaging (Atluri et al., 2013) and electroencephalography (Yener and Basar, 2013) are methods used for measuring physiological correlates of abnormal behavior. As the accuracy, spatial and temporal resolution of these methods improve, the challenge will still be to understand what is causing the defects. We are still left with this basic ethology problem of determining how behavior emerges from groups of cells, and how pathological function arises.

Chapter 2 documents behavioral and electrophysiological characterization of larval mechanosensory responses, revealing that 1) tactile input is weighted differently along the anterior-posterior axis, 2) tactile responses are affected by specific genes, and 3) tactile responses are affected by the functional state of the CNS. This knowledge of larval tactile responses is then used in Chapter 3 to characterize mechanosensory habituation, revealing developmental and genetic aspects of activity-dependent plasticity in a mechanosensory circuit. The modulatory effects of dopamine on mechanosensation are characterized in Chapter 4, followed by an analysis of dopamine-related gene expression and its effects on behavior in Chapter 5. Chapter 6 puts all of those findings

into perspective and suggests future directions that would have the biggest impact on the field.

The last three chapters consist of reports on topics that address dopaminergic modulation and neural circuit function but fall outside the scope of mechanosensory plasticity. Chapter 7 deals with dopaminergic modulation of larval cardiac function. The significance of this study is that is describes pharmacological properties of substances that alter dopamine signaling in *D. melanogaster*, showing that vertebrate agonists and antagonists, and a diacyl-glycerol analogue have an effect on heart rate. In Chapter 8 I discuss experiments that were done to characterize the neurobiological functions of *dSerf*, a conserved protein coding gene that has no known function. Those experiments show that *dSerf* is nonessential for viability or nervous system function. The final chapter describes teaching modules that I have developed for undergraduate physiology and high school classrooms. The first module use *D. melanogaster* optogenetics to teach principles of recombinant DNA technology and nervous system function at an advanced high school level. The second module is an optogenetics exercise that allows undergraduate students to explore the principles of neurophysiology and genetics.

CHAPTER TWO

Anatomical and genotype-specific mechanosensory responses in *Drosophila melanogaster* larvae.

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INTRODUCTION

Experience, environment, metabolic state, and genotype are factors that influence how an animal responds to different stimuli. We want to know how these factors are weighted for responses to a given stimulus, and how the information is integrated in neural circuits. There are a number of sensory systems that could be used to address this problem; we chose mechanosensation in *Drosophila melanogaster* larvae because the detection of tactile stimuli in this organism has been characterized down to the molecular level (Kim et al., 2012, Yan et al., 2013), the specific subsets of neurons that detect mechanical stimuli have been identified (Hwang et al., 2007, Yan et al., 2013), and genetic tools can be used to control neural activity in these neurons (Venken et al., 2011). The purpose of this study was to further characterize behavioral and physiological responses to localized tactile stimuli as a prelude to investigations on plasticity in this system.

Given that some mechanical stimuli will likely have a different meaning to the larva in the sense of impending danger or harm to the animal, one would expect varying sensitivity in responsiveness. Such differences in the behavioral responses indicate the uniqueness of the neural circuitry involved in eliciting these behaviors. Sensory input is known to influence the development of larval sensorimotor circuits (Kohsaka et al., 2012,

Fushiki et al., 2013). Sensory commands for responses to mechanosensory stimuli and the central pattern generator (CPG) for locomotion are known to communicate with each other (Hughes and Thomas, 2007, Song et al., 2007), however, the precise synaptic and neural architectural details remain to be determined. Modulation of this circuitry is of interest as it can provide insight into the finer behavioral regulation the organism possesses, regulatory elements which are likely to be common among animals that have a brain (Strausfeld and Hirth, 2013).

In *Drosophila* larvae, mechanosensory input evokes a few relatively stereotyped behaviors that depend on the intensity and location of the stimulus. Behaviors typically observed in response to a light brush on the thoracic segments while the animal is crawling include: pause, reverse contraction restricted to the anterior segment, full reverse contraction and subsequent change in crawling direction, or several consecutive reverse contractions (Kernan et al., 1994). These behaviors have been quantified with a numerical score based on intensity of the response. More forceful touches to the abdomen evoke a nocifensive rolling behavior that is performed by the animal to evade parasitoid wasps (Hwang et al., 2007, Robertson et al., 2013). Though the sensory and motor neurons are clearly arranged in a somatotopic CNS configuration, how the different behaviors are selected with respect to the stimulus location or pre-stimulus activity levels is not known.

In this study we present a series of behavioral and electrophysiological experiments that show how responses to tactile stimuli vary with respect to anatomical region, genotype, and specific subtypes of sensory neurons. By stimulating crawling larvae with moderately harsh touches to the head, abdomen, and tail, we show that sensitivity to mechanosensory stimuli in the three general regions varies, is highly consistent among genotypes, but can vary between genotypes. The effect of tactile

stimuli on neural activity was studied during fictive crawling and when motor circuits were inactive.

MATERIALS AND METHODS

Fly Maintenance and Stocks

D. melanogaster stocks were kept in standard cornmeal fly food medium at 23°C and 75% humidity on a 12hr light/dark cycle. The following strains were used: *Canton S* (wild type); w^{1118}, smn^{E33}, *D42*-GAL4 (all motor neurons), *elav*-GAL4 (pan-neuronal), *ppk*-GAL4 (class IV and class III dendritic arborization neurons), *nompC*-GAL4 (class III sensory and chordotonal neurons), *iav*-GAL4 (chordotonal neurons in the pattern of the *iav* gene (Gong et al., 2004, Kwon et al., 2010), and GAL4 driver specific for all multidendritic neurons and chordotonal neurons (Pw[+mW.hs]=GawB109(2)80, Pw[+mC]=UAS-mCD8::GFP.LLL5), which all were obtained from the Bloomington *Drosophila* Stock Center. UAS-shi^{ts} line was from the Kitamoto lab (Kitamoto, 2001).

Larva Touch Assay

General procedure

Larvae (5-10 at a time) were placed on an 8cm agar dish (1% agar, 33% apple juice to stimulate crawling). Crawling larvae (early third instar) were prodded three times with an insect pin (Fine Science Instruments, 0.2mm diameter), once on the tail, abdomen, and then head. All touches were directed to the dorsal midline at a 45° angle. Head and tail touches were aimed 0.5mm from the end of the animal, abdominal touches were in the longitudinal center of the animal. While watching the larvae through a stereomicroscope at 20x magnification, an observer recorded all behavioral responses evoked by the stimulus, e.g., no response (NR), pause, etc. When crawling speed was measured before performing the sensory assay, the number of peristaltic waves was

counted for 15 seconds. Those data are reported as the number of body wall contractions per minute.

Force calibration

Three approaches were combined to measure the stimulus force. First, a third instar larva was secured to a glass microscope slide with double-sided tape. The prod (insect pin fixed to a writing pen with modeling clay) was then advanced towards the animal using a micromanipulator. We measured the rotation length and speed required to indent the cuticle a distance comparable to indentations generated manually by an experimenter. This stimulus was then applied with the preparation resting on an analytical balance to measure the force. Applying the stimulus with the micromanipulator, or manually, generated forces in the range of 0.5-2.0g (4.5-19.6mN). Lastly, to determine that our force measurements were accurate, we constructed a Von Frey-like instrument to match previously described specifications (Zhong et al., 2010). With this fiber, measurements made manually and with the micromanipulator were in the range of 4.5-5.5g (44.1-53.9mN), indicating that our apparatus is accurate. Using filaments in the 5-20mN range or the insect pin yielded data that were statistically indistinguishable.

Inactivation of specific neurons using the temperature labile shibire^{ts1} allele

Females from the UAS-*shibire^{ts}* line were crossed with males expressing GAL4 in specific subsets of sensory neurons or motor neurons (positive control, *D42*-GAL4, which expresses the *shibire^{ts1}* in all motor neurons and paralyzes the animal at restrictive temperature). To inhibit chemical transmission in specific neurons, the animals were incubated in a water bath above the restrictive temperature for the *shibire^{ts1}* (dynamin) dominant-negative allele. Five larvae were placed in standard fly vials with 0.5mg

standard fly food and 0.5mL distilled water. Vials were incubated for ten minutes in a 37°C water bath, and then the animals were transferred to an agar-lined petri dish (8cm diameter) resting on a hot plate that maintained an agar temperature of 37°C.

Extracellular field potential recordings

Electrophysiology was performed on a partially-dissected early third instar larva preparation. The dissection is a modified version of the flat filet preparation (Parton et al., 2010) in which the anterior and posterior segments are left fully intact, i.e., 1) the animal is pinned on the lateral edges and 2) the dorsal midline dissection is stopped before reaching the anterior or posterior ends. This allows for visceral organs to be removed and gives access to the ventral ganglion and nerves with minimal damage to the CNS or dorsal PNS structures where the stimulus is delivered. Dissections and recordings were made in modified HL3 saline, NaCl 70 mM, KCl 5 mM, $MgCl_2.6H_2O$ 20 mM, $NaHCO_3$ 10 mM, trehalose 5 mM, sucrose 115 mM, $CaCl_2.2H_2O$ 1 mM, and BES 25 mM and pH 7.1 (Stewart et al., 1994). All saline components were purchased from Sigma Aldrich.

Glass micropipette suction electrodes (7-10µm inner diameter) were filled with saline and attached to an AxoClamp 2B amplifier. Signals were sampled at 10 kHz, digitized with a Powerlab 4SP A/D board (ADI), and visualized on a PC running LabChart7 (ADI). Mechanical stimulations were applied with the same insect pin described above by advancing the instrument with a micromanipulator. Electrical stimulations were delivered to posterior segmental nerves in filet preparations with the CNS intact as described in earlier work (Dasari and Cooper, 2004). A 10-pulse (40Hz) stimulus was applied to the nerve and intracellular recordings were obtained from m6. Firing frequency and duration were measured in LabChart7. Spontaneous bursting period was defined as the time from the first peak of one burst to the first peak of the

next burst. Burst duration was defined as the time from the first peak of one burst to the last peak of that burst. Burst frequencies were measured with the cyclic measurement tool in LabChart7, with counts being detected based on a threshold height set above the noise level.

Statistical analysis

Sample sizes (n) represent the number of individual animals tested for each genotype. The fraction of animals responding to each type of stimulus was compared between genotypes and between stimulus locations using Chi-square analysis of the proportion of animals responding and not responding to the stimulus. Post hoc analysis of the Chi-square results was performed using the COMPPROP procedure for multiple comparisons in SAS (v9.3). This is a Tukey-type test that compares proportions from a 2 x c contingency table as (Zar, 1996, SAS, 2013). When significant differences were observed in the fraction of flies responding to a stimulus, the frequency of specific behavioral responses was compared. This was done using one-way ANOVA because the Chi-square test was not valid when the number of specific responses was below 5 for multiple groups. The Kruskal-Wallis one-way ANOVA test was used to compare groups that were determined to have non-normal distributions (Shapiro-Wilk test). Multiple comparisons were performed with Tukey's method when sample sizes were identical, with Dunn's method when sample sizes were different. This ANOVA procedure was also used to compare crawling speed between different genotypes, and to compare normalized neural activity levels in response to stimuli delivered to different regions. All ANOVA procedures were performed with SigmaPlot (v12.3). Mean differences in neural activity before and after tactile stimuli were compared using paired Student's t-test. Those tests, and linear regression analysis of the data in Figure 4 were performed with Microsoft Excel-Plus (2013).

RESULTS

Larval behavioral responses to sharp mechanosensory stimuli

Previous authors have described the behavioral repertoire evoked by mechanosensory stimuli in *D. melanogaster* (Kernan et al., 1994). Light brushes moving from anterior to poster on the lateral thoracic segment cause the animal to stop crawling, then either turn and continue crawling, crawl in the reverse direction, or simply resume crawling in the same direction. Stronger stimuli (50mN) applied to the dorsal midline near abdominal segment 4 can evoke a rolling behavior described as “nocifensive” (Zhong et al., 2010). Here we applied a lighter tactile stimulus (20mN) to the tail, abdomen, and head regions using the sharp end of an insect pin (Figure 2.1A). Touching the tip of the tail evokes an escape behavior that causes the animal to increase crawling speed. We delivered stimuli to the last body segment, at the base of the tail. Fewer than 30% of Canton S larvae respond to this stimulus, whereas more than 60% respond to abdominal touches, and over 95% respond to head touches (Figure 2.2A). The responses were indistinguishable when the stimulus was applied tail first, head first, or in a random order.

When comparing the types of behaviors evoked by stimuli at different locations, it is clear that the responses are specific to the location of the stimulus. In addition to the pause, turn, reverse, and rolling behaviors that have been described, we also observed a full body bend, which we call a c-bend, and a posterior bend, which we call a tail flip. The c-bend is observed almost exclusively in response to head stimuli (Figure 2.2B), whereas the tail flip was mostly evoked by tail stimulations. Head touches also evoke reverse contractions and turns. In general, the head region is more sensitive and generates a greater variety of response behaviors than the abdomen or tail. To further characterize these mechanosensory response behaviors we delivered the gentle brush stimulus described by Kernan et al. (1994). The response index we measured was

comparable to the scores reported in that study (Figure 2.3A), and the same limited repertoire of responses was observed, i.e., pause, turn, and reverse (Figure 2.3B). Thus the diversity of response behaviors to a tactile stimulus is positively correlated with the force of the stimulus.

Next we began to assess different genotypes, beginning with a mutant that is homozygous for a hypomorphic allele of the *smn* gene, smn^{E33} (Rajendra et al., 2007). *Smn* is an RNA binding protein that is involved in splicing and RNA localization. In flies, as in humans, mutations in *smn* cause early lethality through defects in RNA processing (Chang et al., 2008). Motor neurons are particularly susceptible to *smn* mutations and degeneration of motor neurons leads to muscle atrophy and motor defects. In flies, *smn* loss of function mutants exhibit a decrease in locomotion but an increase in synaptic output at the larval NMJ (Imlach et al., 2012). We observed slower crawling speeds in the smn^{E33} mutants (Figure 2.4A), and compared to Canton-S larvae, the smn^{E33} mutants were also more responsive to tail stimuli (Figure 2.4B). Within the greater number of responses there were more tail flips specifically. Compared to larvae with a loss of function mutation in the *white* allele (w^{1118}; smn^{E33} allele is in a $w^{-/-}$ background), smn^{E33} mutants are only slightly more responsive to tail touches, but execute nearly twice as many tail flips. The mechanosensory phenotype in smn^{E33} was rescued by crossing it with the w^{1118} line, but crawling speed in smn^{E33}/w^{1118} crosses was not restored to wild type levels, or to levels that were observed in smn^{E33}/CS larvae (Figure 2.4). We also observed that Smn^{E33} larvae are more sensitive than w^{1118} to the light touch paradigm used by Kernan et al. (1994), in this case responding with more reverse contractions (Figure 2.3). To test for a correlation between baseline motor activity and mechanosensory responsiveness, we used linear regression to analyze the responses to tactile stimuli with respect to crawling speed within genotypes. No correlation was

observed between the rate of crawling and the probability of responding to the stimulus at any of the regions tested (Figure 2.5).

Three conclusions can be drawn from these results. First is that mutations can affect mechanosensory responses to tactile stimuli in specific anatomical regions. *White* mutations, which are commonly used as a selectable marker in mutagenesis or transgenesis, behave differently than the common wild type strain, Canton-S. This was true for responses to 20mN tail stimuli and light brushes to the anterior segments. *White* mutants and *smn* mutants had similar responses to 20mN head stimuli, but the *smn* mutant was more sensitive to light head brushes. These results show that mutations can affect mechanosensory responsiveness at different thresholds, though these experiments do not determine whether threshold for detecting the stimulus, or threshold for activating the motor output is affected. Lastly, the results suggest that *smn* mutants have an increased responsiveness to mechanosensation, which could be due to enhanced excitability in motor neurons that innervate the body wall muscles (Imlach et al., 2012).

Anatomical localization of specific sensory neuron subtypes that transduce tactile stimuli

Sensory neurons that tile the larval body wall are remarkably well-characterized in terms of function and morphology. For gentle touch and noxious stimulus assays, the stimulus is typically delivered to the regions where the arrangement of sensory neurons is highly ordered between segments. However, notice that the arrangement in the head and tail regions is highly irregular compared to the abdominal region (Figure 2.1B). Our aim here was to determine which sets of sensory neurons are needed to respond to tactile stimuli in the head and tail regions. We used a temperature sensitive dominant negative dynamin mutant (UAS-*shibire*^{ts}) to block chemical neurotransmission, and

disrupt specific subsets of sensory neurons with publicly available GAL4 lines. To determine if high temperature influences mechanosensation in general, we tested our Canton-S line. Elevated temperature only affected Canton-S responses to abdominal touches, where sensitivity was slightly increased (Figure 2.6A). As a negative control for heat inactivation of neural activity, we tested the parental UAS-*shibire*^{ts} line at restrictive temperature in each experiment (Figure 2.6B). Also, we tested the sensory neuron driven UAS-*shibire*^{ts} lines at permissive temperature (Figure 2.6C_{1-3}). For a positive control we observed that the UAS-*shibire*^{ts} line crossed to a motor neuron GAL4 driver (*D42*-GAL4) caused paralysis.

This approach was used to inhibit class III and class IV multidendritic neurons, chordotonal neurons, a combination of class III and chordotonal neurons, and a combination of all multidendritic neurons and chordotonal neurons. Compared to the UAS-*shibire* background controls, only inhibition of all multidendritic neurons and chordotonal neurons had a significant effect on mechanosensory behavior (Figure 2.6B). Inhibiting chordotonal neurons alone, or in combination with class III neurons slightly reduced responses to abdominal touches, both compared to UAS-*shibire* at restrictive temperature (Figure 2.6A) and to the isogenic controls tested at permissive temperature (Figure 2.6C_{1-2}). However the only statistically significant reduction in mechanosensation was observed when inhibiting chordotonal neurons in combination with multiple subsets of multidendritic neurons (Figure 2.6C_3). Therefore response to 20mN tactile stimulation on the dorsal midline requires a combination of sensory neurons.

The majority of responses to abdominal touches in MD+CH > *shi*^{ts} larvae were pauses (Figure 2.6D_2). Therefore it appears that the phenotypic mechanosensory response at restrictive temperature is caused by reduced input to inhibitory neurons that stop crawling, rather than a direct synapse on motor neurons that would normally evoke a response behavior. This may not be the case in the head region as head touches

evoke reverse contractions, turning behavior, and pauses at permissive temperature, each of which is reduced at restrictive temperature (Figure 2.6D_3).

Neural activity associated with mechanosensory stimuli

To extend our characterization of mechanosensory responses to the cellular level, we used *en passant* electrophysiological recordings from posterior segmental nerves (Fox et al., 2006). In partially dissected preparations (Figure 2.7A) we observed several minutes of bursting activity that resembles fictive crawling in other animals. The period between bursts was ranged from 10-25s. Presentation of tactile stimuli was often followed by a pause in the endogenous rhythm (Figure 2.7B). The average spontaneous burst period after the stimulus was significantly longer than the average of the three previous periods, regardless of whether the stimulus was delivered to the head, abdomen, or tail (Figure 2.7C_1). The change in bursting interval did not vary between anatomical regions (data not shown), and the stimulus did not affect the duration or frequency of activity during the bursts (Figure 2.7$C_{2,3}$).

In preparations that did not exhibit rhythmic bursting, tactile stimulations were delivered either during spontaneous activity (Figure 2.8) or between bursts of spontaneous activity (Figure 2.9). For stimuli delivered during activity we compared the frequency over 5s prior to the stimulus, to the frequency measured 5s after the stimulus. Consistent with the bursting data, spontaneous activity decreased following tactile stimuli (Figure 2.8A). The decrease was statistically significant for all three anatomical regions (Figure 2.8B), but there was no difference in the change between regions (Figure 2.8C). Tactile stimuli that were delivered when the nerve was inactive were followed by a burst of activity (Figure 2.9A). The average frequency of bursts in response to stimuli in the three different regions were, head: 9.3 ± 1.0Hz, abdomen: 5.7 ± 0.6Hz, and tail: 8.9 ± 1.2Hz. The average duration of the bursts were, head: 14.1 ± 1.8s, abdomen: 8.2 ± 1.7s,

and tail: 7.22 ± 1.5s. To compare the response to touches in different regions, values from each animal were normalized to the average response to head stimuli in that animal, then the normalized values were compared using one-way ANOVA. The duration (Figure 2.9B) and frequency (Figure 2.9C) in response to abdominal stimuli were lower than responses to head and tail stimuli ($p<0.05$).

Sensory-motor responses in *Drosophila* larvae can also be evoked by stimulating segmental nerves with an electrode (Dasari and Cooper, 2004). This paradigm mimics sensory input to the CNS and is used to investigate CNS output to a single muscle cell. A 10-pulse stimulus (40Hz) typically evokes a burst of activity that is comparable in duration to the responses evoked by tactile stimuli (Figure 2.10A). The average frequency of bursts recorded in seven larvae was 5.4±1.1 Hz, which is comparable to the firing frequency of the phasic motor neuron MNSNb/d-Is observed during fictive crawling (Chouhan et al., 2010). This frequency was also comparable to the extracellular frequencies that were evoked by tactile stimuli (of course the extracellular recordings contain compound action potentials from several neurons firing out of phase). All of these frequencies were below the average frequency of bursts recorded during fictive crawling (Figure 2.10B). These data suggest that the sensory-motor response is mediated by the type Is motor neurons, though these experiments alone do not rule out the possibility that input also comes from MNSNb-d-Ib neurons firing below their maximum frequency.

DISCUSSION

The neural circuitry for larval motor output consists of segmentally reiterative motor neurons in the ventral nerve cord that send axons away from the CNS to body wall muscle fibers, and segmentally reiterative sensory neurons located in the body wall, which send axons back to the CNS. Given this symmetry, it was surprising to observe

robust differences in mechanosensory responsiveness along the anteroposterior axis (Figure 2.2). However, the results are ethologically relevant, as observations of wasp attacks on *D. melanogaster* larvae revealed that responses varied depending on the location of the attack (Robertson et al., 2013).

As one might predict, the head region is the most sensitive to 20mN tactile stimulation, and head touches generated the biggest diversity in behavioral responses. The c-bend and roll behaviors were observed in response to 20mN tactile stimuli (Fig 2.2B), but not light brushing to the anterior segments (Figure 2.3B). Rolling behavior is known to be a response to noxious stimuli (Zhong et al., 2010), it would appear that the bending behavior is also a nociceptive response. However, the frequency of bending behavior did not decrease significantly when the nociceptive neurons (*nompC*-GAL4, Figure 2.6C_2) were inhibited. One possible explanation is that those neurons are fatigued or habituated from the heat stimulus that was used to acutely inhibit chemical transmission. A transient receptor potential (TRP) ion channel that detects heat is also expressed in those neurons (Tracey et al., 2003), and the bend behavior does appear to be an abbreviated form of the nocifensive roll.

Zhou and colleagues (2012) show that the degree of turning is also correlated with stimulus intensity, i.e., stronger stimuli cause a greater turning angle. This suggests a graded or analog relationship between afferent and efferent neurons for this behavior, but the process of switching to a different behavior in response to a tactile stimulus is not understood. Without input from the brain, larvae can crawl and react to light, but are unable to perform goal-directed movements like chemotaxis (Berni et al., 2012). Inhibiting a small subset of neurons in the brain changes the turning direction in response to an innocuous head touch (Zhou et al., 2012). It is unclear how descending input modulates mechanosensory input from the abdomen and tail, and whether it modulates motor circuits for rolling, reverse, or tail flex behaviors. It is also unclear how

the circuits underlying these behaviors overlap with the central pattern generating circuits for crawling behavior. That is something that we attempted to address by measuring changes in neural activity in response to tactile stimulations during fictive locomotion. The pause in bursting after the stimulus was to be expected, *in vivo* the larvae stopped crawling before executing a response behavior. After the delay, rhythmic activity resumed with the same burst waveforms observed during crawling (Figure 2.7C). This could mean that the posterior nerves do not convey impulses involved in other response behaviors, or that those behaviors are inhibited when the animal is pinned down and submerged in saline. To be sure, one would need to record from more anterior nerves during bursting activity.

Another feature of mechanosensory response behavior that became apparent from extracellular recordings was that the change in activity after a tactile stimulation depends on whether or not the nerve is active when the stimulus is delivered. When active, tactile stimuli caused the frequency of activity to decrease (Figure 2.8), when inactive, tactile stimuli generated bursts of activity (Figure 2.9). The duration of the bursts (~10s) are similar to the duration of bursts recorded in muscles after evoking CNS activity through the segmental nerve with an electrode (Dasari and Cooper, 2004). The latter approach is now being used to identify the neural circuit that mediates this response and characterize plasticity in the circuit.

Response to input from the environment is a prominent behavioral characteristic that can have an immediate impact on an animal's survival. Ultimately we want to describe the physiological basis of these responses with the goal of understanding how they are affected by experience, genetic variation, and other factors. To investigate how neural circuits process information, the *Drosophila* larval nervous system is ideal because of the tools for controlling and measuring neural activity (Pfeiffer et al., 2008), and the extensive literature on behavioral genetics (Sokolowski, 2001).

Mechanosensation and its associated behaviors in larvae are an attractive system because of the abundance of research that has been done on the NMJ and peripheral nervous system. Deciphering the CNS circuits that integrate this information has been a more difficult task (Iyengar et al., 2011), and will require genetic techniques to control and measure neural activity, in combination with electrophysiology and pharmacology to identify cellular and molecular features of decision-making. The simple decision addressed in this study was how to react to a tactile stimulus. It is not surprising that afferent neurons in different regions show different sensitivity and evoke different behaviors, but how is one behavior chosen instead of another in response to the same stimulus? Our results show that when motor circuits are active, their output in response to afferent stimulation is not the same as when motor circuits are inactive. We are trying to determine if this difference is due to intrinsic properties of cells within the circuit or modulatory input from other cells.

Conclusion

Though the sensory and motor neurons of the larval peripheral nervous system are symmetrically arranged into segments, behavioral responses to mechanical stimuli in the anterior and posterior regions are quite different. The motor response recorded in segmental nerves also varies between anatomical regions, and depends on whether motor circuits are active when the stimulus is delivered.

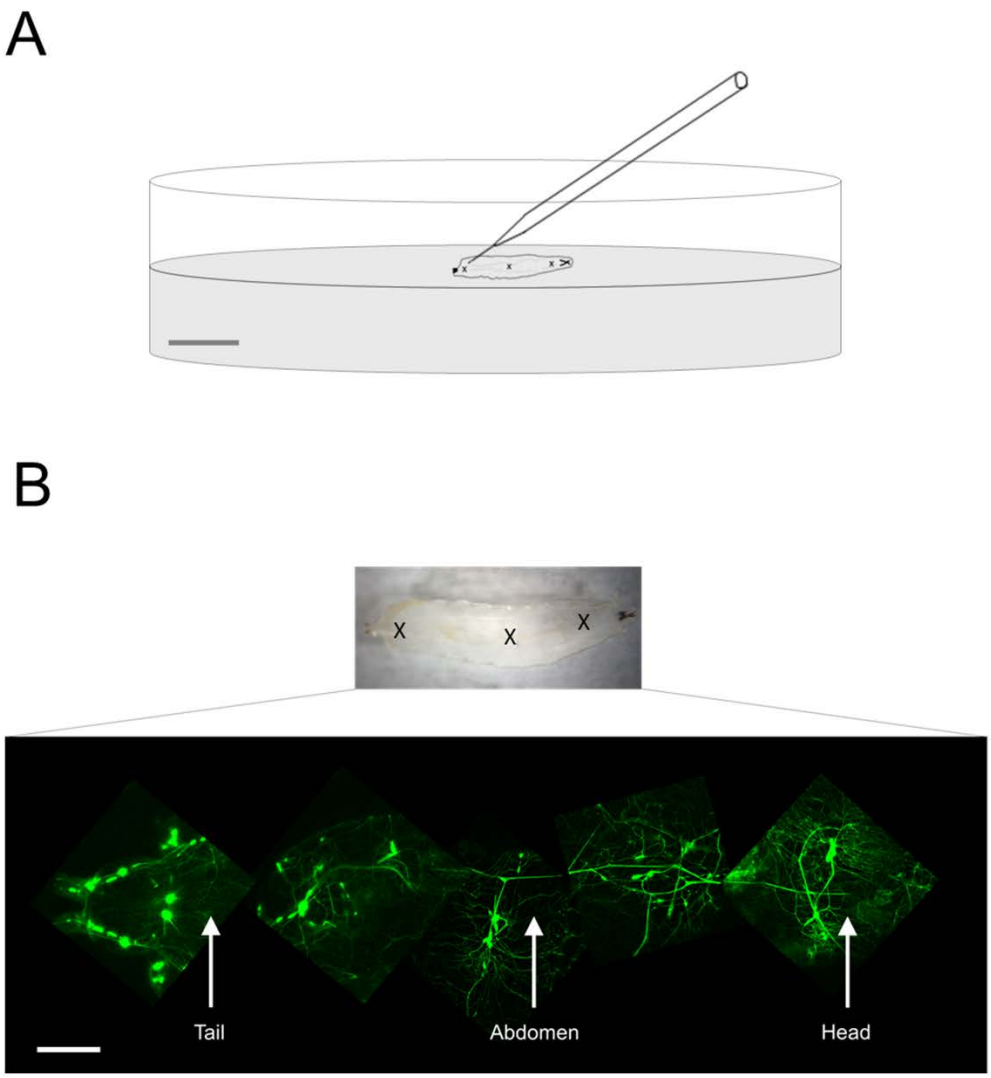

Figure 2.1. Overview of the mechanosensory assay and larval mechanosensation. *A*- Mechanical stimuli are delivered to the dorsal cuticle near the midline as the fly crawls on an agar-lined dish. The type of response, pause, roll, etc., was recorded from each stimulation. *B*- Peripheral nervous system of a third instar larva. The three localized regions that receive stimuli are shown (white arrows). The image is from a live animal expressing GFP tagged to a membrane-bound protein (elav>mCD8::GFP; 40x objective). Scale bars, 5mm (*A*) and 100µm (*B*).

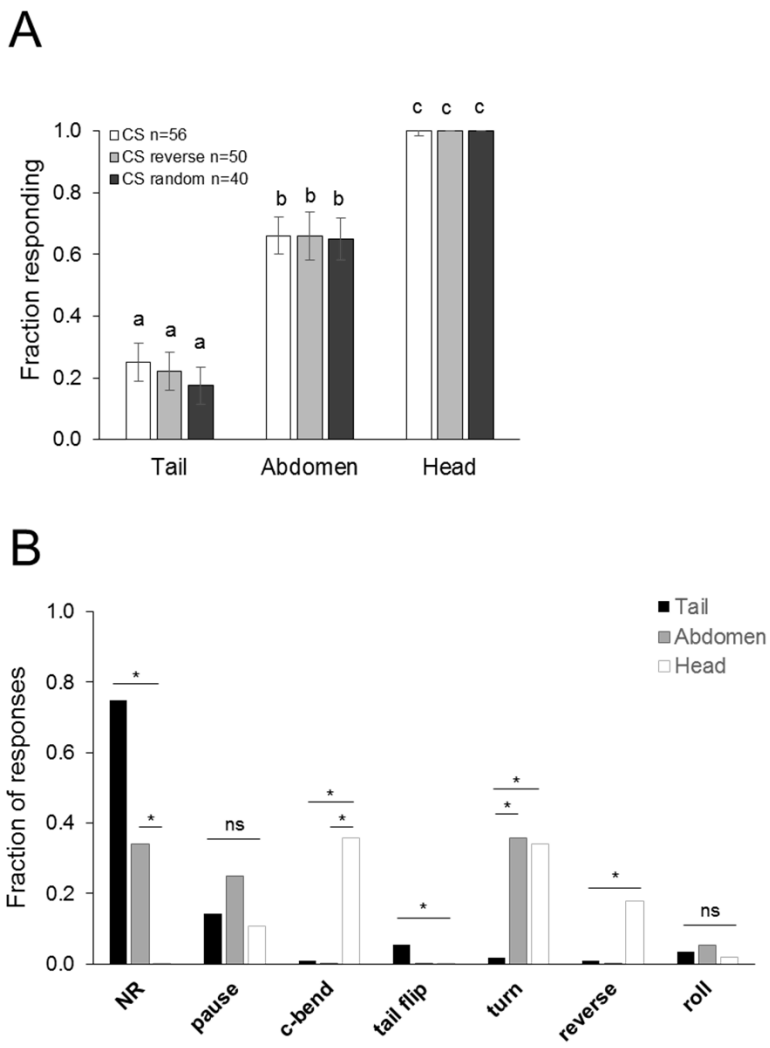

Figure 2.2. Canton S (CS) larvae are more sensitive in the anterior and abdominal regions than in the posterior region. *A*- Responsiveness is independent of the stimulus order (mean ± SEM; letters indicate similarity). *B*- Evoked behavior depends on location of the stimulus. No response (NR) is most commonly observed when the tail is touched. C-bends and reverse contractions are observed almost exclusively in response to head stimuli. Turns were observed more frequently in response to head and abdomen stimulations than tail stimulations. Pauses and rolling behavior were observed at relatively similar frequencies in response to all stimuli. (* $p < 0.05$).

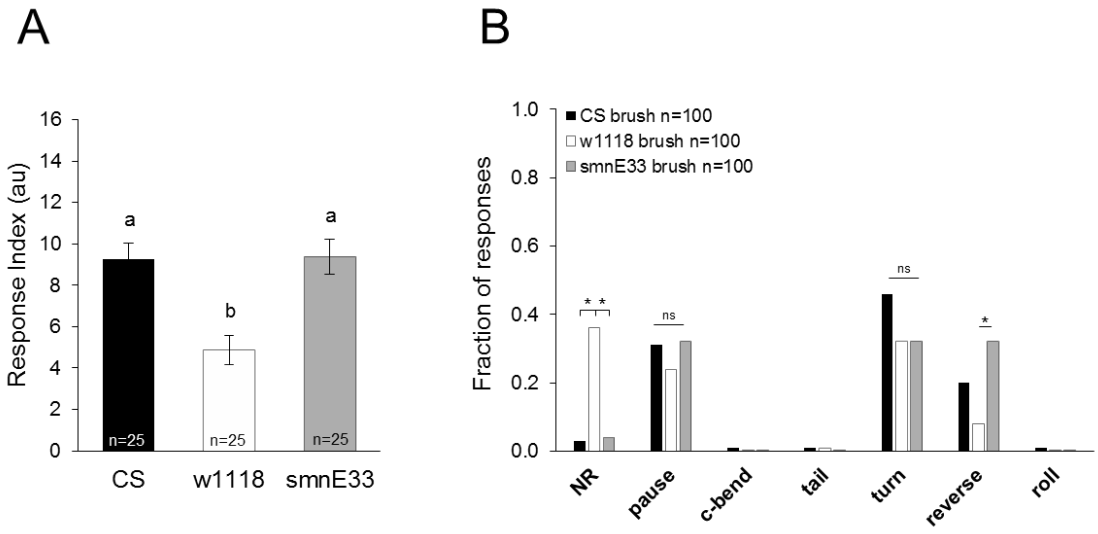

Figure 2.3. Mechanosensory responses to gentle anterior brushes in white, and smn mutants. *A*- Mechanosensory responses to gentle touch are shown using the scoring index from Kernan et al., (1994). Overall, *white* mutants were less responsive to these stimuli than CS or *smn* mutants (mean ± SEM; letters indicate similarity; n=number of animals tested). *B*- Distribution of responses to gentle innocuous stimuli. *Smn* mutants responded with reverse contractions more frequently (* $p < 0.05$; n=number of brushes, 4/animal).

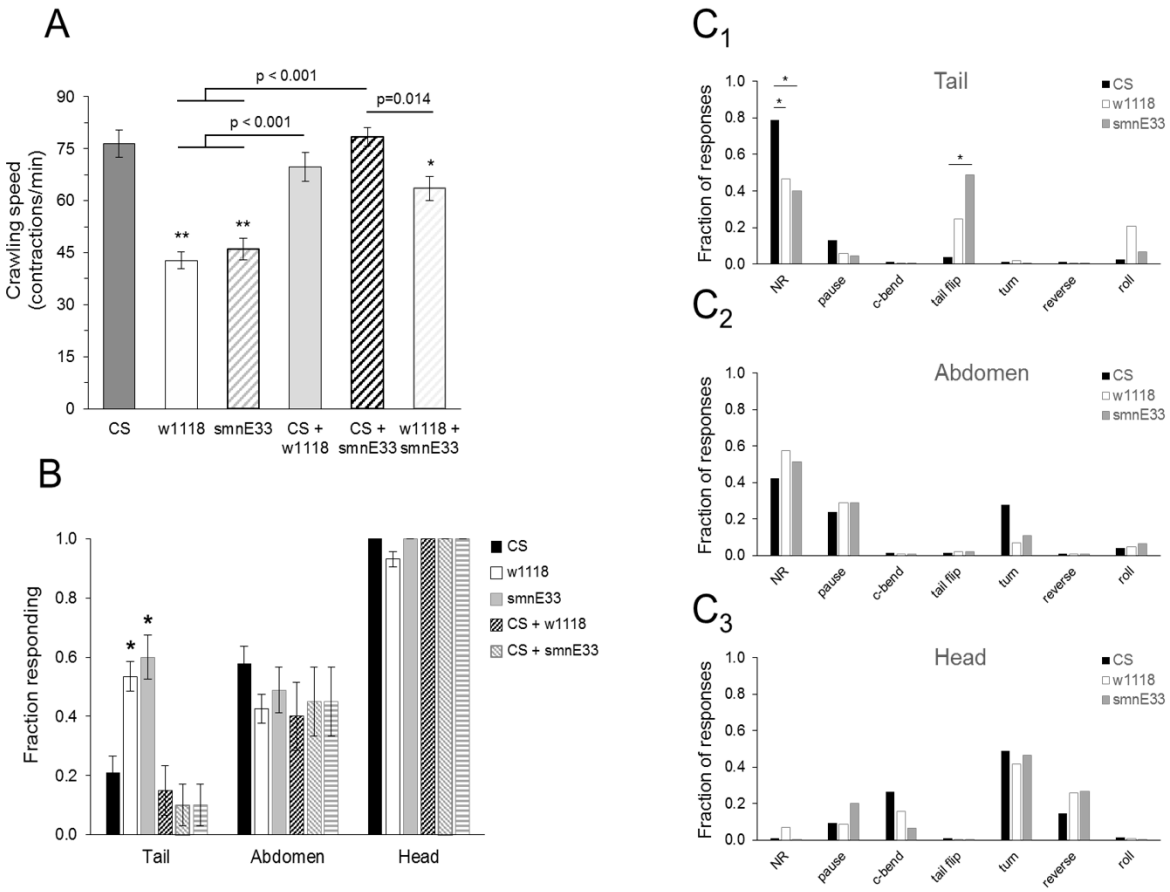

Figure 2.4. Mechanosensory responses and crawling speed in white and smn mutants. *A*- The *white* and *smn* mutants crawl slower than CS larvae. Combining either mutant with CS completely rescues the phenotype. Combining the *white* and *smn* mutations only partially rescues the phenotype. *B*- *Smn* and *white* mutants are more responsive to tactile stimuli on the tail. $C_{1\text{-}3}$ – Distribution of mechanosensory responses in w^{1118} and smn^{E33}. In response to tail stimuli, *smn* mutants perform more tail flips than *white* mutants or CS larvae. (* $p < 0.05$, ** $p < 0.005$; n=25 larvae for each genotype).

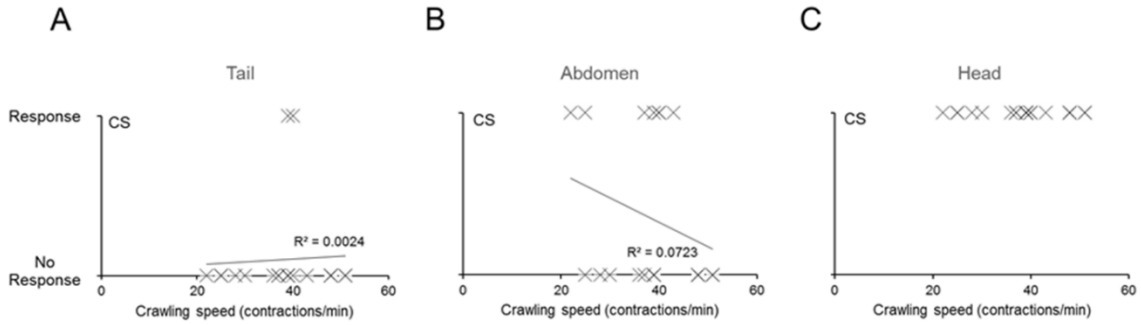

Figure 2.5. Mechanosensory responses are not correlated with crawling speed. A-C Data points indicate the crawling speed (x-axis) and whether or not the larva responded to 20mN tactile stimuli (y-axis). Linear regression analysis shows that there is no correlation between crawling speed and responsiveness in CS. The same was also true for smn and white mutants (not shown).

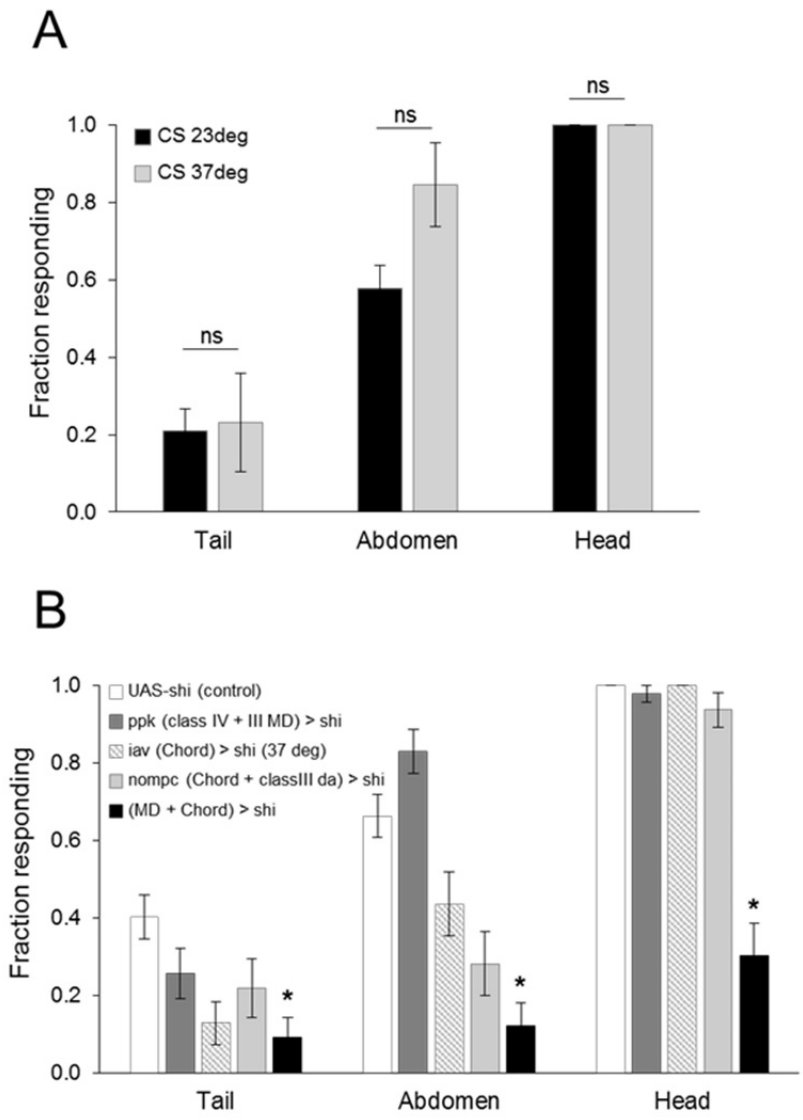

Figure 2.6. Neuronal silencing reveals specific types of neurons involved in mechanosensation at different anatomical regions (continued on next page).

