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# MODULATORY ACTIONS OF SEROTONERGIC SYSTEM IN CARDIAC FUNCTION, BEHAVIOR, AND SENSORIMOTOR CIRCUIT ACTIVITY IN DROSOPHILA MELANOGASTER

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SEROTONERGIC SYSTEM IN CARDIAC FUNCTION, BEHAVIOR,  
AND SENSORIMOTOR CIRCUIT ACTIVITY IN DROSOPHILA MELANOGASTER

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DISSERTATION

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A dissertation submitted in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy in the  
College of Arts and Sciences  
at the University of Kentucky

By  
Zana Rafiq Majeed

Lexington, Kentucky

Director: Dr. Robin Lewis Cooper, Associate Professor of Biology

2016

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## ABSTRACT OF DISSERTATION

### MODULATORY ACTIONS OF SEROTONERGIC SYSTEM IN CARDIAC FUNCTION, BEHAVIOR, AND SENSORIMOTOR CIRCUIT ACTIVITY IN *DROSOPHILA MELANOGASTER*

In this dissertation, I have focused on the role of serotonin (5-HT) as a modulator in heart rate, feeding and locomotion behaviors as well as sensorimotor circuit activity in *Drosophila melanogaster*. A general overview in the actions of the serotonergic (5-HTergic) system on the larval heart and nervous system in larvae and adults is reviewed in Chapter One. I sought to further study the actions of serotonergic system to provide additional insights into cellular and molecular underpinnings in the actions of 5-HT. In Chapter two, I present studies on mechanisms of action by 5-HT in larval cardiac system. For this purpose, genetic and pharmacological approaches were used. The transgenic flies used expressed hM4Di receptors (designer receptors exclusively activated by designer drugs (DREADDs)) which were employed to manipulate the activity of Gai heterotrimeric protein through activation of engineered G-protein coupled receptors hM4Di DREADD. The activation of hM4Di DREADD receptors by clozapine-N-oxide (CNO) arrested the heart beat; however, pharmacological manipulation of adenylyl cyclase activity and cAMP levels had no significant effect on heart rate. In Chapter Three the role of various 5-HT receptor subtypes that mediate 5-HT action in larval cardiac tissue is addressed. In this study, various 5-HT agonists and antagonists were employed. The pharmacological results demonstrate that a 5-HT<sub>2</sub> agonist significantly increases the heart rate. Furthermore, 5-HT<sub>2</sub> antagonist, markedly reduces the effect of 5-HT. In addition, I employed genetic approaches to corroborate the pharmacological results.

In addition, I investigated the role of the 5-HTergic system in locomotion and feeding behaviors as well as in modulation of sensorimotor circuits. This study is delineated in Chapter Four. The 5-HT biosynthesis was dysregulated by feeding *Drosophila* larvae various pharmacological agents. 5-HT receptor subtypes were manipulated using RNA interference mediated knockdown and 5-HT receptor insertional mutations. Moreover, synaptic transmission at 5-HT neurons was blocked or induced in both larvae and adult flies. The results demonstrate that disruption of components within the 5-HT system significantly impairs locomotor activity and feeding behavior in larvae. In addition, acute activation of 5-HT neurons disrupts normal locomotor activity in adult flies. In Chapter Five, I addressed direct actions of fluoxetine on synaptic transmission at neuromuscular junctions (NMJs), neural properties, and cardiac function unrelated to fluoxetine's action as a selective 5-HT reuptake inhibitor using *Drosophila*, crayfish and primary neurons in mouse model system. Fluoxetine application blocked action potentials in crayfish axons, enhanced occurrences of spontaneous synaptic vesicle fusion events at NMJs of both *Drosophila* and crayfish. In rodent primary neurons, fluoxetine application resulted in increase of cytoplasmic Ca<sup>2+</sup>.

I also developed teaching modules, which are presented in Chapter Seven, to guide students how to exploit a vast array of genetic tools, such as optogenetics in *Drosophila* to manipulate various neural circuits and to observe their effects on behavior and sensorimotor circuit activity. I also developed a module to teach college level students a hands-on experiment regarding proprioception and tension receptors in crab limb, which is detailed in Chapter Eight.

**Keywords:** serotonin, 5-HT receptors, heart rate, locomotion and feeding, sensorimotor circuit

Zana Rafiq Majeed  
April 11, 2016

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# CHAPTER ONE

## INTRODUCTION

### **An overview of neuromodulation actions in organisms**

#### **Neuromodulation in physiology and behavior**

The nervous system is a complex network of neurons and neuroglia where the neurons are wired in a sophisticated manner that can generate a functional network to produce various distinct behaviors and control many physiological aspects of a biological system. The neurons in the nervous system communicate with each other through chemicals and/or electrical signals. The chemical substances used for communication between two neurons and for modulation in the strength of synaptic connections and electrical excitability are called neurotransmitters and neuromodulators, respectively. These chemicals are indispensable to fine tune various physiological actions in the nervous system and peripheral organs. The neuromodulatory substances help organisms to exhibit appropriate behavioral responses to accommodate the demands that are imposed by internal and external challenges and to keep homeostatic condition. The homeostasis is essential for the survival of animals; therefore, animals take advantage of various signaling pathways to keep the homeostatic state of the body. These signaling pathways are regulated by many chemical factors, which are called neurohormones (e.g. oxytocin and vasopressin), and neuromodulators. Neuromodulation is an important biological process that is found in most animals across the phyla (Hamood and Marder, 2014). Most organisms use the same chemicals as neuromodulators, such as, dopamine (Jay et al., 2015), acetylcholine (Picciotto et al., 2012) and serotonin (Dayan and Huys, 2009). The neuromodulators can exert their actions at local synapses or on distant targets far from their release site, which is called volume transmission (Agnati et al., 1995). Neuromodulators can alter the activity of intracellular and intercellular molecular pathways which, in turn, leads to changes in physiological actions in cells, organs and

behavior, for instance, synaptic strength, heart rate, intestine motility, and psychological state of an organism; therefore, dysregulation of the action of neuromodulators can result in many pathophysiological and neuropsychiatric disorders (McCormick and Nusbaum, 2014).

Neuromodulators are known to be involved in many neural processes in diverse animals across the phyla. For example, the stomatogastric ganglion neural network in lobster and crab that generates a pattern of rhythmic burst activity called central pattern generator (CPG); the physiological impacts of neuromodulators on CPG are extensively investigated (Marder and Bucher, 2007; Hamood and Marder, 2015; Städele et al., 2015). The studies in crustaceans (crab, lobster, crayfish) (Wiese, 2002; Marder, 2012) and other invertebrate neural networks such as *Drosophila melanogaster* (Wasserman et al., 2015), cockroach (Jung et al., 2013), and leech (De-Miguel et al., 2015) have advanced our understanding of neuromodulators actions in neural networks in general.

### ***Drosophila melanogaster* as a model system**

The fruit fly *Drosophila melanogaster* is an excellent model organism to answer the biological inquires and provide insights into the function of genes that are implicated in molecular mechanisms of human diseases (Bier, 2005). *Drosophila* has a striking repertoire of genetic tools that can be employed to manipulate neurons underlying specific behavior and genes that are expressed by a subset of neurons (Venken et al., 2011). The most widely useful tool in *Drosophila* is the UAS-Gal4 binary expression system, which is employed to target the expression of effector gene of interest to a subset of neurons in a spatial and temporal controlled manner. The UAS-Gal4 binary expression system consists of two elements, yeast Gal4 transactivator and upstream activating sequence (UAS), which drives the expression of reporter or effector gene (Brand and Perrimon, 1993). In this study, I used *Drosophila* system as

a model to investigate the modulatory actions of serotonin on cardiac physiology, locomotion and feeding behaviors, and sensorimotor circuit physiology.

### ***Drosophila melanogaster* cardiovascular system**

The *Drosophila* cardiovascular system is consisted of a dorsal tube, which is divided into heart and aorta subdivisions. The *Drosophila* cardiovascular system is an attractive experimental preparation to obtain a better understanding of the underlying molecular mechanisms of heart development and physiology (Medioni et al., 2009) and to study genes that are related to cardiac development (Alex et al., 2015a) and human cardiac diseases (Piazza and Wessells, 2011; Vogler and Bodmer, 2015). The anatomical details of the *Drosophila* cardiovascular system has been studied well at various developmental stages (Lehmacher et al., 2012). *Drosophila* as a model has advanced our knowledge in the field of metabolic disorders, such as obesity, and how it relates to cardiovascular diseases (Hardy et al., 2015). Recently, innovative tools such as optogenetics are employed to control the heart rate (Alex et al., 2015b; Zhu et al., 2016) which opens up venues for application of optogenetic tools to control the heart rate in patients with arrhythmic heart disorder. The action of most neuromodulators on heart are probably mediated through activation of G-protein coupled receptors (GPCRs). The GPCRs play important physiological and behavioral roles. In Chapter Two I have elucidated the role of engineered GPCR receptors, which are recently developed, to further investigate the physiological actions of GPCRs in cardiac function. Designer receptors exclusively activated by designer drugs (DREADDs) approach was used to understand the signaling pathway of positive chronotropic action of 5-HT in *Drosophila* larval heart. This novel chemogenetic tool broadens our knowledge regarding the cardiac physiology. The findings of these studies can be extended to mammals including humans (Becnel et al., 2013; Majeed et al., 2013).

Furthermore, our group has extensively studied the modulatory actions of various neuromodulators such as octopamine (King et al., 2013; Vaughn et al., 2014), dopamine (Titlow et al., 2013), serotonin (Majeed et al., 2014) and acetylcholine (Malloy et al., 2016), on *Drosophila* heart rate. 5-HT increases the heart rate; although, the 5-HT receptor subtypes that mediate positive chronotropic action of 5-HT remained unknown; therefore, I sought to study the characterization of 5-HT receptor subtypes in *Drosophila* larval heart by using both pharmacological and genetic tools (Chapter Three). The genome of *Drosophila* contains five genes that encode for five different 5-HT receptor subtypes: 5-HT1A, 5-HT1B, 5-HT2A, 5-HT2B, and 5-HT7 (Gasque et al., 2013). The data show that 5-HT2 receptor subtypes are involved in 5-HT action in heart rate modulation in *Drosophila* larval stage (Majeed et al., 2014).

### ***Drosophila melanogaster* nervous system**

In the present era of genomics, transcriptomics, and proteomics our understanding of neural circuit functions and wiring diagrams is still in early stages due to the complexity of vertebrate nervous systems such as mouse and human. Also, it has been proven that obtaining the human brain wiring diagram at high resolution is not attainable with currently available techniques yet but alternative techniques of low resolution have been employed to show the connections among different parts of the human brain by using functional magnetic resonance imaging (fMRI) (Kohl and Jefferis, 2011). The endeavor to map the connectivity of human brain and to understand the behavioral outcomes is called the Human Connectome Project (HCP) (Smith et al., 2015). The neuroscientists use a reduced nervous system of invertebrate models to study the anatomical and functional characteristics and connectivity maps of the nervous system at high resolution of neuronal and synaptic levels. This newly emerging discipline is called connectomics. One of the widely used invertebrate models in neuroscience is fruit fly *Drosophila melanogaster*, which has a relatively simple nervous system consists of about 10,000 neurons in larval stage (Ohyama et

al., 2015) and about 100,000 in adult stage (Peng et al., 2011; Kohl and Jefferis, 2011; Chiang et al., 2011). Besides, versatile genetic and molecular tools have been developed in *Drosophila* system, for instance, optogenetics (Hidehiko et al., 2014) and thermogenetics (Bath et al., 2014), which are powerful tools to manipulate the activity of neurons and to understand the functionality of neural circuits. The beauty of *Drosophila* genetic tools is that expression of various effector genes can be targeted to a subset of neurons by using different Gal4 lines (Pauls et al., 2015). Also, the *Drosophila*'s relatively simple neural circuits, which manifest complex behaviors, make this model organism an ideal system to study the function of neural circuits (Lemon et al., 2015) and mapping wiring diagrams (Chiang et al., 2011: these data are available online which is called Flycircuit. Using this data set, one can navigate the location of various neuronal cells in adult *Drosophila* brain). The advantage of using the *Drosophila* nervous system to decode the neural circuits and interrogate the function of genes is that despite less complicated nervous system, they also have a diversity of neuronal cell types (Venken et al., 2011). In addition, the molecular signaling pathways and genes that are active and expressed during brain development in *Drosophila* are evolutionarily conserved (Reichert and Bello, 2010). Moreover, as for vertebrates, the neurotransmitters used in *Drosophila* are histamine, GABA, glutamate, acetylcholine, dopamine, serotonin and two other neurotransmitters, octopamine and tyramine, which are similar to adrenaline and noradrenaline in vertebrates (El-Kholy et al., 2015). However, the neurotransmitter that mediates the synaptic transmission in fly neuromuscular junction (NMJ) is glutamate, and the majority of sensory neurons and central neurons use acetylcholine as a neurotransmitter to relay information from the presynaptic neurons to postsynaptic neurons during synaptic transmission, which is generally opposite in vertebrates (Venken et al., 2011).

The ultimate goal of neuroscience is to relate a specific neural circuit function to a distinct animal behavior. A recent study has mapped a wiring diagram of rolling behavior in *Drosophila* larvae, which is induced during parasitoid wasp attack (Ohyama

et al., 2015). Furthermore, new imaging techniques have been devised to study the neural circuits in *Drosophila* larvae. Using this imaging techniques, one can observe the activity of neurons in the CNS which helps discovering the function of neural circuits that are implicated in a specific behavior (Lemon et al., 2015; Pulver et al., 2015; Fosque et al., 2015). The recent technological advances have made the study of brain connectomics faster and achievable by using electron microscopy (EM) sectioning and automatically re-combining thousands of EM sections to show the connectivity of neurons in a neural network (Wang et al., 2015). The connectivity map is an important step toward the understanding of the nervous system and related behaviors. In addition, *Drosophila* has been widely used to unravel the molecular mechanisms of human neuropathological disorders (Pandey and Nichols, 2011), for instance, amyotrophic lateral sclerosis (Chai and Pennetta, 2015; Sanhueza et al., 2015), Alzheimer's disease (Bouleau and Tricoire, 2015), Parkinson's disease (West et al., 2015), and spinal muscular atrophy (Imlach et al., 2012).