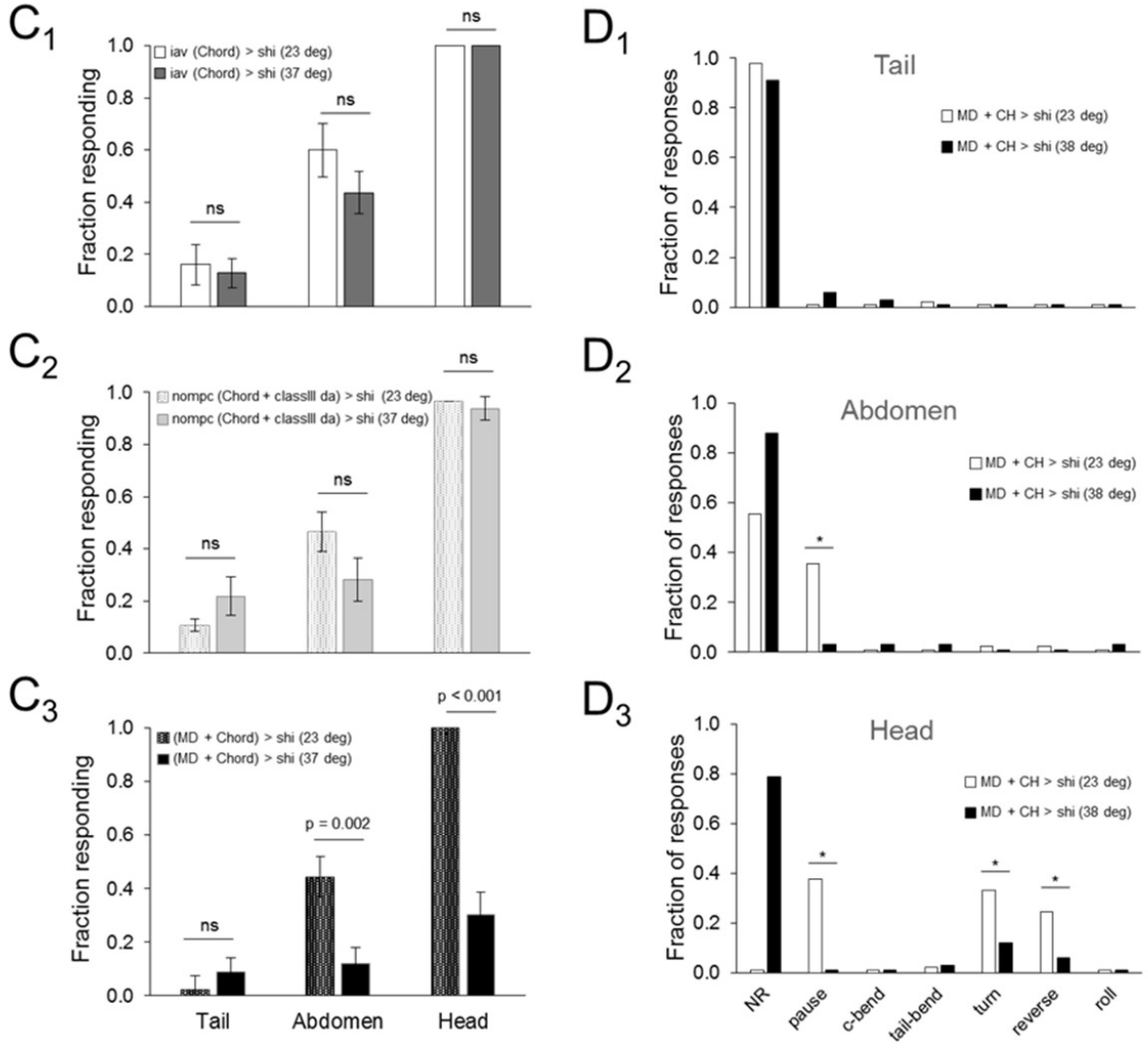

Figure 2.6. Neuronal silencing reveals specific types of neurons involved in mechanosensation at different anatomical regions. A- At high temperature (37°C), larvae are slightly more sensitive to tactile stimuli in the abdominal region, but not the head or tail region. B- Inhibiting chemical transmission in specific subsets of sensory neurons has distinct effects on mechanosensory responsiveness. Response probability is significantly reduced by silencing multidendritic and chordotonal neurons ($p < 0.05$ relative to UAS-shits1). C1-3 – When the sensory neuron driven GAL4 lines are compared at permissive and restrictive temperature, the only statistically significant effect was observed when silencing multidendritic and chordotonal neurons (mean ± sem; UAS-shits1 n=77 larvae, ppk>shits1 n=47 larvae, iav>shits1 n=39 larvae, nompC>shits1 n=32 larvae, MD+Ch>shits1 n=33 larvae). D1-3 In response to abdominal touches in the MD+Ch>shits, pauses were significantly reduced, whereas pauses, turning behavior, and reverse contractions were also reduced in response to head touches (* = $p < 0.05$).

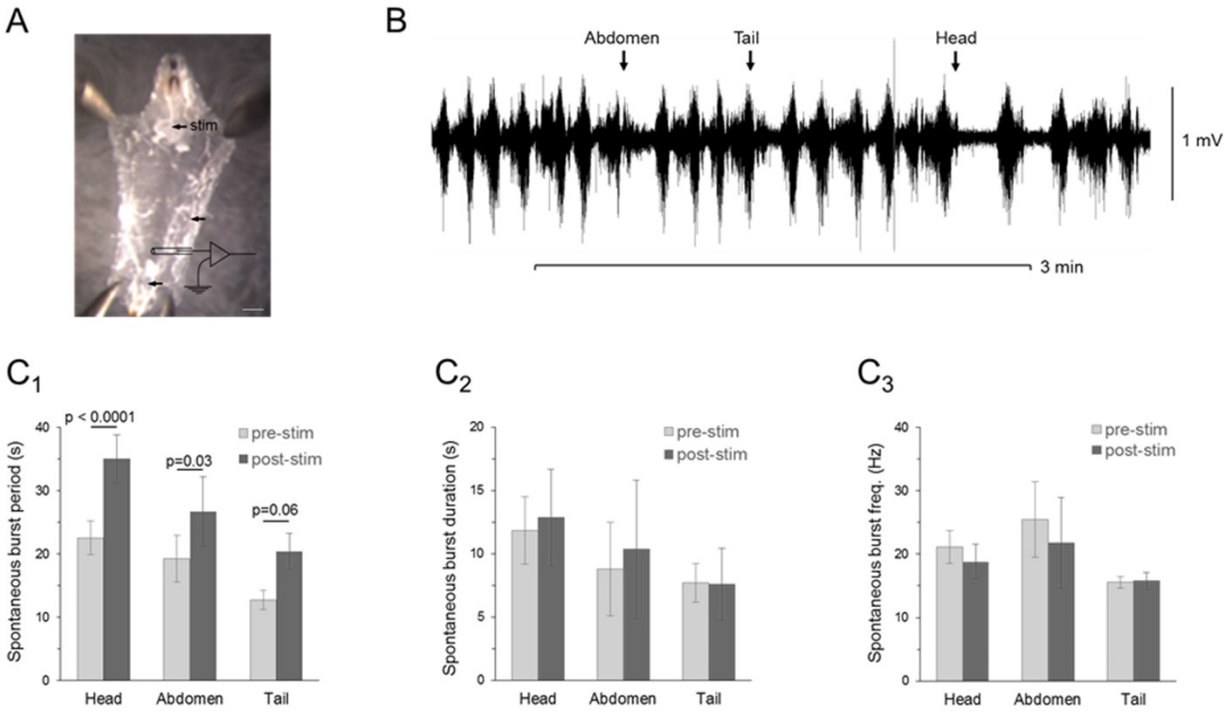

Figure 2.7. Extracellular recordings from a segmental nerve during fictive crawling. A- By not extending the longitudinal incision during dissection to the most anterior and posterior regions, the peripheral nervous system remains largely intact. En passant extracellular recordings were taken from a posterior segmental nerve, and stimuli were applied to the head, abdomen, and tail (black arrows) by advancing the insect pin prod with a micromanipulator. B- Extracellular recordings show rhythmic activity in the nerve. Stimulations (black arrows) typically caused the period between bursts to increase. C1- The average of three intervals before the stimulus (pre-stim) were compared to the interval following the stimulus (post-stim). The interval increased in response to touches in each region. There were no significant changes in spontaneous burst duration (C2) or burst frequency (C3). Scale bar = 500um. Tail stimuli n=6, abdomen stimuli n=6, head stimuli n=19.

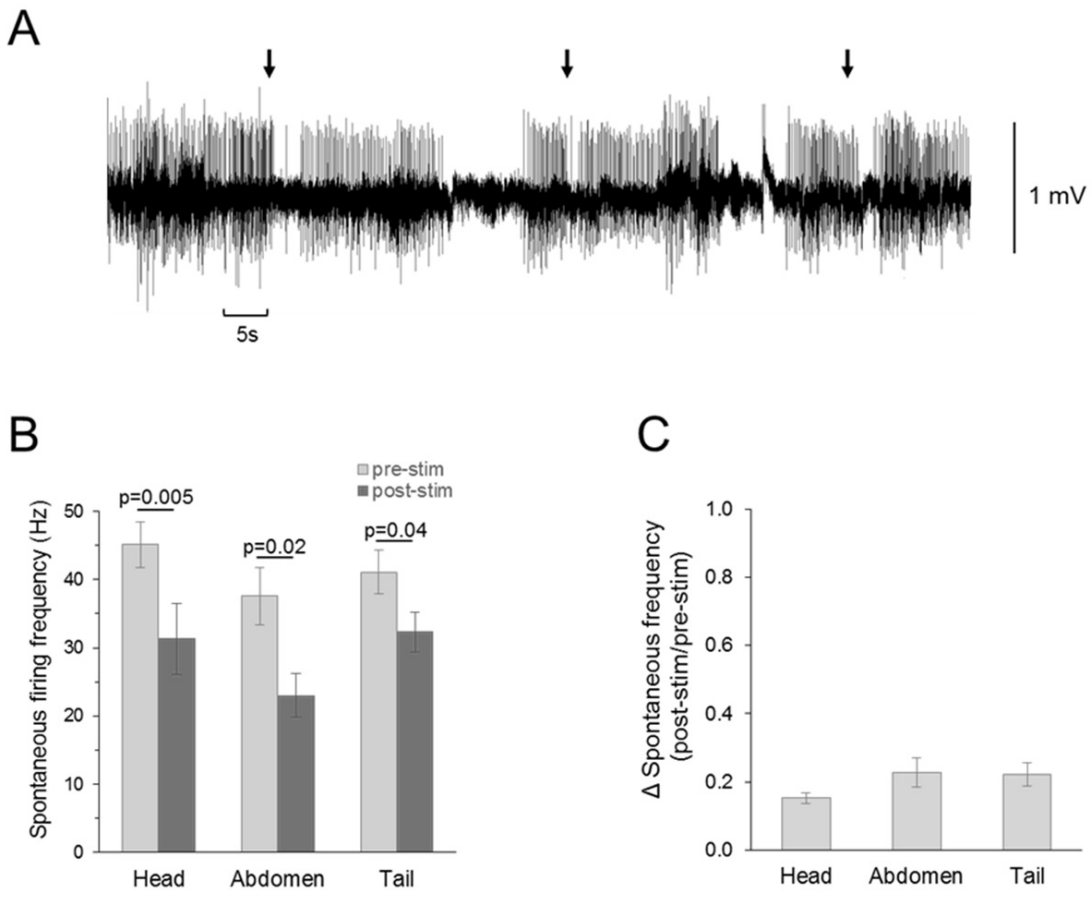

Figure 2.8. Extracellular recordings from a segmental nerve during non-rhythmic spontaneous activity. A- Extracellular recording that shows a typical decrease in spontaneous activity following mechanical stimulations (black arrows). B- Spontaneous activity decreased in response to tactile stimuli delivered in each anatomical region. Firing frequency was measured for 5s before (pre-stim) and 5s after (post-stim) the stimulus was delivered. C- The change in spontaneous frequency was the same for each anatomical region. Tail stimuli n=27, abdomen stimuli n=19, head stimuli n=15.

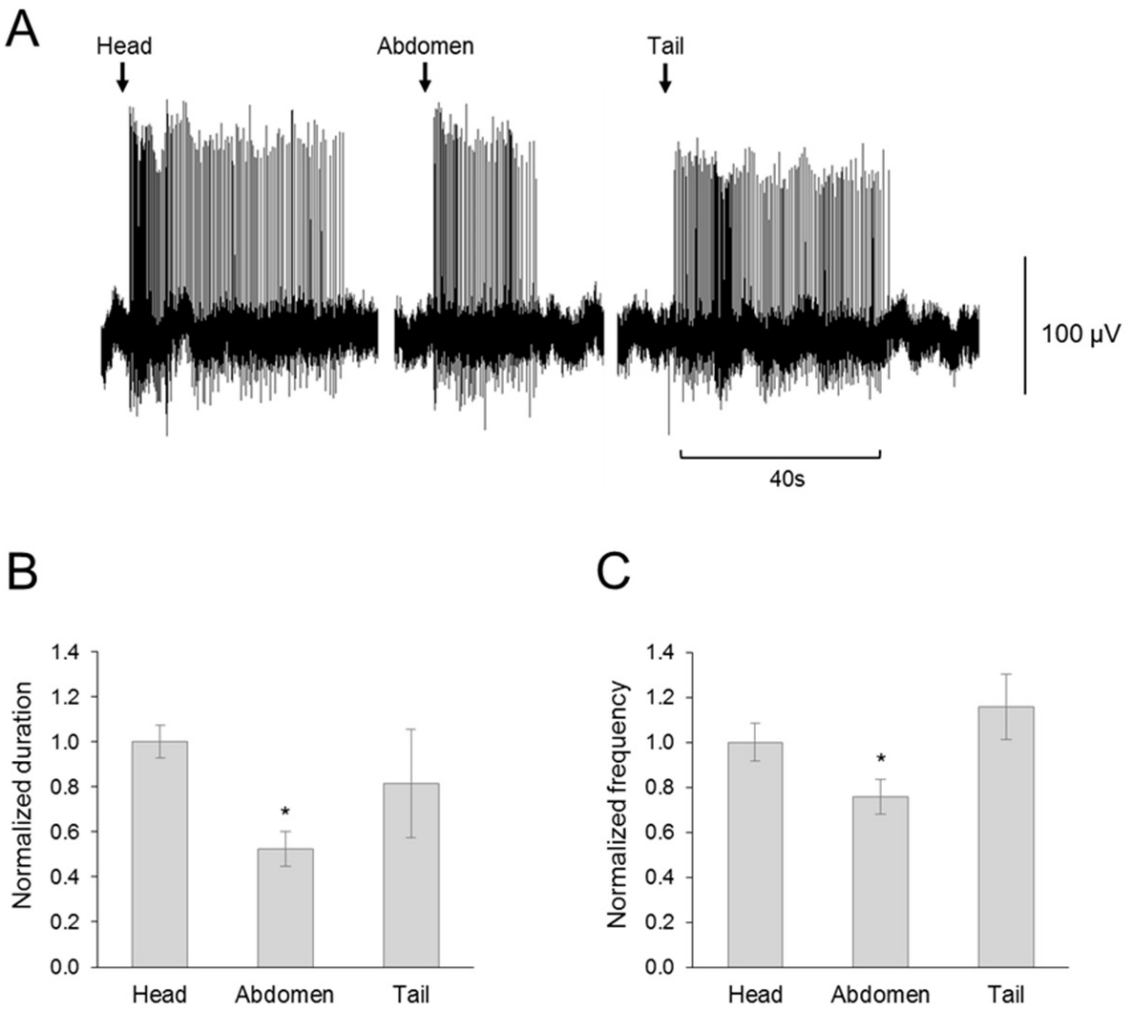

Figure 2.9. Extracellular recordings from a segmental nerve during inactive periods. A- Extracellular recording shows bursts of activity following three different stimuli (black arrows) in the same preparation. The duration and frequency of these bursts were normalized to the response to head stimulations in each prep. Normalized values were used to compare responses to stimuli delivered to the specific anatomical regions. On average, abdominal responses had a shorter duration (B) and slower frequency (C) than head and tail responses (* $p < 0.05$). Tail stimuli n=27, abdomen stimuli n=30, head stimuli n=31.

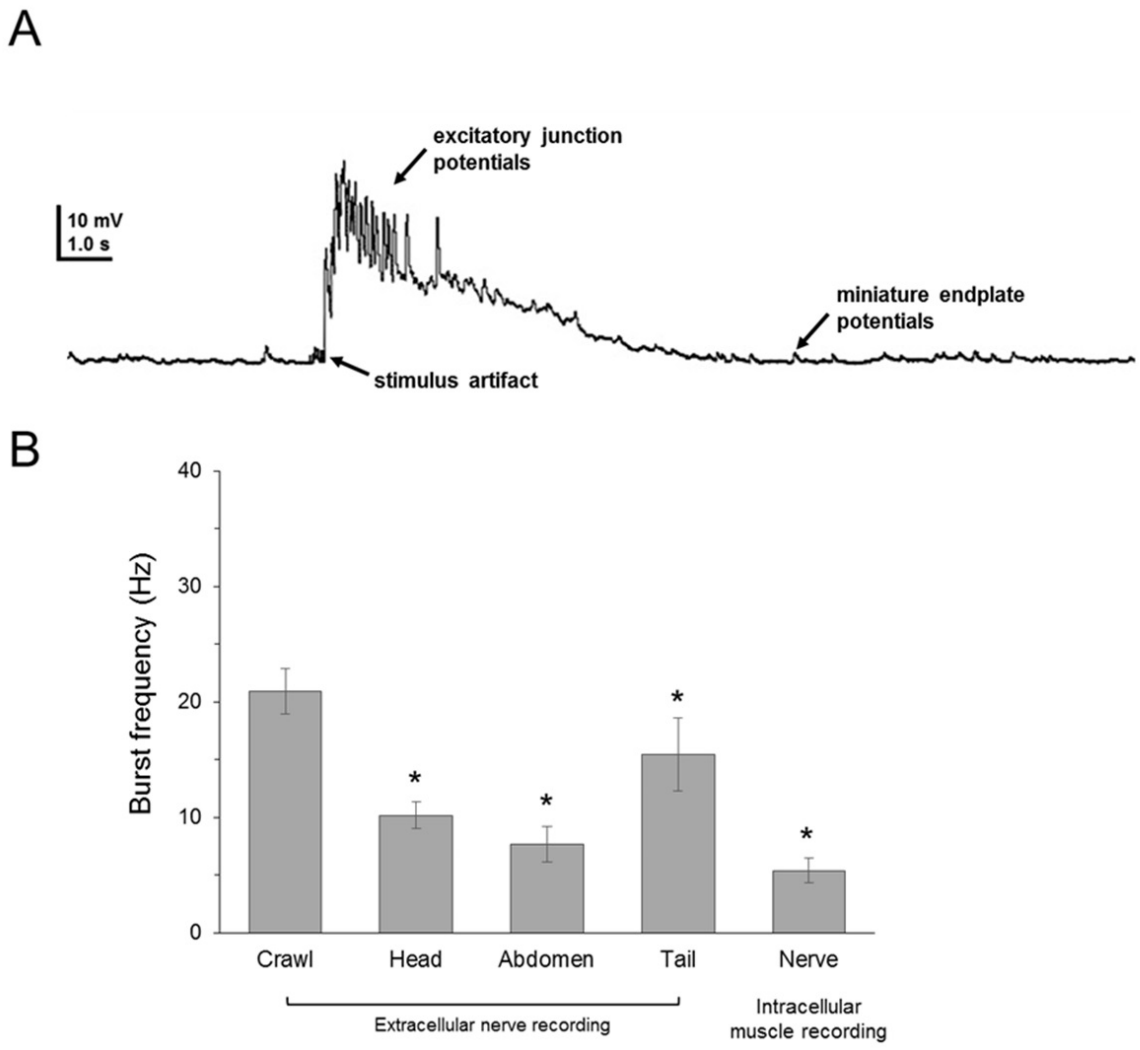

Figure 2.10. Differences in frequency between endogenous bursting and mechanosensory responses. A- Intracellular recording from muscle 6, segment 3, representative of activity that is evoked by stimulating segmental nerves in the posterior region with an electrode. B- Average frequency of endogenous activity and mechanosensory activity. Note that crawling and mechanosensory activity were recorded with an extracellular electrode, whereas nerve-evoked activity was a single cell recording from muscle 6. Endogenous bursts n=31, tail stimuli n=27, abdomen stimuli n=31, head stimuli n=31, nerve-evoked bursts n=7 (* $p < 0.05$).

CHAPTER THREE

Mechanosensory habituation in *Drosophila melanogaster* larvae.

*This chapter has been submitted for publication to Learning and Memory and is currently in review. Ms. Stephanie Biecker collected data that led to figures. I collected data, analyzed all of the data, and wrote the manuscript. Dr. Cooper edited the manuscript.

INTRODUCTION

Habituation is a simple form of cellular learning that is common among invertebrate and vertebrate organisms, and it is not actually simple. Changes in synaptic morphology and molecular organization are required to dampen an animal's response to irrelevant repetitive stimuli, and distinct changes may occur at different synapses throughout a given circuit (Paranjpe et al., 2012). Determining how this plasticity is distributed throughout multiple layers of the CNS is necessary to fully comprehend learning. Different experimental systems present unique advantages to addressing this problem and allow us to assess the generality of mechanisms.

Habituation at the neural circuit level has been described in the *Drosophila* olfactory response (Das et al., 2011) and proboscis extension reflex (Paranjpe et al., 2012). These studies, and others from crayfish and *Aplysia* (Stopfer and Carew, 1996, Shirinyan et al., 2006), show that potentiation of inhibitory neurons within the circuit is an important aspect of habituation. For the proboscis extension reflex (PER), adenylate cyclase-dependent cAMP mediates potentiation of GABAergic neurons that project to areas of the brain involved in PER (Paranjpe et al., 2012). In the olfactory circuit, potentiation of local inhibitory neurons causes habituation between projection neurons and olfactory sensory neurons in the antennal lobe (Sudhakaran et al., 2012). This involves synapsin in the pre-synaptic neurons and translation in both pre-synaptic and

post-synaptic compartments (Sadanandappa et al., 2013, Sudhakaran et al., 2014). An analogous circuit mechanism for habituation was also demonstrated for olfactory habituation in *D. melanogaster* larvae (Larkin et al., 2010). It is not clear how these central mechanisms apply to different sensory modalities in *D. melanogaster* or other organisms.

Though there is extensive knowledge of mechanosensation and motor output in *D. melanogaster* larva, mechanosensory habituation has not been investigated. Larvae respond to tactile stimuli with a limited repertoire of behaviors (Kernan et al., 1994; Titlow et al., In Press). The sensory neurons and molecular mechanisms that transduce tactile stimuli have been exquisitely characterized (Kernan et al., 1994, Tracey et al., 2003, Zhong et al., 2010, Kim et al., 2012, Robertson et al., 2013). The functional and anatomical identity of motor neurons involved in larval motor behavior have also been identified (Sink and Whitington, 1991, Kurdyak et al., 1994, Landgraf et al., 1997, Baines and Bate, 1998). Although the neural circuit for larval motor behavior is not completely known, several interneurons have been identified (Iyengar et al., 2011, Zhou et al., 2012) and it is clear that larvae crawl without input from the brain (Berni et al., 2012). As a complement to ongoing efforts that use larvae to understand molecular mechanisms of mechanosensation (Tsubouchi et al., 2012, Zhou et al., 2012, Ohyama et al., 2013), our aim was to dovetail that research into a system for investigating plasticity.

In the current study we delivered repetitive tactile stimuli to investigate plasticity in larval mechanosensory responses. Stimulus paradigms were modeled after *C. elegans* short-term habituation experiments (Sanyal et al., 2004), and there are some similarities between the two organisms in the habituation curves and signaling pathways involved (Kindt et al., 2007). Surprisingly, the results from our electrophysiology experiments reveal that larval sensorimotor circuits may also become sensitized to

activation of afferents in the segmental nerve. These data lay the groundwork for a new system to study plasticity at the neural circuit level.

MATERIALS AND METHODS

Animals

D. melanogaster were cultured in standard cornmeal fly food medium at 23°C and 75% humidity on a 12hr light/dark cycle. The following strains were used: Canton-S (wild type); Dop1R1 ^{f02676} (Lebestky et al., 2009); Dop2R^{f05621} (Liu et al., 2012); *dnc*^{1} (Byers et al., 1981); and *rut*^{1} (Levin et al., 1992). *Dnc* and *rut* mutants were obtained from Bloomington *Drosophila* Stock Center, dopamine receptor mutants were a gift from Dr. Kristin Scott.

Larvae were tested at the early third instar stage unless noted otherwise. When younger animals were used, larvae from all three stages were taken from the same vial to control for environmental effects. Vials were populated by 10-20 adults for a maximum of four days to avoid crowding stress.

Tactile stimulus paradigm for short-term habituation

The gentle touch assay has been described previously (Kernan et al., 1994). Individual larvae were touched with a single paint brush fiber as they crawled on a petri dish lined with agar (1% agar, 33% apple juice). The touch stroke was applied laterally across the first 2-3 anterior segments at intervals specified in figure legends. If not specified, a 5s inter-stimulus interval (ISI) was used to provide the most robust habituation. Responses were observed through a microscope and recorded during the experiment. Scores were binary for the majority of experiments, a score of 1 was given if the larva stopped crawling or executed any type of behavioral response to the stimulus,

0 was given if the stimulus did not interrupt crawling. Vials were coded to prevent experimenter bias when testing genotypes.

Responses from more forceful tactile stimuli are also reported and those procedures were performed as previously described (Titlow et al., In Press). For those experiments larvae were repeatedly prodded on the dorsal midline with an insect pin (0.2mm diameter) with enough force to indent but not puncture the cuticle. This force has been calibrated using an analytical balance (20mN; Titlow et al., In Press).

Recording evoked sensorimotor circuit activity

Electrophysiology experiments were performed as previously described (Dasari and Cooper, 2004). A modified HL3 saline was used for dissection and recording: NaCl 70 mM, KCl 5 mM, MgCl2.6H2O 20 mM, NaHCO3 10 mM, trehalose 5 mM, sucrose 115 mM, CaCl2.2H2O 1 mM, and BES 25 mM and pH 7.1. The CNS and segmental nerves were left intact while the larva was filleted open from the dorsal midline. EPSPs were evoked by stimulating 2 or 3 posterior segmental nerves with a glass pipette suction electrode. Stimulus trains were 10 pulses at 40Hz delivered at ISIs indicated in the figures. Intracellular recordings were obtained from ventral longitudinal muscle 6 in either the 3rd or 4th abdominal segment. Signals were amplified with an AxoClamp 2B, sampled at 10 KHz and digitized with a Powerlab 4SP A/D board (ADI). Data were collected and analyzed offline using LabChart7 (ADI). The number of EPSPs evoked by each stimulus was counted automatically with a cyclic measurement tool that detects the number of peaks above a specified threshold. The evoked EPSPs were counted from the beginning of one stimulus to the beginning of the next stimulus.

Data analysis

To compare mechanosensory response probability within a single condition, binary responses from the last three trials with each larva were pooled together and compared to the average binary response over the first three trials using Students paired t-test. To compare mechanosensory response probability between different treatments, the average binary response over the last three trials of each larva was normalized to the average response from the first three trials of that larva. The normalized responses from each larvae were pooled and compared to other treatments or genotypes using one-way ANOVA. A Shapiro-Wilk test was used to check for normality. Data that failed this test were compared using Kruskal-Wallis ANOVA on ranks with Dunn's post-hoc test for comparisons between each treatment (SigmaPlot v 12.3). To compare distributions of responses, the proportions of each response were compared using Chi-square analysis with Tukey-type multiple comparisons (SAS v9.3).

The number of evoked EPSPs in each response was normalized to the average of the first five responses for each larva, and data from individual larvae were pooled for each ISI (mean ± SEM). To compare the responses within a given ISI, we systematically compared the baseline average (first five responses) to averages of three subsequent responses throughout the experiment using Student's paired t-test, i.e., average of 1-5 was compared to 6-8, and to 7-9, 8-11, etc.

RESULTS

Drosophila larvae habituate to innocuous tactile stimuli

Crawling larvae alter their motor pattern in response to a light brush on the anterior segments (Kernan et al., 1994, Tsubouchi et al., 2012). We used this experiment to determine if larvae exhibit plasticity in response to tactile stimuli (Figure 3.1A). Initially, 93.7% of animals responded to the stimulus. Over multiple trials, the

probability of an animal responding to the stimulus decreased by more than 50% (Figure 3.1B). Some individuals stopped responding after 15-20 stimuli, but the majority of animals responded at lower frequencies. Figure 2A shows representative sequences of responses from two different larvae. Reverse contractions, turning, bending, and pausing were observed more frequently in response to the first 5 stimuli, whereas the most common behavior is no response (NR) in responses 10-25. A distribution of the responses from 25 larvae stimulated 25 times is shown in Figure 3.2B. Note the steady increase in NR and decrease in turning behavior. There was no obvious pattern in the other responses. From 750 total stimulations, 45.8% exhibited NR, 33.4% turned after pausing, 13.0% paused and continued in the same direction, 9.9% executed reverse contractions, and fewer than 1% executed a whole body bend (Figure 3.2C). Interestingly, of the 33% that turned after pausing, the majority of larvae turned away from the stimulus, but 30% turned toward the stimulus. After 14 stimulations the turning direction became less predictable (Figure 3.2D).

One way to demonstrate that the decrement in overall response probability is due to habituation is by showing that the decrement is not caused by sensory fatigue. In these experiments the first 25 stimuli were delivered to the right side of the anterior segments, then five additional stimuli were delivered to the left side. Sensory neurons on the contralateral side would not have been activated by the initial stimuli, yet the probability of response to those stimuli was significantly less than the initial response, suggesting that sensory fatigue does not cause the larvae to be less responsive. The second aspect of the response that is indicative of habituation is the effect of ISI (Thompson and Spencer, 1966, Davis, 1970). As shown previously in other sensory systems, stimulating *D. melanogaster* larvae at a slower rate resulted in slower, less prominent habituation (Figure 3.1B).

Spontaneous recovery is a common feature of habituation. After 30 light tactile stimulations at 5s ISI, larvae spontaneously recover from habitation rather quickly. Response probability began to increase after 10s, and by 5min the probability returned to 90% of the initial value (Figure 3.3A). To determine if the response decrement was caused by motor fatigue, and to determine if the circuit could be dis-habituated (Kupfermann et al., 1970, Rankin et al., 1990), we delivered a more noxious stimulus to the abdomen between the 25th and 26th stimuli. Responses after the dis-habituating stimulus returned to initial levels (Figure 3.3B), providing further support that the gradual decrease in responsiveness is a form of habituation.

Drosophila larvae do not habituate to 20mN tactile stimuli

We have shown that larvae are also responsive to stronger tactile stimuli on the dorsal midline near the head (20mN), but fewer than 20% of animals respond to the same stimuli applied near the tail (Titlow et al., In Press). Here we tested both stimuli repeatedly (20 stimuli with 5s ISI) to determine if the response changes over time. The average probability of response to the first three stimuli was the same as the last three stimuli, indicating that mechanosensory responsiveness was not affected by repetitive stimulations (Figure 3.4A). It is surprising that the 20mN stimulus was too strong to enable habituation in the head region, yet too innocuous to cause sensitization in the tail region. One possibility is that the caudal mechanosensory circuit is not capable of sensitization, or that sensitization requires a different strength or stimulus paradigm. For the rostral mechanosensory responses, we also quantified the different types of response behavior over the course of repeated stimulations and found that the distribution of behaviors did not change (Figure 3.4B). Instead, action selection maintained a probabilistic distribution from the first stimulus to the last.

Developmental differences in mechanosensory habituation

We hypothesized that plasticity in the mechanosensory circuit would decrease throughout larval development. To test this hypothesis, the tactile habituation assay was performed on 1st, 2nd, and 3rd instar larvae. Surprisingly, the magnitude of habituation increased throughout larval development (Figure 3.5A), with 3rd instars showing nearly a two-fold greater decrease in response probability than earlier stages (Fig. 5B).

Signaling pathways involved in larval mechanosensory habituation

As a first step towards characterizing the molecular mechanisms of mechanosensory habituation in larvae, we tested mutants for four different genes that have known associations with learning and memory. The type-1 dopamine receptor plays a role in olfactory associative memory in *Drosophila* (Berry et al., 2012, Li et al., 2013b), and in *C. elegans* (Sanyal et al., 2004). The type-2 dopamine receptor is more closely associated with motor behavior in flies, but in mammals there have been associations with the type-2 receptor in various types of learning (Piray, 2011). The *D. melanogaster* adenylate cyclase gene, *rut*, and the phosphodiesterase gene , *dnc*, are involved in both associative and non-associative forms of learning in flies (Engel and Wu, 2009). We acquired previously characterized mutants for each of these genes and generated habituation curves for each of the mutants. The type-2 dopamine receptor mutant $Dop2R^{f05621}$ carries a hypomorphic allele that exhibits a 74% decrease in transcript level (Liu et al., 2012). $Dop2R^{f05621}$ larvae habituated to repetitive tactile stimuli (Fig. 6A), but the average decrease in response probability was less than half as much as habituation exhibited in wild type larvae (Figure 3.6C). The type-1 dopamine receptor mutant $Dop1R1^{f02676}$ is a strong hypomorph that reduces transcript abundance by 95% (Lebestky et al., 2009). This mutant did not exhibit an habituation phenotype (Figure 3.6A,C). The dnc^{1} (hypomorph) and rut^{1} (loss-of-function) ethane methylsulfonate

mutants were also tested in this habituation paradigm (Byers et al., 1981, Levin et al., 1992). The *rut*^{1} mutant exhibited a decreased habituation phenotype comparable to the type-2 dopamine receptor mutant (Figure 3.6B,C). The *dnc*^{1} mutant did not exhibit an habituation phenotype (Figure 3.6B,C), however tactile sensation was significantly reduced in these mutants (Fig. 6D). Baseline tactile sensation was unaffected in the other mutant backgrounds. These data implicate the type-2 dopamine receptor and Ca2+/calmodulin dependent adenylate cyclase in larval mechanosensory habituation.

Effect of repetitive nerve root stimulation on sensory-motor output in larvae

Segmental nerves carry afferent and efferent connections between the larval CNS and the body wall. Our lab has developed an electrophysiological assay to measure sensory-evoked motor output in the larva fillet preparation (Dasari and Cooper, 2004). Here we used this assay to determine if mechanosensory habituation could be observed at the cellular level. Ten-pulse trains (40Hz) of suprathreshold stimulation to the posterior segmental nerves evoked short bursts of activity (43.4 ± 10.7 EPSPs at ~ 10Hz; Figure 3.7B). The prediction was that the number of evoked EPSPs would decrease after repeated stimulation. Using ISIs of 5s, 10s, or 60s, the evoked output rarely decreased, in fact when multiple preparations from each ISI were pooled together and normalized, the number of evoked EPSPs typically increased after multiple stimulations (Figure 3.7C-E). A similar pattern emerged when stimulations were resumed after a 10min recovery period in each experiment (data not shown).

Given that individual habituated larvae occasionally respond to the repeated stimulus, an alternative prediction for the electrophysiology experiments might have been that the response intensity doesn't change overall, but that the number of failed responses increases after multiple stimulations. There were occasional failed responses in the electrophysiology recordings (24 failures/390 stims for 5s ISI; 1/210 for stims for 10s ISI; and 2/195 stims for 60s ISI), and the proportion of failures at 5s ISI was

significantly higher than the proportions of failures at longer ISIs ($p < 0.05$, Chi-square), but the failures do not correlate with the number of previous stimuli, they appear to occur randomly throughout the experiment. Therefore we favor the interpretation that short-term sensitization is occurring in response to this stimulation paradigm.

DISCUSSION

Habituation provides a window into the mechanisms of activity-dependent neural plasticity. Working out the molecular details of habituation in different circuits across phyla gives us an understanding of which mechanisms are general principles, and which mechanisms are unique to a specific circuit. Here we introduce mechanosensory habituation in *Drosophila* larvae, which has unique assets for investigating learning and memory. This system exhibits many of the hallmarks of habituation found in other organisms and some unique aspects that will require further investigation.

Larval mechanosensory habituation meets established criteria for habituation

Nine criteria put forth nearly 50 years ago continue to serve as adequate criteria for defining habituation (Thompson and Spencer, 1966), though slight modifications have been suggested (Christoffersen, 1997). The following criteria from those works were observed in larval mechanosensory habituation, 1) responses decline after repeated stimuli, 2) responses recover spontaneously after repeated stimuli, 4) increased frequency of stimulation increases the amount of habituation, 5) the rate of habituation decreases with increasing stimulus strength, and 8) a strong stimulus different from the habituating stimulus can cause dis-habituation. Though we didn't test for generalization of depression for other types of sensory input, we did show that the response to the same stimulus applied to different sensory fields was habituated. Responses from stimuli on the contralateral side remained attenuated relative to initial

responses, but there was a slight increase in response probability. This suggests that there are lateralized and centralized layers to habituation (Bristol et al., 2004).

Christoffersen (1997) also suggests distinguishing between fast and slow habituation to separate processes where the response decreases by 50% within the first 5 stimulations, e.g., crayfish tail flip (Krasne and Bryan, 1973), from processes where habituation occurs more gradually, e.g., sea slug tentacle withdraw (Horn et al., 1970). Differences in kinetics may be due to the ethological importance of the behavior, with fast habituation being associated with reflexes that endanger the organism or require a substantial amount of energy, and slow habituation being associated with less costly maneuvers. Larval *Drosophila* mechanosensory habituation has a fast phase that decreases by 40% within the first 5 stimuli, and then slowly continues to decrease (Fig. 1B, white circles). The unique aspect of this behavior is that it is not a simple reflex, rather the stimulus evokes different behaviors ranging from a short pause to reverse contractions. How these different outputs emerge from the underlying motor circuitry is unknown, but we suspect that they are evoked by different patterns of activity from interneurons that receive afferent input. Any of these synapses or local feedback circuits could be involved in producing habituation (Sudhakaran et al., 2012). With recently developed high-throughput methods to quantify larval mechanosensory behavior (Ohyama et al., 2013), it will be feasible to determine the sets of interneurons and molecular pathways that are involved.

How is mechanosensory habituation integrated with central pattern generation?

Larval mechanosensation occurs in the context of rhythmic crawling behavior. Afferent input is presumably superimposed onto sets of neurons with rhythmically oscillating patterns of activity (Caldwell et al., 2003, Song et al., 2007). While crawling, feedback of sensory neurons modulates rhythmic activity by telling the CNS that

muscles in that segment have contracted (Hughes and Thomas, 2007), which then allows motor neurons in the next segment to activate. Activation of motor neurons at the forefront of the contractile rhythm are required to activate neurons in subsequent segments (Inada et al., 2011). This means that to respond to tactile input, mechanosensory neurons have to stop the contractile rhythm, and then either activate a distinct set of motor units or alter the activity pattern of motor units involved in crawling. Chordotonal neurons and class II and III multidendritic neurons are known to be gentle touch sensors (Tsubouchi et al., 2012, Zhou et al., 2012). The neurons that relay tactile information from these neurons to motor neurons have not been identified, but mechanosensory habituation is likely to occur in interneurons that can stop peristalsis. Our lab has previously shown that applying a tactile stimulus during fictive crawling causes an increase in the period of spontaneous bursting patterns (Titlow et al., In Press). It is possible that during habituation the cells mediating this response undergo synaptic changes in response to repetitive stimuli.

The ethological relevance of habituation to tactile stimulus is arguably similar to habituation in other contexts. Larvae need not respond to every gentle stimulus they encounter, the danger being that the response behaviors are fixed action patterns that could easily be predicted by predators. This would be especially important to regulate in later stages of larval development when larvae leave their food source to pupate, and is one possible explanation for why younger larvae exhibited less habituation in this study (Figure 3.5). Another reason for less habituation in earlier stages could be more proximal, e.g., developmental differences in molecular or morphological arrangement of the mechanosensory circuit. Nonetheless, random distribution of response behaviors is intuitively beneficial for avoiding predation, and these gentle touch response behaviors have been observed in response to parasitoid wasps (Robertson et al., 2013).

In the current study, turning behavior was the most frequent response observed and turns were executed in response to less than 50% of the stimuli. The majority of turns were away from the stimulus, but 30% were toward the stimulus. Though this seems like a high instance of miscalculation, turns toward the stimulus are not uncommon in the animal kingdom (Eaton and Emberley, 1991, Domenici et al., 2009, Domenici et al., 2011). Light-evoked nocifensive responses in larvae were directed toward the stimulus over 90% of the time (Hwang et al., 2007). Surprisingly, the directional bias for light touch in the current study decreased after several stimuli (Figure 3.2D). Additional experiments are underway to determine if this change in bias was due to habituation or stress from the “attacks”.

Adenylate cyclase and dopamine signaling are involved in mechanosensory habituation

It comes as no surprise that adenylate cyclase and dopamine receptor mutants exhibit mechanosensory habituation phenotypes in larvae. The rutabaga adenylate cyclase has been associated with several different forms of non-associative and associative learning in flies (Duerr and Quinn, 1982, Tempel et al., 1983, Engel and Wu, 2009, Paranjpe et al., 2012). Dopamine signaling has also been widely associated with learning in flies (Kim et al., 2007, Berry et al., 2012), although typically the type-1 dopamine receptors mediate learning through a cAMP-dependent protein kinase pathway. The type-2 dopamine receptor is more frequently linked to locomotor activity (Draper et al., 2007, Riemensperger et al., 2013) and arousal (Andretic et al., 2005, Lee et al., 2013). These functions are typically addressed in the brain, so it is unclear how dopamine is involved in plasticity within the ventral nerve cord (VNC). There are both paired and unpaired rows of dopaminergic neurons in the VNC (Selcho et al., 2009) with widely distributed axon terminals that provide numerous possibilities to interfere with

mechanosensory habituation. From light-level immunohistochemistry, it appears that many of the cells expressing dopamine receptors in the VNC are interneurons (Draper et al., 2007, Selcho et al., 2009).

Electrophysiology reveals evidence for sensitization in larval mechanosensory circuit

Our aim was to find a cellular correlate of habituation using a standard electrophysiology protocol. However, the stimulus paradigm we used did not induce habituation in the motor circuit that innervates m6 (Figure 3.7). The only variable that was manipulated was ISI. It is possible that altering the stimulus frequency or the stimulus duration could generate different results. It is also possible that stimulating a different combination of nerves could cause habituation. Though the stimulus was delivered to anterior segments in behavior experiments, posterior segmental nerves were chosen for electrophysiology because they gave the most robust and reliable response. Given that the responses typically increased after multiple stimulations, and segmental nerves carry all classes of sensory neurons, it is likely that broad activation of segmental nerves sensitizes the CNS. The occurrence of sensitization and habituation in this reflex arc would not be uncommon (Prescott, 1998), as both forms of plasticity are seen in the *Aplysia* gill withdrawal (Kandel and Schwartz, 1982) and *P. clarkia* tail flip escape (Krasne and Glanzman, 1986).

Comparison with tactile habituation in other organisms

Invertebrate species are invaluable for studying neural circuit plasticity because of their identified neurons that are correlated with specific behaviors. Crayfish reflex arcs provide electrophysiological evidence of multi-layered habituation. Synaptic depression

between tail afferent neurons and first order interneurons is modulated by extrinsic input (Krasne and Bryan, 1973, Krasne and Teshiba, 1995) and by feedback from giant fiber activation (Bryan and Krasne, 1977). Insight into how habituation and other forms of plasticity emerge from within a single mechanosensory circuit has been gained from the *Aplysia* siphon withdrawal reflex arc (Bristol and Carew, 2005, Hawkins et al., 2006). Molecular mechanisms of habituation are also well-characterized in this system (Ezzeddine and Glanzman, 2003, Esdin et al., 2010). Work in the *Caenorhabditis elegans* tap response circuit has also contributed a number of mechanistic details on molecular pathways (Timbers and Rankin, 2011, Li et al., 2013a) and different forms of mechanosensory habituation (Bozorgmehr et al., 2013). Like *C. elegans*, *D. melanogaster* larvae have mechanosensory neurons with different thresholds that innervate their skin to detect tactile stimuli. *Hirudo medicinalis* has a similar body type and innervation pattern that exhibits mechanosensory habituation and sensitization (Burrell and Sahley, 1998). A key difference in these mechanosensory pathways is a few orders of magnitude in the number of neurons. As we learn more about the cellular and molecular mechanisms of habituation in *D. melanogaster*, we can draw comparisons between them and *C. elegans* and *Hirudo medicinalis* to see how plasticity scales with neural network size throughout evolution.

Conclusion

At the level of single synapses, mechanisms for habituation have been described for sensory systems in a variety of organisms. The next challenge is understanding how plasticity emerges at the level of heterogeneous neural circuits. Mechanosensory behavior in *Drosophila* larvae is an attractive system to study neural circuits because behavioral experiments can be combined with optogenetics, electrophysiology, and a

large array of mutants. Here we show that larval mechanosensory habituation is regulated by canonical signaling pathways and developmental timing.

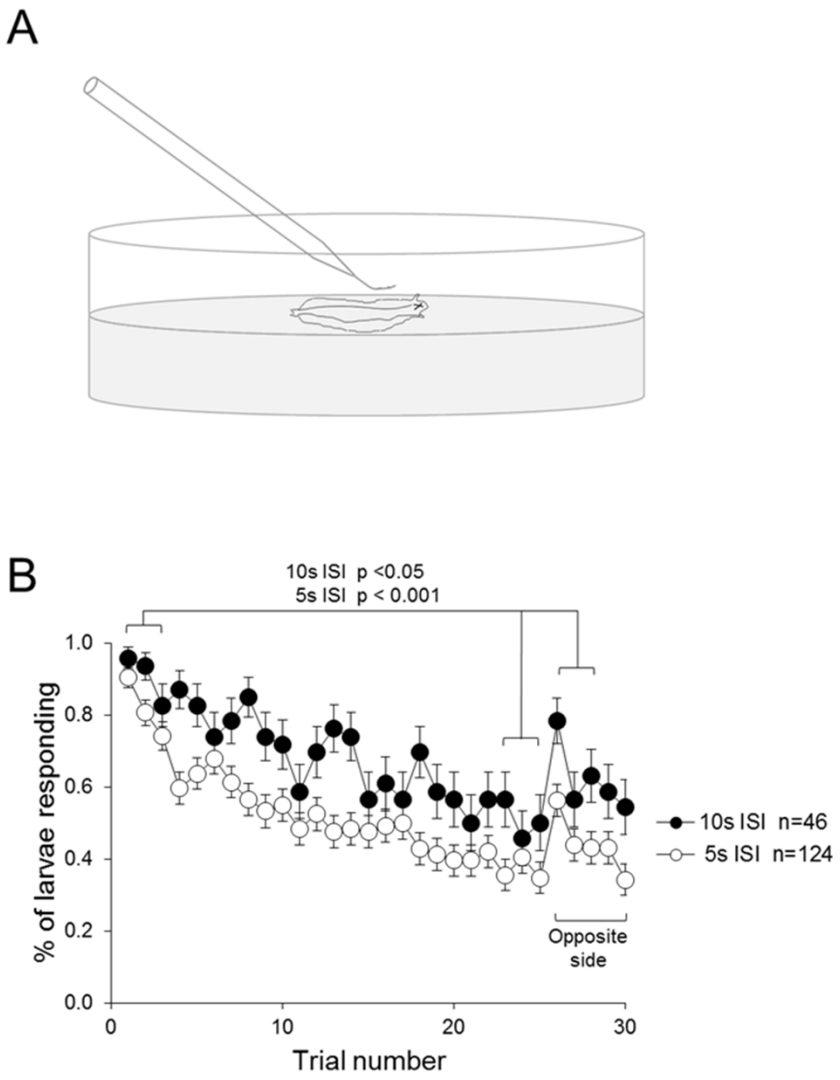

Figure 3.1. Short-term mechanosensory habituation in Drosophila larvae. *(A)*- Larvae were repeatedly brushed across the lateral anterior segments with a single paint brush fiber to evoke behavioral responses. *(B)*- The probability of evoking a response diminishes over time, and is not due to sensory fatigue, as responses to stimulations on the contralateral side remain attenuated. The magnitude and rate of habituation is correlated with the inter-stimulus interval (ISI), as shorter ISI (5s, white circles) induces more prominent habituation than longer ISI (10s, black circles).

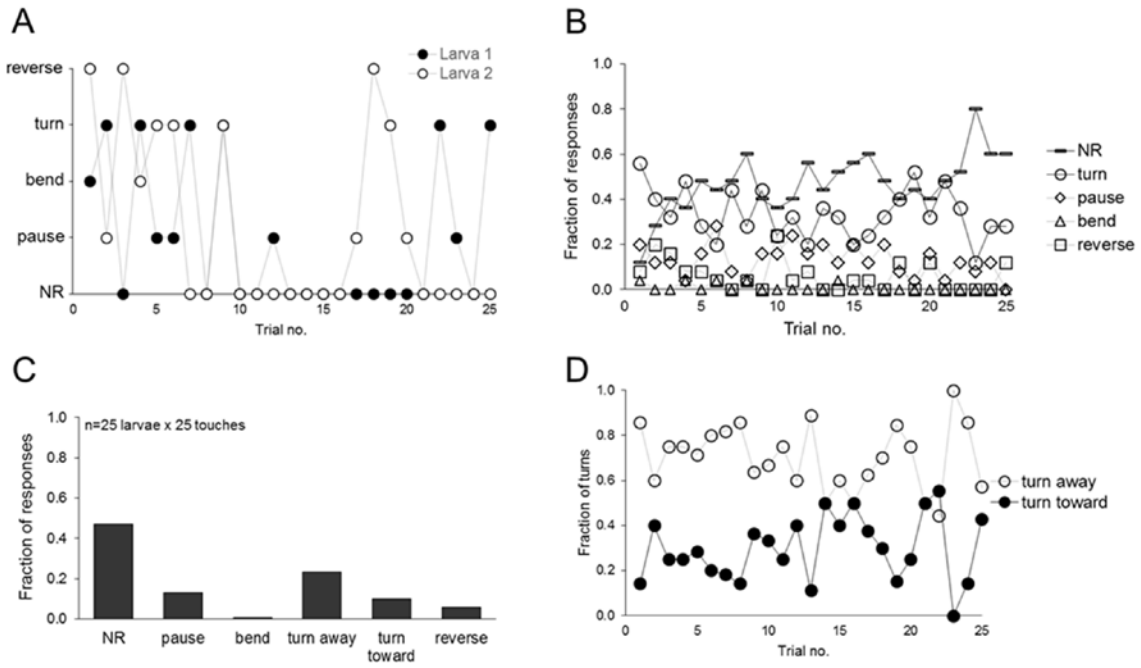

Figure 3.2. Types of mechanosensory responses observed. *(A)*- Response sequence of two larvae showing the typical pattern of behaviors exhibited in response to repetitive light touches. *(B)*-Average distribution of mechanosensory responses exhibited during habituation experiments (n=25 larvae). *(C)*- Total distribution of behaviors (n=750 responses from n=25 larvae). *(D)*- Fraction of turns toward and away from the direction of the stimulus.

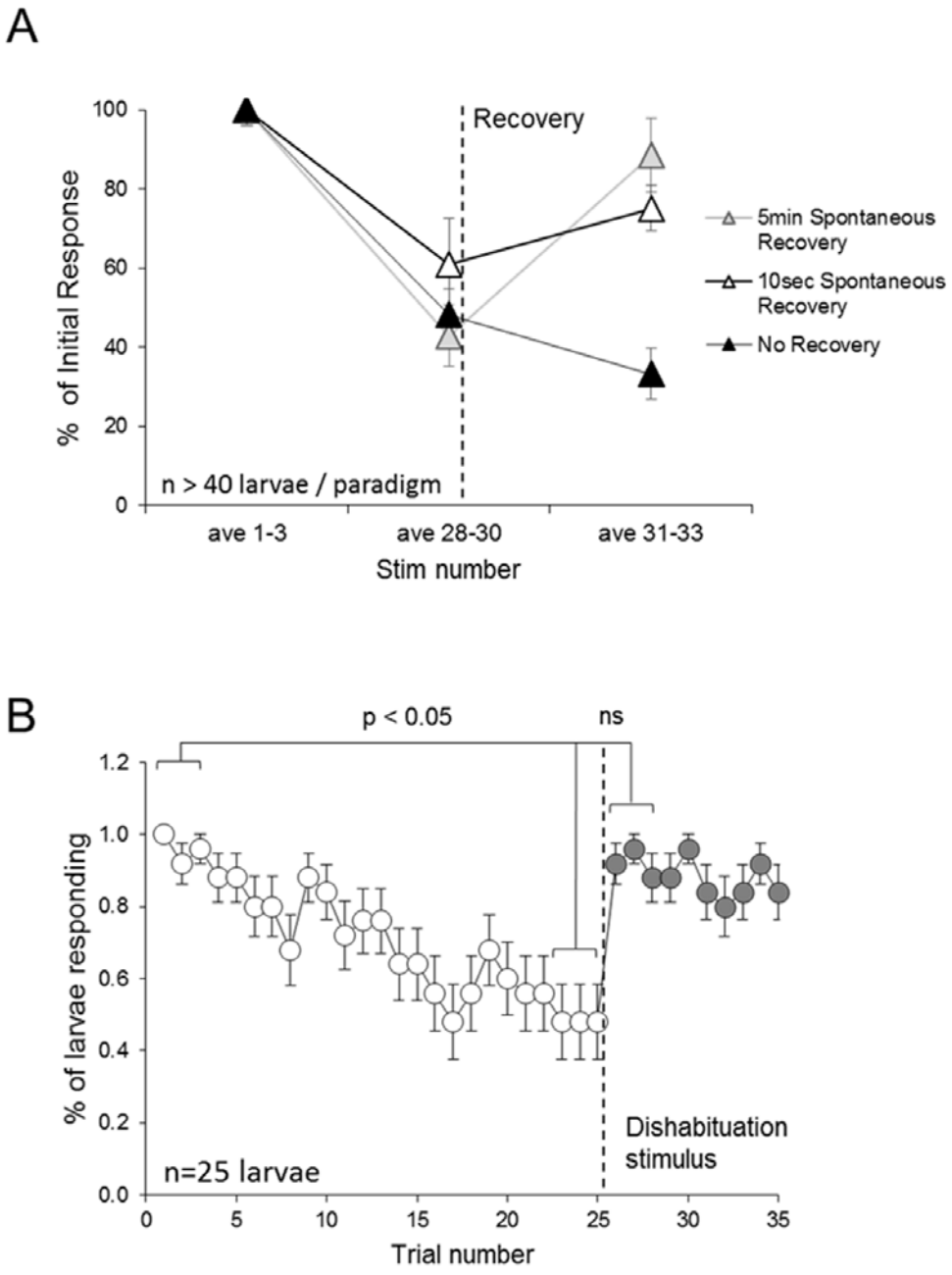

Figure 3.3. Spontaneous recovery and dis-habituation. *(A)*- Larvae quickly recover from habituation, with response probabilities returning to normal within 5min (gray triangles). *(B)*-A stronger tactile stimulus (20mN) delivered with an insect pin to the abdomen can cause dis-habituation. In these experiments the stimulus was applied after 25 touches (dotted line), and subsequent responses were applied to the same side.

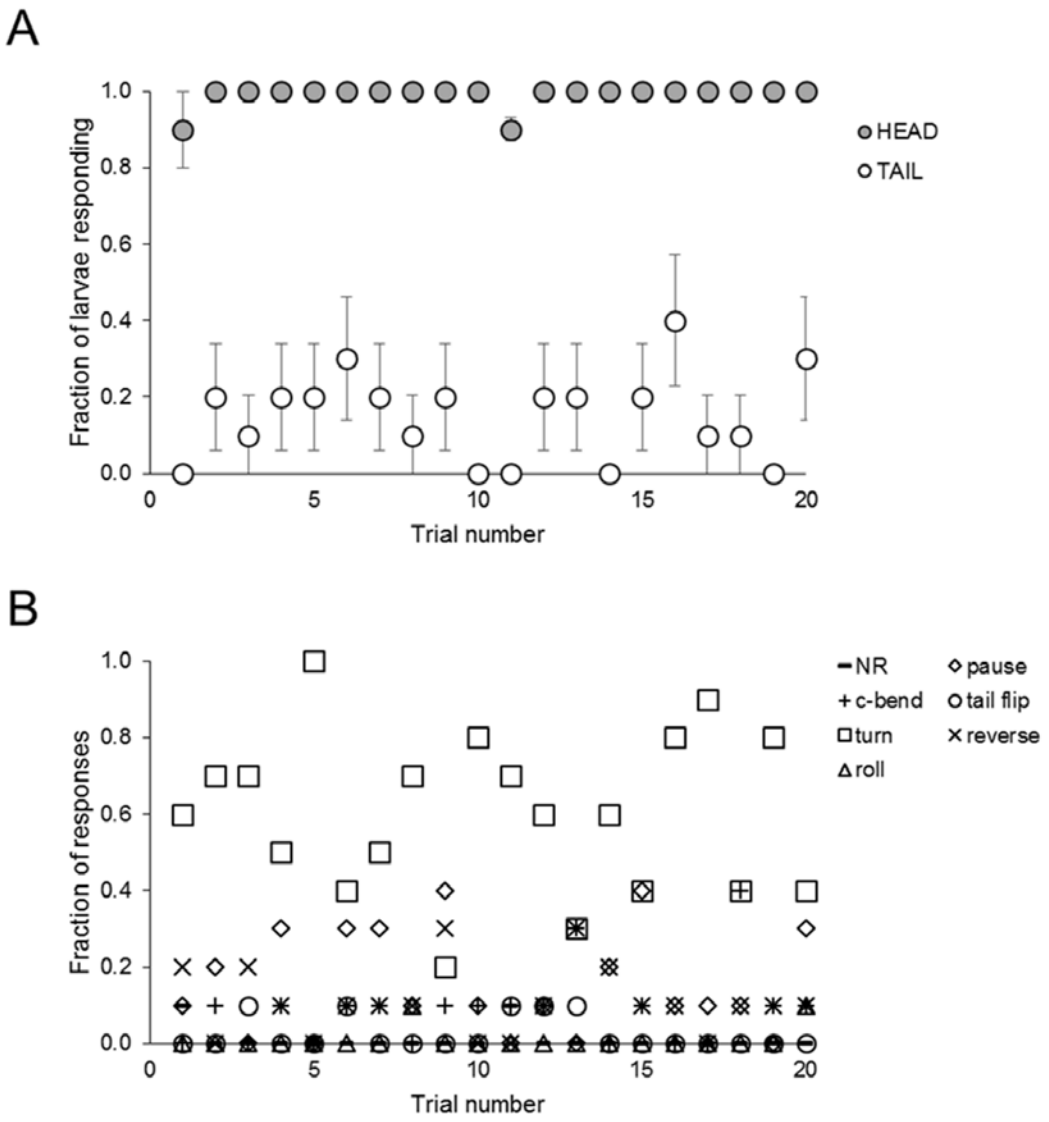

Figure 3.4. Larval response to repetitive 20mN tactile stimuli does not change over time. *(A)*- Slightly noxious pokes to the head of crawling larvae almost always evoke a response (gray circles), tail jabs rarely evoke a response (white circles). We predicted that the animals would either habituate to the head stimulus or sensitize to the tail stimulus, but they maintained a consistent level of responsiveness over the course of 20 trials. *(B)*- Head touches evoke at least 7 distinct mechanosensory responses. The relative distribution of those different behaviors did not change after multiple stimulations.

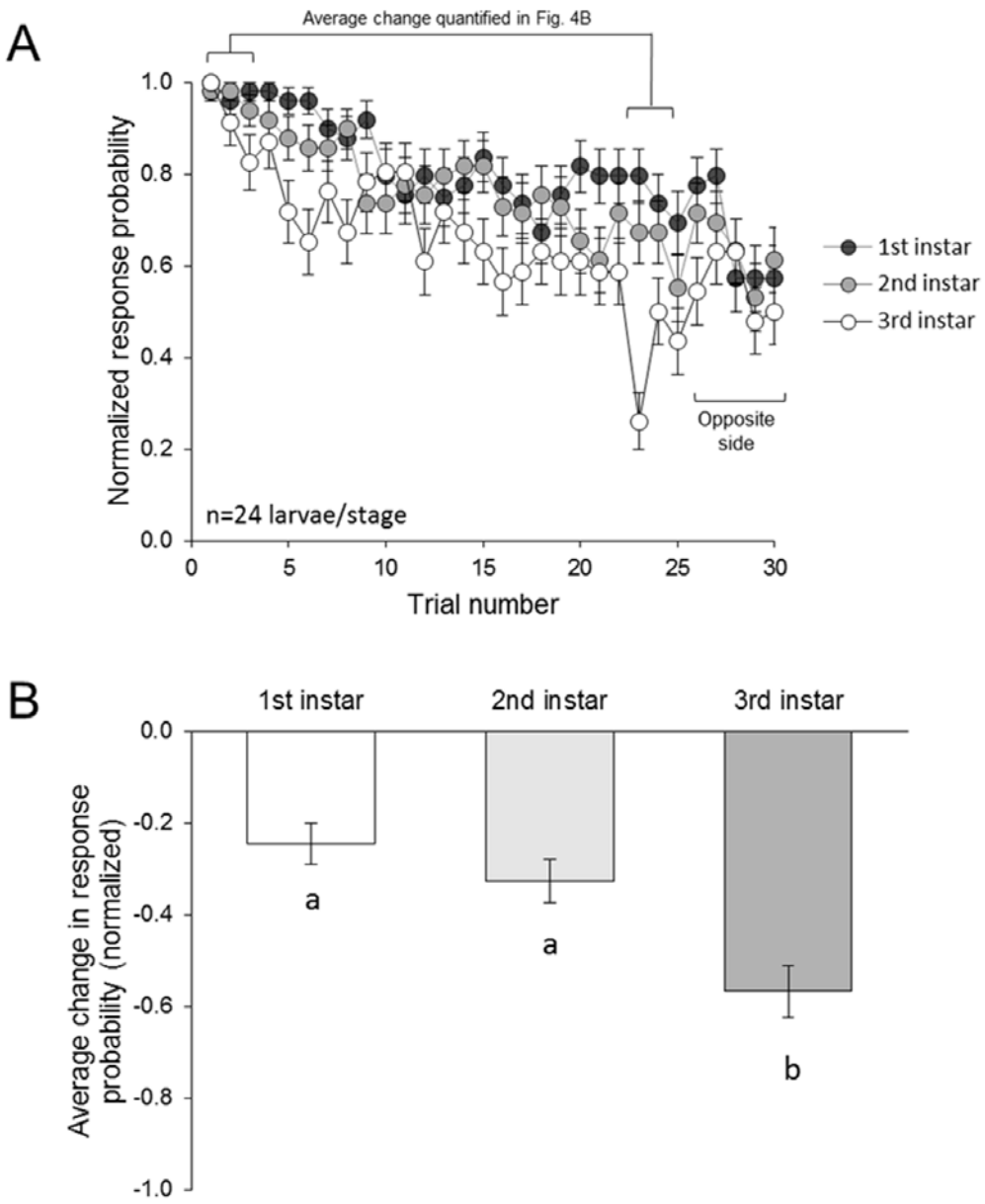

Figure 3.5. Plasticity in mechanosensory circuits at different stages of development. Habituation curves were generated for Canton-S larvae at different stages of larval development (A). The magnitude of habituation of was significantly less in 1st and 2nd instars (B).

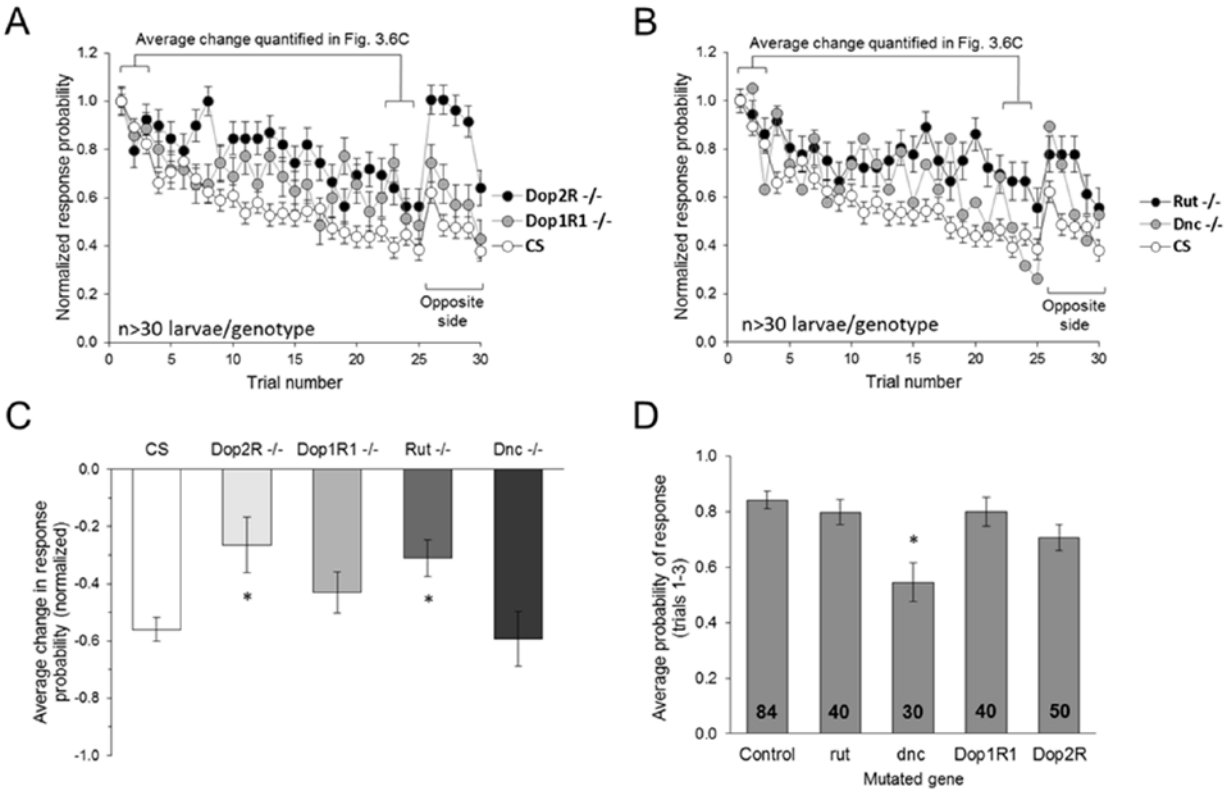

Figure 3.6. Habituation is reduced in dopamine receptor and adenylate cyclase mutants. Habituation curves were generated for two dopamine receptor mutants *(A)* and two signal transduction mutants *(B)*. The average magnitude of habituation is significantly less in type-2 dopamine receptor mutants (Dop2R) and the *rutabaga* adenylate cyclase mutant larvae *(C)*. Baseline sensitivity is significantly lower in the *dnc* mutant, but normal in the other mutants *(D)*.

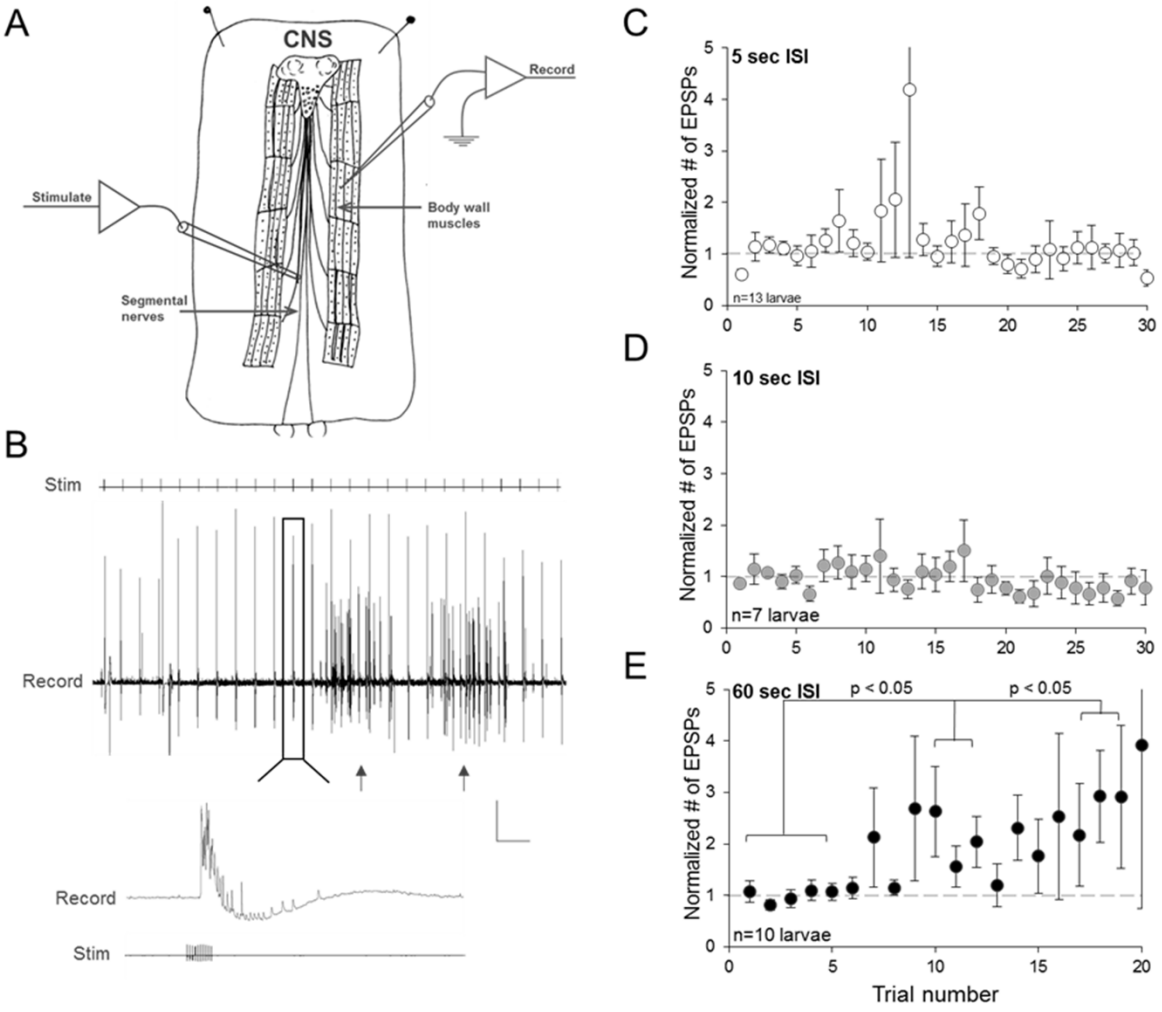

Figure 3.7. Sensory circuit-evoked EPSPs in larvae. (A)- Schematic of the dissected larva preparation showing the positions of electrodes. (B)- Representative traces from a 60s ISI experiment. Top trace shows all of the responses in a single experiment, note the increase in response in the later stimulations (arrows). The bottom trace is magnified to show a single response. (y-scale is 5mV for the top trace, 10mV for the bottom trace; x-scale is 2min for the top trace, 2s for the bottom trace). (C-E)- Data points represent an average of the number of evoked EPSPs from each stimulus normalized to the first five responses in each experiment. The number of animals that were pooled is indicated on each graph (mean ± sem). Note that habituation is not observed in any of these experiments, instead the response tends to increase throughout each of the experiments, most prominently in (E).

CHAPTER FOUR

Specific mechanosensory defects caused by manipulating dopamine pathways in *Drosophila melanogaster* larvae

*This chapter is being submitted for publication to a peer-reviewed journal. Mr. Douglas Potts and Ms. Jordan Rice collected data that led to figures. I collected data, analyzed all of the data, and wrote the manuscript. Dr. Cooper edited the manuscript.

INTRODUCTION

Vertebrate and arthropod dopaminergic circuits are functionally conserved and exhibit homology in their molecular mechanisms of development (Strausfeld and Hirth, 2013). Dopamine acts through its metabotropic membrane receptors to modulate the strength of synapses or the intrinsic activity of neurons, neurons which usually function in learning or motor output (Beaulieu and Gainetdinov, 2011). Disruption of dopamine pathways causes aberrant brain development and cognitive impairment (Jia et al., 2013). Spinal cord development (Reimer et al., 2013) and function (Humphreys and Whelan, 2012) are also influenced by dopamine, meaning that dopamine pathways can modulate pain reflexes and spinal central pattern generators (Clemens et al., 2012, Keeler et al., 2012, Viisanen et al., 2012). If dopamine modulates those processes in the *Drosophila melanogaster* ventral nerve cord, which is functionally analogous to the vertebrate spinal cord, then the tools for mapping neural circuits and molecular pathways in *D. melanogaster* would be useful for understanding basic principles for how these circuits develop and function.

D. melanogaster larva exhibit rhythmic contractions of longitudinal body wall muscles to propel the animal forward or backward (Heckscher et al., 2012). These contractions occur in the absence of input from the brain (Berni et al., 2012), suggesting that a central pattern generator for locomotion exists in the ventral nerve cord.

Modulation of endogenous bursting rhythm by biogenic amines has been measured in intact animals and from segmental nerves and single muscle fibers in dissected preparations (Budnik et al., 1990, Cooper and Neckameyer, 1999, Fox et al., 2006, Selcho et al., 2012). In the dissected third instar larva preparation, exogenous dopamine disrupts spontaneous rhythmic activity in the motor neuron that innervates m6 (Cooper and Neckameyer, 1999). Inhibiting dopaminergic neurons during development causes an abnormal circular crawling phenotype (Suster et al., 2003). Methylphenidate (MPH), which increases synaptic dopamine by blocking the membrane dopamine transporter, causes hyperlocomotion (Pizzo et al., 2013) and depletion of CNS dopamine levels by pharmacologically inhibiting dopamine synthesis causes akinesia (Neckameyer, 1996). Localization of dopaminergic neurons and dopamine receptors in the ventral nerve cord support these functional roles in motor behavior (Draper et al., 2007, Selcho et al., 2009), but is unclear if dopamine modulates pattern generating circuits or sensory feedback, which has been shown to affect crawling (Caldwell et al., 2003, Song et al., 2007, Inada et al., 2011).

A tactile sensory stimulus causes larvae to stop crawling and execute a stereotypical response from a limited repertoire of behaviors (Kernan et al., 1994, Kim et al., 2012). This well characterized sensory transduction pathway resembles mammalian nociception at the molecular level (Tracey et al., 2003, Im and Galko, 2012, Kim et al., 2012), making it an attractive system to study the role of dopamine modulation in mechanosensory pain reflexes.

In this study we used pharmacology and mutant lines to disrupt dopamine signaling in *D. melanogaster* larvae. Using behavioral and electrophysiological assays we found that these disruptions impair specific aspects of mechanosensation.

MATERIALS AND METHODS

Animals

A Canton-S strain that has been isogenic in the lab for several years was used for all experiments. The flies were cultured in bottles at medium density and fed standard cornmeal (Bloomington stock center recipe). Flies were kept on 12 hr light:dark cycle with experiments being performed during the “day”. The Dop1R1 and Dop2R mutants are described in the Methods section of Chapter 3. ple-GAL4 and UAS-mcd8:GFP flies were obtained from Booming *Drosophila* Stock Center.

Pharmacology

Alpha-methyl-p-tyrosine-methyl-ester (AMT), L-3,4-dihydroxyphenylalanine (l-dopa), SKF38393, and methylphenidate were purchased from Sigma Aldrich (St. Louis, MO). Dopamine-HCl and each of the saline salts were also purchased from Sigma. DA was weighed out and prepared from fresh stock solutions daily. The receptor agonists and antagonists were prepared from 0-3 day old stock solution daily.

AMT was administered to early third instars by placing them into normal food vials with 0.5mg standard fly food and the indicated concentration of AMT plus 0.5mL water (controls received only water in their food). Animals that remained in the food were used for experiments.

Development assay

Adult Canton-S flies were allowed to lay eggs on a petri dish filled with agar. Ten eggs were then collected and placed into normal fly food vials containing the following treatments: water, AMT, AMT+L-DOPA, L-DOPA, or methylphenidate. Five vials were used for each treatment. Vials were monitored every four hours from the time the first pupa was observed until the last pupa eclosed.

Quantitative analysis of varicosity number from stacks of confocal images

To visualize dopaminergic neurons in the larval VNC we crossed ple-GAL4 males to UAS-mcd8:GFP virgin females and collected larvae at the early third instar stage. Larvae were then treated for 24hr in vials of 10mg/mL aqueous solution of AMT and fly food, or fly food and water. Treatments were coded to eliminate experimenter bias. 5-10 brains were dissected from larvae in each vial and fixed in 2x10 minute washes of 4% formaldehyde in PBS. Brains were rinsed in PBS, cleared with an ethanol series, mounted in xylene, and imaged the same day.

Images were obtained using a Leica TCS SP confocal microscope. 5µm thick sections were taken at 63x magnification to include 2-3 segments of the anterior abdominal region. A region of interest was then drawn to include a single hemisegment for analysis. The number of varicosities were counted using a voxel counting plug-in in Image-J (Wouterlood et al., 2008). I wrote custom macros and scripts to automatically step through the stacks of images and count the number of voxels across the entire range of pixel values. Those values generated a bi-modal curve (Figure 4.2D), with the first peak representing the ideal threshold and providing the number of varicosities within the image area.

Behavior assays

Larvae were pre-treated with MPH, dopamine, AMT and/or L-DOPA by placing 20-30 animals into 0.5mg standard fly food plus 0.5mL of aqueous drug solution or water. After 24h the larvae were transferred to an agar-lined petri dish (1% agar, 33% apple juice to evoke crawling) and scored for crawling or mechanosensory behavior. Crawling speed was measured by counting the number of strides per 15s as soon as the larva started crawling. We didn't observed any differences in crawling speed as the

larvae adapted to the dish. Mechanosensory behavior was measured as described in Chapter 2.

Electrophysiology

Flies were dissected in several drops of HL3 saline as described previously for recording EPSPs in body wall muscles. The brain and nerve cord were left intact with only the most posterior segmental nerves severed to reduce movement. Anterior segmental nerves from one side were pulled into a microcapillary glass suction electrode to activate motor axons, and a sharp glass electrode (resistance = 5-20 MΩ) was placed into a longitudinal muscle on the opposite side to record excitatory junctions potentials (EJPs) from the muscle fiber. Ten-pulse trains (40 Hz) were delivered to the segmental nerves every ten seconds to monitor evoked CNS activity. EJPs were collected with an Axoclamp 2B amplifier and digitized using the Powerlab-2SP (AD Instruments). Traces were stored and analyzed using LabChart for Windows.

Data analysis

Means for each treatment were compared using t-tests or one-way ANOVA where applicable. When data were pooled across multiple trials from different experimenters a two-way ANOVA was used to confirm that the effects were not due to handling or environment. Rank tests were used when data were not normally distributed.

For the development assay the percentage of pupated or eclosed flies for each treatment was plotted over time and compared using a Kaplan-Meir analysis. The Mantel-Haenszel logrank test was then used to compare the survival curves with p-values less than 0.05 considered significant.

To analyze eEPSPs, the number of eEPSPs from each stimulus in each preparation was normalized to the last 6 stimuli before adding dopamine. Normalized

values from the treatments were then compared to the baseline values using paired t-tests.

RESULTS

Manipulating dopamine levels delays D. melanogaster development

An array of developmental and behavioral assays was used to characterize systemic effects of AMT (tyrosine hydroxylase inhibitor). Depleting dopamine levels with this pharmacological approach at the 1st larval instar stage killed 100% of the flies. When AMT-treated flies were given L-dopa (1mg/mL) the number of larvae that reached pupation was no different than controls. However it took nearly 10 days for 50% of these rescued animals to reach pupation, whereas half of the untreated animals pupated within 7 days (Figure 4.1). Treating the flies with L-dopa alone (1mg/mL) or with methylphenidate alone (1mg/mL) extended larval development by 1 day and did not significantly decrease pupation rates. L-dopa had a more severe effect on metamorphosis. Only 13.3% of pupae treated with L-dopa eclosed, and none of the AMT+L-dopa-treated pupae eclosed. All of the non-treated pupae eclosed and 96.0% of the MPH-treated pupae eclosed. In summary, dopamine is necessary for larval development and systemic increases in dopamine during larval development strongly inhibit metamorphosis.

Next we wanted to determine if changes in dopamine levels could cause changes in the morphology of dopaminergic neurons. This was tested with AMT treatment because it had the greatest effect on developmental timing. Given the lethality of AMT treatment beginning at the 1st instar stage we decided to use a 24-hour treatment beginning in the early 3rd instar stage. This treatment made the flies slightly lethargic but lethality was less than 10%. The larval VNC dopaminergic neurons are shown in Figure 4.2A. Most of the fibers seen in this image are axons and the puncta on

the axons are varicosities, open synapses where dopamine is released onto various neuropils through volume transmission. To measure morphological changes we counted the number of varicosities using voxel identification algorithms.

Figures 4.2B and 4.2C show representative regions of anterior abdominal segment neuropil from control larvae and larvae given 24hr AMT treatment. Typically the AMT treatment caused an increase in the number of observable puncta, though with variation in the limited sample size the difference did not reach statistical significance.

Crawling behavior is affected by pharmacological and genetic manipulations in dopamine signaling

To determine how dopamine influences crawling circuitry we analyzed crawling speed in Canton-S larvae that were pre-treated for 24hr with either AMT, L-dopa, MPH, or dopamine. Each drug was dissolved in distilled water then mixed with a fly food to a concentration of 10mg/mL, a concentration that is commonly used for feeding experiments with these drugs. In our hands this concentration causes behavioral changes without causing lethality. Only AMT caused behavioral changes at a lower dose (1mg/mL). The behavioral readout for crawling speed was the number of strides observed while larvae crawled on an agar dish for 15s.

Feeding larvae drugs that raise CNS dopamine levels (MPH and l-dopa) or lower CNS dopamine levels (AMT) caused the larvae to crawl significantly slower (Figure 4.3). In the type-1 and type-2 dopamine receptor mutants we did not observe any differences in crawling speed (Figure 4.4A). This suggests that neither of those receptors alone is essential for normal development or function of the crawling circuit, but dopamine levels in the CNS need to be within a homeostatic range to maintain proper function. That the type-1 dopamine receptor mutant spends more time crawling (Figure 4.4B) could be caused by aberrant sensory feedback to the crawling circuit (Caldwell et al., 2003, Song et al., 2007).

Mechanosensory impairments caused by changes in dopamine signaling

Mechanosensation was also tested in drug-treated larvae and in both types of dopamine receptor mutants. Pre-treating Canton-S larvae for 24h with AMT or L-dopa decreased responsiveness to abdominal touches (20mN; Figure 4.5A). L-dopa also decreased responsiveness to head touches, whereas dopamine treatment increased responsiveness to tail and abdomen touches, and MPH did not have a significant effect on mechanosensory behavior. The dopamine receptor mutants were tested for gentle brush (Figure 4.6A) and 20mN touch responses (Figure 4.6B-C). Consistent with the pharmacological treatments, both mutants exhibited reduced mechanosensory response phenotypes, specifically in the tail region (Figure 4.6C).

Dopamine modulates evoked neural circuit activity in D. melanogaster larvae

To characterize the modulatory effects of dopamine on cellular mechanosensory responses I recorded motor output from single muscle fibers in dissected larvae. As described in earlier chapters, 10-pulse trains of 40Hz stimulus applied to the segmental nerve roots evokes a burst of activity from the CNS, which on average consists of about 30 EPSPs (Figure 4.7A). Occasionally the number of eEPSPs increased shortly after switching to dopamine saline, but due to high variability this response was not statistically significant (Figure 4.7B,C). The more profound effect was depression of this response that occurred after 20 stimulations. This effect was larger at a higher concentration, and it was reproduced by the type-1 dopamine receptor agonist SKF38393 (Figure 4.8).

DISCUSSION

We show that dopamine modulates crawling and mechanosensory behavior in *D. melanogaster* larvae. The interesting thing about these responses is that multiple

different fixed action patterns were executed following tactile stimulation. Quantifying the frequency of specific types of behavior provided insight into which part of the mechanosensory circuit was affected by changes in dopamine signaling.

Both dopamine receptor mutants exhibited increased responsiveness to gentle brush stimuli, i.e., fewer NRs were observed (Figure 4.5A). By looking at the distribution of behavioral responses in Figure 4.5A, it is clear that the number of pauses is significantly higher, and the frequency of active behaviors is reduced, i.e., fewer turns and fewer reverse contractions. The same is true for responses to 20mN touches (Figure 4.5B). NRs are normal, yet there are significantly more pauses and fewer c-bends and turns. Since the larvae clearly detect the stimulus and crawling speed is normal (Figure 4.3A), it appears that the integration of sensory input into motor commands is affected in these mutants. That such effects were not observed in the 24hr pre-treated larvae suggests that they are caused by aberrations in the development of the circuit. Indeed we also showed that manipulating the dopaminergic system beginning at an early larval stage affected development timing (Figure 4.1) and nervous system morphology (Figure 4.2). Those data are consistent with an effect observed in serotonergic neurons, where increases in serotonin decreased the number of serotonergic varicosities in the larval VNC (Sykes and Condrin, 2005). In primary cultures, dopamine was shown to protect *D. melanogaster* dopaminergic neurons from chemical-induced neurodegeneration through Dop2R autoreceptors (Wiemerslage et al., 2013). Dopamine was also shown to exhibit neurotrophic actions on *D. melanogaster* serotonergic neurons *in vivo* (Necameyer and Bhatt, 2012). Further experiments are necessary to determine which other types of neurons undergo structural modifications in response to changes in dopamine levels, and what mechanisms mediate those changes. Structural modifications and changes in synaptic strength are likely to be important for dopamine's role in learning and other cognitive functions.

With regard to changes in synaptic strength, my electrophysiology data show that dopamine ultimately depresses sensory-evoked motor output through *Dop1R*. There is an initial increase in excitability after applying dopamine, and such biphasic effects are not uncommon within a single cells, e.g., in pyramidal neurons from the mouse cortex (Goode, 1972). So it is not clear whether dopamine is depressing excitatory neurons, facilitating inhibitory neurons, or both. Though these data provide strong evidence for type-1 dopamine receptor-mediated modulation, additional experiments are needed to rule out the possibility that type-2 dopamine receptors also modulate the response. It would not be surprising if they did, given that mutants for both receptor subtypes have mechanosensory behavioral phenotypes.

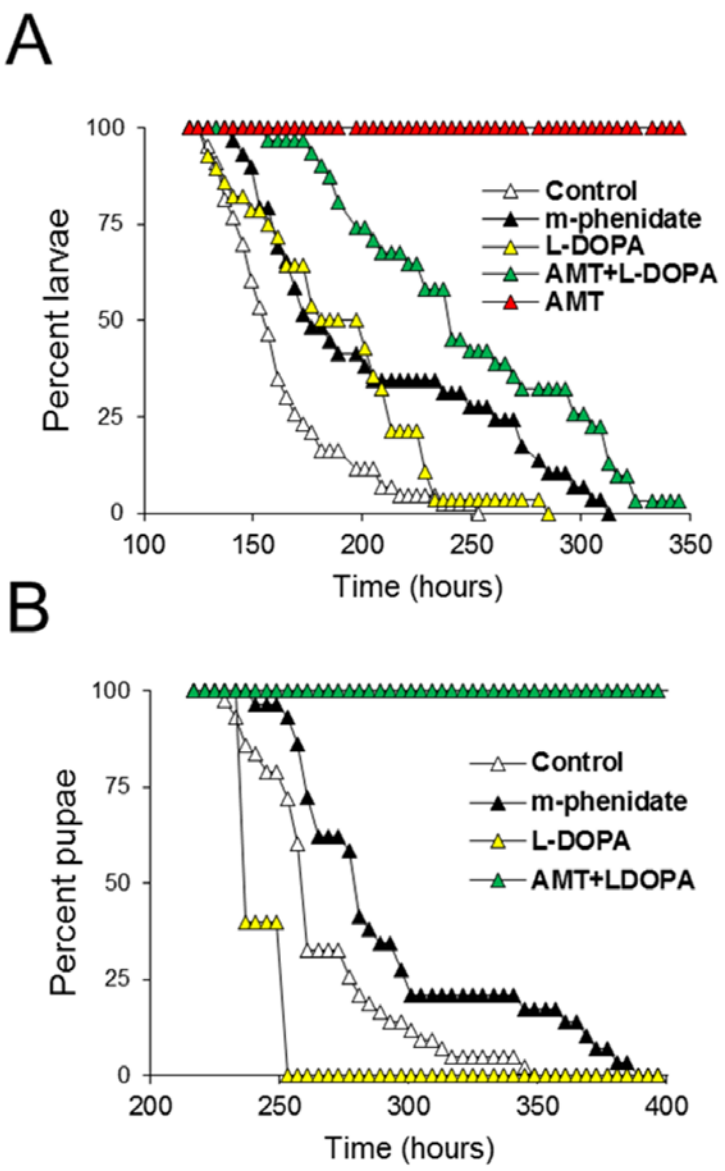

Figure. 4.1. Developmental timeline of flies treated with dopamine-altering drugs. Time points represent the percentage of flies in the larval stage out of the total number of flies that pupated (*left*), or the percentage of flies in the pupal stage out of the total number that eclosed (*right*). Flies treated with AMT did not survive to pupation. Flies treated with AMT and L-DOPA pupated but did not eclose. All treatments significantly increased the time to pupation over water alone (Mantel-Haenszel logrank test, $p < .001$).

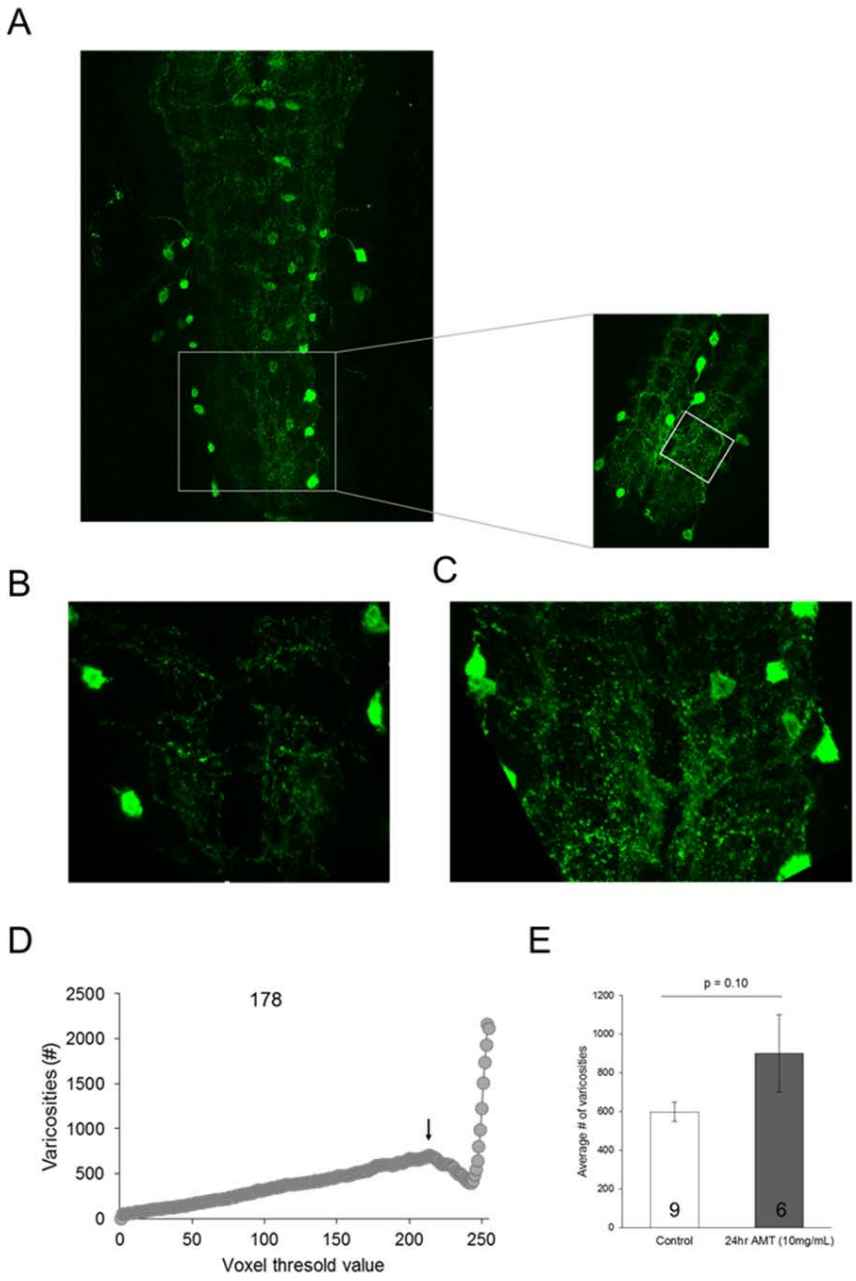

Figure 4.2. Effects of CNS dopamine depletion on dopaminergic neuron morphology. A- Dopaminergic neurons in the larval VNC. Box shows the ROI that was imaged at high power. Box on the inset image shows an example of a hemisegment that was selected to count varicosities. B- Representative image of a control neuropil and C- AMT-treated neuropil. D- Representative graph of voxel threshold data from the analysis algorithm. Plots of the number of voxels across a range of thresholds generated a bimodal curve, the first peak represents the number of voxels at the ideal level, i.e., the number of varicosities in the stack of images. E- Pharmacologically depleted dopamine levels caused a slight increase in the number of varicosities on dopaminergic neurons.