The electrophysiological properties of *Drosophila* central neurons and neuromuscular junction (NMJ) are relatively well studied (Rohrbough and Broadie, 2002; Choi et al., 2004; Chen et al., 2009; Imlach and McCabe, 2009; Marley and Baines, 2011; Ryglewski and Duch, 2012). However, there is a gap in our knowledge of how neuromodulators change the strength of synapses in neural circuits. Even if we obtain the wiring diagram of the whole nervous system, it does not provide us with a thorough picture of neuromodulator actions on synapses, which consequently changes the way animals behave. In Chapter Four, I will show data on how serotonin alters feeding and locomotion behaviors as well as sensorimotor circuit activity. These data give us a rationale to further study actions of neuromodulators in neural networks to obtain a better understanding on how neural circuits generate behavior and how neural circuits fine tune their activity to adapt to internal and environmental challenges.

## **Serotonergic system in *Drosophila melanogaster***

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine neurotransmitter and neuromodulator that is synthesized from tryptophan, which is catalyzed by two enzymes, tryptophan hydroxylase (TRH) and aromatic-L-amino acid decarboxylase (AADC). 5-HT plays a role in a wide array of physiological and behavioral aspects of insects (Vleugels et al., 2015). The 5-HTergic system has been well investigated in the genetic model system *Drosophila melanogaster*. 5-HT plays essential roles in many physiological functions and behaviors in *Drosophila*, for instance, circadian rhythm regulation (Yuan et al., 2005), olfaction (Dacks et al., 2009), sleep (Yuan et al., 2006), feeding behavior (Gasque et al., 2013: in larval stage; (Albin et al., 2015: in adult stage), heart rate modulation (Dasari and Cooper, 2006; Majeed et al., 2013; Majeed et al., 2014), courtship and mating (Becnel et al., 2011), aggression (Zwarts et al., 2011; Alekseyenko and Kravitz 2014), insulin signaling regulation (Luo et al., 2014), locomotion (Silva et al., 2014; Majeed et al., 2016), learning and memory (Johnson et al., 2009), ecdysteroid hormone regulation (Shimada-Niwa and Niwa 2014), turning behavior in larval stage (Okusawa et al., 2014), modulation of feeding apparatus motor pattern (Schoofs et al., 2014a) as well as evoked sensorimotor circuit modulation (Majeed et al., 2016). Moreover, there are already many behavioral and physiological assays that can be carried out readily in *Drosophila* to investigate the influence of the 5-HTergic system disturbance on behavior paradigms to correlate with neurophysiological measures.

In addition, *Drosophila* has a relatively simple 5-HTergic system that consists of 96 5-HT neurons in larval central nervous system (CNS), and 106 5-HT neurons in adult stage; most of the larval stage 5-HT neurons are kept in adult stage during development (Vallés and White, 1988; Huser et al., 2012). The anatomical and morphological characteristics of *Drosophila* larval CNS 5-HTergic system are well documented; even the morphological features of single 5-HT neurons were elucidated by using genetic tools to haphazardly label single 5-HT neuron and study its

morphology (Huser et al., 2012). Various techniques have been developed to investigate neural circuitry and single neuron features, such as MARCM system (Luo, 2007). Furthermore, fluorescence microscopy techniques can be used to label a subset of neurons with various fluorescent proteins (del Valle et al., 2011; Hadjieconomou et al., 2011; Nern et al., 2015), which open up venues for our understanding in the function of small subsets of 5-HT neurons and studying their presynaptic and postsynaptic targets. Most of the 5-HTergic neurons in the CNS are interneurons (Huser et al., 2012); however, the 5-HT neurons connectomics map in the CNS neural network is still lacking. It has been shown that when most of the 5-HTergic neurons are ablated by expressing genes, such as head involution defective (*hid*) and *reaper*, which are inducing apoptosis in 5-HTergic neurons, no observable abnormal phenotype was noticed in larval behaviors, for instance olfactory chemotaxis, appetitive and aversive olfactory learning, gustation, and phototaxis. These results show that 5-HTergic neurons function as a neuromodulator that can change a specific behavior according to the sensory signal inputs from the environmental cues (Huser et al., 2012). Another study has demonstrated that a subset of three pairs of 5-HT neurons from subesophageal ganglion (SOG) of the CNS innervates prothoracic gland (PG), which is part of the ring gland, and regulates the synthesis of growth-related hormone ecdysone. The activity of this subset of 5-HT neurons is dependent on the nutrient state; when the nutrient is abundant, this subset of 5-HT neurons send signals to PG to accelerate the ecdysone production that plays essential role in molting processes and in the transition from larval stage to pupal stage. Furthermore, they have shown that 5-HT signaling acts through activation of 5-HT7 receptor subtype, because the knockdown of 5-HT7 receptor delayed the pupal formation stage; this phenotype can be rescued by 20-hydroxyecdysone administration (Shimada-Niwa and Niwa 2014). A recent study has shown that a subset of four subesophageal (SOG) 5-HT neurons are part of the enteric nervous system of *Drosophila* larvae that plays role in modulation of feeding behavior by innervating part of the enteric system such as

pharynx, esophagus, and proventriculus. Activation of CNS 5-HT neurons increases the motility of esophagus but no noticeable change was observed in proventriculus and midgut (Schoofs et al., 2014b).

5-HT receptors in *Drosophila* are G-protein coupled receptors (GPCR) (Yuan et al., 2006). 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> are coupled with G $\alpha$  inhibitory (G $\alpha$ i). Therefore, activation of this 5-HT receptor leads to the reduction of cytosolic cAMP level through inhibition of adenylate cyclase (AC) activity. 5-HT<sub>2</sub> is coupled with G $\alpha$ q heterotrimeric G-protein. When 5-HT binds to 5-HT<sub>2</sub> receptor, it will activate phospholipase C (PLC) enzyme. Moreover, 5-HT<sub>7</sub> is coupled with G $\alpha$  stimulatory (G $\alpha$ s). When 5-HT<sub>7</sub> is activated, it will activate AC that leads to the increasing of cytosolic cAMP concentration. These receptors are expressed in various neurons and nervous system regions (Johnson et al, 2011). After release of 5-HT into the synaptic cleft at nerve terminals by synaptic vesicles fusion, it will be taken up into the nerve terminals again by the action of serotonin transporter (SERT) (Sanders-Bush and Nichols, 2012). Serotonin transporter (SERT) is an important target for many anti-depressant agents (Rodríguez et al., 2003). *Drosophila* genome contains a gene that encodes for dSERT, which is homologous to vertebrate SERT (hSERT and rSERT). It has been demonstrated that dSERT can be inhibited by a variety of chemicals, such as, fluoxetine, which is a potent inhibitor of the SERT and used as an antidepressant drug (Corey et al., 1994; Demchyshyn et al., 1994). In Chapter 5, I will discuss the new findings regarding the actions of fluoxetine in various models: *Drosophila* larvae, crayfish and primary neurons in mouse.