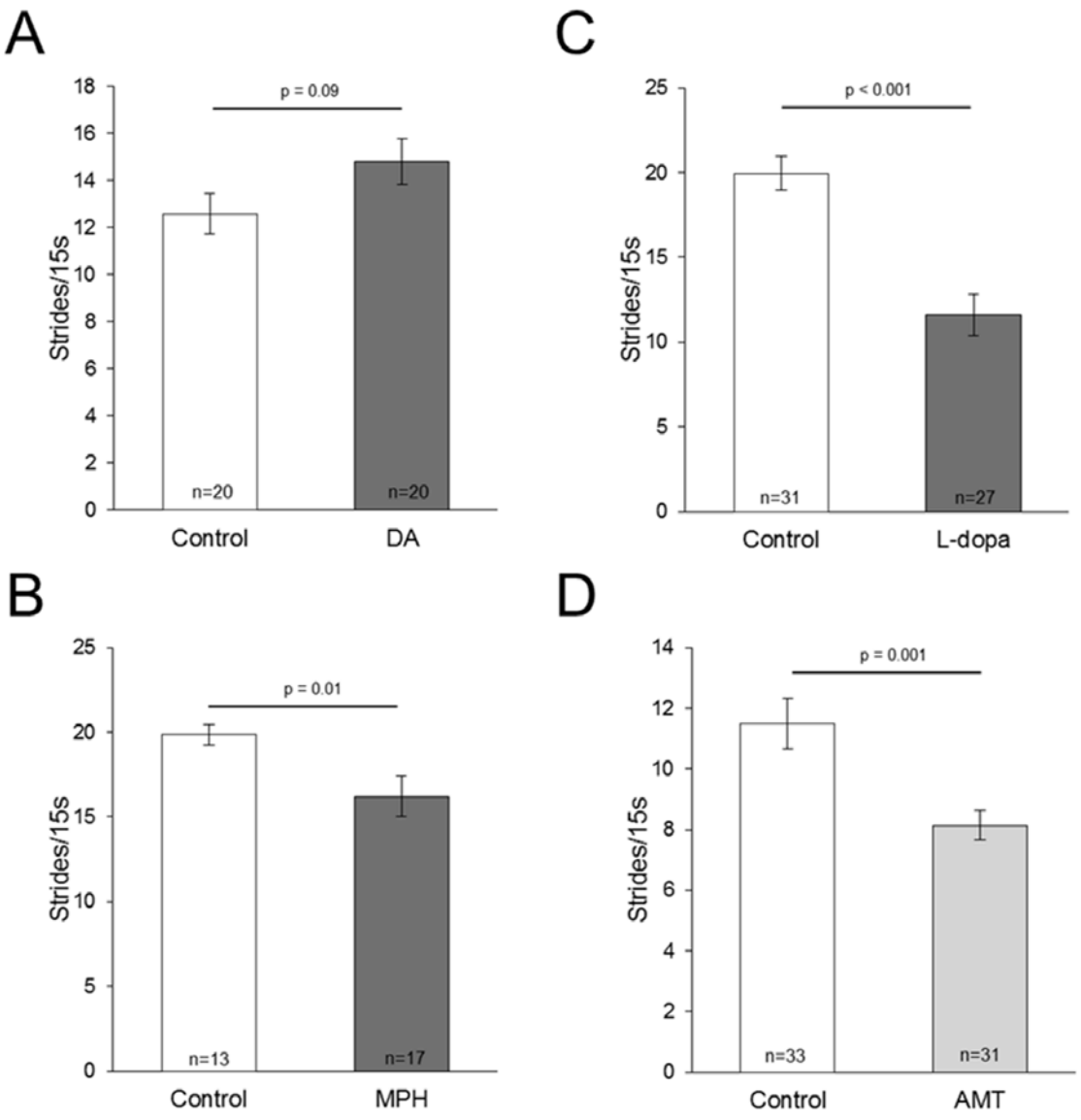

Figure 4.3. Inhibiting DA synthesis reduces locomotor activity in 3rd instar larva. Animals were generally lethargic after feeding on AMT and standard fly flood for 30 hours. Lethality was observed more frequently in the treatment vials. These standard behaviors were quantified on two separate occasions and pooled together for analysis (n=32 for control and n=31 for AMT).

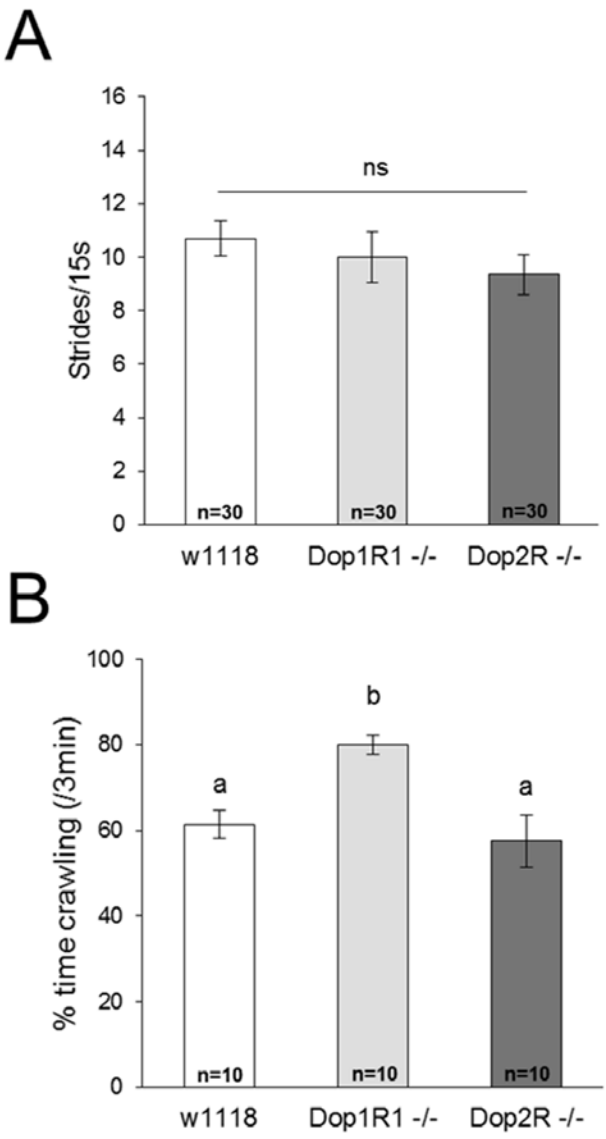

Figure 4.4. Effects of dopamine receptors mutations on larval locomotion. *A*- Relative to isogenic controls (w^{1118}), dopamine receptor mutants do not exhibit normal crawling behavior. The type-1 dopamine receptor mutants spend significantly more time crawling than controls or type-2 dopamine receptor mutants.

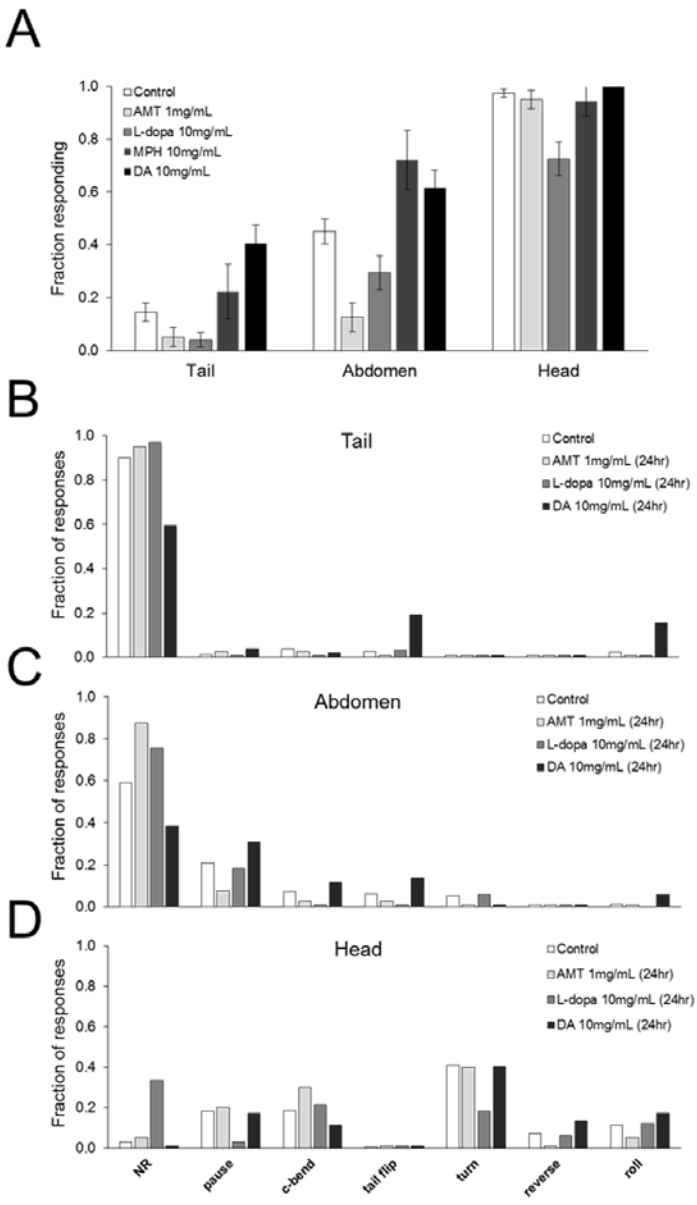

Figure 4.5. Effects of pharmacological manipulation of dopamine signaling on 20mN mechanosensory responses. *A*- AMT and L-dopa decrease sensory responses to abdominal stimuli. *B-D*- Distribution of responses to with respect to anatomical region.

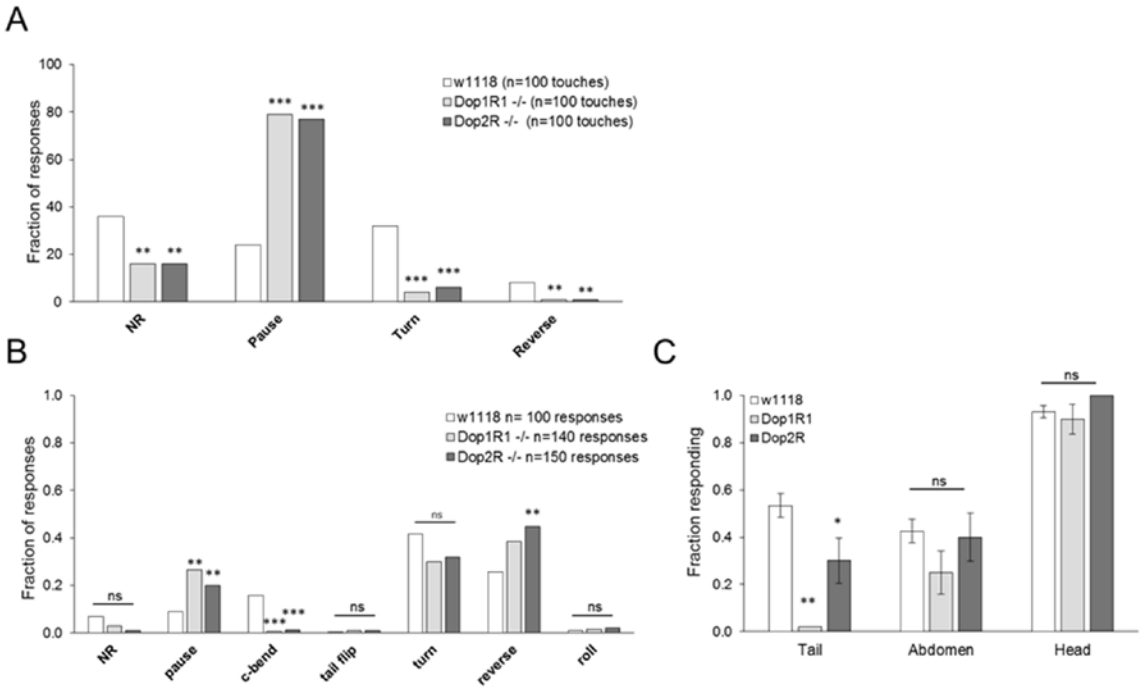

Figure 4.6. Mechanosensory behavior phenotypes in dopamine receptor mutants. *(A)*- Gentle brush assay, both type-1 and type-2 dopamine receptor mutants are significantly more sensitive to this stimulus, i.e., fewer NRs. These data indicate that though they are more sensitive to the stimulus, the input does not reliably evoke motor output. *(B)*- 20mN touch assay- shown here is the response distribution to head touches. Note that there are no differences in sensitivity, but pauses are more frequent than sensory-evoked behaviors. *(D)*- The dopamine receptor mutants are less responsive to tail stimuli than controls.

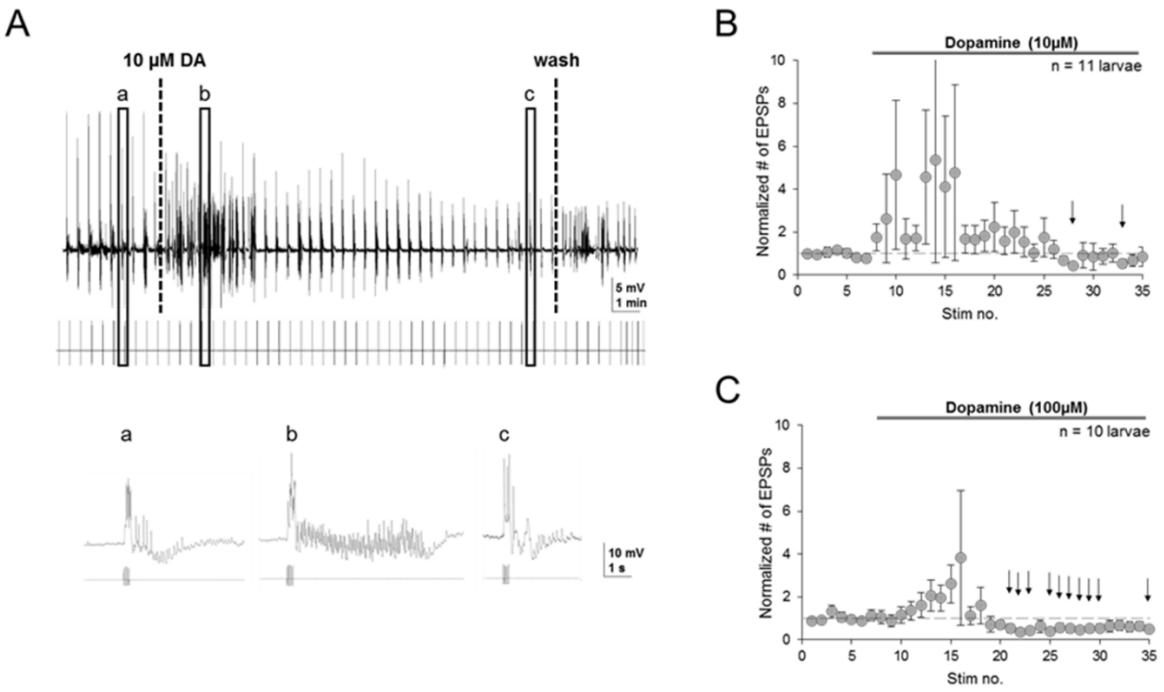

Figure 4.7. Evoked CNS activity initially increases but is then depressed by DA. (A)-Typical EPSP traces from body wall muscles during baseline and after DA application. Before DA was applied the number of EPSPs was consistent for at least five stimulations. (B,C)-The average frequency for that baseline period was divided into the frequency of EPSPs from each stimulation throughout the experiment. Normalized frequencies from third instars that were fed AMT or water for 30 hours are plotted with respect to DA application. After applying DA the number of EPSPs typically increased by 2-5 times the average baseline frequency (by 36 times in one experiment). This increase was far more subtle in larvae that were treated with AMT for 30 hrs before the experiments. In both sets of experiments CNS activity was blocked 2-3 minutes after applying the modulator. Arrows indicate average responses that were significantly less than baseline.

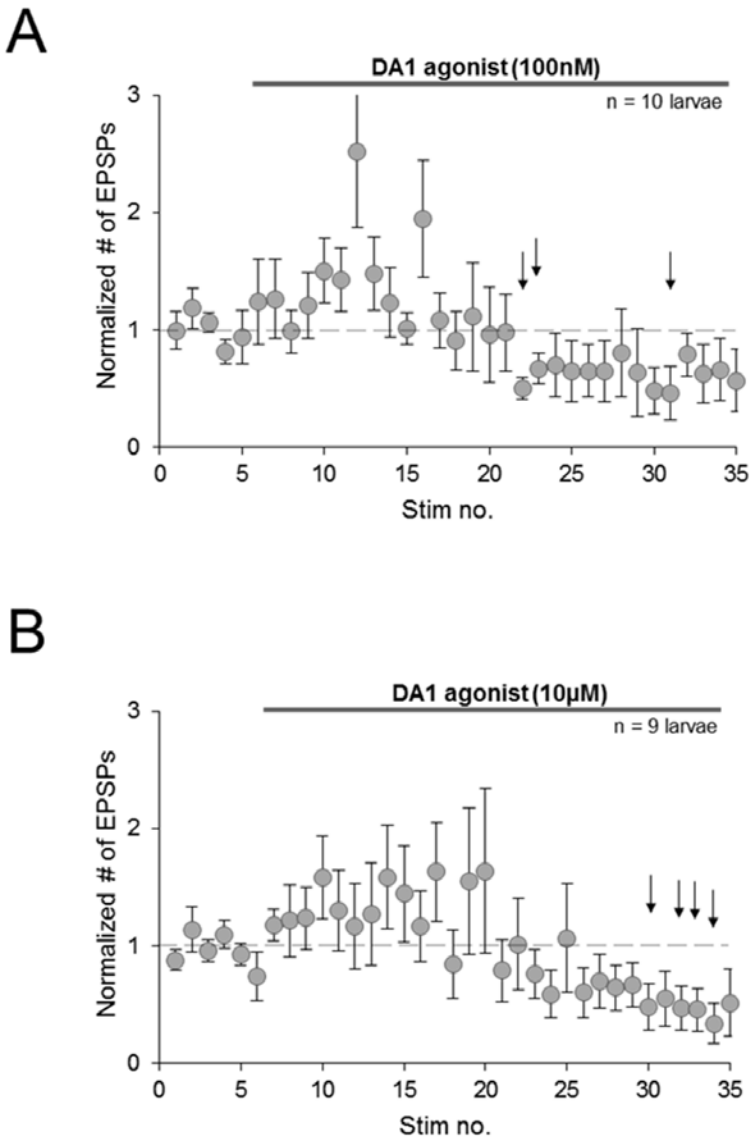

Figure 4.8. Dose-dependent effects of the type-1 dopamine agonist SKF38393 on sensory-evoked motor output at the larval NMJ. As with exogenous dopamine, the higher concentration of this drug ultimately depresses output more prominently. Arrows indicate average responses that were significantly less than baseline.

CHAPTER FIVE

Targeted eQTL analysis reveals dopamine-related gene regulation in *Drosophila melanogaster* populations.

*This chapter is being submitted for publication in a peer-reviewed journal. Ms. Emily Rayens collected data that contributed to Figure 3. Ms. Jordan Rice collected data that contributed to Figure 4. Mr. Zana Majeed performed part of the bioinformatic analyses that contributed to Figure 5. Dr. Jeramiah Smith and Dr. Robin Cooper helped design experiments and edit the manuscript. All other experiments, data analysis, and writing were done by Mr. Josh Titlow.

INTRODUCTION

Deviations in dopamine signaling have been associated with abnormal behavior in model organisms and in man. When dopamine receptor knockout mice were first generated in the 1990's they exhibited obvious locomotor and hyperactive phenotypes (Drago et al., 1994, Baik et al., 1995, Accili et al., 1996). Later it was shown that dopamine receptor mutants also exhibited more complex behavioral phenotypes, e.g., learning (El-Ghundi et al., 1999), memory (Glickstein et al., 2002), anxiety (Steiner et al., 1997), and alterations in neural plasticity (Calabresi et al., 1997, Matthies et al., 1997). Drug sensitivity and addiction phenotypes were also reported in dopamine receptor mutants (Rubinstein et al., 1997, El-Ghundi et al., 1998, Ralph et al., 1999). The association between dopamine signaling and abnormal behaviors led to the hypothesis that variation in dopamine-related genes predisposes humans to neurological disorders.

Dopamine receptor alleles have been identified as causative variants in neurological disorders through genetic testing and genome wide association studies (GWAS). The Ser9Gly variant in DRD3 is a gain of function allele that causes essential tremor (Jeanneteau et al., 2006). In the Chinese Han population, a polymorphic DRD2 allele is associated with vulnerability to schizophrenia (Fan et al., 2010). The DRD2 rs1800497 polymorphism is associated with mood disorder (Zhang et al., 2014). Several

studies have also addressed the role of dopamine receptor variants in addiction (Gorwood et al., 2012) and attention deficit disorder (Polanczyk et al., 2010, Stergiakouli and Thapar, 2010). Thousands of SNPs have been associated with risk loci in schizophrenia, a disorder which has an estimated heritability of 70% (Schwab and Wildenauer, 2013). One purpose for understanding the genetic contribution to these disorders is to use genomic data to inform treatment regimens. This pharmacogenetic approach is complicated by the fact that the disorders are polygenic, dopamine homeostasis is polygenic, and dopamine-related genes are pleiotropic. Environmental variables are also an issue in human behavior studies. Because of these factors, laboratory studies on fully-sequenced inbred animal populations are an effective strategy to understanding complex interactions between the genome, neurochemistry, and behavior.

Two systems have been developed to accommodate this quantitative genetics approach, one for *Mus musculus* (Cross, 2012) and one for *Drosophila melanogaster* (Mackay et al., 2012). Here we use the *D. melanogaster* system to assess the influence of natural genotypic variance on dopamine-related gene expression and sensitivity to drugs that alter dopamine homeostasis. The *Drosophila* Genetic Reference Panel (DGRP) is a collection of 198 naturally derived *Drosophila melanogaster* populations that have been fully sequenced (Mackay et al., 2012). For 39 of those lines, expression data is available for over 90% of protein coding genes in the *D. melanogaster* genome (Ayroles et al., 2009). Microarray data showing the effect of exogenous dopamine (and twenty other pharmacological and environmental factors) on the expression of those genes have also been generated (Zhou et al., 2012). We combined those datasets to analyze natural variation in dopamine-related genes and identify factors that regulate the expression of those genes.

Treating gene expression as a quantitative trait and determining genetic variants that influence gene expression is the basis for expression quantitative trait loci (eQTL) analysis. Brem and colleagues were one of the first groups to perform this type of analysis, using a two-locus mapping procedure in *Saccharomyces cerevisiae* (Brem et al., 2002). Similar studies soon followed in plants and mammals (Schadt et al., 2003), with the majority of eQTL studies to date focusing on specific human or mouse tissues. Surprisingly there have been just two eQTL studies in *Drosophila melanogaster*, for which there is an abundance of genetic and phenotypic data to complement statistical associations. The first study discovered multiple *trans* eQTL that regulated blocks of genes in response to lead toxicity in the roo recombinant inbred lines (Ruden et al., 2009). The second study focused primarily on identifying structural variants (SVs), e.g., indels and duplications, and their association with gene expression in the 39 DGRP lines that were included in an earlier microarray study. To assess the relative impact of SNPs on the SV eQTL, Zichner and colleagues performed an eQTL analysis with SNP data (Zichner et al., 2013). Those analyses showed that of the 129 SV eQTL, 91 had stronger associations with nearby SNPs, and 38 had no association with nearby SNPs.

A steady increase in the number of eQTL studies and the diversity in experimental designs has created a demand for creative statistical procedures to analyze these data (Li and Deng, 2010, Scott-Boyer et al., 2012). The multiplicity effect and limited statistical power due to a relatively small sample size are two fundamental problems in genome-wide association studies (Zhang et al., 2012). These effects have been neutralized by permutation and bootstrap procedures along with false discovery rate considerations (Benjamini and Hochberg, 1995), but the effect of interactions between transcript levels is an additional concern in eQTL mapping (Imholte et al., 2013). We used two different statistical approaches to search the DGRP datasets for SNPs that correlate with the expression levels of 19 dopamine-related transcripts, one

that accounts for interactions (Imholte et al., 2013), and one that treats each gene expression level as an individual trait (Mackay et al., 2012). To assess the physiological relevance of variation in dopamine-related genes we performed meta-analysis of published behavioral data on the DGRP lines and performed new behavioral experiments with pharmacological manipulation of dopamine pathways.

MATERIALS AND METHODS

Data acquisition

Microarray expression data were downloaded as .CHP files (Ayroles study; E-MEXP-1594) or .CEL files (Zhou study; E-MTAB-639) from the ArrayExpress repository. Experimental design files were created using .sdrf.tx files included with the raw data sets, and the Drosophila 2.0 array library file. File sets from the experiments were then imported into JMP-Genomics (V. 5.1) using the Affymetrix expression workflow, and stored as a SAS dataset. Experimental design and quality assessment of the data can be found in the original reports (Evans and Maqueira, 2005, Zhou et al., 2012). Genes that were associated with quantitative traits or sensitive to dopamine were identified in the supplementary data from those reports. SNP data were downloaded as .csv files from the DGRP website (http://dgrp.gnets.ncsu.edu/data/). Information on sequencing and SNP calls can be found in the original DGRP paper (Mackay et al., 2012).

Targeted eQTL mapping with the DGRP online GWAS pipeline and iBMQ

Curators for the DGRP maintain an online bioinformatics pipeline to analyze GWAS datasets (Mackay et al., 2012). Simple regression calculations were performed with this pipeline using Affymetrix array data that was normalized to median values across sexes. For the analyses, phenotypic data included average gene expression values from 2 replicates of adult males per line (39 lines). A total of 19 separate

calculations were performed to generate SNP-transcript associations for each transcript. The 19 transcripts were chosen for their direct relation to dopamine signaling, i.e., receptors, transporters, and metabolic enzymes

An integrated Bayesian model was also used to calculate SNP-transcript associations (Scott-Boyer et al., 2012, Imholte et al., 2013). The iBMQ package was ran in R-3.0.2 with the RIS parameter set to FALSE, i.e., the data included heterozygous values. Transcript levels were from the normalized array data described above (Ayroles et al., 2009).

The total number of SNPs downloaded from the DGRP website (http://dgrp.gnets.ncsu.edu/data/) was 5,524,211. The 2,723,459 SNPs that had missing values and 423,233 SNPs that had "N" calls were discarded to accommodate the iBMQ package. The 21,918 tri-allelic SNPs were also discarded. Of the 1,771,775 SNPs used in the analysis, 211,320 had at least one heterozygous line. SNPs were coded on a 1-3 scale, with 1 representing homozygous for the major allele, 2 representing heterozygous alleles, and 3 representing a homozygous minor allele. The same 19 transcripts were used for genome-wide eQTL analysis. A Markov chain Monte Carlo procedure was used to estimate the posterior probabilities of association (PPA) for eQTL mapping. We chose to use 10,000 iterations with a burn-in of 5,000. This function ultimately generates a matrix that is used to calculate a threshold with a specified false discovery rate (FDR). The FDR was set at 10%.

Behavioral assays

The proboscis extension response (PER) assay was performed as described previously (Inagaki et al., 2012). Ten to twenty adult flies (5-7 days post eclosion) were pre-treated for 48h with either aqueous sucrose solution (89mM) or aqueous sucrose

solution with l-dopa (3mg/mL). Five groups of ten flies were tested for each genotype. Data shown are the mean of each group average ± SEM.

To test for dopaminergic modulation in larvae we used a mechanosensory assay that quantifies behavioral responses to tactile stimuli (Titlow et al., In Press, Chapters 2-4). Third instar larvae were pre-treated systemically with dopamine-HCl (Sigma-Aldrich) or alpha-p-methyltyrosine (Sigma-Aldrich) by dissolving the drugs in 0.5mL of distilled water and 0.5g standard fly food.

Meta-analysis of RNAseq data

RNAseq data were generated from tissue collected at various time points throughout *D. melanogaster* development (Graveley et al., 2011). Raw data files were obtained from the Short Read Archive and referenced against the refMrna.fa.gz *D. melanogaster* sequence from http://genome.ucsc.edu. The reference sequence was prepared and mRNA levels were quantified using bowtie (0.12.8) and RSEM (1.1.21). The number of dopamine-related transcripts per 1 million transcripts at each developmental stage was normalized to the number of beta-tubulin transcripts at that stage.

RESULTS

Natural variation in the expression of dopamine-related genes

Ayroles et al. (2009) quantified the expression of 18,800 transcripts from 40 DGRP lines using the *Drosophila* 2.0 Affymetrix chip. We sorted those data to analyze natural variation in the expression of 19 dopamine-related genes, including receptors, transporters, and genes involved in the metabolism of dopamine (Table 5.1). Variation in mRNA levels between the lines was generally higher for the receptor genes than for metabolic enzymes or dopamine transporters (Figure 5.1). Expression of three of the six

dopamine receptor transcripts varied by more than 2-fold between the highest and lowest expressing lines, both for males and females (Ayroles et al., 2009)

The level of dopamine-related gene expression varied within lines. The line RAL_820 expressed all of the genes at relatively high levels, whereas RAL_707 expressed some genes at high levels and others at low levels (Figure 5.1). Expression of all the dopamine-related genes at high levels is suggestive of a modular regulation mechanism with the expression of several genes being controlled by a common transcription regulatory pathway, whereas the variable levels of dopamine-related gene expression are suggestive of transcriptional control that involves feedback and compensation. Based on mRNA levels in the 39 DGRP lines, neither of these regulatory mechanisms seem to be used exclusively. Instead, it appears that a combination of these mechanisms and other trans-acting regulatory elements are involved in modulating the expression of dopamine-related genes.

SNPs that are associated with the expression of dopamine-related genes in D. melanogaster

Given the variation in dopamine-related gene expression observed in the 39 DGRP lines, we used GWAS and eQTL models to identify SNPs that could be affecting gene expression. Gene expression values from calculations described above were analyzed using the online GWAS pipeline developed for the DGRP (Mackay et al., 2012). This approach called 38 ± 10 statistically significant SNPs per transcript ($p<10^{-5}$), all of which were over 1kb away from dopamine-related genes with the exception of a cluster of SNPs in close proximity to *Vmat*, the vesicular monoamine transporter. For comparison, starvation resistance, startle response, and chill coma recovery generated an average of 175 significant SNPs per trait using this approach (Mackay et al., 2012).

Therefore the number of SNPs associated with dopamine-related gene expression is reasonable, if not conservative.

The number of significant SNPs per chromosome was significantly correlated with the size of the chromosome ($R^2 = 0.78$; Figure 5.2A), but not with the size of the gene ($R^2 = 0.02$). There were twice as many SNPs located in introns than in any other gene region (Figure 5.2B). Functional annotation of the non-synonymous polymorphisms are shown in Table 5.1. Transcription factors (TFs) and calcium handling proteins are the most enriched protein functions observed in this list. This may point to specific TFs that are involved in regulating dopamine receptor expression level.

The main hypothesis we wanted to address with this meta-analysis is that common signaling pathways regulate the expression of numerous dopamine-related genes. The prediction was that certain SNPs would be associated with the expression level of multiple dopamine-related genes. Of the 702 significant SNPs identified, three were associated with multiple dopamine-related genes, each with at least one other nearby SNP that was also associated with each gene. On the X chromosome, a synonymous mutation in the *vanin-like* gene was associated with *Dop1R2* and *ple* expression levels, a mutation in an intron of the *NK7.1* gene was associated with *Dop1R2* and *Dop2R* levels, as was a mutation in an intergenic region of chromosome 3. *Vanin-like* is a hydrolase for nitrogen metabolism that is associated with foraging (Riedl et al., 2005) and *NK7.1* is a homeobox containing transcription factor (Sakoyama et al., 2002). While it makes sense that these genes could impact dopamine signaling, these data do not support the notion that there are master regulators for dopamine signaling genes.

eQTL analysis with a model that accounts for interactions

To further address SNP-transcript associations in these lines we analyzed the data with a statistical package that incorporates all of the transcript levels into a single model. This integrated hierarchical Bayesian model is called iBMQ (Scott-Boyer et al., 2012). After cleaning the data to remove missing values and tri-allelic SNPs we tested for associations between 1,771,775 SNPs and the 19 transcripts. This analysis identified 1,415 unique SNPs that were associated with the expression of dopamine-related genes. Only three of those SNPs were cis-eQTLs (within 1Mb of the gene), each located in intergenic regions several kb upstream and downstream of *Dop2R*.

This analysis identified several SNPs that affected multiple genes. Four SNPs were associated with 3 different genes (Table 5.2), and 43 SNPs were associated with two different genes. The four SNPs associated with three dopamine-related genes could affect genes that influence dopamine signaling. Genes near those markers include a phosphodiesterase and serotonin receptor gene, *for*, and a homeobox containing transcription factor. Among the SNPs affecting 2 genes, there was a pair of SNPs on the third chromosome that was associated with four different dopamine-related genes, *ple*, *Dat*, *Dop2R*, and *Vmat*. Both of these SNPs lie within a putative brain enhancer region, GMR_Brain_exp_1, which was identified in an enhancer screen (Pfeiffer et al., 2008).

Precise agreement between the models was very poor, as none of the SNPs were called by both methods. However, 71% of the correlated SNPs identified with the DGRP GWAS method were within 10kb of SNPs called by the iBMQ algorithm. One technical reason accounts for part of this discrepancy. SNPs with significant correlations are positioned in clusters, and the algorithms have different methods for handling these clusters. Both packages only call a fraction of clustered SNPs, so it is likely that the packages highlight different polymorphisms within the same cluster based on how significance values are integrated.

Behavioral relevance of variation in dopamine-related gene expression

To determine if gene expression levels are representative of dopamine receptor function, we compared l-dopa-induced sensitization of the proboscis extension reflex (PER) in different DGRP lines. Dopamine signaling is required in the PER neural circuits to modulate sucrose acceptance behavior (Marella et al., 2012). Wet-starved flies typically exhibit increased sensitivity to a sucrose stimulus. This change in sensitivity is mediated by *DopEcR* expression in gustatory neurons and can be reproduced in fed flies by supplementing the food with l-dopa (Inagaki et al., 2012). Here we tested the hypothesis that dopaminergic modulation of PER behavior is associated with the level of dopamine receptor expression in DGRP lines.

L-dopa-induced sensitization of PER was demonstrated in Canton-S females (Figure 5.3A) and males (Figure 5.3B). Females treated with l-dopa were more sensitive to 50mM and 100mM sucrose stimuli, as reported previously (Inagaki et al., 2012). We also observed that males treated with l-dopa were more sensitive to higher sucrose concentrations, and that their baseline sucrose sensitivity was significantly lower than females (Figure 5.3C).

Lines RAL_208 (low overall expression of dopamine receptors) and RAL_820 (high overall expression of dopamine receptors) were chosen to assess the two extremes of dopamine receptor expression in the DGRP lines. These lines were identified as having the highest and lowest rank sum of dopamine receptor expression levels among the four dopamine receptor subtypes. RAL_820 has higher mRNA levels than RAL_208 for all four dopamine receptor subtypes.

L-dopa treated RAL_208 females were more sensitive to 400mM sucrose stimulus than fed controls (Figure 5.3D). The prediction was that the increased dopamine receptor expression in RAL_820 females would make this line hypersensitive to l-dopa treatment, causing an increased PER over a wider range of sucrose

concentrations, or a larger increase in sensitivity to the same sucrose concentrations. The latter prediction was observed, i.e., the significance levels for increased sensitivity in RAL_820 were an order of magnitude higher than RAL_208 ($p=0.002$ compared to $p=0.03$; two-way ANOVA). However the more striking result is the difference in baseline sensitivity between the two populations. Fed RAL_820 females were far less sensitive to sucrose than RAL_208 females (Figure 5.3F). Male RAL_820 flies were also less sensitive to sucrose than RAL_208 males (Figure 5.3G). These results indicate that the different levels of dopamine receptors cause homeostatic differences in the regulation of PER circuitry. However the data do not strongly support or reject the notion that the differences are caused by differences in sensitivity to the l-dopa treatment. Therefore we chose a second assay to test the hypothesis that dopamine receptor mRNA levels for one genotype are representative of their sensitivity to exogenous dopamine compared to another genotype.

First we determined that dopamine modulates behavioral responses to tactile stimuli in crawling larva. This was done using a mechanosensory assay that tests responsiveness to moderate (20mN) tactile stimuli applied to three regions of the dorsal midline, the head, abdomen, and tail (Titlow et al., In Press). Pre-treating third instar Canton-S larvae with dopamine for 1h significantly increased their sensitivity to tail touches, and moderately increased sensitivity to abdomen and head touches (Figure 5.4A). We then used RAL_820 (high dopamine receptor expression) and line RAL_730 (low dopamine receptor mRNA for males and females), to determine if the populations respond differently to exogenous dopamine. Compared to untreated controls, RAL_820 flies pre-treated with dopamine for 1h were significantly more sensitive to tactile stimuli in all three anatomical regions tested (Student's t-test, $p<0.005$). Whereas dopamine-treated RAL_730 flies were not significantly more sensitive to tactile stimuli than untreated controls. It appears that the increased levels of dopamine receptor expression

in RAL_820 resulted in increased sensitivity to acute exogenous dopamine treatment. This suggests that mRNA levels are indicative of dopamine receptor function in flies. Together these data also support the idea that relative dopamine receptor mRNA levels between these two populations are maintained from the third instar larva stage to adult stage, as the predictions are based on microarray data obtained from adult tissue.

Developmental variation in the expression of dopamine receptors

We employed additional bioinformatic analyses to investigate the genetics of dopamine signaling during *D. melanogaster* development. Zhou et al. (2012) showed that treating *D. melanogaster* with exogenous dopamine (47mM) during embryogenesis and larval development delays eclosion by an average of 36 hours. Inhibiting dopamine synthesis during development has also been shown to affect nervous system development both in larval stages and in adult behavior (Neckameyer et al., 2001, Neckameyer and Bhatt, 2012). Systemic dopamine levels start off high and decrease throughout larval development (Cooper and Neckameyer, 1999). To determine how this coincides with receptor levels at various developmental time points we compiled RNAseq data from a public repository (Graveley et al., 2011). From these data we observed that the dopamine receptors are expressed at different levels throughout development (Figure 5.5). *DopEcR* is the first receptor expressed beginning midway through embryonic development, and is then expressed at substantially higher levels at all other stages. *Dop1R2* exhibits the second highest expression levels, followed by *Dop2R* and *Dop1R1*. This relative order of expression levels is maintained throughout life. From early to late larval stages the expression level of each receptor decreases, which parallels the decrease in systemic dopamine levels during this time frame (Cooper and Neckameyer, 1999). Together these data emphasize the fact that dopamine pathways are dynamically regulated throughout development.

Environmental variation in dopamine-related gene expression

Findings reported in the literature and the experiments described above make it clear that manipulating dopamine levels causes nervous system aberrations. Zhou et al. (2012) provided molecular insight into the mechanisms of dopaminergic modulation by performing genome-wide expression analysis on flies that were treated with exogenous dopamine (47mM). This treatment affected the expression of 257 genes, only one of which is known to be directly involved in dopamine signaling (*ebony*). Many of the genes have not been functionally characterized. To learn more about this dopamine-modulated gene network we cross-referenced those genes with QTL data from the Ayroles study where we obtained microarray data (Ayroles et al., 2009). In that study QTL mapping was performed for several behavioral and fitness traits, including copulation latency, lifespan, fitness, locomotor reactivity, chill coma response, and starvation resistance. Each of these traits was influenced by at least five genes whose expression level was also sensitive to exogenous dopamine (Figure 5.6). Cold shock response was influenced by the most genes (22), and several genes were pleiotropic, with fitness and starvation resistance having the largest number of shared influential genes (5). QTLs were also mapped to dopamine-related genes for these traits, providing further insight into how dopamine signaling interacts with these gene networks to influence specific traits.

DISCUSSION

Studies with the DGRP lines have shown that there is considerable phenotypic and genotypic variation between the lines. Part of the goal of this Chapter was to simply emphasize the dopamine-related quantitative genetics data from those studies. Cross-referencing the Zhou et al. (2012) study with the Ayroles et al. (2009) study elucidates potential gene networks related to dopamine and provides a way to rank which interactions are most likely to be real.

Pulling out the expression values for dopamine-related genes shows how this trait varies in genetically diverse populations. To develop hypotheses as to what causes that variation I performed SNP-trait analyses and identified about 2,000 potential markers in over 1,800 different genes. To determine which ones were likely to be involved in dopamine signaling I cross-referenced that list with genes that were shown to change expression levels in response to exogenous dopamine (257 total). This decreased the list to 22 genes likely to be involved in dopamine-related genetic variation (Table 5.2). Based on their known biological function it is difficult to determine why the expression of those genes would be linked to exogenous dopamine levels, but matching those genes together with the phenotype graph in Figure 5.6 indicates that CG3264 is within a QTL for both copulation latency and fitness, and carries an eQTL for *skeletor*. This approach not only provides predictions based on statistics, but it also shows which trait is most likely to reveal the interactions.

The other interesting and potentially relevant hit identified in this eQTL cross-referencing scheme was *cheerio (cher)*, a filamin actin binding protein that has known roles in motor neuron axon guidance (Zheng et al., 2011) and olfactory learning in flies. There are 7 intronic SNPs and a synonymous coding SNP within this gene that are all associated with *ple* expression. *Cher* expression levels decrease in response to exogenous dopamine. The question then is how do *cher* polymorphisms affect dopamine homoeostasis and plasticity?

Compensation for low expression of subtypes by increased expression of other subtypes was not consistent. Such adaptive compensatory mechanisms are equally unpredictable in *murine* dopamine receptor mutants. A D3 receptor knockout does not exhibit changes in any of the other dopamine receptor subtypes in the spinal cord (Zhu et al., 2008), yet D3 receptors compensate for the loss of D2 receptor function in the D2

knockout mouse (Jung et al., 1999). This is something that would be interesting to address in this system at the sub-brain level using RNAseq.

A modular network of genes that drive dopamine homeostasis

My hypothesis was that there is a master regulator of dopamine signaling like LEAFY is for plants (William et al., 2004). If there is such a thing for dopamine signaling in the *D. melanogaster* CNS then it must work by upregulating some dopamine-related genes and downregulating others. Between different DGRP lines, the relative levels of the different genes are not consistently high or low, rather each line has a mixture of high and low expressed genes that do not correlate with their known role in dopamine homeostasis. Obtaining brain dopamine levels in each of the DGRP lines would be a powerful way to investigate how physiology of the dopamine system feeds back into the regulatory mechanisms for dopamine-related genes. It is also unclear whether the sequence variants associated with dopamine-related gene expression act through physiological feedback loops or if those loci are cis-regulatory elements for the dopamine-related genes. The only classification for calling the eQTLs trans-acting was based on distance from the gene, 1MBp, which was simply modeled after earlier eQTL studies.

This study identifies several transcription factors that could regulate dopamine-related gene expression. *esg* was identified in the Zhou et al. study (2012) and several were found here to have SNPs that are correlated with dopamine-related gene expression levels. Experiments are needed to determine if these associations are found in the CNS or systemically. *Ebony* was the only gene affected by exogenous dopamine treatment in the Zhou study. *Ebony* encodes a beta-anlanyl-dopamine synthetase that is expressed in the nervous system and cuticle (Berry et al., 2012). An important difference between dopamine catabolism in *D. melanogaster* and humans is that dopamine is not

degraded by monoamine oxygenase in flies, instead it is used in scleritization of the cuticle with the cuticle acting as a dopamine sink (Wright, 1987).

Genes that indirectly affect dopaminergic function

One aspect of this study was to test hypotheses related to gene products that interact directly with dopamine in the nervous system. But an additional goal was to identify new candidate genes that contribute to complex traits related to dopaminergic function. The list of 257 genes differentially expressed in response to dopamine treatment are potential candidates. The nine genes associated with copulation latency and five genes associated with locomotor reactivity are even stronger candidates given that these traits have already been associated with dopamine signaling in the earlier microarray experiment (Evans and Maqueira, 2005), and using traditional reverse genetics and pharmacological techniques (Riemensperger et al., 2011, Stansley and Yamamoto, 2013). The majority of these genes do not have a known biological function, which presents its own challenge for gene discovery. The characterized genes that are sensitive to dopamine treatment and involved in copulation latency are *uro*-urate oxidase, *TTLL3B-a* tubulin-tyrosine ligase, *PGRP-SC1a-a* peptidoglycan binding protein involved in the innate immune response, and *LCP1-a,* larval cuticle protein. A simple explanation exists for why these genes are associated with exogenous dopamine, i.e. they are associated with scleritization and nitrogen compensation. But the role of these genes in reproductive behavior is somewhat peculiar.

The dopamine-sensitive genes associated with locomotor reactivity were *Arc1*, a zinc-binding protein associated with starvation response (Wright, 1987), *Cyp6d2*, a cytochrome, and Ptp52f, a fibronectin involved in axon guidance (Draper et al., 2007). The former are likely associated with dopamine biochemistry and have a peculiar association with the trait as mentioned for the genes above. But Ptp52f is interesting

from a neurobiological perspective. Dopamine is known to act as a neurotrophic factor during development (Neckameyer and Bhatt, 2013, Chapter 4), so it would be interesting to see what the role of this protein is in the mature nervous system and how it is related to dopamine. One possibility is that the gene is downstream of *escargot*, a well-characterized transcription factor that was up-regulated in response to dopamine treatment. At the mRNA level there was a positive correlation between *escargot* and type-2 dopamine receptor levels among the 40 DGRP lines ($p=0.03$).

Dopaminergic neurons innervate numerous regions of mammalian brains (Ko and Strafella, 2012) and every neuropil in the *Drosophila* brain (Riemensperger et al., 2011). This complexity has made it difficult to fully comprehend dopamine function and pathology at the organism level. Behavioral phenotypes have been identified using loss of function mouse (Eells, 2003) and fly mutants (Porzgen et al., 2001, Riemensperger et al., 2011) that are defective in some aspect of dopamine signaling, i.e. synthesis, degradation, transport, or signal transduction. But it is unclear how natural allelic variation in these genes contributes to complex traits in wild type populations. The fields of pharmacogenomics and personalized medicine stand to benefit from quantitative genetic analysis of dopamine signaling and behavior, as human alleles related to dopamine signaling genes have already been correlated with abnormal behavior (Kieling et al., 2010).