Dysregulation of 5-HT concentration in critical periods of development leads to abnormal development of neural circuitry that consequently results in the long-lasting neuropsychiatric disorders. 5-HT plays role in development of the 5-HTergic neurons in the CNS of *Drosophila* larval brain (Sykes and Condrón, 2005). 5-HT<sub>2</sub> receptor expression was observed in *Drosophila* embryo during the formation of segments (Colas et al., 1995). However, the importance of all 5-HT receptor subtypes in

development of CNS has yet to be investigated and elucidated. It was already shown that 5-HT increases the evoked excitatory post synaptic potentials (EPSPs) in body wall muscle fibers in stimulated sensory-CNS-motor circuit (Dasari and Cooper, 2004). Also, 5-HT acts on the 5-HTergic neurons through activation of 5-HT autoreceptors to orchestrate the branching pattern and density of neurites in 5-HTergic neurons (Sykes and Condrón, 2005; Daubert and Condrón, 2010). When the 5-HT concentration is altered during critical periods of development, 5-HT receptors would be more or less activated that causes the abnormal formation of organism's neural circuits. In Chapter Four, I present a detailed explanation on how various 5-HT receptor subtypes impact feeding and locomotion behaviors in *Drosophila* larvae.

#### **Sensorimotor circuit in *Drosophila melanogaster* larvae**

The *Drosophila* larval motor neurons receive synaptic inputs from sensory and interneuron presynaptic partners. During development, the number of synapses increases as the larva growing from embryonic stage to third instar stage (Couton et al., 2015). The *Drosophila* larvae have many types of sensory neurons, which gather information about the surrounding environment and sending signals into the CNS. The sensory information is processed and integrated in the CNS. The input from the sensory neurons modulates the activity of central pattern generator (CPG) (Song et al., 2007); in turn, the motor output will be changed in response to the environmental demand. The morphological characteristics of various types of sensory neurons and the projections of most of them in the CNS of *Drosophila* larvae are well established (Grueber et al., 2002; Grueber et al., 2007; Halachmi et al., 2012). Some of the sensory neurons, which are developed during embryonic stage, are maintained and preserved during developmental stages into adult stage (Williams and Shepherd, 1999). The sensory neurons, which are classified to external sensory neurons (es), chordotonal neurons (cho), and multidendritic (md) neurons, are located on *Drosophila* body wall musculature. The md neurons are subdivided, which is based on the way they form

their dendritic arborization (da), into bipolar dendritic (bd) neurons, and four morphologically distinct types of Class I-IV dendritic arborization neurons. Each body wall hemisegment has 43 sensory neurons, which gather and relay signals about specific hemisegment to CNS (Kohsaka et al., 2012). A recent study has demonstrated that dorsal bipolar dendritic (dbd) sensory neurons in *Drosophila* larvae respond to stretch stimuli; therefore, they are called stretch receptor neurons. Besides, the activity of this subset of sensory neurons are dependent on stretch-activated channels called Piezo channels (Suslak et al., 2015). A study has shown that the input from class II multidendritic (md) sensory neurons are indispensable for the CPG neural network activity. When the activity of Type II sensory neurons was inhibited by expressing *shibire<sup>ts</sup>*, which is a temperature-sensitive mutation, in these neurons and after exposing the larvae to restrictive temperature, the CPG activity was abolished (Song et al., 2007). This result indicates that the activity and feedback from sensory neurons are required for the CPG to function properly.

*Drosophila* larvae show a rhythmic peristaltic locomotion behavior, and the contraction of the musculature of the body wall starts from the posterior end and spreads toward the anterior part of the body. Our understanding of the sensorimotor circuit is progressing in *Drosophila* model system. The *Drosophila* has stereotypic motor neurons that innervate body wall musculature. The whole body of a *Drosophila* larva consists of 10 segments. In each body wall hemisegment there are 30 muscle fibers; these muscle fibers are innervated by 40 motor neurons (Kohsaka et al., 2012). Motor neurons have distinct dendritic morphology and project their axons outside the nervous system through segmental nerves (SN), intersegmental nerves (ISN) or transverse nerves, to reach and make synaptic contacts with their targets, body-wall muscle fibers. The morphological features of motor neuron dendritic organization in the ventral nerve cord (VNC) is well investigated in *Drosophila* larvae (Kim et al., 2009). Nonetheless, the elaborate structural characteristics of distinguishable motor neuron

axonal terminals at the neuromuscular junction of identifiable muscle fibers are well studied (Hoang and Chiba, 2001).

The genetic factors in the CNS regulate the position of motor neuron dendrites. For instance, Netrin-Frazzled pathway attracts dendrites while Roundabout (Robo)-slit mediates dendrite repulsion. The input from presynaptic partners onto motor neurons governs the growth of dendritic arborization to accommodate the plasticity of the neural circuits to motor output. If the synaptic input on the motor neurons is increased, the motor neuron dendrites will have less branches and vice versa (Kohsaka et al., 2012). It has been revealed that the alteration of biogenic amine concentrations (octopamine and tyramine), due to the mutation in tyramine  $\beta$  hydroxylase gene, results in the abnormal phenotype of locomotor activity. This result demonstrates that biogenic amines are essential for the normal locomotion activity (Fox et al., 2006). Interestingly, octopamine positively modulates locomotion behavior; although, tyramine has negative effects on locomotion system (Saraswati et al., 2004).

### **Serotonergic neurons wiring diagram within sensorimotor neural networks**

Our understanding of the morphological features of both sensory and motor neurons is well advanced in *Drosophila* larvae; however, our knowledge on how interneurons make synaptic connections with sensory and motor neurons in locomotor neural networks is still incomplete. Most of the 5-HTergic neurons are interneurons and the features of single identifiable 5-HT neurons are well established (Huser et al., 2012). However, how 5-HTergic neurons make synaptic contacts with presynaptic and postsynaptic partners and how activity of various 5-HT receptor neurons is implicated in modulation of various aspects of different behaviors still have to be further investigated. Various pharmacological agents have been employed to observe how the CNS motor neurons in *Drosophila* larvae respond to various neurotransmitters while the electrophysiological recording was being performed. The results show that GABA or glutamate reduces motor neuron excitability; however, when exogenous

acetylcholine was applied, it increased the electrical excitability of motor neurons (Rohrbough and Broadie, 2002). These data indicate that motor neurons should probably make synaptic contacts with GABAergic, glutamatergic, and cholinergic neurons. Some VNC inhibitory interneurons can establish synaptic contacts with motor neurons; some of these interneurons, such as GABAergic and glutamatergic interneurons, make inhibitory synaptic connections with motor neurons (Kohsaka et al., 2014; Itakura et al., 2015; Fushiki et al., 2016); although, other interneurons, such as cholinergic interneurons, can make excitatory synaptic contacts with motor neurons (Heckscher et al., 2015). All these various neurons: sensory neurons, interneurons (inhibitory and excitatory), and motor neurons generate a neural network to drive and control the locomotion behavior in *Drosophila*. In Chapter Four, I present data on how 5-HTergic neuron activation alters locomotion behavior and sensorimotor circuit activity. However, important questions regarding 5-HTergic system in CNS still have to be addressed. For instance, how 5-HTergic neurons make synaptic contacts with other elements in neural network and how they work together to orchestrate locomotory neural networks are still unknown. Future studies should further investigate the functional connectivity of small subsets of 5-HTergic neurons in the CNS and probe their role in various behaviors.

### **Serotonergic system in mammalian nervous system**

In a mammalian nervous system such as in a mouse or human, 5-HTergic neurons are found in the raphe nuclei which send axons to most parts of the brain and are involved in many physiological and behavioral processes (Vadodaria et al., 2016). Also, many neurons from different parts of the brain, such as forebrain and cortex, extend their axons to make synaptic contacts with 5-HTergic neurons in mice (Pollak et al., 2014). In a recent study, it has been shown that a subset of 5-HT neurons is involved in reward signaling; 5-HT neurons in dorsal raphe nucleus (DRN) are activated when mice are given rewards such as sucrose (Li et al., 2016). 5-HTergic

system dysregulation is related to various mental illness conditions such as depression, anxiety, learning and memory impairment, and autism (Sodhi and Sanders-Bush, 2004). It has been revealed that autistic patients have high 5-HT concentration in their blood; in addition, 5-HTergic neuron terminals are reduced in those patients (Whitaker-Azmitia, 2010). Genetic variations in genes that encode serotonin transporter (5-HTT) and monoamine oxidase A (MAOA), which degrades 5-HT, might be related to some of psychological disorders. Some of the polymorphic variations are located in the regulatory sequences of these genes. The polymorphism might not be the only factor that can be related to neuropsychiatric disorders; the interactions between genetic and environmental factors might result in some of the neuropsychiatric disorders as well (Nordquist and Oreland, 2010). In a recent attempt, researchers have been successful in generating 5-HTergic neurons from human fibroblasts, which are called induced 5-HTergic neurons (Vadodaria et al., 2016; Xu et al., 2016). This groundbreaking finding opens up many avenues for the discovery of potential therapeutic interventions for those patients who are not benefited from the available anti-depressant drugs, such as selective serotonin reuptake inhibitor (SSRI), fluoxetine (Prozac). A lot of progress has been made in studying the physiological and pathophysiological mechanisms of 5-HT neuron actions in various bodily systems by using a wide range of model organisms from fruit fly to mice, which have provided novel insights into the molecular mechanisms of some of the neuropsychiatric disorders in humans.

### **Specific aims**

The main objectives of this dissertation are:

1. To study the molecular signaling pathways that mediate positive chronotropic action of 5-HT in *Drosophila* larval heart by using pharmacological and chemogenetic, which is called Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), approaches.
2. To investigate the modulatory role of various 5-HT receptor subtypes that mediate acceleratory action of 5-HT in *Drosophila* larval heart rate by using pharmacological and genetics tools.
3. To provide insights into the role of 5-HTergic system in feeding and locomotion behaviors as well as sensorimotor circuit activity in *Drosophila melanogaster*.
4. To obtain a better understanding on how fluoxetine (Prozac) affects neuronal activity in various model systems, crayfish, *Drosophila* and primary cortical neurons in mouse through targeting non-canonical pathways.

## CHAPTER TWO

### 5-HT stimulation of heart rate in *Drosophila* does not act through cAMP as revealed by pharmacogenetics

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#### Abstract

The fruit fly, *Drosophila melanogaster*, is a good experimental organism to study the underlying mechanism of heart rate (HR) regulation. It is already known that many neuromodulators (serotonin, dopamine, octopamine, acetylcholine) change the HR in *Drosophila melanogaster* larvae. In this study, we investigated the role of cAMP-PKA signaling pathway in HR regulation and 5-HT positive chronotropic action. In order to obtain insight into the 5-HT mechanism of action in larvae cardiomyocytes, genetic and pharmacological approaches were used. We used transgenic flies that expressed the hM4Di receptor (designer receptors exclusively activated by designer drugs; DREADDs) as one tool. Our previous results showed that activation of hM4Di receptors (modified muscarinic acetylcholine receptors) decreases or arrests the heart from beating. In this study, it was hypothesized that the positive chronotropic effect of serotonin (5-Hydroxytryptamine, 5-HT) are mediated by serotonin receptors coupled to the adenylyl cyclase pathway and downstream cAMP and PKA activity. Activation of hM4Di by clozapine-N-oxide (CNO) was predicted to block the effects of serotonin by inhibiting adenylyl cyclase activity through G $\alpha$ i pathway activation. Interestingly, we found here that manipulation of adenylyl cyclase activity and cAMP levels had no significant effect on HR. The ability of hM4Di receptor activation to slow or stop the heart is therefore likely mediated by activation of GIRK channels to produce hyperpolarization of cardiomyocytes, and not through inhibition of adenylyl cyclase.

**Keywords:** 5HT, M4D receptor, CNO, adenylyl cyclase, PKA.

## **Introduction**

The heart in the fruit fly, *Drosophila melanogaster*, is a model for investigating the underlying mechanism of cardiac diseases which are related to human cardiac disorders, such as congenital heart diseases (Vogler et al., 2012) and dilated cardiomyopathy (Ma et al., 2010). Although, the *Drosophila* heart is simple and consists of a heart tube at the caudal region and anterior aortic region (Cammarato et al., 2011), it is been established as a model system to study the ionic basis of myocardiocytes (Desai-Shah et al., 2010). The myogenic heart of *Drosophila* larvae also is useful to examine modulators and their mode of actions, because modulators directly act on the myocardiocytes as there is no direct innervation on the pacemaker region. It has been previously shown that  $Ca^{2+}$  and  $K^{+}$  ions play a crucial role in generation of the cardiac action potential. Inhibition of L-type calcium channels or potassium channels by specific blockers significantly reduce the HR whereas the  $Na^{+}$  channel blocker, tetrodotoxin, does not have any effect on HR in *Drosophila* (Gu and Singh, 1995). To investigate the underlying mechanisms of modulation, ionic and second messenger cascades need to be determined.