Conclusion

The DGRP lines exhibit diversity in dopamine signaling that is evident at the levels of DNA sequence and gene expression. This study identifies specific sequence variants that are likely to affect the expression of dopamine-related genes and it shows that those molecular differences are correlated with differences in the animal's behavior at different developmental stages.

Table 5.1. Non-synonymous SNPs that influence dopamine-related gene expression levels.

Affected gene	Effector Gene	Effector Gene Function
DATT	GM130	Golgi organization (Rab GTPase binding)
Dop1R1	CG12517	Cytokine (chorion egg shell forming)
Dop2Ra	c-cup	Meiosis
Dop2Ra	CG43209	Unknown
Dop2Ra	CG8027	Notch domain
Dop2Ra	CalpB	Proteolysis (Ef hand Ca binding)
Dop2Ra	CG13003	Unknown
Dop2Rb	Sur	ABC transporter ATPase
esg	Tom70	Protein targeting to mitochondrion
esg	Cpn	Sequesters Ca
esg	CG10841	Ef hand Ca binding
esg	fog	Developmental signal
ple	CG15356	EMSY (transcriptional repressor)
ple	GalNac-T2	Oligosaccharide biosynthesis
Skeletor2	Elp2	Transcription elongation factor
Tbh	CG7362	Glycoylsis (pyruvate kinase)
Vmat1	CG1784	Synaptojanin (phosphoric ester hydrolase)
Vmat1	CG9624	Unknown
Vmat4	mTerf5	mito TF
Vmat4	E(spl)m8-HLH	TF

Table 5.2. Genes affected by exogenous dopamine in the DGRP lines that also carry SNPs in dopamine-related genes. *GWAS, otherwise identified using iBMQ.

Gene name	Function
CG4562	ABC transporter- nucleotidehydrolase
CG18173	acyltransferase
CG3264*	alkaline phosphatase
CG8785*	amino acid membrane transporter
CG32473	aminopeptidase
CG8745	aminotransferase
CG13659	choline kinase
LKR	dehydrogenase- histone regulation
CG14495	DUF725 unknown function
Cher*	filamin- actin binding
CG10912	glycoprotein
CG14963	insect allergen-related
yellow-e	isomerase- melanin biosynthesis
CG7702	leucine rich protein
CG15120	parkin co-regulated
CG9673	serine peptidase
CG3344	serine peptidase
CG32483	serine peptidase
vg	TF
Ugt86Dc	UDP-glucosyltransferase- metabolism
CG13482	unknown

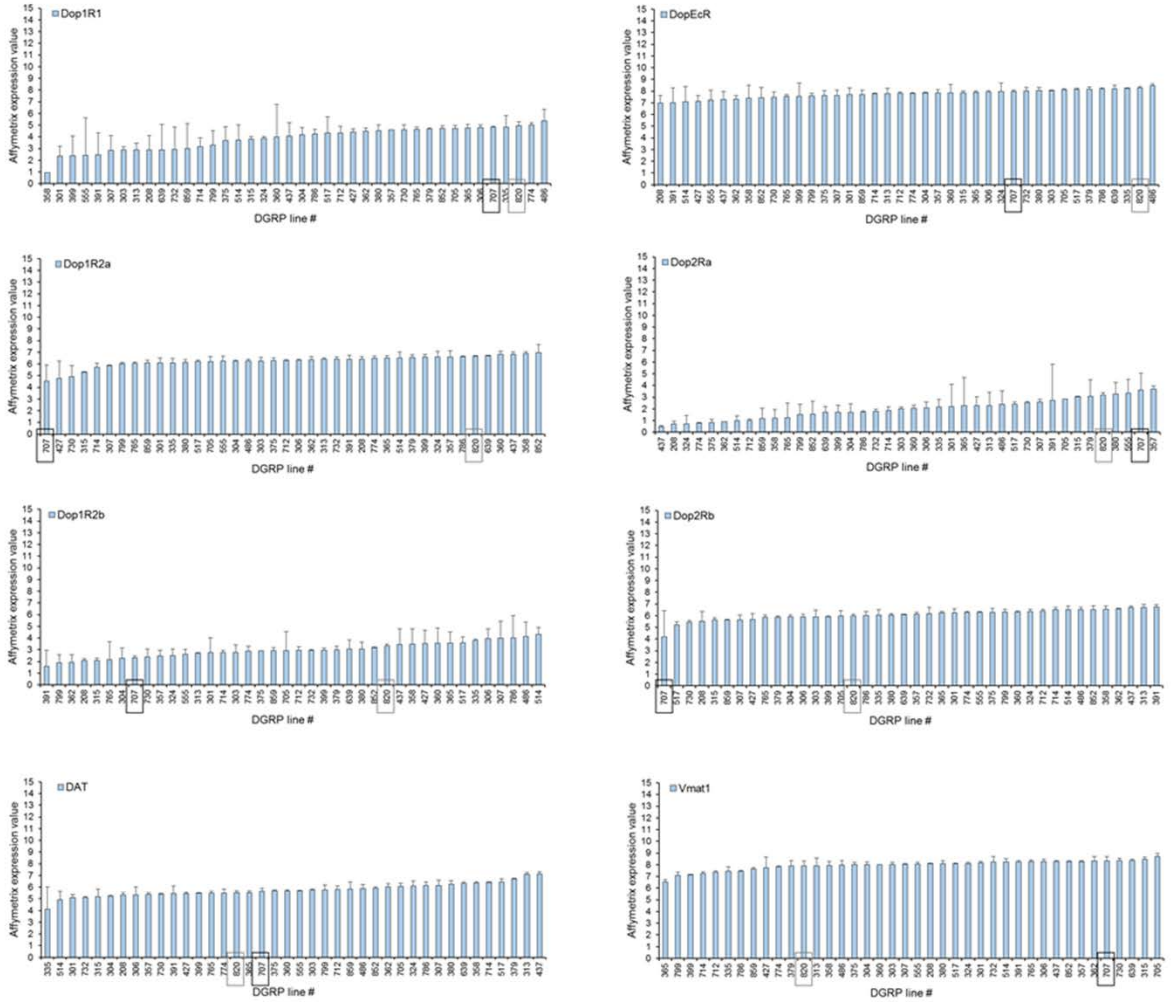

Figure 5.1. Variation in the expression of dopamine-related genes among 39 DGRP lines. Expression values were derived from the median $\log_2$ signal intensity of *Drosophila* 2.0 Affymetrix probes (Ayroles et al., 2009). Average expression values from two samples of 3-5 d-old adult males (25 flies/sample) are shown for each line (± SD). Note the differences in the range of expression values for *Dop1R*, *Dop1R2b*, and *Dop2Ra*, compared to the transporters, *DAT* and *Vmat1*. Two lines are highlighted to demonstrate mostly high (black circles) and highly variable (gray squares) levels of dopamine-related gene expression in specific lines. Plots of expression values for all 24 dopamine-related genes in males and females are shown in the supplemental data spreadsheet.

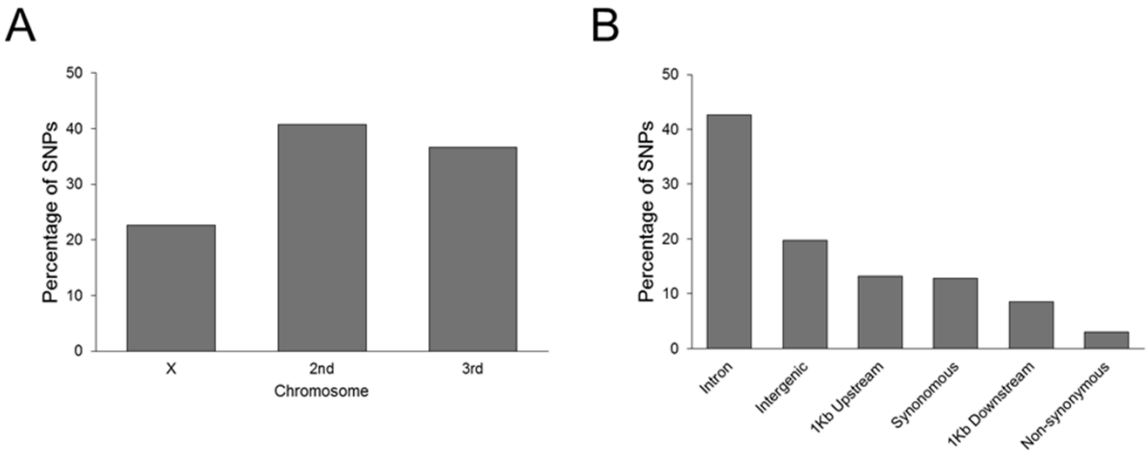

Figure 5.2. Characterization of SNPs that were correlated with dopamine-related gene expression with the DGRP GWAS pipeline. *(A)*- Distribution of significant SNPs by chromosome (n= 702 total SNPs). *(B)*- Functional classification of dopamine-related SNPs.

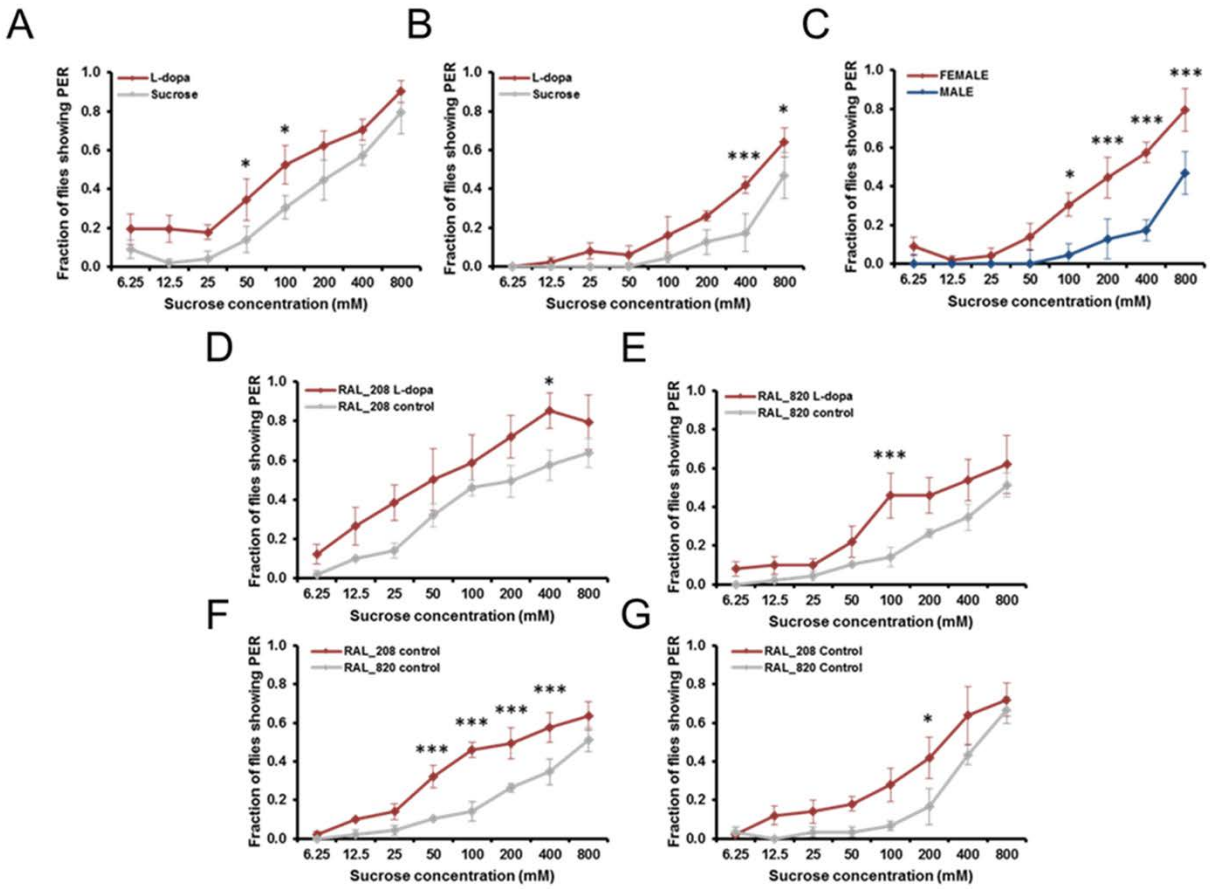

Figure 5.3. Individual differences in a dopamine-modulated behavior are correlated with gene expression data. *(A-B)*- L-dopa-treated flies (red lines) are more sensitive to sucrose stimuli applied to their proboscis than fed controls. Females (*A*) are significantly more sensitive to 50mM and 100mN, males (*B*) are significantly more sensitive to 400mM and 800mM. Fed females have a higher baseline sensitivity to sucrose stimuli than males (*C*). L-dopa-mediated sensitivity is not remarkably different in a low dopamine receptor expression line (RAL_208, *D*) compared to a high dopamine receptor expression line (RAL_820, *E*). RAL_820 has significantly reduced baseline sucrose sensitivity for females (*F*) and males (*G*).

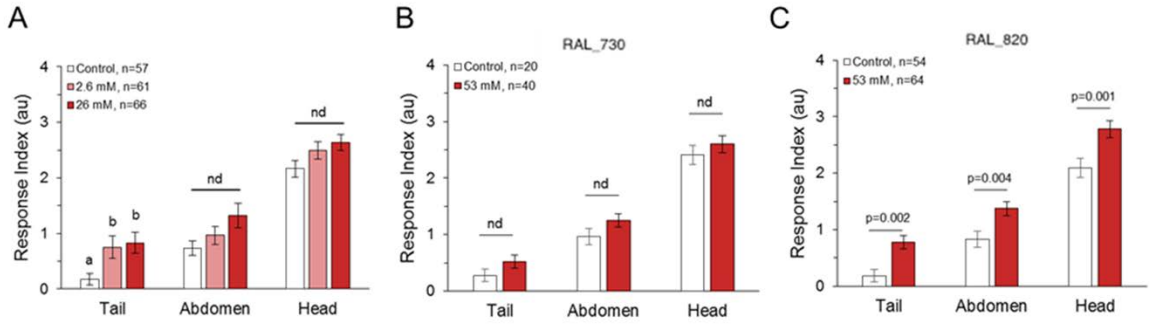

Figure 5.4. Effects of dopamine on mechanosensory behavior are genotype specific. A- Canton-S larvae were treated for 1h with increasing concentrations of dopamine. Treated animals were more significantly more responsive to tail touches (ANOVA on Ranks; different letters indicate $p<0.05$; mean ± SEM). B- Inhibiting dopamine synthesis by 24h pretreatment with alpha-p-methyltyrosine significantly decreased responsiveness to abdomen and head touches. C- Treating a low dopamine receptor expression line (RAL_730) for 1h with dopamine did significantly increase responses to tactile stimuli. D- The high dopamine receptor expression line (RAL_820) was significantly more responsive to all three stimuli when treated with dopamine.

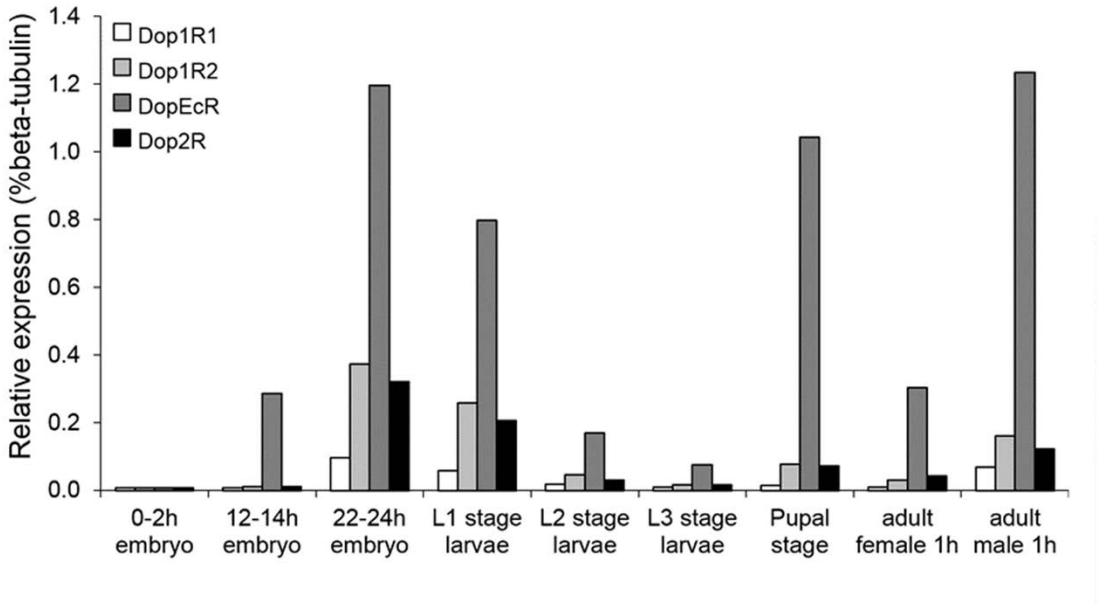

Figure 5.5. Changes in the expression of dopamine receptors throughout development. RNAseq data were used to compare dopamine receptor expression at various developmental stages (Graveley et al., 2011). The relative proportion of each mRNA for the receptors remains fairly constant. Between L3 and adult stages, which were the focus of our experiment, only *DopEcR* expression is remarkably different.

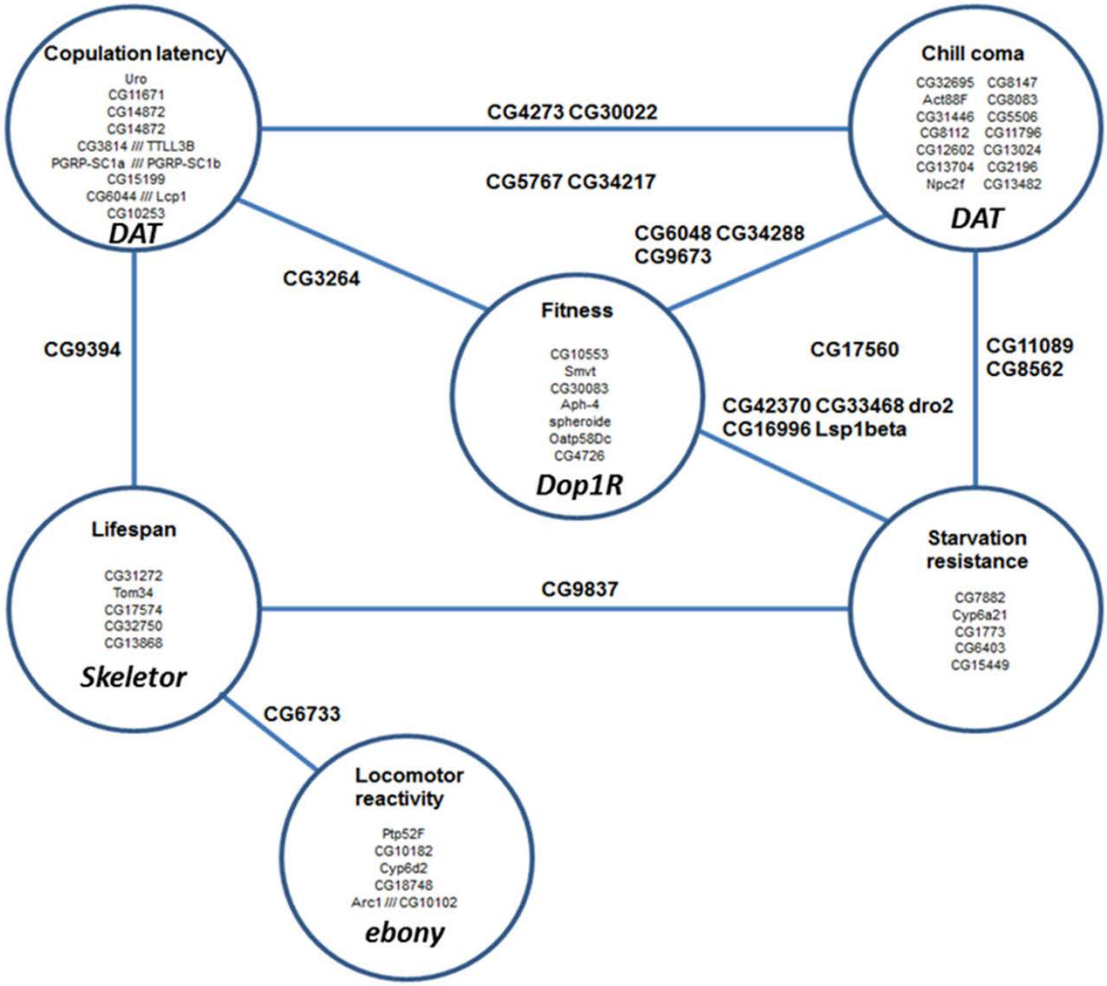

Figure 5.6. Drosophila genes associated with quantitative traits. Expression levels of these genes were affected by dopamine in the Zhou study (Zhou et al., 2012). Dopamine-related genes that were associated with these traits are in large bold font. Nodes represent phenotypic traits characterized in the Ayroles study (Ayroles et al., 2009). Several of these genes were pleiotropic (genes positioned between multiple nodes).

CHAPTER SIX

Impact and Future Directions

Impact on the field of neural circuit biology

Several new features of larval mechanosensory behavior were described throughout this work that have general implications for neural circuit function and plasticity. The observation of spike-timing dependent mechanosensory responses was the most compelling finding in Chapter 2 (Figures 2.7-2.9). Spike-timing dependent plasticity is basic Hebbian logic (neurons that fire together, wire together) and is well known from synaptic long term potentiation and long term depression studies in mammalian brain slices (Feldman, 2012). The mechanisms of spike-timing dependent synaptic responses are not as well understood, but they have been modeled for individual cells based on electrophysiological data from globus pallidus neurons (Schultheiss et al., 2010). These data emphasize the different effects of somatic and dendritic synaptic inputs on spiking parameters. Based on physiologically realistic phase response curves, representations of how inputs affect the neuron's spike cycle, the incidence of skipped or added responses depends on the input phase and its relation to the spike cycle (Schultheiss et al., 2012). My *in vivo* data indicate that spike timing dependence at the neural circuit level operates under a similar principle. Further analysis of this phenomenon awaits elucidation of the interneurons involved in the circuit and knowledge of their intrinsic firing properties (discussed below).

I think the electrophysiological data also provide the most compelling results in Chapter 3 (Figures 3.7CE). Habituation in that system is completely novel and accessible for more detailed follow-up on the neural circuit mechanisms, but I would be most excited to follow-up on the electrophysiology data. It was surprising to observe that

the 5s ISI did not have an effect on the average number of eEPSPs per stimulus. Our lab has performed enough of these experiments to know that an occasional increase in the number of EPSPs occurs at 10s ISI, but not enough to affect to the baseline. Based on those observations I expected to see more frequent increases in the 5s ISI experiments. However the 60s ISI actually caused increases, which seem to resemble sensitization. My interpretation of those results is that habituation processes and sensitization processes were simultaneously activated upon activation of all of the sensory neurons within a segmental nerve. Interaction between the two pathways neutralized the effects, resulting in a steady baseline, the so called dual-process theory (Prescott, 1998). However at the 60s ISI, habituation pathways were relatively weak, allowing sensitization to dominate. This is a testable hypothesis. I predict that EPSPs evoked by activating nociceptive neurons would exhibit a similar increase in response to repetitive stimulations, and that the response to repetitive activation of class III gentle touch neurons would eventually habituate. If this is true, then this system could be useful for testing pain medications.

The work on dopaminergic modulation of mechanosensation in Chapter 4 and dopamine-related genes in Chapter 5 fits in with a recent study by Hodges *et al*. (2013) that describes how functional polymorphisms in the *Drosophila* dopa-decarboxylase gene (*Ddc*) confer fitness traits against wasp parasitism (Hodges et al., 2013). The homozygous TCG haplotype has an increased ability to avoid being parasitized by *L. boulardi* both in the lab and in the field (Hodges et al., 2013). Compared to larvae with the CAT and heterozygous haplotypes, the TCG morph also exhibits increased foraging activity and feeding rates, which are believed to be secondary to reduced levels of endogenous dopamine. Though Hodges *et al*., (2013) show that the different morphs exhibit differences in motor behavior, it is unclear how these behavioral differences influence interactions between larvae and wasps. The straightforward approach to

assessing how larvae with the different alleles are able to evade predation would be to directly quantify behaviors observed and success rates for encounters between the larvae and *L. boulardi* females (Robertson et al., 2013). Those findings could then be complemented with the methods described in Chapters 2 and 3 to compare the behaviors at the neural circuit level. Do larvae with the different *Ddc* alleles exhibit differences in mechanosensation or in habituation? Do they exhibit differences in synaptic transmission or morphology at the NMJ? How do the altered levels of dopamine affect the activity of dopaminergic neurons? These naturally occurring alleles would also be an asset for investigating feedback loops at the molecular level. How do the differences in dopamine levels influence the expression of other genes involved in dopamine signaling, e.g., receptors, transporters, tyrosine hydroxylase and other metabolic enzymes?

Impact on pharmacogenetics and personalized medicine

A major goal of human genomics research is to identify DNA sequence abnormalities that cause diseases. Genes related to dopamine have been implicated in a number of these association studies for diseases of the nervous system (see Chapter 5- Introduction). My work on associations between SNPs and transcript abundance provides evidence for pathways that could be driving variation in the dopamine-related gene network (Chapter 5- Results and Discussion). It is possible that similar epistatic gene interactions are also present in human cells, in which case the associations found in this study may point to variants that contribute to aberrant dopamine signaling and related neurological disorders. Using *Drosophila* transgenics these hypothetical correlations can be verified at the molecular and physiological level. Meta-analysis of human GWAS data can then be used to determine if variants exist in human

homologues of the genes and whether they have been associated with disorders caused by aberrant dopamine signaling.

Future directions

Identifying the neural circuit for mechanosensation in D. melanogaster larvae

Identifying interneurons that process mechanosensory input should be high on the list of things to do if this system is to reach its full potential for elucidating mechanisms of neural circuit function. The first question to ask is simply which interneurons are activated by mechanosensory input. To determine which cells and synapses are modulated by dopamine and habituation, we need to determine which cells are activated by sensory input in the first place. We would also need to determine the basic synaptic responses in those cells, i.e., how long are the cells activated in response to sensory input, what is the firing frequency and spike amplitude? From there we would be able to determine how those responses are modulated by dopamine or habituation.

The most straight forward approach to identify neurons in this circuit would be to screen the Rubin collection of neuronal GAL4 lines that drive transgene expression in small subsets of interneurons (Jenett et al., 2012). Those lines could be crossed with lines that carry the most current version of UAS-GcAMP (Akerboom et al., 2012), and screened for evoked responses to nerve root stimulation in larval fillet preparations. The throughput is low, but it is essentially a reverse genetic screen because the lines are being chosen based on images of the expression patterns. The payoff is big because once the lines are identified, those cells can be controlled with optogenetic stimulation, and the neurons can be genetically silenced to determine their role in behaviors. Dopamine receptors or other genes putatively involved in synaptic plasticity could be

knocked down specifically in those cells, or the cells could be selectively killed to determine their role in the development of mechanosensory circuits.

It will be interesting to see what types of interneurons are included in the mechanosensory circuit, and which types are involved in habituation. Recurrent inhibition through facilitation of GABA-ergic neurons seems to be a prevalent neural circuit level mechanism for plasticity in the *D. melanogaster* brain (Larkin et al., 2010, Das et al., 2011, Paranjpe et al., 2012, Sadanandappa et al., 2013). Is this mechanism recapitulated in the ventral nerve cord, or are there examples of the opposite process, e.g., recurrent inhibition through depression of excitatory neurons.

Characterizing motor activity patterns in response to sensory input

What types of motor unit activity are activated by tactile sensory input? Is it coded as different patterns of activity, different burst durations, or different frequencies? The answer to these questions are important because the motor output tells us what types of activity patterns to expect from sensory integration centers. The change in sensory-evoked motor pattern could also be the functional readout of habituation. Suppose that a typical sensory stimulus evokes a burst of motor activity that initiates a muscle contraction and interrupts crawling. At what point does the motor response fall below the threshold of initiating a behavioral response? The animal often pauses before making a decision, what is the nature of inhibitory input to the motor neurons? These are basic physiological characterizations of the mechanosensory neural circuits that need to be completed before this system will yield mechanistic insight into neural network function.

Acquiring these data is relatively straightforward. Motor neuron somata in the VNC are some of the most accessible neurons in the larval CNS. Intracellular recordings could be taken from individual cells by stimulating interneurons optogenetically, or by

activating segmental nerve roots electrically. An alternative approach would be to express GcAMP or a genetically encoded voltage indicator in motor neurons to monitor sensory-evoked synaptic potentials (Cao et al., 2013).

A tractable system to study ethologically relevant behavior at the molecular level

Activity-dependent modulation of neural circuits is one mechanism through which organisms learn at the cellular level. In the case of tactile sensory habituation, the larvae are ignoring a repetitive innocuous stimulus in favor of enhancing their sensitivity to novel and more salient stimuli. At the cellular level, this form of plasticity requires morphological and physiological changes to the strength of synapses. The input and output nodes of this mechanosensory circuit, and other circuits throughout nervous systems in various animals are resolved at the single cell level. The interesting physiology takes place in the interneurons that store, modulate, and convey signals between afferent and efferent neurons. However we know relatively little about the interneuron nodes, except for in the case of classical command neurons (Olson and Krasne, 1981, Rock et al., 1981, Flood et al., 2013). Such nodes are likely to be discovered in the *Drosophila* larval ventral nerve cord using high-throughput behavioral screening (Ohyama et al., 2013) and genetic control of small subsets of neurons (Jenett et al., 2012). A portion of the interneurons that contribute to larval motor behavior have been described (Iyengar et al., 2011), as a more complete mechanosensory circuit emerges in genetically controllable cells, the macroscopic findings described in this dissertation will have a big impact on how plasticity is addressed in this system.

Potential preparations to study long-term memory in larvae

This work characterized short-term habituation to tactile stimuli in larvae. Moving forward with larval mechanosensory habituation research it will be important to

determine to what extent this circuit acquires long-term habituation. The most effective stimulation protocols typically use serial blocks of treatments with long inter-stimulus intervals. Based on the similarity in habituation kinetics and magnitude between *D. melanogaster* larvae and *C. elegans*, I hypothesize that the following spaced training paradigm will induce long-term memory in larvae: 4 blocks of 20 stimulations (60s ISI), with each block separated by 1h (Rose et al., 2002). In *C. elegans* the attenuated response lasts at least 24h, whereas in *Aplysia*, mechanosensory habituation can last over a week (Carew et al., 1972). It will be interesting to determine how long these mechanosensory memories can last in *D. melanogaster* larvae, or how long olfactory memories could last when larval development is experimentally prolonged (Larkin et al., 2010).

Larval development in *D. melanogaster* takes 4-5 days under normal conditions, i.e., adequate nutrition, 23°C, 75% humidity, which means that the potential for studying long-term memory is limited. However, there are two at least three options for increasing the duration of the third instar larval development stage to investigate long-term habituation. One is an extended third instar stage (ETI) paradigm induced by knockdown of a hormone receptor (Miller et al., 2012). Though the third instar stage triples from 3 to 9 days and extensive synaptic overgrowth occurs, synaptic transmission remains largely unchanged. Other approaches would be to culture the larvae at cold temperatures or with limited nutrition.

After the second molt, larvae could be exposed to the memory-inducing stimulus paradigm, and then be transferred to low temperature or non-nutritional media. It would require a lot of man-hours with our approach, a high-throughput approach that has been developed for testing non-localized mechanosensory responses would be more efficient (Ohyama et al., 2013). Sound vibrations or light air puffs evoke behavioral responses from several larvae simultaneously in a dish, and the responses are analyzed by

tracking software. The behavioral repertoire in response to these stimuli is not as robust, so the larvae may have to be conditioned with this training paradigm and manually tested them after 24hr training. If this form of long-term memory is robust, it could be used as a primary screen for genes that are involved in long-term memory. Given an identified circuit from the experiments proposed above, one could then investigate activity patterns and molecular processes that occur as memories are being formed.

Important experiments that would be more reasonable

The experiments proposed above are relatively complex, high risk experiments. After looking at this work through a low power lens some more reasonable experiments come to mind that would also yield interesting results. The first experiment comes from the habituation work. We found that the *dnc* mutant exhibited a low threshold mechanosensory phenotype, though it didn't exhibit a habituation phenotype (Chapter 3, Figure 6D). This brings up the questions of whether the *dnc* mutation also affects higher threshold tactile responses, and whether the distribution of mechanosensory response behaviors is affected. This could be tested simply by collecting 20mN touch response data with these mutants.

None of the pharmacological treatments that affected mechanosensation were tested for effects on habituation. MPH treatments are particularly interesting given their association with neural plasticity in humans. We didn't observe a mechanosensory response phenotype in larvae treated acutely (1hr) or long-term (24hr) with MPH, however long-term treatment caused decreased locomotor activity and 3hr doses have been shown to increase larval locomotor activity (Pizzo et al., 2013). It would be interesting to see how a 3hr MPH treatment would affect habituation.

Functional MRI studies in humans are accumulating large amounts of data from patients and healthy volunteers that will ultimately point to physiological signatures in regions of

the brain that are affected by different neurological disorders (Turk-Browne, 2013). Those physiological signatures can then be correlated with molecular markers (Thompson et al., 2013).

CHAPTER SEVEN

Pharmacological analysis of dopamine modulation in the *Drosophila melanogaster* larval heart

*This chapter has been published in *Physiological Reports* (Titlow et al., 2013). The work is published under the Creative Commons Attribution License and does not require permission for academic reuse. Ms. Jenna Rufer, Ms. Kayla King and Dr. Cooper helped collect data. The rest of the experiments, data analysis, and writing was done by Mr. Josh Titlow.

INTRODUCTION

Dopamine (DA) is a well characterized neurotransmitter that also exhibits modulatory effects on peripheral tissues. Cardiac function is influenced by DA in several species, e.g., *Periplaneta americana* (Collins and Miller, 1977), *Ligia exotica* (Yamagishi et al., 2004b), *Tapes watlingi* (de Rome et al., 1980), *Drosophila melanogaster* (Zornik et al., 1999), *Canus lupus* (Chen et al., 2007), *Cavia porcellus* (Habuchi et al., 1997), *Mus musculus* (Asghar et al., 2011), and *Homo sapiens* (Cosyns et al., 2013). Chronic use of DA pro-drugs (e.g., L-dopa) has been linked to cardiac valve dysfunction in man (Delgado et al., 2012). Though DA receptors have been identified in mammalian cardiac tissue (Cavallotti et al., 2010; Tonnarini et al., 2011), pharmacological analysis of the effects of DA on heart rate and other aspects of cardiac function are lacking. Doing so will increase our understanding of how the cardiac rhythm is modulated and how it is affected by systemic DA homeostasis.

The larval *D. melanogaster* heart is a myogenic tube that spans the rostral:caudal axis of the animal (Gu and Singh, 1995). Hemolymph is drawn into the heart through ostia in the posterior pump (which is analogous to a ventricle) and circulated through an aorta back into the visceral lumen. Similarities in the developmental genetics (Bodmer, 1995; Bodmer and Venkatesh, 1998) and physiology

(Choma et al., 2011) between *D. melanogaster* and human hearts make the larval heart an insightful model system.

DA has a positive chronotropic effect (meaning change in heart rate) on the adult and pupal heart (Johnson et al., 1997; Zornik et al., 1999). To investigate the molecular mechanisms mediating cardiac dopaminergic effects we used the semi-intact *Drosophila* larva preparation (Cooper et al., 2009). One advantage of this preparation for pharmacological analysis is that the heart is quickly isolated from the nervous system and other sources of modulatory input. Because DA homeostasis is often manipulated systemically to study larval behavior (Neckameyer and Bhatt, 2012) we are also interested in the effects of DA on cardiac function.

In the nervous system, and in smooth muscle, dopaminergic modulation proceeds through canonical G protein-coupled receptor (GPCR) pathways (Neve et al., 2004). Arthropod DA receptors exhibit strong functional and pharmacological similarities to vertebrate receptors (Mustard et al., 2005; Yuan and Lee, 2007). Four DA receptors have been described in *D. melanogaster*. Based on sequence identity and cAMP accumulation assays they can be classified as type-1 (DopR, DopR2, DopEcR) or type-2 (D2R) (Gotzes et al., 1994; Gotzes and Baumann, 1996; Han et al., 1996; Sugamori et al., 1995). Type-1 DA and type-2 DA receptors are either positively or negatively coupled adenylate cyclase through stimulatory and inhibitory G protein alpha subunits. Type-2 DA receptors are also known to function through protein kinase C (PKC) and calcium-dependent pathways (Yan et al., 1999). The degree to which GPCRs activate phospholipase-C and other second messenger cascades in *D. melanogaster* hearts is not completely understood and we are far from understanding how information from multiple signaling pathways is integrated. The aim of this study was to determine if vertebrate drugs targeting DA receptors and second messengers have an effect on this preparation with the long term goal of dissecting interactions between multiple pathways.

MATERIALS AND METHODS

Heart rate assay

A Canton S strain that has been isogenic in the lab for several years was used for all experiments. Flies were maintained on a 12hr light:dark cycle in bottles at medium density and fed standard cornmeal fly food (Bloomington stock center recipe). Early third instar larvae were pinned ventral side up on a glass plate and dissected in a droplet of HL3 saline (Stewart et al., 1994): (in mM) 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 1 $CaCl_2$, 5 trehalose, 115 sucrose, 25 N,N-Bis-(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES). Note the following modifications: pH was decreased from 7.2 to 7.1 and BES buffer was increased from 5.0 mM to 25.0 mM to maintain stable pH. All recordings were made at room temperature (21-23°C) between 9-5pm.

The larva dissection was first described by Gu and Singh (1995). Early third instars were opened by an incision in the ventral midline and visceral organs were removed without touching the heart. After recovering from surgery for five minutes the heart was visualized through a dissecting microscope and the baseline heart rate was measured by directly counting contractions in the posterior “heart” region. The saline was then carefully removed and exchanged with the various drug solutions. Counts in the new solution were taken one minute after the exchange to allow the heart time to adjust after mechanical agitation, and for the ninth minute after applying the solution to determine the duration of modulatory effects. Hearts that did not beat continuously or stopped beating at the end of the experiment were not included in our analyses. As a control for the solution change, heart rate was measured after exchanging saline with fresh saline.

Pharmacology

SCH23390, SKF38393, Quinpirole, SQ22536, and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma Aldrich (St. Louis, MO). Spiperone was

purchased from TOCRIS (Minneapolis, MN). Dopamine-HCl and each of the saline salts were purchased from Sigma. DA was weighed out and prepared daily. The other drugs were prepared from stock solutions. Lipophilic drugs were dissolved in saline solutions containing less than 1.0% DMSO. Saline containing 1.0% DMSO did not have an effect on larval heart rate.

Analysis

Heart rates were determined by counting the number of contractions observed in the posterior region of the heart (between seventh and eight abdominal segments). Contractions were counted by visual inspection through a dissection microscope. The rates measured after drug treatment were normalized to the rate measured before drug treatment (baseline). Normalized values were then pooled for each treatment and the two-tailed Student's t-test (Sigma Plot, 12.0) was used to compare drug treatments to saline treatments (control) and to compare different concentrations of drug treatments. Data points depict the mean and sem. for each treatment at a given one-minute interval during the experiment. Sample sizes for each experiment are indicated in the figure legends.

RESULTS

Dopamine increases larval heart rate.

The average baseline heart rate (HR) measured in dissected third instar larvae was 98.9 ± 2.5 BPM (N=164). The distribution of baseline heart rates in this preparation were skewed towards lower frequencies and the range exhibited 3-fold variation (Figure 7.1A). Surgical and environmental differences explain a portion of this variation. Previous reports in non-dissected flies show that up to 25% of the variation can be

attributed to genotypic differences (Robbins et al., 1999). Statistical analyses were performed on baseline-normalized values to account for this variation.

On average 0.1µM DA increased heart rates 36.02% ± 7.15% above baseline (Figure 7.1). At this concentration heart rate increased in each individual experiment (Figure 7.1B). DA caused a rapid rise in HR upon exposure and maintained a heightened level during the 0.1µM treatment (Figure 7.1C). In most cases the increase persisted for at least 10 minutes and was not immediately washed out by saline after the treatment. The dose-response results (Figure 7.1D) would suggest that 0.1 µM DA reaches a saturation effect in increasing HR as higher concentrations did not produce significantly higher rates.

Type 1 and 2 DA receptors mediate dopaminergic modulation of larva heart rate.

Pharmacological approaches were used to investigate the mechanisms of dopaminergic modulation in this system. Synthetic vertebrate DA receptor agonists and antagonists are known to bind to *Drosophila* DA receptors and have pharmacological effects comparable to vertebrates in heterologous expression systems (Gotzes et al., 1994). In *Drosophila* cell culture (Yuan and Lee, 2007) and in the intact nervous system (Yellman et al., 1997) several common vertebrate DA receptor drugs have been used to correlate specific DA receptors with a modulatory effect. The type-1 and type-2 DA receptor agonists used in these experiments were SKF38393 and quinpirole. SKF38393 was applied to larval hearts at 0.01µM, 0.1µM, and 10.0µM concentrations. At each concentration there was an initial dose-dependent increase (20-70%) in heart rate followed by a return to rates that were 1-36% above baseline (Figure 7.2A).

The type-2 agonist quinpirole caused an initial dose-dependent increase in heart rate that grew during the incubation to 12-53% above baseline (Figure 7.2B). This was in contrast to the chronotropic effect of SKF38393, which diminished during the course of

treatment (p=0.03 at 10µM). Also the acute chronotropic effect of SKF38393 was smaller at higher concentrations. These results suggest that the type-1 DA receptors desensitize in response to prolonged exposure to ligand. Mechanisms of DA receptor desensitization have been described in neuronal tissues (Beaulieu and Gainetdinov, 2011; Rex et al., 2008) but further pharmacological characterization is needed to confirm this phenomenon in *D. melanogaster* cardiac cells.

Antagonists for both DA receptor subtypes were moderately effective at blocking the effect of DA. For those experiments the dissected preparation was pre-treated with either antagonist before adding a solution containing dopamine and the antagonist. Heart rates for the DA treatment were normalized to values recorded at the end of the pre-treatment. The effect of DA (10µM) on heart rate was completely inhibited by antagonists for either DA receptor nine minutes after DA was applied (Figure 7.3A). The type-1 DA receptor antagonist (SCH23390, 10µM) significantly blocked the immediate effect of DA but the type-2 antagonist (spiperone, 10µM) was not as effective at this time point (i.e., two minutes after DA was applied). Spiperone initially increased heart rate during pre-treatment but the rate returned to baseline levels before DA was added (Figure 7.3B). Possible explanations for this result are that the drug has off-target effects or that the drug inhibited the function of a constitutively active DA receptor. Constitutively active DA receptors have been identified in *Aplysia* heart (Barbas et al., 2006) and in mammalian nervous system (Charpentier et al., 1996; Tiberi and Caron, 1994).

Adenylate cyclase and protein kinase C are involved in modulation of heart rate.

To determine if DA acts on the heart through classical stimulatory GPCR pathways, the vertebrate adenylate cyclase inhibitor SQ22536 (SQ) was tested in the same manner as the DA receptor antagonists, i.e., the drug was applied for 10 minutes before applying it in solution with 10 µM DA. Heart rates were measured at the end of

this treatment and later time points were normalized to those pre-treatment rates. Under these conditions the modulatory effect of DA (10µM) was significantly inhibited by 5µM SQ (Figure 7.4A). Oddly SQ was less effective at 500µM and at both concentrations SQ alone had a stimulatory effect on heart rate (Figure 7.4B). Though the drug clearly inhibits dopaminergic modulation in this context, we are unable to rule out the possibility of off-target mechanisms.

The diacylglycerol (DAG) analogue PMA was used to determine if protein kinase C (PKC) is involved in modulation of larval heart rate. This drug consistently increased heart rate and was more effective at 100 µM than at 10µM (Figure 7.5). At both concentrations the effect lasted for 10 minutes and was not immediately washed out.

Calcium ion reduction inhibits dopaminergic modulation of larval heart rate.

To test the hypothesis that Ca^{2+} influx is a factor in dopaminergic modulation of larval heart rate we experimented with various levels of $[Ca^{2+}]_o$. The normal HL3 saline contains 1.0mm $CaCl_2$ (Stewart et al., 1994). At 0.1mm Ca^{2+} the hearts did not beat, but changing the saline to 0.5 mm Ca^{2+} revived them from cardiac arrest (n = 5). In this low calcium solution DA (10µM) did not have an effect on heart rate (Figure 7.6). High calcium saline (2.0mm) did not change the effect of DA at any point. Therefore calcium influx is necessary for dopaminergic modulation of heart rate and it appears that $[Ca^{2+}]_o$ contributes its maximum input at 1mM. Higher $[Ca^{2+}]_o$ is known to substantially increase heart rate and further modulatory effects are difficult to ascertain at higher frequencies (Desai-Shah et al., 2010). After washing away DA in the low calcium solution there was a 40% increase in heart rate. Slight increases after washout were observed in other treatments (Figure 7.1C and Figure 7.3) but this phenomenon was accentuated in this condition. One explanation is that after several minutes without calcium the tissue

developed an increased sensitivity to mechanical stress, causing an elevated response to the solution change.

DISCUSSION

Positive chronotropic effect of DA on D. melanogaster larval hearts.