Some neuromodulators and neurotransmitters, like serotonin, dopamine, octopamine, and acetylcholine are known to modulate heart performance in *Drosophila melanogaster* larvae (Dasari and Cooper, 2006; Johnson et al., 1997; Titlow et al., 2013). 5-HT is an essential neuromodulator that has many behavioral and physiological functions in the fly, such as learning and memory (Johnson et al., 2009), courtship and mating (Becnel et al., 2011), cardiac rate modulation (Dasari and Cooper, 2006; Zornick et al., 1999), and modulation of sensory-motor circuits (Dasari and Cooper, 2004). Moreover, 5-HT is assumed to be a modulator with an old evolutionary history because it is found in simple as well as in complex animals and even plants (Azmitia, 2007). There are four characterized 5-HT receptor genes in the

*Drosophila melanogaster* genome, 5-HT1ADro, 5-HT1BDro, 5-HT2Dro, and 5-HT7Dro (Becnel et al., 2011; Johnson et al., 2009; Luo et al., 2012; Nichols, 2006). Recently, another 5-HT2Dro receptor subtype, 5-HT2BDro, has been identified in *Drosophila* (Gasque et al., 2013). 5-HT1ADro, 5-HT1BDro inhibits adenylyl cyclase (AC) activity (Saudou et al., 1992); 5-HT7Dro increases AC activity (Witz et al., 1990). Exogenous 5-HT application in semi-intact larvae increases the HR, but the underlying signaling mechanism has yet to be elucidated. However, we have shown that activation of 5-HT2Dro receptor mediates the positive chronotropic effect of 5-HT in *Drosophila* larval heart (Majeed et al., 2014).

It is obvious that the *Drosophila* and vertebrates are different morphologically; notwithstanding, *Drosophila* and vertebrates use similar molecular pathways underlying cardiac development (Bodmer and Venkatesh, 1998). Further, *Drosophila* and vertebrate hearts share crucial physiological and dynamic aspects, for example, cardiac output, rate and duration of systole or diastole (Choma et al., 2011). The rationale for *Drosophila* larval heart study is to decode the undiscovered aspects of cardiac physiology and pathophysiology, which in turn can be extended to the physiology of hearts in other animals including humans.

In this study, we sought to elucidate the signaling pathway underlying the positive chronotropic effects of 5-HT in *Drosophila melanogaster* larva by utilizing classical pharmacology with incorporation of a pharmacogenetic approach using DREADD receptors (Becnel et al., 2013). DREADDs are powerful new tools that allow a high degree of spatial and temporal control of neuronal and effector pathway activity. Significantly, DREADD control is reversible, and requires no specialized equipment. We used the UAS-Gal4 binary expression system (Brand and Perrimon, 1993) to express hM4Di receptors, which are positively coupled to Gai, in muscle fibers. The hM4Di is a modified human muscarinic acetylcholine M4 receptor, mutated such that it no longer has affinity for the native ligand, acetylcholine. Instead, this engineered receptor has high affinity for a chemical that is considered physiologically inert,

clozapine-N-oxide (CNO) that has full agonist efficacy at DREADD receptors (Armburster et al., 2007; Becnel et al., 2013; Nichols and Roth, 2009). With this approach one can rule out off-target effects of the natural ligand to specifically and remotely control effector pathway activity in defined target tissues that the DREADD receptor is expressed in.

In the pupal stage of *Drosophila* forskolin does not produce a change the HR (Johnson et al., 2002), indicating that cAMP levels are not important in the pupa; however, in the pupal stage significant alterations in endocrine function accompanied by significant morphological changes are occurring, and mechanisms of cardiac function may be different than in the larva. We previously demonstrated that 5-HT increases HR in larva, therefore we examine here the potential mechanisms of action of 5-HT in the larval stage heart. Our working model was that 5-HT activates G<sub>s</sub>, which activates adenylyl cyclase that in turn increases the level of cAMP. Increased cAMP leads to an increase in active PKA signaling and positive chronotropic modulation of the heart. In this scenario, blockade of adenylyl cyclase activity with pharmacological methods, or activation of G<sub>i</sub> through activation of hM4Di receptors expressed in the heart, would be predicted to decrease cAMP levels and PKA activation and block the positive chronotropic effects of 5-HT on the heart. Our data, however indicate that cAMP levels do not contribute to either modulation of HR or the positive chronotropic effects of 5-HT in the *Drosophila* heart. It has been reported that forskolin does not noticeably change the HR in P1 pupal stage of *Drosophila* (Johnson et al., 2002). This confirms that *Drosophila* HR in larvae also might not be markedly changed by activation of AC-PKA pathway; although, AC-PKA pathway might increase the contractility of heart.

## **Materials and Methods**

### **Transgenic fly strains**

In this study, two fly strains were used, UAS-hM4Di and 24B-GFP (a strain heterozygous for the 24B-GAL4 driver element that expresses in all larval muscle, and is also homozygous for the UAS-GFP element; from Dr. Charles Nichols, LSUHSC). The UAS-hM4Di strain was crossed with 24B-GFP strain in order to express hM4Di (Gai) coupled receptor in muscle fibers, including cardiomyocytes. In the F1 generation, half the flies contained the 24B-GAL4 expression element, detected by GFP expression, and the other half did not. The GFP-positive, hM4Di-expressing larva were used as the experimental animals, and the non-GFP-positive, non-hM4Di-expressing (but UAS-hM4Di containing) 'littermates' were used as controls (background). The flies were reared at room temperature (22°C) in vials containing cornmeal-agar-dextrose-yeast medium.

### **Heart rate measurement**

Third instar larvae were dissected in the ventral side up position to expose the dorsal vessel (heart) in HL3 (saline solution) (NaCl 70mM, KCl 5mM, MgCl<sub>2</sub>.6H<sub>2</sub>O 20mM, NaHCO<sub>3</sub> 10mM, Trehalose 5mM, sucrose, 115mM, BES 5mM, and CaCl<sub>2</sub>.2H<sub>2</sub>O 1mM with the pH adjusted to 7.1). Because heart performance is very sensitive to pH change, the pH was tightly regulated and adjusted as needed. Recently, the effects of various buffers have been probed to optimize the saline for monitoring heart rate (de Castro et al., 2014). Drugs were applied at various concentrations as indicated in the Results. The preparation was left for one minute in saline after dissection, and then heart beats were counted for the following minute. Exceptions are indicated on graphs. The difference in the HR before and after application of drugs was used to measure the effects of the various compounds.

## **Chemicals**

All the chemicals, serotonin hydrochloride (5-hydroxy tryptamine (5-HT)), forskolin, SQ 22,536, N<sup>6</sup>, 2'-O-Dibutyryl adenosine 3',5' cyclic monophosphate sodium salt (dbcAMP), 2', 5' dideoxyadenosine (ddadenosine) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Clozapine-N-oxide was kindly synthesized by Dr. David Nichols (Purdue University). 5-HT stock solution (1mM) was prepared with HL3 saline and immediately before use; a 1  $\mu$ M serotonin solution was made from the stock solution. Forskolin was dissolved in 1% dimethyl sulfoxide (DMSO) to obtain a 1 mM stock solution. The final concentration of DMSO was less than 0.1% (roughly about 0.03%) in 30  $\mu$ M forskolin and 0.5% in 100  $\mu$ M forskolin. 300  $\mu$ M and 1 mM of dbcAMP were also prepared with HL3 saline. 200  $\mu$ M of SQ 22,536 was made with saline from a 1 mM stock solution. A 1mM ddadenosine stock solution was made in DMSO, then 50  $\mu$ M (0.04% final DMSO concentration) and 500  $\mu$ M (0.2% final DMSO concentration) with dilutions in saline.