Endogenous DA levels fluctuate in response to environmental cues and an animal's state of arousal (Noguchi et al., 1995). In insects DA modulates peripheral organs by circulating in hemolymph at concentrations in the micromolar range (Matsumoto et al., 2003). The source of hemolymph dopamine is debatable but it likely originates from hypodermal cells that secrete dopamine to harden the cuticle (Friggi-Grelin et al., 2003; Wright, 1987) or from neurohemal axon terminals (Buma, 1988) and varicose projections within the nervous system (Helle et al., 1995), as is the case in other invertebrates. Regardless of the source, our data show how a sudden increase in DA has a positive chronotropic effect on the semi-intact larval heart in *D. melanogaster*. This effect has not been described in larvae but Zornik and colleagues (1999) reported a positive chronotropic effect in adults and a negative effect in pupae. Two factors that may have led to different findings between the two studies were the developmental stage and genotype. Here, early third instar larvae from the Canton-S line were used whereas the previous study used Oregon-R flies in the "wandering" third instar stage. Using intact P1 pupal stage from the Canton-S line, Johnson et al., (1997) observed that DA has a positive chronotropic effect. Similar developmentally specific dopaminergic effects on heart rate have also been reported in the sea roach, *Ligia exotica* (Yamagishi et al., 2004a). Moreover DA has a positive chronotropic effect on the cockroach heart (Collins and Miller, 1977). The data suggest that DA is regulating heart rate by modulating pacemaker activity in cardiac myocytes (Johnson et al., 2002), but whether modulation occurs directly in the myocardial cell layer or indirectly through the

epicardium is unclear (Su et al., 1999). We did not measure contractile force generated by the cardiac tube but experiments in *Limulus polyphemous* have shown that DA can have a positive ionotropic effect (change in contractile function) on invertebrate heart muscle and that the effect is mediated by cyclic AMP and protein kinase C (Groome and Watson, 1989).

Canonical second messenger pathways involved in fruit fly heart rate modulation.

Using SQ and ion substitution we showed that DA exerts its positive chronotropic effect on heart rate through adenylate cyclase and calcium influx (Figure 7.4 and Figure 7.6). Coupling of a *D. melanogaster* type-1 DA receptor to increases in intracellular Ca^{2+} and cyclic-AMP through G proteins has been demonstrated in *Xenopus* oocytes (Reale et al., 1997). The effect of cyclic-AMP is subtle and confounded by the fact that SQ increased heart rate. In pupal hearts a cyclic-AMP analogue (8-bromo-cAMP) had a very small stimulatory effect (9.5%) and forskolin did not significantly affect heart rate (Johnson et al., 2002). Mutations in adenylate cyclase (*rutabaga*) and cAMP phosphodiesterase (*dunce*) did not significantly alter the stimulatory effects of cardioactive molecules (Johnson et al., 2002). However there is strong genetic evidence that indicates the involvement of calcium and phospholipase C in modulation of heart rate (Johnson et al., 2002) and our pharmacological data are consistent with those findings.

We showed that direct activation of PKC has a dose-dependent stimulatory effect on larval heart rate (Figure 7.5). The PKC pathway could modulate heart rate by targeting calcium channels. In rat ventricular myocytes an L-type calcium current is modulated through a PKC-dependent pathway (Chen et al., 2012). Calcium handling in *Drosophila* myocytes exhibits many of the same physiological properties observed in mammalian myocytes. L-type Ca^{2+} channels enable periodic waves of calcium influx (Gu

and Singh, 1995). Intracellular calcium is in turn buffered by sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (Sanyal et al., 2006) and a sodium/calcium exchanger (Desai-Shah et al., 2010). Larval heart rate is positively correlated with extracellular calcium, e.g., decreasing $[Ca^{2+}]_o$ from 1.0mM to 0.5mM decreases heart rate by over 50% and increasing $[Ca^{2+}]_o$ from 1.0mM to 2.0mM increases heart rate by 40% (Desai-Shah et al., 2010). Although the experiments reported here did not directly address activation of PKC signaling by DA, evidence from rat myocytes (Li et al., 2009) and *Aplysia* sensory neurons (Dunn et al., 2012) indicate that DA modulates the function of those cells through a PKC-dependent pathway.

Though DA and the DA receptor agonists used here were effective at doses that are likely below the threshold to exert off-target effects, our experiments do not completely rule out the possibility that DA was acting through an adrenergic or other aminergic receptor. Norepinephrine and an alpha-adrenergic receptor agonist have been shown to increase pupal heart rate in *D. melanogaster* (Johnson et al., 2002). However there are no true adrenergic receptors in *D. melanogaster (*Evans and Maqueira, 2005). These molecules are believed to act through octopamine or tyramine receptors, which exhibit pharmacological properties similar to adrenergic receptors (Bayliss et al., 2013).

Future studies and impact.

In *D. melanogaster* a leak current from an outward rectifying potassium channel (ORK1) regulates heart rate by controlling membrane excitability and in turn the slow diastolic depolarization phase (Lalevee et al., 2006). It is possible that DA influences heart rate through protein kinases that inactivate ORK1 through phosphorylation. Indeed it was shown that heart rate increased when expression of this channel was knocked down (Lalevee et al., 2006). The current work establishes a system for addressing

hypotheses about the mechanisms of aminergic modulation of the heart using electrophysiological techniques and transgenic flies.

In mammals the effects of DA on cardiovascular function have been studied extensively. Chronotropic, ionotropic, and pressor effects have been demonstrated in guinea pigs, rabbits, dogs, and in humans (Tsai et al., 1967; Wakita, 2007). In several instances the results are contradictory and complicated by the fact that DA acts on smooth muscle as a vasodilator, it modulates parasympathetic innervations to the heart, and can be taken up by neurons and converted to norepinephrine. Nonetheless it has been shown that each DA receptor subtype is expressed in mammalian hearts (Cavallotti et al., 2010; Tonnarini et al., 2011), giving some support to the idea that DA or DA receptor agonists could modulate the mammalian heart directly. A current concern is that extended use of dopamine pro-drugs (e.g., L-dopa) and DA receptor agonists for neurobiological disorders has been associated with cardiac valve dysfunction (Delgado et al., 2012; Trifiro et al., 2012). Though circulating DA levels are typically not high enough to activate DA receptors (Zeng and Jose, 2011), these pharmacological agents seem to influence cardiac function through DA pathways. The larval heart has a pair of intracardiac cells that function as a valve (Lehmacher et al., 2012; Zeitouni et al., 2007), so this system could potentially be used to address the molecular mechanisms that cause this valvular dysfunction associated with DA treatments.

Conclusion

Our pharmacological analysis indicates that the chronotropic effect of DA is mediated by functionally conserved G protein-coupled DA receptors and canonical second messenger pathways. The results also indicate that calcium flux is an important element of dopaminergic modulation in the heart. These data can guide future studies

that address interactions between signaling pathways and homeostatic changes to monoamine signaling using the genetic tools available in this system.

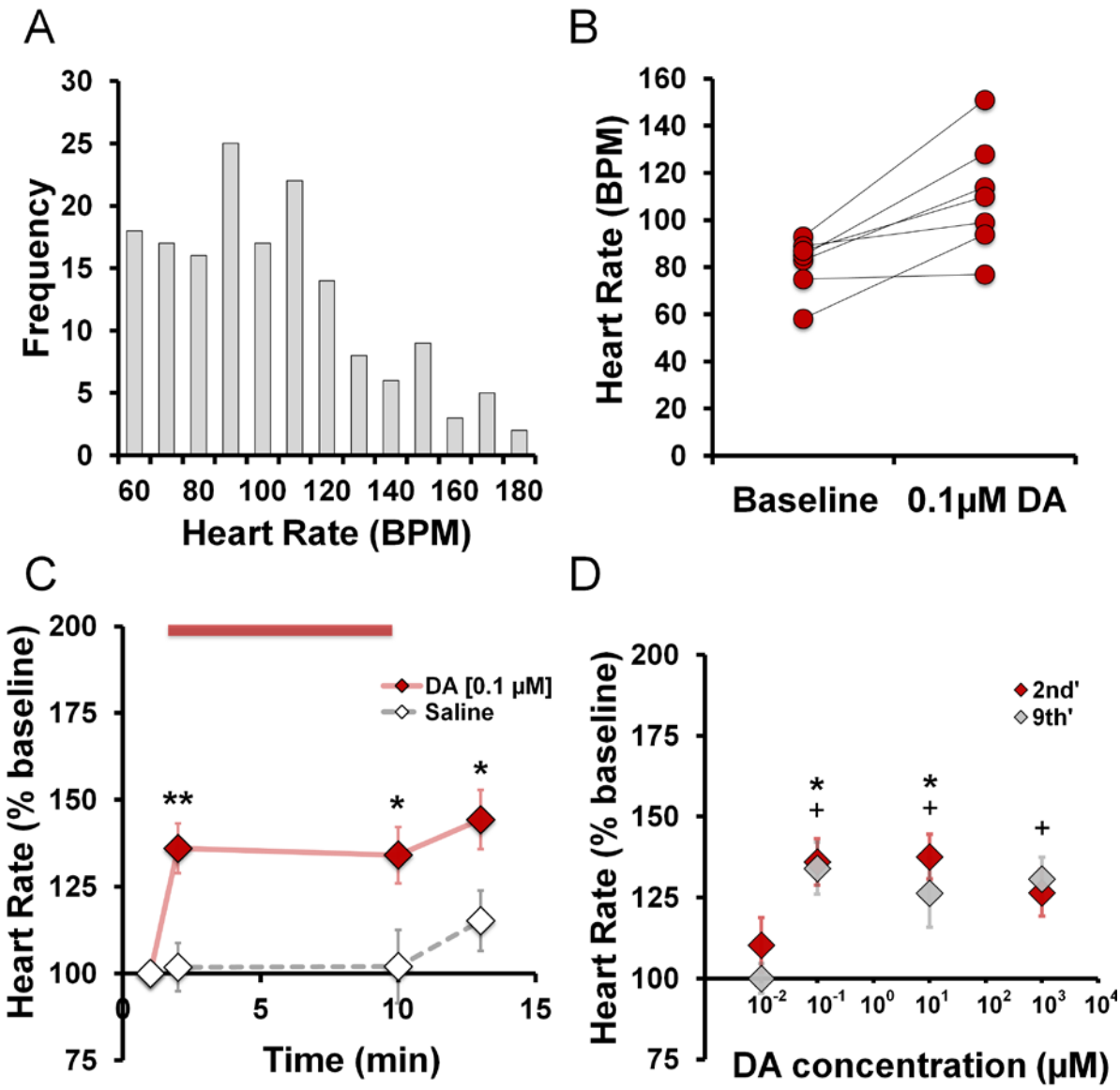

Figure 7.1. Dopamine (DA) has a positive chronotropic effect on larval heart rate. (A) Baseline heart rate counts from all experiments, i.e., five minutes after dissection and prior to application of drugs. (B) Heart rate counts from seven individual experiments, before and two minutes after the saline was exchanged with 0.1µM DA. (C) Mean heart rate (normalized to baseline) plotted with time to show the full time course of the experiments (n=7; ** p=0.005 and * p=0.03 compared to saline changes, Student's t-test). The red bar depicts how long preparations were incubated in the drug solution. (D) Mean heart rate counts from the second and ninth minute of the experiment in response to different DA concentrations (n>7 for each concentration; * $p<0.05$ at 2nd minute, + $p<0.05$ for the 9th minute compared to saline changes at those times, Student's t-test).

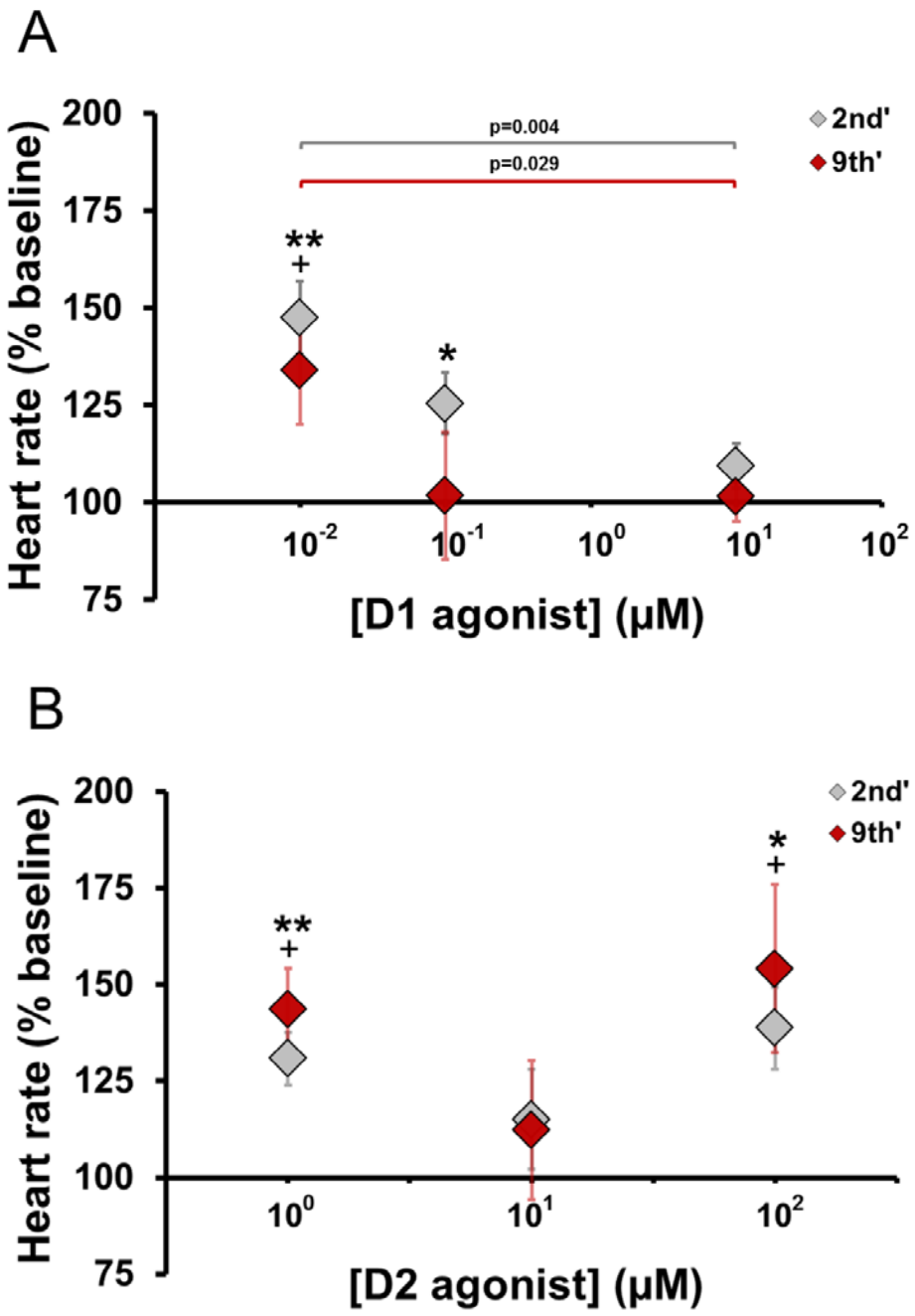

Figure 7.2. Synthetic vertebrate agonists for the two DA receptors increase heart rate in 3rd instar larvae. Agonist application regimens and heart rate counts were identical to DA experiments in Fig. 1C, i.e., change is shown relative to baseline two minutes and nine minutes after the drugs were applied. (A) SKF38393 (type-1 DA receptor agonist) and (B) quinpirole (type-2 DA receptor agonist) have positive chronotropic effects (**p<0.005, *p<0.05 compared to 2nd minute of saline treatment, Student's t-test, +p<0.05 compared to 9th minute of saline treatment, Student's t-test; N>5 different individuals for each treatment). The magnitude of the effects is statistically similar to DA but there are subtle differences in temporal and dose response. Efficacy of the type-1 DA receptor agonist decreased at higher concentrations.

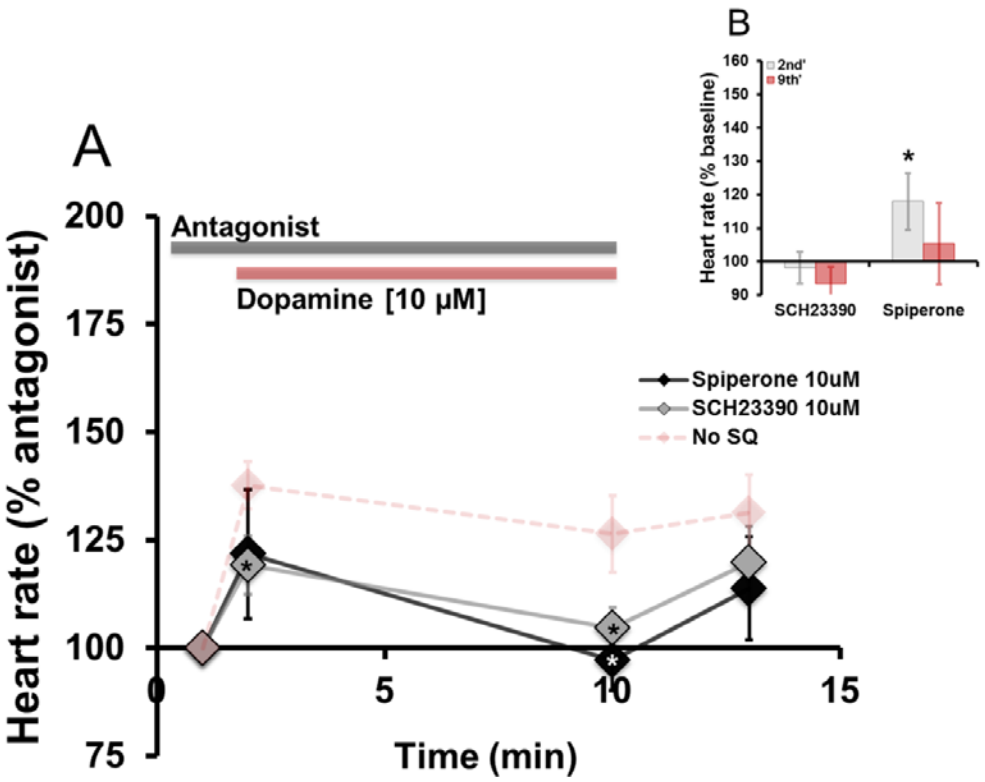

Figure 7.3. Type-1 and type-2 DAR antagonists partially block the modulatory effect of DA on larval heart rate. Dissected larva hearts were pre-treated with a DA receptor antagonist for ten minutes prior to DA application. The dopaminergic increase in heart rate was partially blocked by either drug two minutes after DA application, and almost completely blocked nine minutes after DA application (*$p<0.05$ compared to the effect of dopamine without the antagonist at that time point, Student's t-test, $n>8$ different individuals for each treatment). SCH23390 is a type-1 DA receptor antagonist (gray data points) and spiperone is a type-2 DA receptor antagonist (black data points). The dopaminergic effect without pre-treatment with antagonists is shown in light red. (B) SCH23390 alone did not have an effect on heart rate at either time point. Spiperone caused a slight but significant increase in heart rate that diminished before dopamine was added.

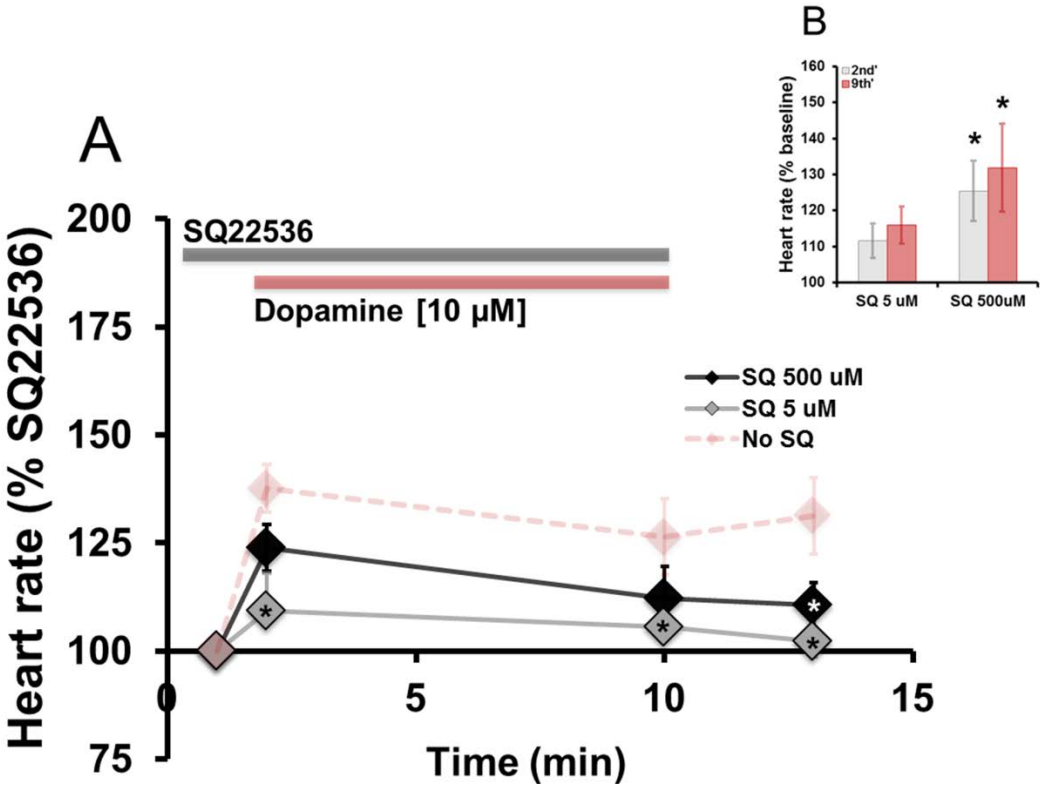

Figure 7.4. Dopaminergic modulation of larval heart rate is mediated by adenylate cyclase. Dissected hearts were pre-treated for ten minutes with SQ22536 (adenylate cyclase inhibitor) as in Fig. 3. Heart rates shown here were normalized to the rate recorded at the end of SQ22536 treatment. (A) At 500µM (black data points) and at 5 µM (gray data points) the drug attenuated the effect of dopamine. Inhibition was only statistically significant for the lower concentration (*p<0.05 compared to dopamine alone (shown in light red), Student's t-test: n>9 individuals for each concentration). (B) At both concentrations SQ22536 alone increased heart rate relative to saline treatment (* p<0.05, Student's t-test, pre-treatment rates from preparations in (A)).

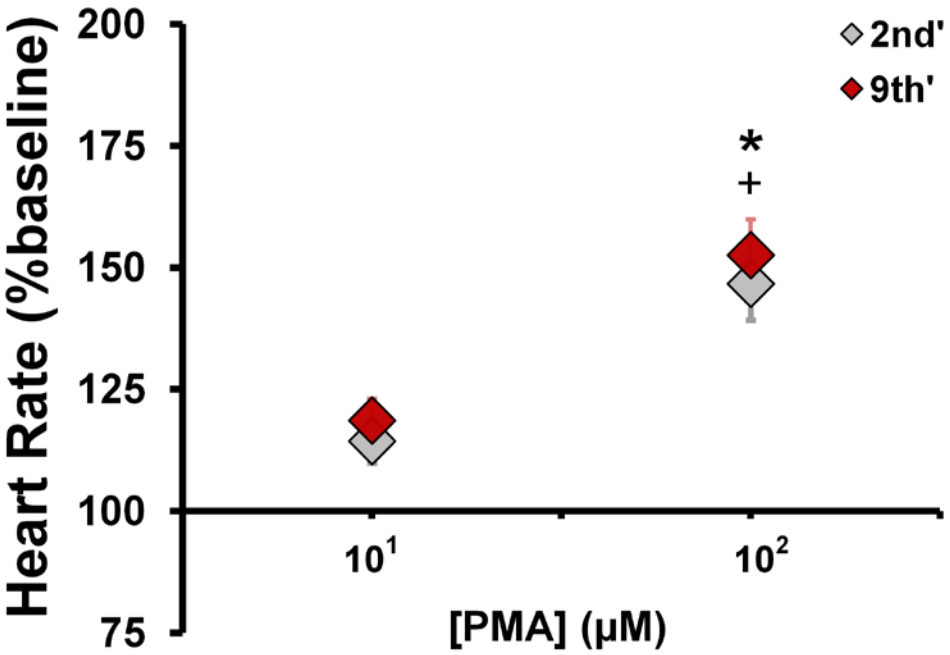

Figure 7.5. Activation of PKC has a positive chronotropic effect on heart rate. Phorbol-myristate-acetate (PMA), a cell permeable diacylglycerol analogue, was used to determine if protein kinase C (PKC) acts as a second messenger in modulation of insect heart rate. The stimulatory effect of PMA was much stronger at the higher concentration (* $p<0.005$ after 2nd minute compared to 10µM, + $p<0.005$ after the 9th minute compared to 10 µM; $n>10$ different animals for both concentrations).

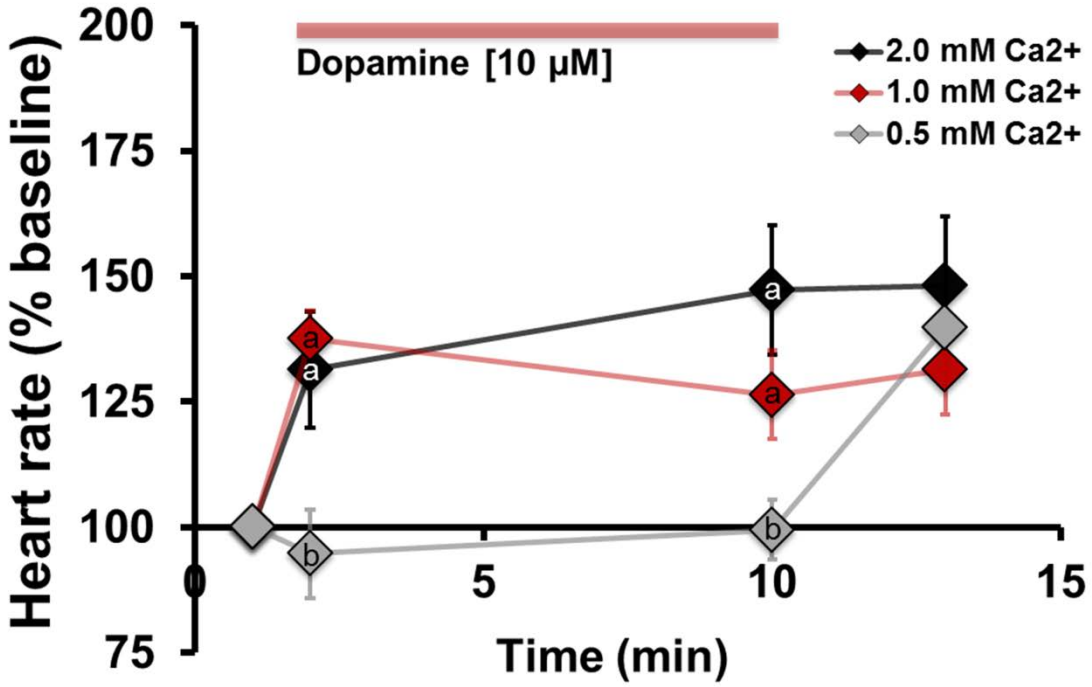

Figure 7.6. The modulatory effect of DA on heart rate is correlated with extracellular Ca2+ levels. 1.0mM (red data points) is the normal [Ca^{2+}] used for HL3 (hemolymph-like) fly saline. In low [Ca^{2+}] conditions (gray data points) the positive chronotropic effect of DA was not observed. In high calcium (black data points) the effect of DA is unaffected, though the persistence of the dopaminergic effect is slightly stronger in this condition. Data points with different letters exhibited significantly different rates ($p<0.05$, Student's t-test; n >6 for each [Ca^{2+}] tested). The solution used for dissection contained the same [Ca^{2+}] as the treatment solution.

CHAPTER EIGHT

Characterization of Serf function in the *D. melanogaster* nervous system

*This work was completed in collaboration with Drs. Rymond and Harrison. Ms. Swagata Ghosh and others in the Harrison lab performed excision screening and generated mutant lines. All data, analysis, and writing were performed by Mr. Josh Titlow.

INTRODUCTION

Serf1 as a Potential Modifier of Spinal Muscular Atrophy

Serf1 was identified as a gene that could modify the SMA phenotype (Scharf et al., 1998). In SMA patients the absence of this gene was correlated with more severe symptoms. Serf1 is a protein coding gene with known homologues in *S. cerevisiae, C. elegans* and *D. melanogaster* (Figure 8.1), but its function is not known in any organism. A study in *C. elegans* suggests a role for Serf1 in protein aggregation, but this study was performed in a sensitized background expressing human proteins prone to aggregation, and null mutations in the gene do not produce an obvious phenotype (van Ham et al., 2010). The notion that the protein intersects with the SMN pathway is supported by the fact that the yeast homologue of Serf co-purifies with Prp8 (Rymond and Boone, unpublished data), a large component of the spliceosome. Synthetic lethality experiments in yeast suggest that Serf interacts with proteins in the ubiquitin pathway. Serf's association with ubiquitin-mediated protein homeostasis and some findings in *C. elegans* relating Serf to protein aggregation and spinal muscular atrophy (Scharf et al., 1998, van Ham et al., 2010), lead us to hypothesize that mutations in *dSerf* exacerbate age-related decline in neuromuscular function. Such a decline was seen in flies expressing human amyloid precursor protein, and the phenotype was rescued by co-expressing a human E3 ligase (Kim et al., 2011).

Drosophila SMA model systems recapitulate the disease

D. melanogaster SMA disease models exhibit a range of phenotypes that correlate with the severity of SMN mutation. smn^{A} and smn^{B} are point mutations that recapitulate the mutations found in SMA patients quite well, i.e. the deletion is in a self-association domain that reduces self-oligimerization. These strains do not live past the third instar larval stage and exhibit synaptic defects similar to those observed in mice and zebra fish. Evoked post-synaptic current is significantly reduced in larval body wall muscles, and synaptic boutons are enlarged with sparse distribution of the type IIA glutamate receptor (Rajendra et al., 2007). There is also a hypomorphic allele, smn^{E33}, which survives into adulthood and is fertile (Rajendra et al., 2007). Severe atrophy of the flight muscles and branching defects of the motor neurons that innervate them were observed in these flies. SMN was also found to localize to the sarcomere almost exclusively in thin filaments. A follow-up study in the severe SMA mouse model revealed morphological defects in the Z-disks of muscle fibers.

To test the hypothesis that Serf modifies the SMN-mediated SMA phenotype we have generated a set of null mutants and rescue constructs in *D. melanogaster* by mobilizing a P-element from the 5'UTR. Several screens are underway to determine if *dSerf* has any phenotypes associated with SMA or protein homeostasis. If Serf function is associated with SMN then gathering more biochemical information on its function could help identify the motor neuron-specific or temporally sensitive pathway that connects SMN to SMA.

MATERIALS AND METHODS

Animals

In *Drosophila melanogaster,* transposable mutagenesis was used to generate alleles with substantial deletions in the coding region of *dSerf.* DNA sequencing of the

dSerf region showed that upstream and downstream sequence was left intact. The absence of *dSerf* transcripts in these mutants was confirmed by Northern blot and hybridization of an RNA probe complementary to exon 2. Thus there are two loss of function alleles of interest, hereafter referred to as 6C and 10A. A precise excision allele with normal *dSerf* expression was also generated, referred to as 13A. The original insertion p-element insertion line, ey09918 was also determined to be a null mutant. A *dSerf* rescue line was generated by cloning *dSerf* cDNA into a vector downstream of the yeast UAS promoter and transfecting embryos. Expression of the *dSerf* transgene by crossing the UAS-*dSerf* line with lines expressing GAL4. *dSerf* knockdown was achieved using GAL4-UAS crosses to drive the expression of a *dSerf-RNAi* transgene. The following *smn* lines were also used: smn^{E33}, and smn^{E33}/smn^{73A0}.

Larval NMJ electrophysiology

Third instar larvae were dissected and intracellular recordings were made in HL3.1 saline containing 1.5mM Ca^{2+}. All evoked EPSPs (eEPSPs) were recorded from m6 in the 3rd or 4th abdominal segment with resting potentials less than -50mV. Single pulses were delivered to a segmental nerve every 5s, and the average of 10 eEPSPs was measured from each specimen.

To evoke EPSPs, a segmental nerve was stimulated through a microcapillary glass suction electrode to activate motor axons, and a sharp glass electrode filled with KCl (3M, resistance = 10-15 MΩ) was used for recordings. eEPSPs were collected with an Axoclamp 2B amplifier and digitized using the Powerlab-2SP (AD Instruments). Traces were stored and analyzed using LabScope (v. 3.9.1) for Windows. The average eEPSP amplitudes from each genotype were compared using One Way ANOVA in Sigma Plot (v. 12.3). Shapiro-Wilk method was used to test for normality, the Holm-Sidak method was used for pairwise multiple comparisons. Miniature EPSP (mEPSP)

frequency and amplitudes were analyzed from a 60s interval after evoking EPSPs. Recordings and analysis were obtained using LabChart (v. 7.3.7).

Intracellular electrophysiology recordings from adult thoracic muscles

The tergotrochanteral muscle (TTM) is the major force generating effector in the mesothoracic limb. The dorsal longitudinal muscles (DLMs) are flight muscles in the wings. Because these are large sets of muscle fibers positioned directly below the cuticle in the distal thorax they are commonly used for intracellular recordings in adult flies. Excitatory post synaptic potentials (EPSPs) and action potentials have been evoked in these muscles by stimulating the giant fiber (GF) pathway indirectly through the brain (Elkins and Ganetzky, 1988, Martinez et al., 2007, Augustin et al., 2011). Anatomy of this circuit has been well characterized Latency, refractory period, and following frequency of the GF pathway to the TTM and DLMs have been characterized in *Drosophila* (Engel and Wu, 1992).

A similar protocol was developed to characterize neurotransmission in *dSerf* and *dSmn* mutants. After carefully removing the legs and wings the animal was placed on its side and pinned through the abdomen and eyes to a dish lined with Sylgard. I use thin insect pins bent 90° near the bottom, this keeps the prep from moving up the pin during dissection and gives a large surface to which I attach the indifferent Ag/AgCl ground wire. A segment of the lateral thorax was then removed with dissecting scissors to expose the TTM and DLM muscles. A small drop of HL-3 saline (in mM) 1.0 $CaCl_2 \cdot 2H_2O$, 20 $MgCl_2 \cdot 6H_2O$, 70 NaCl, 5 KCl, 10 $NaHCO_3$, 5 trehalose, 115 sucrose, and 5 BES (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (Stewart et al., 1994), was immediately added to the exposed muscle. A large drop of saline is placed on the ventral side such that the thoracic region is bathed but the spiracles remain exposed to air. Viability of the preparation decreases when the animal drowns.

When the dish was moved to the rig for electrophysiology recording it was positioned under a dissecting light microscope at 4X with the cervical connective in focus. Ag/AgCl ground wires for the stimulating and recording electrodes were placed on the head pin and saline respectively. Electrodes were pulled from glass capillary tubes (30-60 MΩ) and filled with HL-3 saline and 3M KCl respectively. The stimulating electrode worked well when broken to about 5 um. A stimulus (20 V, .5 Hz) was applied as the stimulating electrode was advanced to the dorsal part of the cervical connective near the head. As the electrode and cervical connective came into contact, muscles of the giant fiber pathway began to twitch and the stimulus was removed until the recording electrode was placed. The recording electrode was inserted deep into the DLM or TTM fibers (-80 mV) as the outer fibers were typically damaged during dissection (-20 mV).

RESULTS

Influence of dSerf on synaptic transmission and the smn mutant phenotype

Increased eEPSP amplitude at the *Drosophila* NMJ in *smn* null mutants was reported by Imlach and colleagues (2012). We have observed a similar phenotype in a hypomorphic mutant, smn^{E33}, and in a transheterozygous mutant, smn^{E33}/smn^{73Ao} (Figure 8.2). Average eEPSP amplitude in the trans-heterozygous line was 23.4% higher than the control genotype (Act-GAL4). Knocking down *dSerf* in the mutant background significantly reduced eEPSP amplitude down to levels indistinguishable from controls. Knockdown of *dSerf* in an $smn^{+/+}$ background caused an insignificant increase in eEPSP amplitude ($p=0.07$). Null *dSerf* mutations and overexpression of *dSerf* do not affect eEPSP amplitude. Miniature EPSP amplitude and frequency in the *dSerf* knockdown line were statistically indistinguishable from in the *dSerf* knockdown line (Figure 8.3). Therefore *dSerf* does not appear to be a critical component of synaptic transmission at

the larval NMJ, but to some extent, the absence of *dSerf* suppresses the *dSmn* mutant phenotype.

Synaptic transmission in the giant fiber circuit is affected by mutations at the Serf locus

High frequency synaptic transmission between the giant fiber circuit and dorsal longitudinal muscles (DLMs) is impaired in all of the Serf mutants: 10a, 10a recombinant, 6c, 6c/tm3, and ey09918 (Figure 8.4). Out of 100 pulses, a control line (*yw*, which have a genetic background similar to the mutants) has DLMs that respond to 84.3 ± 5.5% of 160 Hz pulses. Whereas all of the mutant lines respond to fewer than 60% of 160Hz pulses. Inserting Serf cDNA into the 10a recombinant line improves the average response by 11.8%, but this does not reach statistical significance ($p=0.24$, two-tailed t-test). There are also lines with similar genetic backgrounds and functional copies of Serf (13a and 26b, precise excision alleles from the transposon mutagenesis screen) that exhibited the phenotype. These data suggest that the locus is involved in synaptic transmission, or in a more general nervous system function, but evidence for involvement of the Serf gene specifically is not definitive.

A hypomorphic *smn* mutant, *smnE33*, does not exhibit synaptic impairment in this circuit. This is interesting because *smnE33* is a flightless mutant with a severe neuromuscular morphology phenotype. Given that *smn* may interact with Serf, knockdown of Serf in the *smnE33* background could cause defects in high frequency synaptic transmission.

Effects of Serf mutations on behavior and aging

To determine if Serf function is important in the nervous system I tested Serf mutants in a geotaxis/motor assay. In this general climbing assay I measured the

percentage of flies (out of 6-15 males) climbing 17.5cm up a glass graduated cylinder within one minute of being knocked to the bottom (Figure 8.5). The time required for the first fly to climb that distance, and the time required for half of the flies to climb that distance were also measured. The sign and magnitude of differences between genotype and age groups was similar for each of these measures, so only the % of flies climbing is described here for simplicity. Over 75% of the flies in the *yw* and x37E lines (control animals for genetic background associated with cuticle selection markers) climbed 17.5cm within one minute. A smaller percentage of 10a and 6c Serf mutants climb that well, however the precise excision line, 13a, also climbs faster, and the insertion mutant, ey09918, actually climbs faster.

As flies age, their performance in this assay diminishes for a number of biological reasons related to senescence. As many biochemical pathways that influence aging have been described, we would have an idea of where to look for the molecular function of Serf if Serf mutants exhibit an aging phenotype. Different groups of animals were aged and tested at 2, 3, and 4 weeks post eclosion. The 10a and 6c Serf mutants exhibit the biggest loss of function between the first and second week. The 13a precise excision control, though it is poor climber initially, does not get progressively worse at climbing through 4 weeks. Together, these experiments do not rule out the possibility that Serf is involved in pathways that influence aging or general nervous system function.

Effects of Serf mutations on a stress response

Building on the idea that Serf could be involved in pathways related to aging, the Serf protein has been shown to interact with molecules that are involved in protein homeostasis. Defects in these pathways can manifest as stress response phenotypes, so I tested the Serf mutants and controls in a heat response assay. Ten vials of 15-20 adult flies from a given genotype were kept in an incubator at 39°C until all of the flies died. The

number of flies surviving were counted every 30 minutes to generate survival curves for each genotype (Figure 8.6). The survival curves were then compared using a Mantel-Haenszel logrank test to generate p-values from a chi-square distribution (R-3.0.2). On average, the 10a recombinant mutants were less tolerant of heat stress than the *yw* control ($p=0.02$) or the precise excision control 13a ($p=0.009$). The Serf cDNA transgene improved stress tolerance in the 10a background ($p=0.02$). However, the 6c and ey09918 lines, imprecise and insertion mutants, did not exhibit a stress response phenotype.

DISCUSSION

These experiments provide some evidence to support the notion that Serf is playing a role in nervous system function, though it is clearly not essential. Because of inconsistency between the different types of mutants that were tested, these data alone do not convincingly implicate Serf in either process without additional experiments. One approach being used to address the role of Serf in protein homeostasis is by comparing the abundance of polyubiquitin aggregated structures in the flight muscles, which is associated with muscle aging (Demontis and Perrimon, 2010).

Another approach is to characterize the biological function of Serf by investigating its interaction with other proteins that have known functions. *Smn* is a candidate because of a genetic interaction found between the two genes in SMA patients (Scharf et al., 1998). Various *smn* mutant alleles have been generated in *Drosophila* that phenocopy SMA in humans (Rajendra et al., 2007, Chang et al., 2008). Experiments with these mutants demonstrate synaptic transmission at the larval body wall NMJ is compromised in *smn* mutants.

A related phenotype observed in *smn* mutants is decreased spontaneous CNS activity. This phenotype can be rescued by genetically enhancing CNS activity (even without restoring SMN levels), and in turn the eEPSP amplitudes return to normal

(Imlach et al., 2012). If *Serf* knockdown is acting through a related pathway then we expect to observe an increase in spontaneous bursting patterns relative to the trans-heterozygous mutant. Spontaneous synaptic vesicle release and plasticity at the NMJ are additional functional aspects of motor output that can be investigated to characterize the function of *Serf* and its interaction with *smn* (described below). *Drosophila smn* mutants also exhibit anatomical defects. The mutants have significantly smaller body wall muscle fibers than wild type animals, a phenotype that is also rescued by genetically enhancing CNS activity or transgenic *smn* expression (Imlach et al., 2012). Lastly, synaptic bouton size and bouton number are typical assays of NMJ homeostasis that can be included in this study. Obtaining these measurements in the *Serf*:RNAi; $smn^{E33/73Ao}$ genotype will allow us to further address the relationship between *Serf* and *smn* function in synaptic transmission.

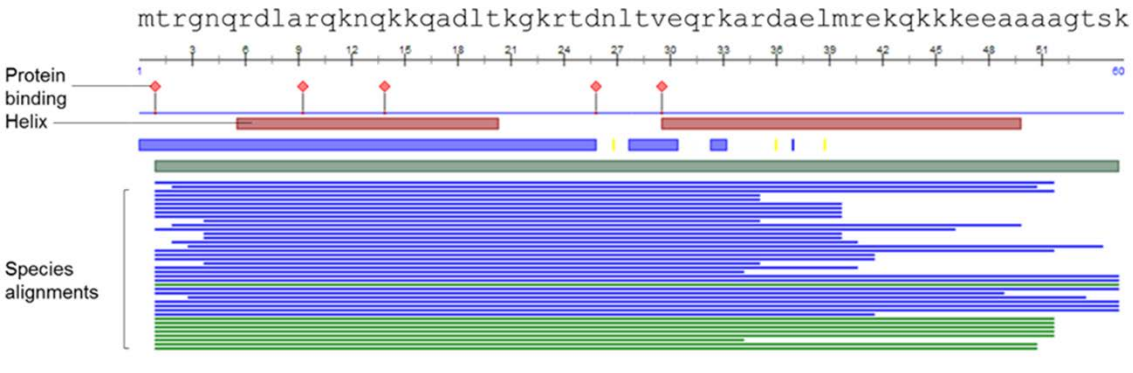

Figure 8.1. Primary amino acid sequence and functional domains of the Serf protein.

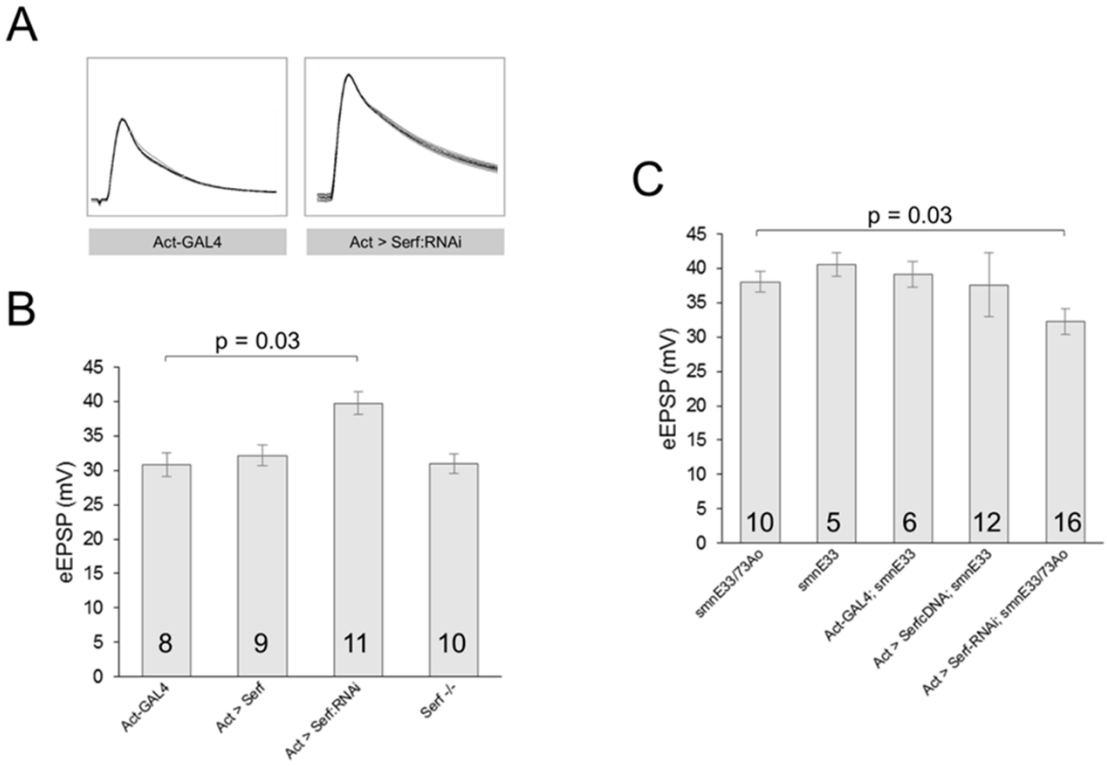

Figure 8.2. Serf knockdown interferes with an NMJ phenotype in smn mutants. A- Overlay of 10 traces recorded from a representative specimen. B- Average evoked EPSP amplitudes from each of the dSerf manipulations. C- eEPSP amplitudes from smn mutants combined with dSerf RNAi.