## **Statistical analysis**

All data are expressed as mean  $\pm$  SEM. The paired t-test (before and after) was used to compare the difference of HR change after exchanging solution with saline containing chemicals. T-test was used to compare between background (UAS-hM4Di) and hM4Di expressing (UAS-hM4DiX24B-Gal4) larval HRs (SigmaPlot version 11.0).  $P \leq 0.05$  is considered as statistically significant. The number of asterisks are considered as  $P \leq 0.05$  (\*),  $P \leq 0.02$  (\*\*),  $P \leq 0.001$  (\*\*\*).

## **Results**

### **Effects of media change on heart rate**

We previously found that the larval HR is sensitive to the physical exchange of the bathing media (Dasari and Cooper, 2006). In order to control for the exchange of the bathing media when exposing the heart to various compounds, a series of control

experiments of saline bath exchanges was conducted. Also, because some of the drugs contained small concentrations of DMSO, the effect of low concentrations of DMSO alone and the effect of switching the DMSO bathing media were examined (Figure 2.1). In addition, the HR was measured with an additional exchange of the bathing media twice for the same period as the first one, left for one minute, and then examined for the following minute. Although the larvae were already exposed to saline during the dissection, there was still an average of 20 BPM increase in HR with the first saline change. After another two minutes the heart was not as sensitive to the exchange of the saline, as only an average of 10 BPM was noted (Figure 2.1B). Exchanging bathing media that contained DMSO did not produce as large of an increase in HR as saline. The second exchanges also resulted in a smaller alteration in HR (Figure 2.1D).

#### **CNO concentration-dependently inhibits heart rate in hM4Di expressing larva**

Increasing concentrations of CNO (10nM, 100nM, and 1 $\mu$ M) were applied to the exposed heart of semi-intact larval preparations. Application of CNO decreases the HR in a concentration dependent manner in the UAS-M4D X 24B-GFP larvae (Figure 2.2). CNO application at the highest concentration does not significantly change the HR in the non-expressing UAS-M4D (control) larvae (Figure 2.2). In this study, 500nM CNO was chosen to use for the primary experiments because 1 $\mu$ M stopped the heart from beating in all UAS-M4D expressing preparations. We observed that most of the hearts started to beat again when the saline + CNO was exchanged with saline only (not shown).

#### **The presence of CNO to activate hM4Di in expressing larvae blocks the positive chronotropic effect of 5-HT.**

In flies that do not express hM4Di receptors (background), 5-HT markedly increases the HR, even in the presence of CNO (Figure 2.3A, C). These results confirm

the positive chronotropic effects of 5-HT, and that CNO does not block the plasma membrane receptor binding site for 5-HT. Only when hM4Di DREADD receptor expression was induced did CNO have an effect on the positive chronotropic effect of 5-HT (Figure 2.3B, C). We observed that CNO by itself slightly decreased HR in background larvae (Figure 2.3A, C). This may be due to leaky expression of the UAS-GAL4 element present in the background genotype, rather than a direct effect of CNO on the heart. When the CNO + 5-HT containing saline was exchanged with saline (4→5), the HR significantly increased in both background and hM4Di-expressed larvae. This might be due to the 5-HT residual effect on the HR. The increase in HR on this final wash was 2-fold greater in the hM4Di expressing larva suggests a rebound effect occurring from the removal of the repressing CNO mediated hM4Di signaling.

#### **After 5-HT is applied to increase HR, the addition of CNO reverses the positive chronotropic effects of 5-HT.**

5-HT was applied prior to exposure of CNO in order to see if CNO can also reverse the effects of 5-HT in addition to block, as demonstrated above. 5-HT markedly increases the HR (Figure 2.4). The addition of CNO reversed the effects of 5-HT (Figure 2.4). When CNO + 5-HT saline was exchanged with saline alone (4 →5), the HR increased again, as observed in the experiments shown in Figure 2.3, indicating a possible rebound effect from removal of the repressing hM4Di signaling.

#### **CNO blocks the effects of forskolin**

In order to know more about the effect of hM4Di expression on cardiac muscle fiber second messengers, forskolin, an AC activator was examined. In the presence of CNO forskolin increased the HR in background larvae (Figure 2.5A). The presence of CNO blocked the positive chronotropic effect of forskolin in hM4Di-expressing larvae. Furthermore, not only was the HR completely arrested by CNO application, but forskolin was unable to overcome this blockade (Figure 2.5B, C).

Forskolin (30 $\mu$ M) alone was observed to increase the HR (Figure 2.6). The subsequent addition of CNO had no inhibiting effect in the background larva, but dramatically halted the HR in the hM4Di expressing larva (Figure 2.6). Since forskolin was not able to overcome the effects of hM4Di activation this may indicate that cAMP levels alone are not the primary mediator of HR. The effect forskolin did not show a significant difference between two genotypes (Figure 2.6C).

A higher concentration of forskolin (100  $\mu$ M) was used to determine whether or not the 30  $\mu$ M concentration was maximal or near maximal and thus producing a saturating effect. We also incubated with the higher concentration of forskolin for 10 minutes to observe if there was a time dependent effect present on heart rate for forskolin exposure as compared to an acute exposure. We observed that forskolin at 100  $\mu$ M increased the HR at the beginning; however, it was not greater than that of 30  $\mu$ M, and no further increase the HR occurred during the 10 minute incubation time (Figure 2.7 A, B), indicating that our original 30  $\mu$ M concentration was likely saturating for forskolin.

To further probe the role of cAMP, we used the AC inhibitor SQ 22,536 (SQ). Application of SQ had no effect in the first minute (Figure 2.8). Because no inhibitory effect of SQ on the HR was observed within the first few minutes, we prolonged the interval to nine minutes. At the ninth minute of incubation the HR decreased significantly ( $P < 0.02$ ), but it did not stop (Figure 2.8). This decrement in HR might be due to the lengthy incubation period, rather than the drug. However, it was shown in the semi-intact heart preparation (Canton-S flies) that were incubated inside the saline (pH 7.00) for 10 minutes, the HR do not show a marked change (data not shown). Taken together, these results show that SQ has a slightly inhibitory effect on the HR. When the SQ solution was exchanged with SQ + 5-HT solution, HR significantly increased (Figure 2.8). These results indicate that SQ cannot override the positive chronotropic effect of 5-HT. Either the SQ is not effective in blocking *Drosophila* AC as

it for mammalian AC, or perhaps manipulating cAMP levels alone is not sufficient to mediate an increase in HR, in agreement with our results using forskolin.

We next tried another AC inhibitor, dideoxyadenosine. Application of ddadenosine (50 $\mu$ M and 500 $\mu$ M) did not produce a noticeable change in HR in hM4Di expressing larvae (Figure 2.9 and 2.10). By the second minute HR showed a very slight decrease in hM4Di expressing larvae. However, when ddadenosine (50 $\mu$ M or 500 $\mu$ M) solution was exchanged with ddadenosine + 5-HT, a marked increase in the HR occurred (Figures 2.9 and 2.10).