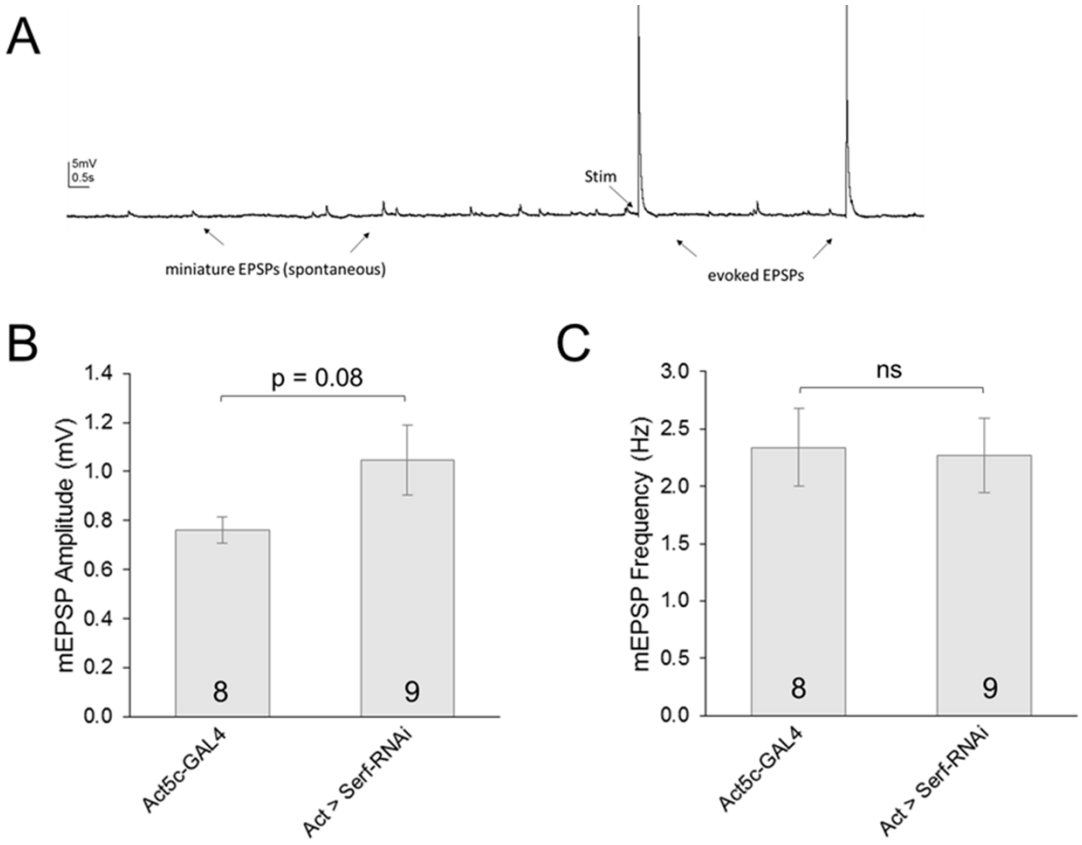

Figure 8.3. mEPSP amplitude in the larval NMJ was slightly increased by knocking down dSerf. A- Representative trace illustrating the difference between mEPSPs and evoked potentials. B- Difference in mean amplitude of mEPSPs in dSerf knockdown mutants and controls. C- mEPSP frequency was unaffected by dSerf knockdown.

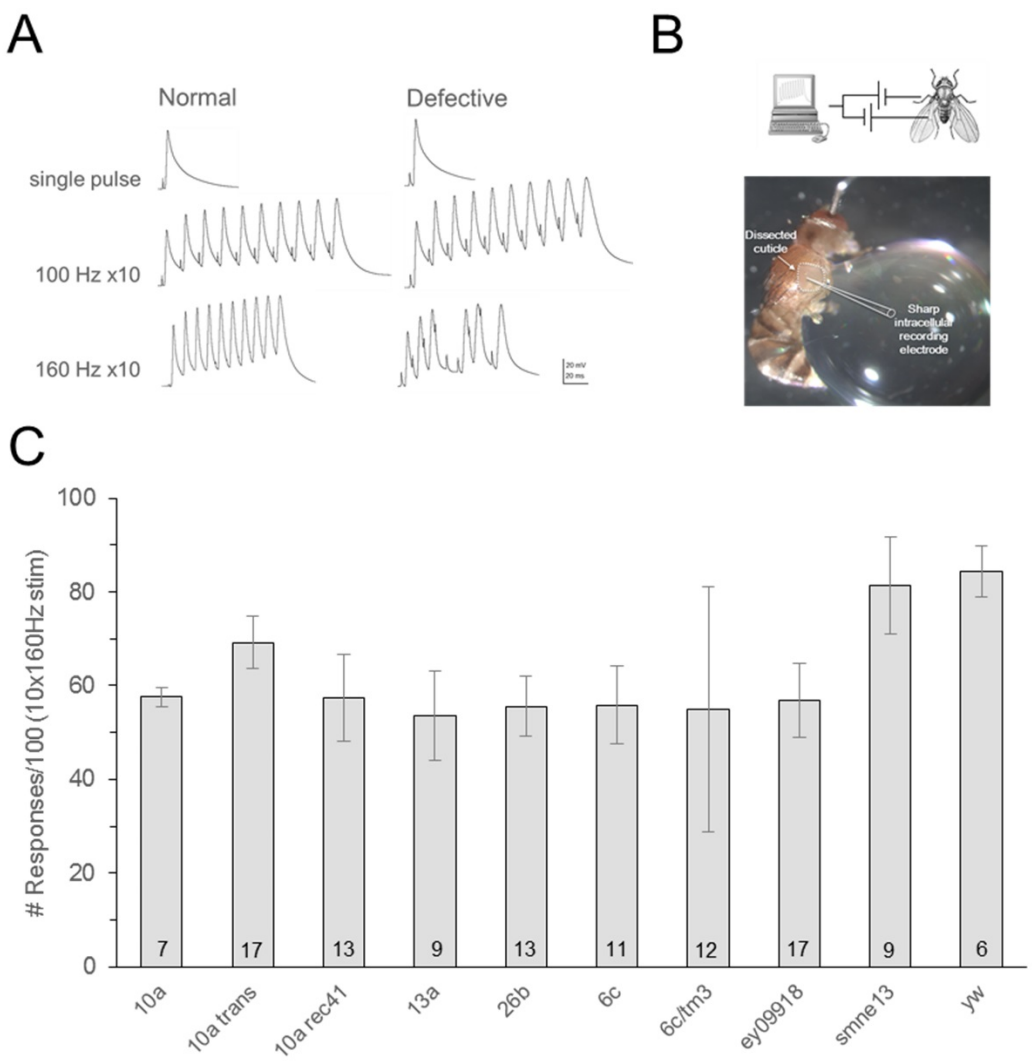

Figure 8.4. Effects of Serf and SMN mutations on synaptic transmission in the adult thorax. Fidelity of muscle response to high frequency giant fiber stimulation provides a quantifiable measure of synaptic function. Single pulses are given to measure EPSP amplitude, 100Hz and 160 Hz 10-pulse trains are given to measure high frequency output. Common phenotypes are changes in EPSP amplitude, or a high percentage of failed responses (A). A preparation is shown in (B), where the dissected cuticle and insertion of the intracellular electrode is shown. Stimulations are delivered through the cervical connective. Healthy flies respond to over 80% of 160 Hz stimuli (C), and over 90% of 100Hz stimuli (not shown). Number of responses to 10 trains of 10x160Hz stimulations is shown, with the number of male flies indicated in the bar for each genotype.

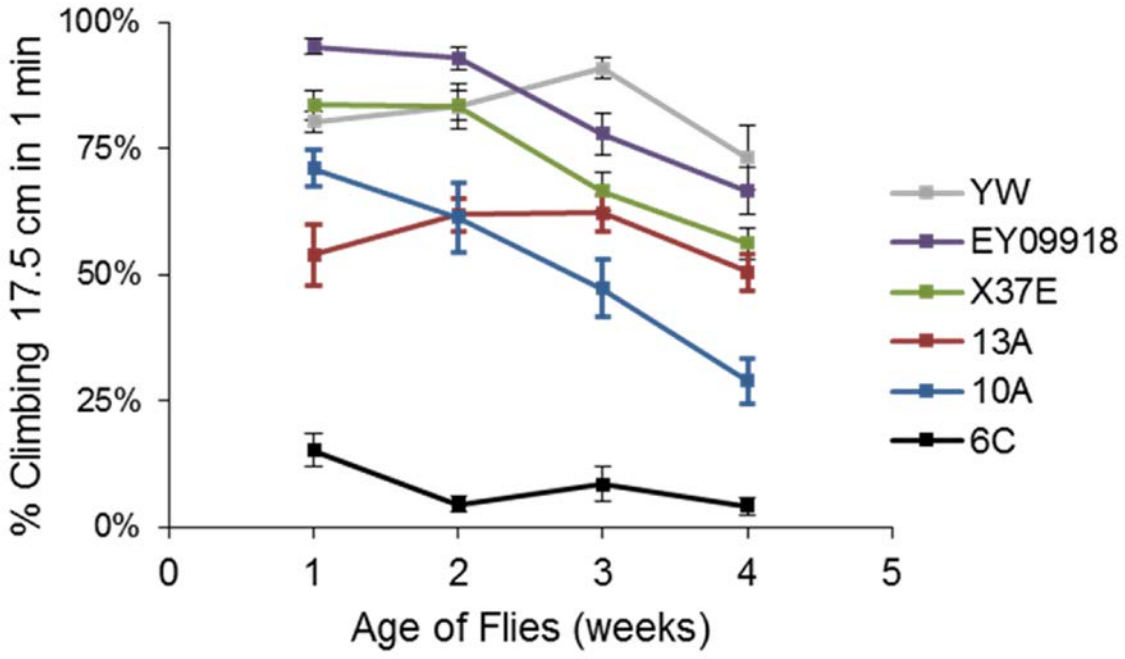

Figure 8.5. Geotactic/motor responses in Serf mutants with respect to aging. Several groups of 6-14 male flies were tested at each time point.

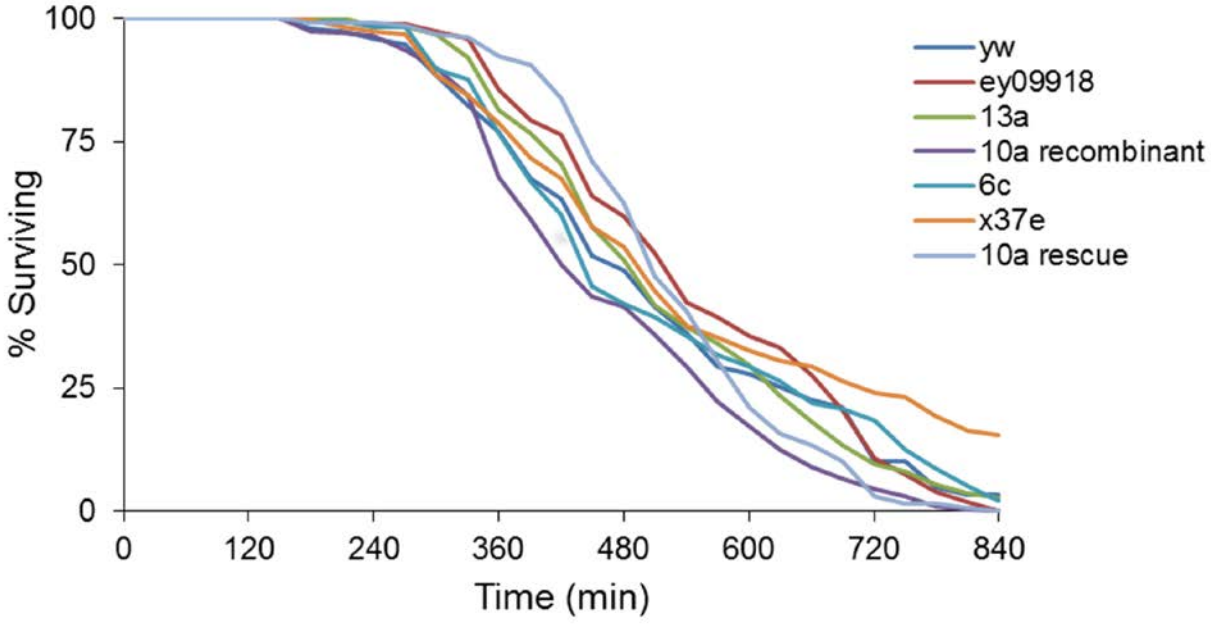

Figure 8.6. Effect of Serf mutations on response to heat stress (39°C). Survival curves show the percentage of flies observed every 30min until all of the flies were dead. Data are the average from two separate experiments (except for 10a rescue and 10a recombinant, which were only used once).

CHAPTER NINE

Teaching prospectus

Lights and Larvae- Using optogenetics to teach recombinant DNA and neurobiology

*Parts of this chapter have been submitted for publication in The Science Teacher. The original manuscript and associated documents were drafted by Mr. Josh Titlow, and edited by Mrs. Heidi Andersen and Dr. Robin Cooper.

INTRODUCTION

Swapping genes between algae and animals to control the animal's brain using lasers lights may seem like science fiction, but this technique is used daily in neuroscience research. The technique called optogenetics combines recombinant DNA technology with a controlled light source to help researchers address biomedical questions in the life sciences. Optogenetics has gained the most traction in the field of neurobiology, where specific wavelengths of light can be used to control and measure the activity of neurons in transgenic organisms. These optical recordings and stimulation techniques provide precision readings in nervous system preparations ranging from individual cells in culture to whole organism, where the observations and data collected have been used to determine which neurons are involved in specific animal behaviors. In this article and subsequent student activity, we describe an inexpensive *Drosophila* experiment that can be used to teach principles of the nervous system, genetics, and bioengineering.

Fruit flies receive an algae gene

Algae and other microorganisms have been known to sense and emit light (Foster and Smyth, 1980). Advances in molecular biology techniques near the end of the

20th century enabled researchers to determine which proteins were involved in phototaxis, clone the respective genes, and transfer them into new species for research. The proteins themselves are called channelrhodopsins and are transmembrane ion channels that reside in a closed state to convey a non-specific ion flux when the channel is activated by a specific wavelength of light. Channelrhodopsin-2 (ChR2) is sensitive to blue light, by introducing point mutations into the gene, researchers have been able to alter the light sensitivity and optimize ion conduction in these channels. By 2006, a ChR2 gene was being expressed in specific subsets of neurons to study associative learning in *Drosophila melanogaster* (Schroll et al., 2006).

Expression of ChR2 in Drosophila is restricted to motor neurons

A huge breakthrough in *Drosophila* transgenics occurred when the yeast GAL4-UAS binary expression system was introduced into the fly genome (Brand and Perrimon, 1993, Duffy, 2002). Thus the transgenic ChR2 could be controlled in specific subsets of cells using this standard binary expression system. The ChR2 transgene is controlled by the yeast upstream activation sequence (UAS) for a galactose-induced transcription factor (GAL4), meaning that the transgene is in every cell, but is only expressed where GAL4 is expressed. Expression of the GAL4 gene is controlled by a promoter sequence either from a nearby promoter in the fly's genome, or a specific promoter that is added to the GAL4 transgene sequence. By adding a promoter to the GAL4 sequence that is expressed specifically in the nervous system, e.g., a gene that codes for an enzyme involved in synthesis of a neurotransmitter, the GAL4 gene only gets expressed in the nervous system thus activating expression of the ChR2 transgene in the nervous system.

The promoter used to drive expression of GAL4 in the flies used for this activity is called OK371-GAL4. The promoter element is from the *Drosophila* vesicular glutamate

transporter gene (DVGLUT) that is expressed almost exclusively in neurons that release glutamate (Mahr and Aberle, 2006). Glutamate is the neurotransmitter that stimulates skeletal muscles in insects, as opposed to acetylcholine in vertebrate nervous systems, therefore in the OK371 > ChR2 line, shining a blue light on the whole animal will activate all of its motor neurons at once thus making a visual and observable behavior for students to measure response to stimuli.

When introducing this experiment to students, we ask them to imagine that all of their motor neurons have this light-activated ion channel, and then ask them to predict what would happen if they walked under a powerful blue light. This leads into the first part of the activity, which is to simply shine blue light on the flies and have students describe their behavioral response to changed stimulus.

Building an LED controller and behavior arena

Drosophila larvae have a thin and translucent cuticle that allows light to penetrate relatively well. To increase penetrance, light from an LED is focused through a 10X ocular objective lens. This is a technique introduced by Pulver et al. 2011 in their teaching article, which describes the use of this system in an undergraduate physiology lab). Here, we reduce their light apparatus to the bare minimum components and provide a set of behavioral experiments that are appropriate for the high-school classroom.
The following materials are needed to perform this optogenetics experiment:

<u>Materials for LED setup:</u>

-side emitting LED (488 nm emission (blue)

-small heat sink (to disperse heat from the LED)

-10x ocular objective lens (taped to the heat sink holding the LED, cover back part of the light)

-Lab adhesive tape

-9V battery-9V battery snap connector

-solder (or adhesive to connect the 9V battery wire to the LED)

Materials for each lab station:

1 petri dish with lid (8.5cm dia)

1 paper filter (8.5cm dia)

1 wooden dowel (or small paint brush for maneuvering the larva)

1 disposable plastic dropper (3mL)

1 stop watch

1 data sheet

1 stereomicroscope (10x ocular/4x objective)

3rd instar larvae (OK371>ChR2 fed retinal, control is the same flies not fed retinal, see below)

Materials for Instructors:

Apple juice (50mL for 20 groups)

Yeast paste (1 mL yeast/10mL water)

Scoopula (to transfer larvae)

Water bottle (to transfer larvae)

Drosophila melanogaster larvae:

-OK371 > ChR (expresses channel rhodopsin in motor neurons) The OK371-GAL4 and UAS-ChR2 lines can be obtained separately from the Bloomington Stock Center (OK371-GAL4: http://flybase.org/reports/FBst0026160.html and UAS-H134R-ChR2-mcherry. In this case virgin females from one line should be crossed with males from the other line, giving progeny that express that transgene.

-All-Trans retinal (co-factor that is fed to the flies >24hr before the experiment; we dilute according to label, then aliquot 100uL into centrifuge tubes (makes about 30 vials). One

vial can be mixed with one standard vial of fly food. For details of this procedure in video form see (Hornstein et al., 2009).

Larval optogenetics experimental procedure

Before start of laboratory class, the teacher constructs light apparatuses and cultures the larvae in fly food with retinal. Several small 3rd instar larvae should be transferred into the retinal food source 24h before the experiment or the adults can lay eggs directly into the retinal food source 1 week before experimentation as it will take 5-7 days for the eggs to reach the third instar stage in retinal medium.

Effects of motor unit photo-activation on crawling behavior

The activity can be completed in one 90-minute laboratory session. Students separate into small groups (2-4) and prepare their own behavioral arena by 1) placing a paper filter (8.5cm dia) into a petri dish (8.5cm dia) and 2) dampening it with a few drops of apple juice. This will provide a moist surface for the larva to crawl on, and the smell of apple juice encourages them to crawl. The teacher passes out 2-3 control larvae (OK371>ChR2 not fed retinal) to each group. Students then observe the larvae under a dissecting microscope, noting how they crawl and what happens when the blue light is shined on them. Next, the student looking in the microscope counts the number of crawling strides the larva makes in 15 seconds, while another student keeps track of time. This number is recorded in the data sheet (“On the web”) as # of crawling strides / 15s under the column for before stimulation. The same measurement is then repeated, this time with blue light focused on the larva. The number of strides is recorded in the column for during stimulation. Immediately after counting the strides in blue light, the students should take an additional measurement to determine if the light had a lasting effect. This value is recorded as after stimulation.

Effects of motor unit photo-activation on feeding behavior

A second behavioral experiment that can be conducted under the same premise is to count the number of mouth hook movements that occur in 15 seconds before, during, and after the light stimulus. The teacher should tell the students that the mouth hooks are the black cuticle structures at the front of the animal, and that they move much faster than body contractions. It may help to show them the video of mouth hook movements ("On the web"). They should observe 30-50 movements in 15s.

To perform the mouth hook experiment, students use the dropper to form a small drop of yeast solution on the lid of their petri dish. The solution should be rather dilute, as the students will need to observe the larva while it is submerged in the solution. Students carefully transfer the larva into the yeast solution and observe their behavior under the microscope. As they did with the crawling strides, they will count the number of mouth hook movements that occur in 15s, then again while focusing blue light on the larva, then again immediately after the light is removed. After recording these measurements, the data sheet should be complete and the students can answer the questions either alone or as a group.

Students can analyze the data in a second session to characterize the effects of the stimulus. If time allows, the groups can test multiple larvae and analyze their own data. Alternatively, the data from each group can be pooled together, and then each student can analyze the pooled data set. The crawling data are arranged into 6 columns in a spreadsheet, before, during, and after for all of the control values and experimental values. The feeding data are also arranged into the same 6 columns. Students can graph the means and variance for each set of data, and then apply statistical tests. One statistical test would be to determine if the light had a significant effect on the behavior. A one-way ANOVA comparing values before, during, and after light stimulus within a

given treatment would address this question. A post-hoc test (e.g., Tukey's) could be used to determine which groups had different values.

Assessment

There are several nuances to this dataset, enough to allow each group to give an oral or written presentation on a different aspect of it. Within the crawling stride data, one group could discuss the before stimulation data, noting how that is an appropriate control, and why the retinal treatment could have caused differences in the animal's baseline behavior. The second group could discuss how the light stimulus affected the behavior, comparing the experimental before stimulation data to the during stimulation data. In doing so, how the crawling speed changed, how the light caused this change, and how they were able to tell that effect was due to the transgene and not the blue light in general. The third group could then present that after stimulation data. They would state whether the crawling speed returned to normal, or whether the effect of the light persisted after the light was turned off. Then they should give explanations for why they observed that particular result. The other three groups could then present the same arguments for the feeding behavior data.

Activity modifications and extensions

This activity can easily be modified to incorporate a student inquiry component by allowing students to experiment with the animals before they are given a protocol. Students are then told that this is new technology and they need to determine 1) if it is working and 2) if the stimulations have any lasting effects on the animals. The assignment is to develop hypotheses and design experiments to address these two points. This option hasn't been explored, so it will be interesting to see how the students approach this topic. Some other ideas that we have considered are increasing the

stimulus time and taking After stimulus measurements at later time points, e.g., immediately after, 5 mins after, 1 day after, etc.

There are many behavioral assays for larvae that could be combined with the stimulus to determine if activating this large group of neurons impairs mechanosensation, phototaxis, chemotaxis, or olfactory learning. This idea is one of the discussion points that we typically bring up in a group discussion after the experiments. The students are asked to think about how these experiments could be applied to biomedical research questions. One way for them to think about this is in the context of epilepsy. During a seizure, large groups of neurons (often glutamatergic neurons) are activated randomly to cause uncontrolled motor activity. By simulating this in fruit fly larvae, researchers could dissect the brains to determine what effects the seizure had on the physiology of those neurons.

Lastly, the activity can be more multidisciplinary if students are allowed to participate in the experimental setup. If the classroom is oriented towards engineering, students can be given the opportunity to construct their own light apparatus. If students are already familiar with basic concepts of genetics, students can setup the OK371-GAL4 x UAS-ChR2 fly crosses and collect their own progeny. There are also sensory neuron drivers that the students could test to determine how activating different sets of sensory neurons can drive behavior. The blue light doesn't penetrate adult cuticle very well, but we are in the process of testing red-shifted channelrhodopsins for adult optogenetics experiments. Thermogenetic control of adult fly behavior with heat sensitive rather than light sensitive channels also provides very robust behavioral changes.

Conclusion

Fruit flies are commonly used in high school laboratories to teach principles of genetics. This activity builds on those basic principles by applying fruit fly genetics to neurobiology. Students have fun controlling fruit fly behavior with LEDs, they get to conduct behavior experiments with a genetically modified organism and in doing so they learn how optogenetics and recombinant DNA technology works.

On the web

We built a webpage that contains the datasheet, a supplies list with prices and order numbers, links to Bloomington stock center to order specific fly lines, links to important freely available articles on optogenetics in teaching, and links to videos to demonstrate optogenetic behavior. Here is the web address: http://joshtitlow.wordpress.com/lights-larvae/

Lights and Larvae Data Sheet

PART 1- Observation of basic behavioral response to light-activated neural impulses.

1) Place a ChR2 larva on the agar dish and observe the fly under the microscope.
2) Turn on the blue LED by plugging the connector into the 9V battery.
3) Determine how far away the light needs to be from the dish to be focused on the fly.
4) Focus the light on the fly as you look into the microscope.
5) Note what happens when the light is focused on the fly, and what happens to the fly when you remove the stimulus.

Basic behavioral observations:

PART 2A- Effects of light-activated neural impulses on locomotion behavior.

1) Place a new ChR larva onto the agar plate.
2) In the microscope, observe the crawling behavior, note how a wave of muscle contraction begins from the tail and ends at the head as the animal crawls. One complete cycle is referred to as a body wall contraction. Count the number of body wall contractions that occur in 15s. Multiply that number by 4 to get the number of body wall contractions per minute.
3) Again, count the number of body wall contractions when the animal is being stimulated with blue light.
4) Count the number of body wall contractions that occur in 15s after the animal is stimulated with blue light.
5) Repeat the experiment with a Control fly.
6) Record your data in the table below as the number of body wall contractions per minute.

	# of crawling strides / 15 seconds								
	Before stimulation			**During stimulation**			**After stimulation**		
	Trial 1	**Trial 2**	**Trial 3**	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 1**	**Trial 2**	**Trial 3**
Experimental ChR2 larva									
Control ChR2 larva									

PART 2B- Effects of light-activated neural impulses on feeding behavior.

1) Form a small droplet of water in a petri dish.
2) Add 3-5 pellets (a pinch) of yeast to the droplet.
3) Place a ChR larva into the droplet.
4) Under the microscope, observe the black mouth hooks that are rapidly retracting. Count the number of mouth hook movements that occur in 15s. Multiply that number by 4 to get the number of mouth hook movements per minute.
5) Then, focus the LED on the fly, and count the number of mouth hook movements that occur in 15s while the neurons are being activated by light.
6) Lastly, count the number of mouth hook movements that occur in 15s after the stimulus is applied.
7) Repeat the experiment with a Control fly.
8) Record your data in the table below as the number of mouth hook movements per minute.

	# of mouth hook movements / 15 seconds								
	Before stimulation			**During stimulation**			**After stimulation**		
	Trial 1	**Trial 2**	**Trial 3**	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 1**	**Trial 2**	**Trial 3**
Experimental ChR2 larva									
Control ChR2 larva									

Addressing the Next Generation Science standards

This optogenetics activity addresses the following national standards:

National Science Education Standards (NRC 1996):

Science Content Standard A: Science as Inquiry (p. 173)

Science Content Standard C: Life Science (p. 181)

Science Content Standard F: Science in Personal and Social Perspectives (p. 193)

Science Content Standard G: History and Nature of

Science (p. 200)

Principles and Standards for School Mathematics

(NCTM 1998):

Problem Solving

Communication

Connections

Representation

Student learning objectives

-Students apply optogenetics to the study of locomotion and feeding behavior in *Drosophila* larvae.

-Students observe firsthand how optogenetics can be used to activate motor units in a live genetically modified organism.

-Students practice observational skills and work as a team to obtain measurements of behavior.

-Students input data into spreadsheets and use software to analyze and graph the data.

-Students collaborate within a group to explain important aspects of the experiments to their peers.

Student guidance questions and key concepts.

-How does light affect crawling behavior and feeding behavior in the ChR2 larva?

-Were there any long-term effects of optogenetic stimulation on the larva?

-What causes the larva to respond to light?

-What is a channelrhodopsin? In what organism did channelrhodopsin originate?

-Which cells in the fly express channelrhodopsin?

-If you wanted to see what happens when sensory neurons are photo-activated in the fly, how could you generate a fly that expresses ChR2 in sensory neurons?

-What would you expect to happen at the neuromuscular junction if the ChR2 larva was stimulated with blue light for several minutes?

-How could this optogenetics system be used in biomedical research to address a clinically relevant problem?

-In people with severe spinal cord injuries, neural stimulation often helps the spinal cord recover. How could you use optogenetics photo-activate neurons in a human spinal cord?

Photo-activation of flight and jump motor neurons in adult flies: **A teaching laboratory to study high frequency synaptic transmission and action potential waveforms**

*Parts of this chapter are being submitted for publication to Advances in Physiology Education. The lab materials were written by Josh Titlow, with input from Drs. Stefan Pulver, Bruce Johnson, and Robin Cooper.

Purpose

One purpose of this lab is to learn how neural circuits and muscles control insect flight. While doing this you will get a sense of the heterogeneity in active membrane properties and action potential waveforms in the muscles. And you will learn how neuromuscular junctions respond to motor neuron stimulation. This lab also teaches the quantal nature of synaptic transmission through recording of miniature end plate potentials. Lastly, this lab demonstrates the advantages of genetic model systems and optogenetic techniques in physiological inquiry.

INTRODUCTION

Flight is one of the insect's most interesting behaviors and has been intensely studied for several years. With modern imaging and physiological techniques, researchers have been able to characterize the precise muscle contractions and neuronal firing patterns that generate aerial locomotion. This "basic" curiosity-driven science has provided a system that can be used to apply molecular and pathological aspects of insect neurophysiology to neurological disorders in humans. The foundation of this system comes from more than 100 years of research in *Drosophila melanogaster* genetics. With fruit flies it is almost trivial to insert transgenes into genomes, and control specific neurons with light or heat (Brand and Perrimon, 1993, Venken et al., 2011).

Before studying the experimental objectives and nuances of this laboratory exercise, have a look at this biomedically relevant use of the system and this short review of the literature pertaining to flight muscle electrophysiology in *D. melanogaster*.

Martinez and colleagues were interested in the molecular and cellular basis of functional decline that occurs as animals age (Martinez et al., 2007), a biological phenomenon that is common to all organisms. Specifically they were interested in determining how motor function and synaptic transmission declines during senescence. To address this, they used a common behavioral assay and a version of the flight muscle electrophysiological assay that we are using in today's lab. The obvious variable was age, they tested flies across the adult lifespan. The other variable was genotype, they tested *methusaleh* mutants, which have a mutation in a G protein-coupled receptor, and *fragile-x* mutants, which have a mutation in the *fragile-x* gene that codes for an RNA-binding protein, and is the main cause of inherited mental retardation in humans. These mutants were used because they are long and short–lived respectively, relative to normal flies. Their data showed that the *fragile-x* mutants have a strong electrophysiological phenotype, i.e., when the giant fiber pathway is stimulated at high frequency (130Hz), the flight muscles do not fire action potentials in response to each stimulus like they do in wild-type flies. Interestingly, the jump muscles are capable of following this high frequency stimulus, suggesting that an interneuron synapse in the flight muscle circuit is specifically prone to aging. With this system researchers can now test pharmacological agents to determine how they influence the functional decline in this circuit, or systemically alter different genes to determine the molecular cause of functional decline in this synapse. Knowing the underlying anatomy and physiology of the circuit will be critical in these endeavors.

Much of what is known about the giant fiber circuit and electrical properties of the flight muscles comes from the investigation of mutants with behavioral defects. Fly

strains that became paralyzed at high temperatures or hyperactive under ether anesthesia were isolated after random mutagenesis, and the cause of those phenotypes was traced back to specific ion channels using electrophysiology (Tanouye et al., 1986). What you need to know about the ionic currents in flight muscles is that there are essentially four of them: a glutamate-activated synaptic current, a voltage-gated Ca^{2+} current, a voltage-activated delayed rectifier K^{+} current, and a Ca^{2+} activated transient K^{+} current. Elkins and Ganetzki characterized the potassium currents using mutants and classical ion substitution/pharmacological techniques (Elkins and Ganetzky, 1988). They also describe how the potassium channels influence action potential waveforms and the implications for those ion currents in flight behavior and development. One learning objective for this lab is to be able to explain how variations in ion channel distribution generate the different waveforms and spike frequencies you observe. This can be done by injecting different amplitudes of current into the cells and plotting an F-I curve, i.e., the frequency of action potentials with respect to input. If the firing frequency of a cell is to be tuned to the frequency of its input, in this case the muscles are controlled by motor neurons, then the relationship between frequency and input should be linear. Elkins and Ganetzki (1988) demonstrated how transient potassium currents and calcium-activated potassium currents influence firing frequency in DLMs. In today's experiment, we will calculate the slope of the F-I curve to further characterize the firing frequency of DLMs and other thoracic muscles.

Another physiological principle that will be explored with this preparation is the quantal nature of neurotransmitter release. The notion, for which Bernard Katz was awarded the Nobel Prize in 1970, is that neurotransmitters are released from discrete packets (vesicles) in uniform amount (quanta). Quanta were discovered by analyzing the spontaneous depolarizations that resemble the shape of excitatory junction potentials in intracellular recordings, i.e., fast rise, slow decay. Plotting the frequency of the different

amplitudes of these spontaneous events revealed a discrete probability distribution (Poisson distribution), meaning that the amplitudes were intervals of the lowest amplitude rather than a continuous distribution of all possible amplitudes. Frequency and amplitude of these spontaneous events now serve as metrics for quantifying efficacy and plasticity in synaptic transmission. Baseline values for these metrics have been described in the dorsal longitudinal flight muscles (DLMs), but not the other flight muscles or jump muscles. In the DLMs there are over 1,000 motor neuron synaptic boutons that give rise to as many as 40 spontaneous events per second. Because this is a calcium-dependent phenomenon, and bursts of activity in the motor neuron increase intracellular calcium, a prediction that can be tested with this preparation is that the frequency and amplitude of miniature end plate potentials increases after a burst of activity (Miledi and Thies, 1971).

Anatomy of the giant fiber circuit and flight muscles is fairly straightforward. Coggshal published an elegant paper on anatomy of the dorsal longitudinal flight muscles and their innervations by back-filling the muscles with horseradish peroxidase (Coggshall, 1978). Then there were two papers published in the same issue of Journal of Neurocytology that describe the central circuit (King and Wyman, 1980) and direct innervations of the DLMs (Ikeda et al., 1980). A more contemporary description of the circuit with attractive schematics (Figure 1) was published in the Journal of Visualized Experiments (Augustin et al., 2011). From that description you get the general layout of the circuit, which is bilaterally symmetric giant interneurons descend from the brain into the thoracic ganglion, those cells form 1) electrical synapses with tergotrochanteral motor neurons that innervate the jump muscle, and 2) electrochemical synapses with a peripherally synapsing interneuron (PSI), which in turn forms a cholinergic synapse with the flight muscle motor neurons. That paper is also important to read because it gives an overview of the assay that was discussed in the second paragraph of this section, a

version of which will be used in today's experiment. Though it has not been explored in detail, photo-activation of flight has been demonstrated at the behavioral and physiological levels (Lima and Miesenbock, 2005, Zhang et al., 2007).

Specific Experiments and Questions for this Laboratory Exercise

In today's experiment you will be collecting intracellular electrophysiological recordings from muscles in the fruit fly thorax, mainly flight muscles and jump muscles. The primary goal is to record evoked activity in the muscles through photo-activation of their respective motor neurons. Once you record from one muscle, it is relatively easy to advance the electrode deeper into the thorax to record from other muscles. With recordings from different muscles you can address the following questions about their physiology:

1) What are the maximum spike frequencies of muscles involved in adult fruit fly locomotion?
2) What is the rise/decay time for the muscle spike?
3) What are the passive membrane properties of the cell (e.g., resting membrane potential and membrane resistance)?
4) Does the activity diminish over time?
5) Which muscles are associated with your measurements? How could you be certain?

This experiment is commonly used as an assay to measure synaptic function. One value that is used is the number of action potential failures in response to high frequency stimulation. Here we are applying a constant stimulus and it is unknown how each of the synapses responds. An objective of today's experiment is to quantify the fidelity of each synapse by addressing the following questions:

1) How many times does the synapse fail?

2) Is the failure because of the neuron, muscle, or both? How could you test this?
3) Is the failure biological, or is it due to the transgenic ion channels?

By injecting different amplitudes of current into the muscle and analyzing the spike frequency we can address the following questions:

1) What is the relationship between firing frequency and input?
2) Do the muscles have different F-I relationships? What could that mean?

With this technique it is also possible to record miniature endplate potentials from spontaneous quantal release of neurotransmitter at a single terminal. Today's experiments address the following questions in this system:

1) What is the baseline frequency and amplitude of miniature endplate potentials in the various flight muscles?
2) Does the frequency or amplitude of miniature endplate potentials change after evoking activity in the synapse?

Because this is a novel technique, there are additional questions about the experimental design that can be addressed:

1) Is it necessary to cover the incision with saline?
2) Do chemicals applied through the cuticle incision modulate neural circuits in the thorax?
3) Are there differences in light-evoked and electrically-evoked activity in these circuits?

Procedure

There is a JOVE article that provides background information on the flight muscle system and intracellular recordings (Augustin et al., 2011).

There is also an educational article that describes the use of channel rhodopsin in a teaching laboratory (Pulver et al., 2011).

These articles are “must reads” before coming to lab. It is also highly recommended that you read the paper on potassium channels in flight muscles (Elkins and Ganetzky, 1988) and the paper on mEPPs in flight muscles to help interpret your data.

Setting up the rig

When recording from live tissue it is always best to have the equipment completely ready before sacrificing the animal. For this experiment you need to be sure that you have the software, electrode, and light source ready to go before beginning the dissection. LabChart is the data collection software that will be used for this experiment. You should set it up to record at 10 kHz with a range of at least 500mV. Once you begin recording you can just let the software continue to run like an oscilloscope until the experiment is finished. Adding frequent comments to indicate when you observe particular events will be a tremendous help during data analysis.

Fill a micropipette with 3M KCl to use as an intracellular electrode. BE CAREFUL WITH THE GLASS ELECTRODES, THEY ARE VERY SHARP AND 3M KCL IS A SKIN IRRITANT, MAKE SURE ALL PIPETTES ARE PUT IN THE SHARPS CONTAINER WHEN YOU ARE FINISHED USING THEM. Carefully place the pipette into the electrode holder. Ground the electrode by placing the ground wire into a saline solution, this also serves as a reference to the electrode. Slowly advance the electrode into the bath. Adjust the input offset so that it reads 0mV, this is so you can tell when you advance the electrode into a hyperpolarized cell. At this time you should also balance the bridge to insure that you get accurate recordings (see Capillary tip resistance check in the Appendix).

Dissection

Anesthetize the flies by submerging the entire vial in ice, when they stop moving they are anesthetized. While waiting for the flies to anesthetize, be sure that you have a Sylgard-lined dish with at least two small insect pins, a syringe with fly saline, dissecting tools (forceps, a dingy pair of microdissection scissors, needle holder/needle), and tissue paper to wipe off the tools.

Under the microscope, carefully remove the wings and all of the legs (work fast because they regain consciousness rather quickly). Lay the animal on its side and carefully place insect pins into its abdomen and head to keep it from moving. An image of the arrangement is shown in Figure 2. Notice the small hole in the cuticle in the thoracic region, where you would expect the wings to be. This is the most challenging aspect of the dissection. Use the needle holder (with needle) to poke a small hole in the bottom of what will eventually be a small window into the cuticle. Use the edge of the needle like a scalpel to begin making a circular incision (visualize the cut going from 6:00 to 3:00, to 12:00, on the face of a clock). Once this half circle is cut into the cuticle, you can typically peel away a small segment with your forceps. Grab the piece of cuticle and rotate the forceps as you tear the cuticle away (you may need to stabilize the fly with your other pair of forceps as you make the incision and pull away the cuticle). When you have made a functional window, place a droplet of saline next to the animal, and slowly add saline until the droplet reaches the exposed thorax (do not submerge the entire animal in saline).

Recording

Carefully place the ground wire into the saline droplet, and then slowly advance the pipette into the saline. Adjust the input offset so that the electrode reads 0mV. You are now ready to impale muscle fibers. You have to use the gross anatomy drawings

and your imagination to visualize which set of muscles the electrode is going into. Many of the muscles have very polar resting membrane potentials (-70 to -90mV) so it will be obvious when the electrode is in a cell. The key is to not break the tip of the electrode (use the bridge balance to determine if input resistance has changed), and advance it very slowly. Do not get discouraged if you have to continue changing electrodes, you need a high resistance electrode to get good recordings, so it is necessary to switch them often. After you advance the pipette a cell (i.e., observe that the voltage has dropped below -50mV), determine whether or not there are spontaneous quantal events occurring in the cell. If there is, record this baseline activity for 3 minutes before using light to evoke action potentials. Do this every time you enter a new cell. Also determine the membrane resistance in the cell by balancing the bridge. The idea here is that the injection of a specific amount of current causes a deflection in the membrane voltage. Using Ohm's law you can use these current and voltage values to calculate the membrane resistance (R_m). Record R_m and the resting membrane potential for each cell.

Light activation of motor units

The flies you are using express a light-sensitive cation channel in motor neurons. By directing blue light from an LED to the preparation, you can cause action potentials to fire in motor neurons that innervate the muscle you are recording from (Figure 3). Deliver a series of two-second pulses to each of the muscles you encounter, one pulse every ten seconds for a total of five pulses. As with any neurophysiological preparation, do not over-stimulate the circuit by leaving the light on for long periods of time, as this will fatigue the synapses and desensitize glutamate receptors on the muscles.

Injecting current into the muscles

After evoking activity with blue light, you will evoke activity by injecting current (Figure 4). Depending on the size and health of the cell it will take between 0.150-0.250μA to evoke spikes in the muscle. To do this, make sure the current injection switch is in the OFF position. Then adjust the μA knob to 0.150μA. Flip the switch to MOMEN and hold for 1-2s. If action potentials are observed (as in Figure 4) then repeat this procedure with injection amplitudes that are both smaller and larger, such that you acquire responses at 4 different amplitudes. If responses are not observed, continue to increase the amplitude in small increments until you reach the threshold. Then collect recordings at 3 additional amplitudes. Be sure to note which amplitudes were given for each of the traces.

Learning Objectives

Physiological mechanisms

1. Understand what causes differences in firing frequency in motor circuits.
2. Understand the molecular basis of heterogeneity in action potential waveforms.
3. Understand how optogenetics works.

Evaluation and Analysis of Experimental Data

1. Determine firing frequency of evoked activity in adult fly thoracic muscles. Plot the traces and point out key features related to the ion channel diversity.
2. Characterize passive electrical membrane properties as a function of evoked activity.
3. Quantify fidelity in high output synapses.

Experimental Techniques

1. Activate motor neurons with light from an LED.

2. Intracellular recording from intact flight muscles.
3. Single electrode current injection.
4. Small insect dissection.

Pre-Lab Quiz

1. What is the molecule that enables light activation of motor neurons in this preparation? How does it work?
2. What aspect(s) of the "flight and jump" motor circuits determines the maximum firing frequency (cellular and molecular)?
3. Name some fast human muscle fibers that are comparable to the high output *Drosophila* thoracic muscles. What are some differences between them?
4. What are some advantages (limitations?) of optogenetics over non-genetic optical physiology techniques? What are some advantages (limitations?) of optical techniques over classical electrophysiology?
5. Explain the mechanism of quantal release.

Post-Laboratory Report Content and Questions to Consider

1. Use the posted data to **calculate and report averages and standard deviations** for each of the data collected and posted. Use the averages and standard deviations to address the questions for the overall experiment (what did you find). Results should be presented in the following forms: text and table/graph with caption. Use inferential statistics to identify significant differences in the results, e.g., differences in active and passive membrane properties between muscles (One-way ANOVA is a good choice here), or differences in endplate potentials before and after stimulation (t-test is good here). Follow-up your results with a discussion (what do the results mean). Wrap up your discussion with a clear and concise summary/conclusion

statement. NOTE: DO NOT FOCUS ON TECHNICAL/PROCEDURAL PROBLEMS OF THE LAB EXERCISE in your report. The purpose of the lab report is for you to demonstrate your ability to deduct information from the experimental results, and convey your understanding of the physiological topics of interest. Reports that focus on procedural problems, and do not convey a sense of physiological understanding will be severely penalized.

2. You should try to address as many of the questions as possible from the section above entitled: Specific Experiments and Questions for Today's Laboratory Exercise.

Table 9.1- Passive membrane properties of thoracic muscles. Note the resting membrane potential and membrane resistance of each cell at the beginning and end of each recording.

Passive membrane properties	Membrane potential		Membrane resistance	
	Before stimulation	After stimulation	Before stimulation	After stimulation
Muscle 1				
Muscle 2				
Muscle 3				
Muscle 4				
Muscle 5				

Table 9.2- Miniature end plate potentials in thoracic muscles. Note the frequency and amplitude (average of 3) of miniature end plate potentials from each muscle.

Quantal events	Frequency (events/min)		Amplitude (mV)	
	Before stimulation	After stimulation	Before stimulation	After stimulation
Muscle 1				
Muscle 2				
Muscle 3				
Muscle 4				
Muscle 5				

Table 9.3- Light-evoked activity in thoracic muscles. Note the maximum spike frequency (Hz) from each muscle.

Muscle activity Spike frequency	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Average
Muscle 1						
Muscle 2						
Muscle 3						
Muscle 4						
Muscle 5						

Table 9.4- Synaptic function. Note the number of responses that are graded, i.e., not action potentials.

Synaptic function # of failed events	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Average
Muscle 1						
Muscle 2						
Muscle 3						
Muscle 4						
Muscle 5						

Table 9.5- F-I relationship. Note the amplitude of current injected (A), and the maximum spike frequency (Hz) from each muscle. Use a scatter-plot graph to present these data in your report.

F-I Data Frequency	Amp1 =	Amp2 =	Amp3 =	Amp4 =
Muscle 1				
Muscle 2				
Muscle 3				
Muscle 4				
Muscle 5				

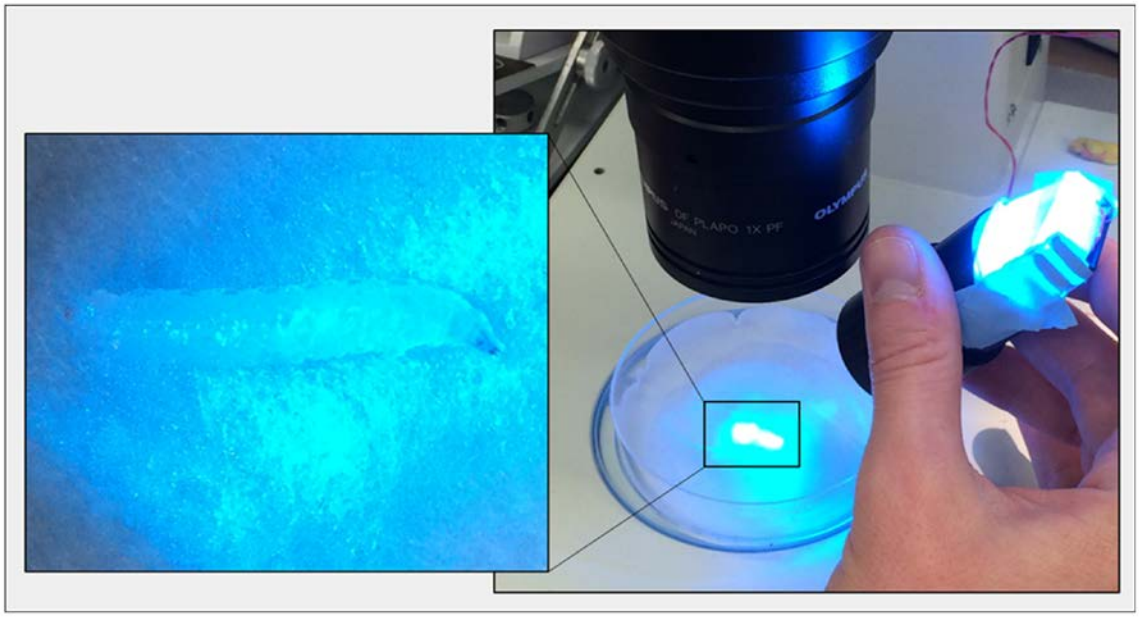

Figure 9.1. Optogenetic activation of fruit fly behavior.

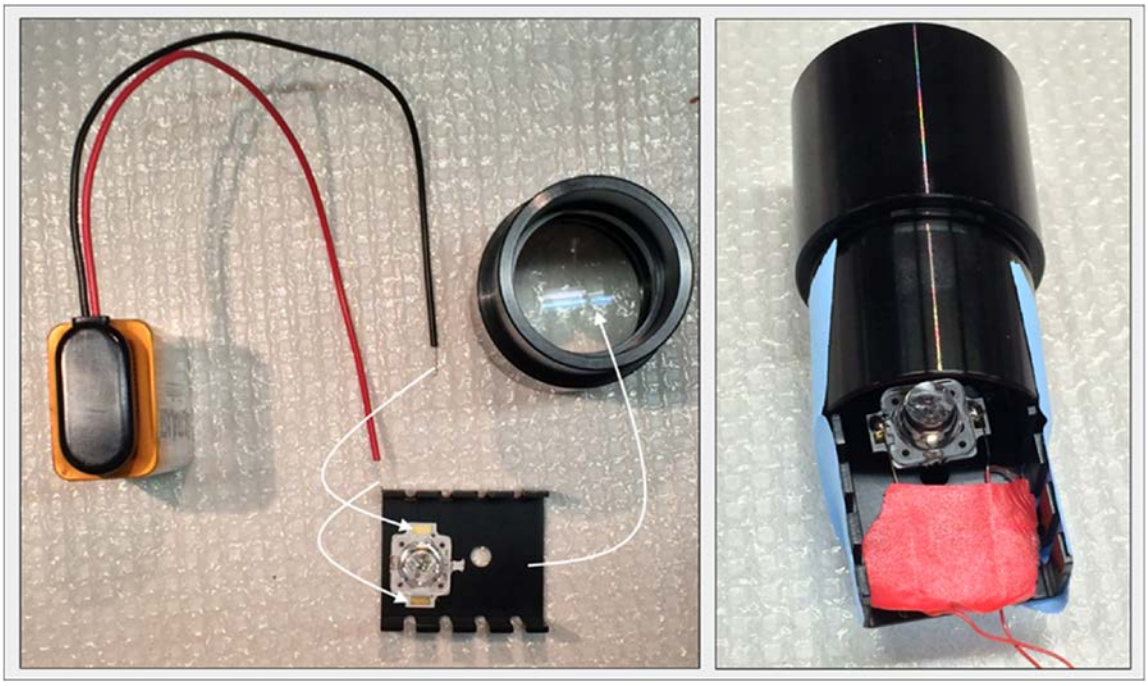

Figure 9.2. LED apparatus.

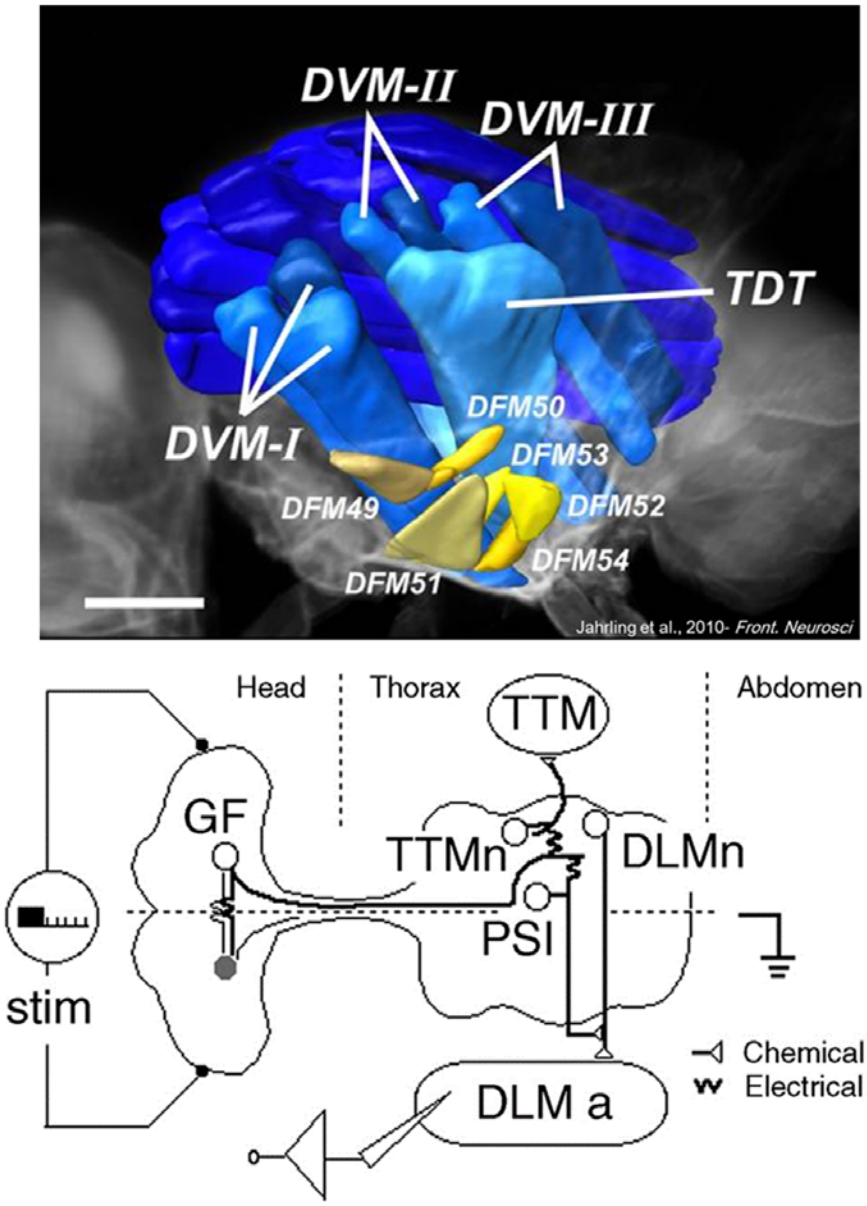

Figure 9.3. Diagrammatic view of the neuromuscular components for flight in D. melanogaster. *Top*- The dorsal longitudinal muscles are the large, dark blue muscles that are unlabeled in this image, deep to the DVMs. The dorsal ventral muscles (DVMs) are another set of indirect flight muscles, meaning they do not move the wings directly, rather they deform the cuticle. TDT is the jump muscle, also called tergotrochanteral muscle. It is unlikely that you will record from the DFMs as they are typically damaged during dissection and not in the electrode trajectory. *Bottom*- Neural circuitry innervating thoracic muscles in the adult fly. Note the differences in synaptic physiology (electric vs. chemical) and the differences in synaptic arrangement (ipsilateral vs. contralateral projections. From (Jahrling et al., 2010).

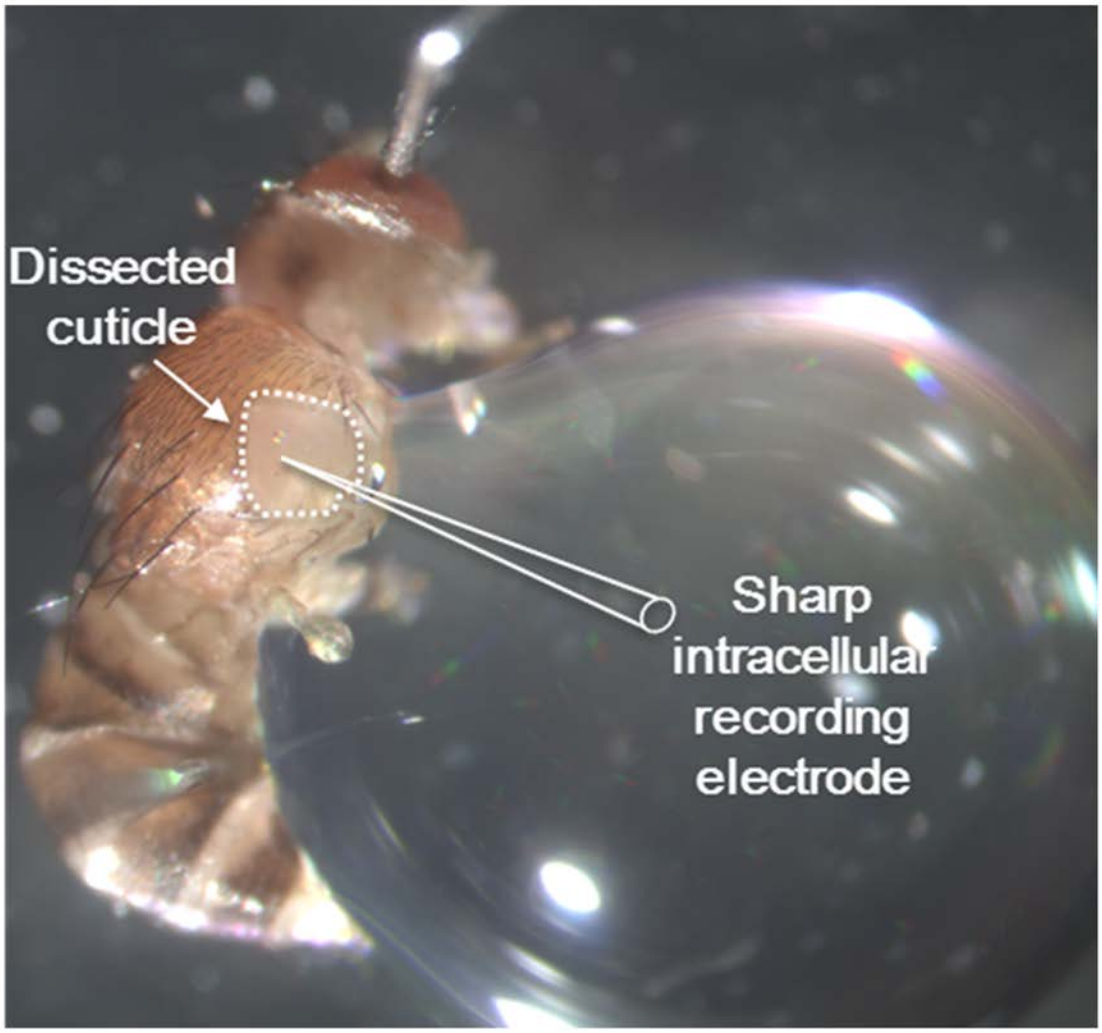

Figure 9.4. Preparation for recording from adult fly muscles. Pins in the abdomen and head keep the animal from moving. A small window of the cuticle (dotted line) is dissected to allow access to the sharp glass electrode. Note the angle on entry for the electrode, this is critical to getting a stable recording. A droplet of saline keeps the tissue healthy and serves as a ground.

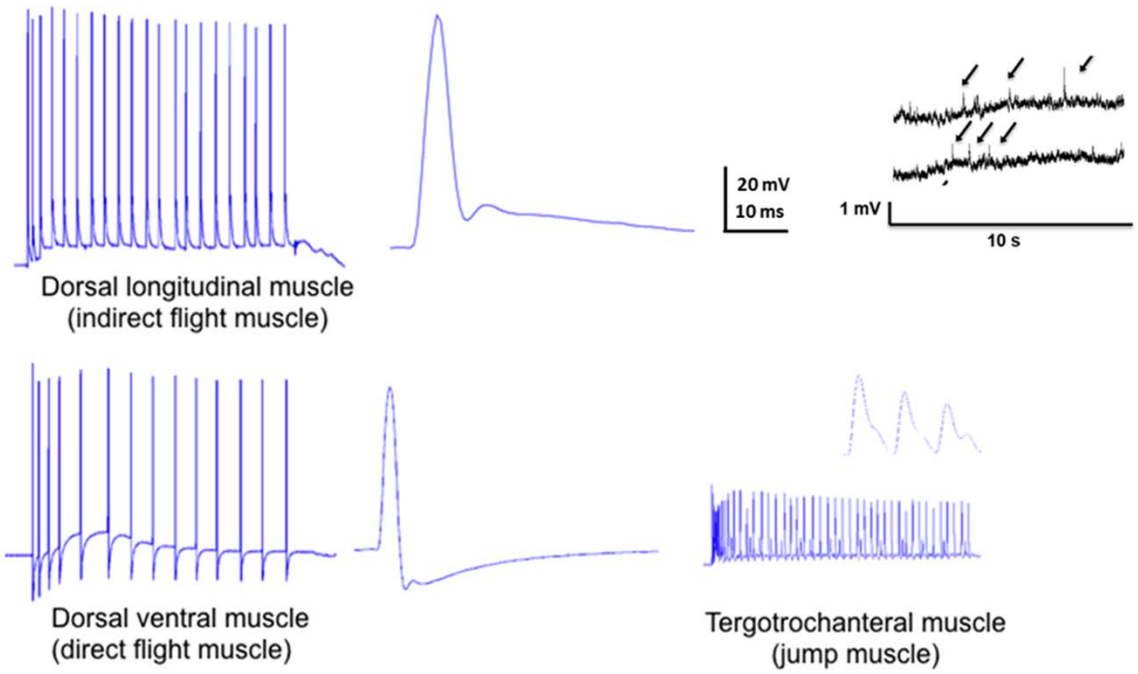

Figure 9.5. Muscle action potentials evoked by light activation of motor neurons. A two second light pulse was delivered to the animal for each recording. Note the differences in action potential waveforms, amplitude, and frequency. In the top-right corner are two separate traces showing miniature end plate potentials.

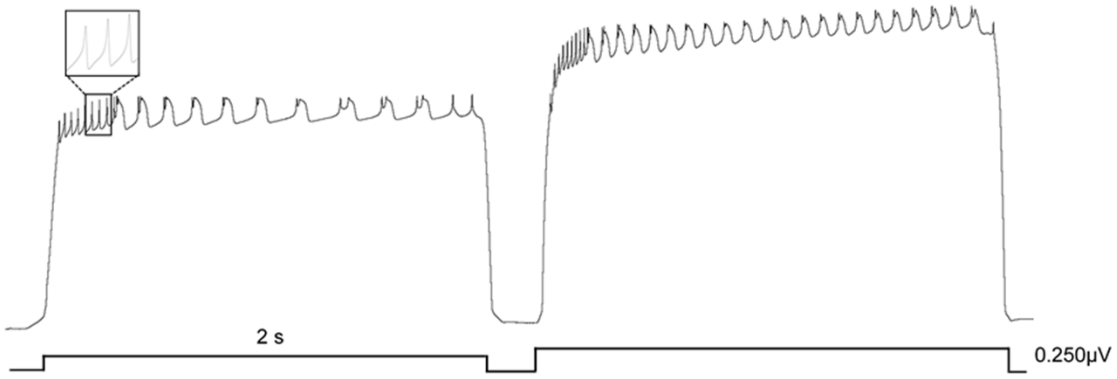

Figure 9.6. Muscle action potentials evoked by current injection. Two second injections of depolarizing current were given at two different amplitudes (200μA and 220μA). Note the difference in frequency with respect to the amplitude of current, and compare this muscle-evoked action potential waveform to the neural-evoked action potential.

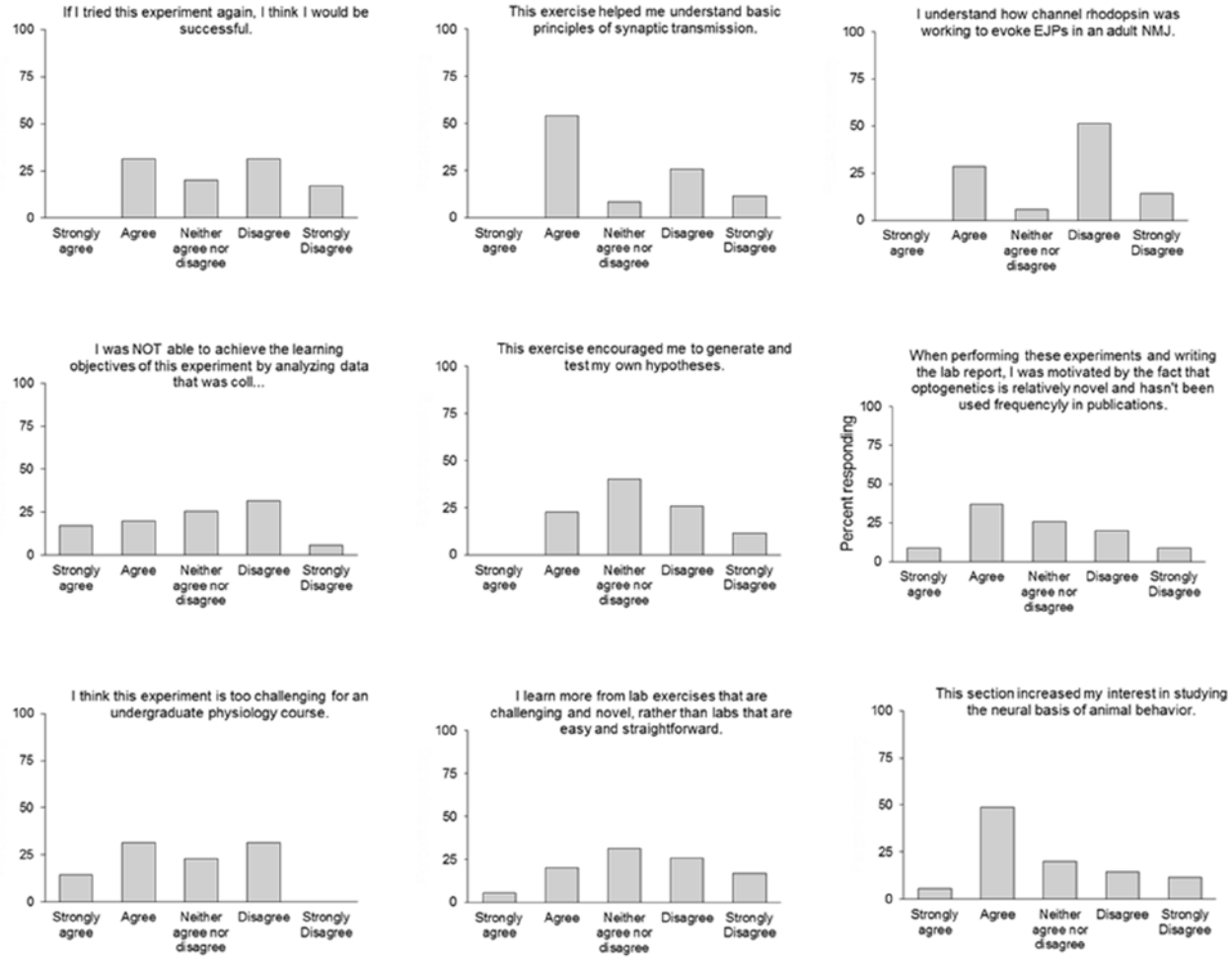

Figure 9.7. Student responses to survey questions. Surveys were filled out online within 2 weeks of performing the lab (n=35).

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CHAPTER EIGHT

Chang HC, Dimlich DN, Yokokura T, Mukherjee A, Kankel MW, Sen A, Sridhar V, Fulga TA, Hart AC, Van Vactor D, Artavanis-Tsakonas S (2008) Modeling spinal muscular atrophy in Drosophila. PloS one 3:e3209.

Demontis F, Perrimon N (2010) FOXO/4E-BP signaling in Drosophila muscles regulates organism-wide proteostasis during aging. Cell 143:813-825.

Imlach WL, Beck ES, Choi BJ, Lotti F, Pellizzoni L, McCabe BD (2012) SMN is required for sensory-motor circuit function in Drosophila. Cell 151:427-439.

Kim C, Srivastava S, Rice M, Godenschwege TA, Bentley B, Ravi S, Shao S, Woodard CT, Schwartz LM (2011) Expression of human amyloid precursor protein in the

skeletal muscles of Drosophila results in age- and activity-dependent muscle weakness. BMC physiology 11:7.

Rajendra TK, Gonsalvez GB, Walker MP, Shpargel KB, Salz HK, Matera AG (2007) A Drosophila melanogaster model of spinal muscular atrophy reveals a function for SMN in striated muscle. The Journal of cell biology 176:831-841.

Scharf JM, Endrizzi MG, Wetter A, Huang S, Thompson TG, Zerres K, Dietrich WF, Wirth B, Kunkel LM (1998) Identification of a candidate modifying gene for spinal muscular atrophy by comparative genomics. Nature genetics 20:83-86.

Stewart BA, Atwood HL, Renger JJ, Wang J, Wu CF (1994) Improved stability of Drosophila larval neuromuscular preparations in haemolymph-like physiological solutions. Journal of comparative physiology A, Sensory, neural, and behavioral physiology 175:179-191.

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CHAPTER NINE- Lights and Larvae

Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401-415.

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Mahr A, Aberle H (2006) The expression pattern of the Drosophila vesicular glutamate transporter: a marker protein for motoneurons and glutamatergic centers in the brain. Gene expression patterns : GEP 6:299-309.

Pulver SR, Hornstein NJ, Land BL, Johnson BR (2011) Optogenetics in the teaching laboratory: using channelrhodopsin-2 to study the neural basis of behavior and synaptic physiology in Drosophila. Advances in physiology education 35:82-91.

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CHAPTER NINE-Optical stimulation of flight

Augustin H, Allen MJ, Partridge L (2011) Electrophysiological recordings from the giant fiber pathway of D. melanogaster. Journal of visualized experiments : JoVE.

Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401-415.

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Miledi R, Thies R (1971) Tetanic and post-tetanic rise in frequency of miniature end-plate potentials in low-calcium solutions. The Journal of physiology 212:245-257.

Pulver SR, Hornstein NJ, Land BL, Johnson BR (2011) Optogenetics in the teaching laboratory: using channelrhodopsin-2 to study the neural basis of behavior and synaptic physiology in Drosophila. Advances in physiology education 35:82-91.

Tanouye MA, Kamb CA, Iverson LE, Salkoff L (1986) Genetics and molecular biology of ionic channels in Drosophila. Annual review of neuroscience 9:255-276.

Venken KJ, Simpson JH, Bellen HJ (2011) Genetic manipulation of genes and cells in the nervous system of the fruit fly. Neuron 72:202-230.

Zhang W, Ge W, Wang Z (2007) A toolbox for light control of Drosophila behaviors through Channelrhodopsin 2-mediated photoactivation of targeted neurons. The European journal of neuroscience 26:2405-2416.

VITA

Josh S. Titlow

EDUCATION AND TRAINING

Ph.D. Biology- University of Kentucky 2011-2014 (Expected in May)
Primary advisor: Dr. Robin Cooper
Co-advisors: Drs. Doug Harrison, Bruce O'Hara, Wayne Cass

Summer Course at Cold Spring Harbor Laboratory- Drosophila Neurobiology: Genes, Circuits, and Behavior. June-July, 2013.
Instructors: Drs. Kate O'Connor-Giles, Gregory Macleod, Adrian Rothenfluh

M.S. Biology- Marshall University 2008-2010
Primary advisor: Dr. Brian Antonsen
Co-advisors: Drs. Eric Blough, Elmer Price, Nadja Spitzer

B.S. Chemistry Marshall University 2004-2008
Advisor: Dr. Brian Antonsen

RESEARCH EXPERIENCE

University of Kentucky

2012-2013	Investigating the effects of dopamine on sensorimotor circuits and development
2012-2013	Identifying eQTLs for dopamine-related genes in *Drosophila*
2011-2013	Characterizing the function of the Serf gene in *Drosophila*

Marshall University

2008-2011	Investigating temporal specifics of neuromodulation in a sensorimotor circuit
2007-2008	Modeling proprioception in AnimatLab

PROFESSIONAL APPOINTMENTS

2011-current **Graduate Teaching Assistant-** University of Kentucky
Courses taught:
Animal Physiology
Histology
Evolution (online)
Intro Biology

2011 **Adjunct Instructor**- Marshall University
Human Physiology

2011 **Laboratory Manager-** Marshall University
P.I. Dr. Brian Antonsen

2009-2010 **Graduate Teaching Assistant**- Marshall University
Human Biology (non-majors) & Human Physiology

PEER-REVIEWED PUBLICATIONS

10. **Titlow, J.S**., Smith, J.J., and Cooper, R.L. (In Manuscript) *Quantitative genetic analysis of dopamine signaling in Drosophila melanogaster.*

9. **Titlow, J.S.** and Cooper, R.L. (In Manuscript) *Dopaminergic modulation of sensorimotor circuits in Drosophila larvae*.

8. **Titlow, J.S**., Biecker, S., Cooper, R.L. (In Review) *Mechanosensory Habituation in Drosophila melanogaster larvae.*

7. **Titlow, J.S**., Anderson, H., and Cooper, R.L. (In Review) *Lights and Larvae: Using optogenetics to teach recombinant DNA and neurobiology*.

6. **Titlow, J.S.,** Rice, J., Biecker, S, Holsopple, E, Majeed, Z.R., and Cooper, R.L. (In Press) *Anatomical and genotype-specific mechanosensory responses in Drosophila melanogaster larvae.* Neuroscience Research.

5. **Titlow**, **J.S.,** Rufer, J., King, K., and Cooper, RL. (2013) *Pharmacological Analysis of Dopamine Modulation in the Larval Drosophila Heart.* Physiological Reports.

4. De Castro, C., **Titlow, J.,** Majeed, ZR., and Cooper, RL. (2013) *Analysis of various pharmacological salines for heart rate, CNS function, and synaptic transmission at neuromuscular junctions in Drosophila melanogaster larvae.* J Comp Physiol A.

3. **Titlow, JS,** Majeed, ZR, and Cooper, RL. (2013) *Neural Circuit Recording from an Intact Cockroach Nervous System*, Journal of Visualized Experiments.

2. Majeed, ZR, **Titlow, JS**, Burns, E, and Cooper, RL. (2013) Proprioception and tension receptors in crab limbs: Student laboratory exercises. Journal of Visualized Experiments.

1. **Titlow, JS**, Majeed, ZR, Nicholls, J, and Cooper, RL. (2013 *Intracellular Recording, Sensory Field Mapping, and Culturing Identified Neurons in the Leech, Hirudo medicinalis*. Journal of Visualized Experiments.

GRANTS AND FELLOWSHIPS

2013 NRSA NIH-Predoctoral Fellowship (Not funded)
2012 G. Flora Ribble Summer Research Grant- University of Kentucky
2010 Summer Thesis Research Grant- Marshall University Graduate College
2008 WV EPSCoR SURE Grant- Marshall University

AWARDS AND SCHOLARSHIPS

2013 Dissertation Enhancement Award- University of Kentucky Graduate College
2013 AAAS Program for Excellence in Science
2012 G. Flora Ribble Research Fellowship- University of Kentucky
2012 Travel Award- Marshall University Graduate College
2010 Travel Award- SENN/ SC Neuroscience Consortium
2009 Presentation Award- 35th East Coast Nerve Net, Woods Hole
2009 Presentation Award- Sigma Xi Research Day, Marshall University
2008 NASA Space Consortium Scholarship

2004 West Virginia Undergraduate Promise Scholarship
2004 Undergraduate Presidential Scholarship- Marshall University

PEER REVIEW- SCIENTIFIC JOURNALS

American Journal of Physiology
Journal of Experimental Biology
Behaviour
Hormones and Behavior
Journal of Neurophysiology
Journal of Neuroscience Methods
Physiology and Behavior
Synapse
Fly

PEER REVIEW- TEXTBOOKS

Human Physiology- An Integrated Approach, Pearson Higher Education
Principles of Human Physiology (5th Edition), Pearson Higher Education

PROFESSIONAL AFFILIATIONS

2010-current Society for Neuroscience
2012-current Kentucky Academy of Science
2013-current American Physiological Society
2013-current AAAS
2009-current Sigma Xi

SELECTED CONFERENCE ABSTRACTS AND PRESENTATIONS (out of 34)

#Undergraduates mentored

Titlow, J.S., #Biecker, S. and Cooper, R.L. (2014). Firing-rate plasticity in sensory-evoked motor output. 2nd Annual meeting of the Ky chapter of the American Physiological Society, Univ. of Louisville, Ky. March 31, 2014.

#Potts, D., **Titlow, J.S**. and Cooper, R.L. (2014). Drosophila dopamine receptor mutants exhibit locomotion abnormalities in response to light-touch stimuli. 2nd Annual meeting of the Ky chapter of the American Physiological Society, Univ. of Louisville, Ky. March 31, 2014.

#Biecker, S., **Titlow, J.S**., #Rice, J., Majeed, Z.R., #Holsopple, E. and Cooper, R.L. (2014). A novel system to investigate sensory habituation. 2ndAnnual meeting of the Ky chapter of the American Physiological Society, Univ. of Louisville, Ky. March 31, 2014.

DeCastro, C., **Titlow, J**., Majeed, Z.R. and Cooper, R.L. (2014). Maintaining the Drosophila larval heart for physiological measures: Modulators and cocktails. 2nd Annual meeting of the Ky chapter of the American Physiological Society, Univ. of Louisville, Ky. March 31, 2014.

#Rice, J. Majeed, Z.R., **Titlow, J**. and Cooper, R.L. (2014) Development of the Jordan HAT assay to study mechanosensation: Selective modulation of a neural circuit in larval Drosophila melanogaster. NCUR-National Council on Undergraduate Research. April 5-7. Univ of KY, Lexington, KY.

#Rayens, E., #Holsopple, E., **Titlow, J.,** and Cooper, R.L. (2014) Differences in Drosophila dopamine receptor expression drive metabolic regulation of a gustatory circuit. NCUR-National Council on Undergraduate Research. April 5-7. Univ of KY, Lexington, KY.

#Potts, D., **Titlow, J.S**. and Cooper, R.L. (2014) The Role of Dopamine Homeostasis on Dopaminergic Neuron Morphology, Function, and Fate in Drosophila. NCUR-National Council on Undergraduate Research. April 5-7. Univ of KY, Lexington, KY.

#Biecker, S., #Holsopple, E., **Titlow, J**., Cooper, R.L. (2014). Transmitters and second messengers involved in Drosophila melanogaster habituation to tactile stimuli. NCUR-National Council on Undergraduate Research. April 5-7. Univ of KY, Lexington, KY.

Vaughn, M., #King, K., Majeed, Z.R., **Titlow, J.S**. de Castro, C., and Cooper, R.L. (2014). The effects of combined modulators on Drosophila melanogaster heart physiology: dopamine, octopamine and serotonin. NCUR-National Council on Undergraduate Research. April 5-7. Univ of KY, Lexington, KY.

Titlow, J.S., #Biecker, S. and Cooper, R.L. (2014) Firing-rate plasticity in sensory-evoked motor output. Bluegrass Chapter of Society for Neuroscience annual meeting. Lexington, KY. March, 27.

#Potts, D., **Titlow, J.S.** and Cooper, R.L. (2014) Drosophila dopamine receptor mutants exhibit locomotion abnormalities in response to light-touch stimuli. Bluegrass Chapter of Society for Neuroscience annual meeting. Lexington, KY. March, 27.

#Biecker, S., **Titlow, J.S**. and Cooper, R.L (2014) A Novel System to Investigate Sensory Habituation. Bluegrass Chapter of Society for Neuroscience annual meeting. Lexington, KY. March, 27.

Titlow, J.S., #King, K.E., Majeed, Z.R. and Cooper, R.L. (2013) Additive stimulatory effects of monoamines on Drosophila melanogaster heart rate. University of Kentucky Gill Heart Institute Cardiovascular Research Day.

Titlow, J. and Cooper, R.L. (2013). Photo-activation of flight and jump motor neurons in adult flies: A teaching lab to investigate synaptic transmission and action potential waveforms. Annual meeting of the Kentucky Academy of Sciences. Nov. 8-9, 2013 at Morehead Univ. KY.
-Platform Talk

#Rice, J. Majeed, Z.R., **Titlow, J**. and Cooper, R.L. (2013) Development of the Jordan HAT assay to study mechanosensation: Selective modulation of a neural circuit in larval Drosophila melanogaster. Annual meeting of the Kentucky Academy of Sciences.Nov.8-9, 2013 at Morehead Univ.

#Rayens, E., #Holsopple, E., **Titlow, J**. and Cooper, R.L. (2013) Differences in Drosophila dopamine receptor expression drive metabolic regulation of a gustatory circuit. Annual meeting of the Kentucky Academy of Sciences. Nov.8-9, 2013 at Morehead Univ.

#Potts, D., **Titlow, J.S**., and Cooper, R.L. (2013) The Role of Dopamine Homeostasis on Dopaminergic Neuron Morphology, Function, and Fate in Drosophila. Annual meeting of the Kentucky Academy of Sciences. Nov. 8-9, 2013 at Morehead Univ. KY.

#Holsopple, E., #Rayens, E., **Titlow, J**. and Cooper, R.L. (2013) Genetic and pharmacological modulators of mechanosensory behavior in fruit fly larvae, Drosophila melanogaster. Annual meeting of the Kentucky Academy of Sciences. Nov. 8-9, 2013 at Morehead Univ. KY.

#King, K., Majeed, Z.R., **Titlow, J.** and Cooper, R.L. (2013) Additive stimulatory effects of octopamine and serotonin on Drosophila melanogaster heart rate. Annual meeting of the Kentucky Academy of Sciences. Nov. 8-9, 2013 at Morehead Univ. KY.

De Castro, C., Majeed, Z.R., **Titlow, J**. and Cooper, R.L. (2013). Analysis of various physiological salines for heart rate, CNS function, and synaptic transmission at NMJs in Drosophila melanogaster larvae. University of Kentucky Gill Heart Institute Cardiovascular Research Day

Cooper, R.L., Majeed, Z.R., **Titlow, J.,** Stacy, A., #King, K., #Rufer, J.M., Nichols, C.D. (2013) Pharmacogenetic approaches in altering heart rate in Drosophila larvae. The American Physiological Society Annual Meeting. Boston, MA, USA.

#Potts, D**., Titlow, J.S**., and Cooper, R.L. (2013) Dopamine's influence on nervous system anatomy during juvenile development. Annual meeting of the Ky chapter of the American Physiological Society, Univ of Kentucky, Lexington, KY, USA.

Titlow, J.S., Majeed, Z.R., Nicholls, J.G. and Cooper, R.L. (2013) Teaching with leeches- An undergraduate neurophysiology module. Annual meeting of the Ky chapter of the American Physiological Society, Univ of Kentucky, Lexington, KY, USA.

Majeed, Z.R., **Titlow, J.S**., Hartman, H.B. and Cooper, R.L. (2013) Teaching with crabs- An undergraduate physiology module. Annual meeting of the Ky chapter of the American Physiological Society, Univ of Kentucky, Lexington, KY, USA.

#Browne, J, **Titlow, J.S**., and Cooper, R.L. (2013) What fruit fly behavior teaches us about dopamine homeostasis, and vice versa. Undergraduate Showcase of Scholars. University of Kentucky, Lexington, KY, USA.

#Potts, D, **Titlow, J.S**., and Cooper, R.L. Dopamine's influence on nervous system anatomy during juvenile development. Undergraduate Showcase of Scholars. University of Kentucky, Lexington, KY, USA.

Titlow, J.S., Majeed, Z. R., Nicholls, J.G. and Cooper, R.L. (2013) Teaching with Leeches- An Undergraduate Neuroscience Module. Spring Neuroscience Day, University of Kentucky, Lexington, Kentucky, USA.

#Browne, J, **Titlow, J.S**., and Cooper, R.L. (2013) What fruit fly behavior teaches us about dopamine homeostasis, and vice versa. Spring Neuroscience Day, University of Kentucky, Lexington, KY, USA.

#Potts, D., **Titlow, J.S**., and Cooper, R.L. (2013) Dopamine's influence on nervous system anatomy during juvenile development. Spring Neuroscience Day, University of Kentucky, Lexington, KY, USA.

Titlow J.S., Cooper, R.L (2012) Come discuss DA's involvement in Drosophila behavior and development. Society for Neuroscience Annual Meeting, New Orleans, LA, USA.

Cooper, R.L., **Titlow J.S**., and Majeed, Z.R. (2012) Introduction of a new neurophysiology laboratory for students at the University of Kentucky. Society for Neuroscience Annual Meeting, New Orleans, LA. USA.

Titlow, J.S., Cooper, R.L. (2012) Behaviors and neural circuits modulated by dopamine. Behavioral Neurogenetics of Drosophila Larvae Conference, HHMI-Janelia Farms, Ashburn, VA, USA.

Titlow, J.S., Cooper, R.L. (2012) Genotyping abnormal behavior- Lessons from the fruit fly. Kentucky Academy of Sciences Annual Meeting, Richmond, KY, USA.
-Platform Talk

#Rufer, J.M., #King, K, **Titlow, J.S**., Cooper, R.L. (2012) Ritalin and other dopaminergic drugs affect CNS function and development in Drosophila larvae. Kentucky Academy of Sciences Annual Meeting, Richmond, KY, USA.

#Keathley, J, **Titlow, J.S**., Cooper, R.L. (2012) Carbohydrate energy considerations for cardiac function in Drosophila melanogaster. Kentucky Academy of Sciences Annual Meeting, Richmond, KY, USA.

#King, K, #Rufer, J.M**., Titlow, J.S**., Cooper, R.L. (2012) Pharmacological analysis of dopamine modulation in the developing fruit fly. Kentucky Academy of Sciences Annual Meeting, Richmond, KY, USA.

Titlow, J.S., Ghosh, S., Cooper, R.L., Harrison, D. and Rymond, B. (2012). A modifier of spinal muscular atrophy may be involved in motor behavior and stress response. Spring Neuroscience Day, University of Kentucky, Lexington, KY, USA.

Cooper, R.L**., Titlow, J.S.,** and Majeed, Z.R. (2012) Introduction of a new neurophysiology laboratory for students at the University of Kentucky. Spring Neuroscience Day, University of Kentucky, Lexington, KY, USA.

Ghosh, S., **Titlow J.S**., Cooper, R.L., Harrison, D., Rymond, B. (2012). Serf1 gene function in Drosophila melanogaster. 53rd Annual Drosophila Research Conference. Chicago, Il, USA.

Titlow J.S. and Brian L. Antonsen, (2010). Temporal Integration of Dopaminergic and Serotonergic Modulation in an Identified Neuron. Society for Neuroscience Annual Meeting. San Diego, CA, USA.

Titlow J.S. and Brian L. Antonsen. (2010), Optical and Electrical Evidence for the Metamodulatory Effects of Amines in a Reflex Circuit. 20th Annual Sigma Xi Research Day, Marshall University, WV, USA.
-Platform Talk

Titlow J.S., Inman, AL and Antonsen, BL, (2010). Combined Modulatory Effects of Dopamine and Serotonin in a Reflex Circuit. Frontiers in Neuroscience- Conference Abstract: 2010 South East Nerve Net and Georgia/South Carolina Neuroscience Consortium conferences, GA, USA.

Titlow J.S., and Brian L. Antonsen, (2009). Neuromuscular Control in a Postural Perturbation Model. 19th Annual Sigma Xi Research Day, Marshall University, WV, USA.

Titlow J.S. and Brian L. Antonsen. (2009). Interactions Between Serotonergic and Dopaminergic Modulation of the Crayfish Lateral Giant Circuit. 35th East Coast Nerve Net, Woods Hole, MA, USA.

Titlow J.S. and B.L. Antonsen. (2008). Refining Neuromuscular Control in a Postural Perturbation Model. The Inaugural Research Symposium of the Cell Differentiation and Development Center, Marshall University, WV, USA.

Titlow J.S. and B.L. Antonsen. (2008). Designing a Biomimetic Postural Feedback System with Optimal Response to Perturbation. WVINBRE Summer Research Symposium, West Virginia University, Morgantown, WV, USA.
-Invited Presentation

Titlow, J.S. and B.L. Antonsen. (2008). Designing a Biomimetic Postural Feedback System with Optimal Response to Perturbation. Undergraduate Research Symposium, Marshall University, WV, USA.

INVITED LECTURES

2014	Photo-activation of an escape response (Faculty for Undergraduate Neuroscience Workshop- Ithaca University, Ithaca, NY, USA)
2014	Photo-activation of an escape response (Cornell University- Neurophysiology course, Ithaca, NY, USA)
2014	Optogenetics in Neuroscience- Introduction to Physiology and Recombinant DNA (Paul Dunbar High School, Lexington, KY, USA)
2013	Transgenic Applications in Experimental Physiology (University of Kentucky- Animal Physiology course)

UNDERGRADUATE STUDENTS MENTORED

2013-2014	Stephanie Biecker, University of Kentucky (Ribble Research Scholarship awardee)
2013-2014	Douglas Potts, University of Kentucky (Ribble Research Scholarship awardee)
2013	Jordan King, Transylvania University (KBRIN Summer Research Fellowship)
2013	Emily Rayens, Wittenburg University
2013	Emily Holsopple, University of Kentucky
2013	Jessica Brown, University of Kentucky
2012-2013	Kayla King, University of Kentucky
2012	Jenna Rufer, Berea College (KBRIN Summer Research Fellowship)
2012	Tanner Keathley, University of Kentucky
2011	Brian Adkins, Marshall University

HIGH SCHOOL STUDENTS MENTORED

2012-2014 Elizabeth Schwarcz- Sayre School, KY, 10th grade Science Fair
2011 Jackie Maynard- Spring Valley High School, WV, MU TREK Program

PUBLIC SERVICE AND OUTREACH

2013-2014 **Optogenetics in the Classroom**- Dunbar High School and Mercer County High School, Lexington, KY. A neurogenetics laboratory module for AP Biology students. This is a spinoff of an optogenetics lab I developed for undergraduate physiology courses, in which the students are given an introductory presentation on recombinant DNA and synaptic transmission before performing a three-part lab exercise to study behavior. (Contact person: Mrs. Heidi Andersen)

2013 **Thoroughly Science Thursday**, Scott County Public Library- Georgetown, KY. Presented respiratory physiology activities to after school program for 20 4th graders (Contact person: Dr. Melody Danley)

2012-2013 **See Blue STEM Camp-** University of Kentucky, Lexington, KY. Gave two 3-hr laboratory demonstrations to 6-9th graders on human sensory systems and the use of invertebrate model organisms in the biological sciences. (Contact person: Dr. Craig Shroeder).

2012 **Neuroscience in the Classroom-** Beaumont Middle School, Lexington, KY. Provided a half-day to teaching 8th graders **(**2 classes) at Beaumont Middle School on differences between innate and learned behaviors. This was done with lecture and hands on activities. (Contact person: Mr. Patrick Goff, Science teacher at Beaumont Middle School).

2011-2014 **Science fair judge**
Kentucky Junior Academy of Sciences Fair, University of Kentucky, Lexington, KY
Central Kentucky Regional Science, Lexington, KY
Fayette County Science Fair, Lexington, KY
Morton Middle School Science Fair, Lexington, KY
Rosa Parks Elementary School Science Fair, Lexington, KY
Sigma Xi Research Day- Marshall University, Huntington, WV

2008-2011 **Brain Awareness Expo volunteer-** Marshall University, Huntington, WV.

2011 **EEGs in the Classroom-** Belpre Middle School, Belpre, OH. Provided a half-day of teaching to 5th graders (2 classes) at Belpre Elementary School (Parkersburg, WV) on the scientific method and brain electrophysiology. This was done with lecture and hands on activities. (Contact person: Mrs. Suzanne Halterman, Science teacher at Belpre Elementary School).

2010 **Healthy Herd Summer Camp volunteer-** Marshall University, Huntington, WV.

ACADEMIC DEPARTMENTAL SERVICE

2013 **Microteaching mentor for TA orientation**- University of Kentucky Graduate College

2013 **Representative for the Graduate Student Congress-** University of Kentucky, Department of Biology

2012 **Graduate Student Representative for External Review of the College of Arts and Sciences**- University of Kentucky, Department of Biology

2011 **Invited Panelist for Graduate School 101**- MUGC

2011 **Invited Panelist for the Graduate School Teaching Assistant Orientation-** MUGC

2011 **Invited Speaker for the Week of Welcome Orientation-** Marshall University, Department of Biology

2011 **Invited Speaker for the Green and White Days Orientation**- Marshall University, Department of Biology