In a different approach, we attempted to manipulate cAMP levels by using the cAMP analog dbcAMP, which both inhibits phosphodiesterase and mimics the effects of cAMP on downstream effectors like PKA. **dbcAMP** is resistant to cleavage by phosphodiesterase (Henion et al., 1967). Application of dbcAMP (300 $\mu$ M) does not change the HR in background larvae (Figure 2.11A, C). Because no effect was observed with the initial exposure of dbcAMP on HR, the HR was measured for the following second minute. Again, there was no significant change in HR with a longer exposure to dbcAMP (Figure 2.11A, C). When the dbcAMP solution was exchanged with dbcAMP + CNO solution, HR slightly increased in background larvae (Figure 2.11A, C). Application of dbcAMP slightly decreased HR in hM4Di expressing larvae (Figure 2.11B, C). When the dbcAMP solution was exchanged with the dbcAMP + CNO solution, the HR dramatically decreased in the hM4Di expressing larvae (Figure 2.11B, C). dbcAMP did not have a significant difference between two genotypes.

Because there was no significant change after application of 300 $\mu$ M dbcAMP, a higher concentration of dbcAMP (1 mM) was used. Application of dbcAMP at this higher concentration only slightly decreased the HR in the first and second minute (Figure 2.12) to the same degree as with the lower concentration (Figure 2.11). However, when the dbcAMP solution was exchanged with dbcAMP + CNO, the HR markedly decreased (Figure 2.12). Changing dbcAMP + CNO solution with saline alone showed a marked increase in HR (Figure 2.12).

## Discussion

*Drosophila* has been established to be a good model for the study of cardiac physiology in part because of its powerful genetics and conserved development with mammalian heart (Pandey and Nichols, 2011). Moreover, fly and mammalian hearts use many of the similar signaling pathways during development (Vogler et al., 2012). In mammals, 5-HT receptor dysfunction or overactivation leads to abnormal cardiac development and physiological disturbance (Lairez et al., 2013; Nebigil et al., 2000). Clearly, 5-HT and proper activation of relevant 5-HT receptors is critical for normal cardiac development and function in mammals (Berger et al., 2009). 5-HT receptors are all G-protein coupled receptors (GPCRs), with the exception of the 5-HT<sub>3</sub> receptor that is a ligand gated ion channel (Nichols and Nichols, 2008). Modified DREADD GPCR receptors have been shown to be excellent tools to further understand the function of specific GPCRs, like serotonin receptors, and their signaling pathways in normal physiological processes in both fly and mammals (Becnel et al., 2013; Nichols and Roth, 2009). For example, the hM4D1 DREADD receptor we have used here has also been utilized to understand the role of serotonin in respiratory control (Ray et al., 2011), and to determine key structural components of the native muscarinic M4 acetylcholine receptor itself (Nawaratne et al., 2008). These pharmacogenetic approaches have opened up new avenues to further understand the roles of GPCR receptors in physiology, and are promising tools to obtain additional insight into underlying molecular mechanisms of human diseases.

Here, we initiated studies to elucidate effector pathways underlying HR regulation in the *Drosophila* heart, and to further explore the positive chronotropic role of serotonin on the *Drosophila* larva heart. Our original hypothesis was that *Drosophila* larva HR, like mammalian HR, was governed by adenylyl cyclase activity and its downstream effectors and second messengers. Further, that the positive chronotropic effect of 5-HT was due to positive modulation of adenylyl cyclase activity. To address this hypothesis, we used both pharmacological and pharmacogenetic approaches

using a combination of drugs and the modified mammalian G-protein coupled receptor (hM4Di, coupled to G $\alpha$ i) to provide an insight into potential signaling pathways underlying HR and the effects of 5-HT in *Drosophila* larval heart. We initially observed that activation of hM4Di can slow or stop the heart. Moreover, that hM4Di activation can override the positive chronotropic effect of 5-HT. These observations were consistent with our hypothesis, with the prediction that hM4Di activation would activate G $\alpha$ i, reduce adenylyl cyclase activity, and slow the heart.

It has been shown, however, that forskolin does not noticeably change the HR in P1 pupal stage of *Drosophila* (Johnson et al., 2002). We extended the investigation in larvae and showed in general, manipulation of adenylyl cyclase activity and cAMP levels with the dbcAMP, SQ 22,536 and ddadenosine did not have a noticeable impact on the HR. There was a positive effect of forskolin on HR, however the effect was only slight. Also, dbcAMP slightly decreased the HR. This may be due to activation of targets by cAMP that could bring about ion channel activation, such as K $^{+}$  channels. This might result in depolarization of the pacemaker cell membrane potential. Further, adding dbcAMP is not the same as stimulating AC as there are various isoforms of AC that might be differentially activated by forskolin whereas addition of dbcAMP maintains a high level of cAMP, by-passing activation of AC. In rodents adenosine analogs can retard the positive chronotropic effect of norepinephrine (Samet and Rutledge, 1985).

Our findings indicate that cAMP signaling does not contribute to regulation of HR in *Drosophila* larva as it does in mammals, and that cAMP is not a component of the positive chronotropic effects of 5-HT in the fly heart. Supporting this conclusion is that 5-HT is still able to produce a large increase in HR in the presence of AC inhibitors. As an additional tool to probe mechanisms underlying HR we employed the designer receptor hM4Di, which is positively coupled to G $\alpha$ i signaling in both mammals and the fly, as well as the G-coupled inwardly rectifying potassium channel (GIRK) to produce silencing. For example, CNO activation of hM4Di in mammalian systems results in the

hyperpolarization and electrical silencing of hippocampal neurons in culture (Armburster et al., 2007). We previously observed that CNO significantly slowed or stopped the heart when applied to *Drosophila* hearts expressing hM4Di receptors (Becnel et al., 2013), and observe the same effect here. Significantly, pharmacological manipulation of AC or cAMP levels in the presence of activated hM4Di had no effect on the negative chronotropic effects of activated hM4Di. Forskolin was unable to override the repressing effects of CNO. Together, the responses likely indicate that the main effects of hM4Di activation on HR are probably mediated through G $\beta\gamma$  activation of GIRK channels with subsequent hyperpolarization and silencing of cardiomyocytes rather than through negative modulation of cAMP levels through G $\alpha_i$  signaling.

When we applied 5-HT to the larvae preparations, we observed that 5-HT markedly increased the HR, in agreement with our earlier studies. Our original hypothesis was that 5-HT activates AC, which in turn lead to the activation of PKA and phosphorylation of Ca<sup>2+</sup> channels that lead to inward Ca<sup>2+</sup> currents and contraction. If this were correct, then the positive chronotropic effects of 5-HT action could be blocked by decreasing AC activity or levels of cAMP. Since our multiple attempts at manipulation of AC and cAMP levels had no or little effect on HR indicate that HR in the fly is in fact not substantially mediated by cAMP. The inhibitory effect of activation of hM4Di we observed is therefore likely mediated through G $\beta\gamma$  activation of GIRK channels and silencing of cardiomyocytes, rather than inhibition of AC through G $\alpha_i$  to decrease cAMP levels and PKA activity.

Towards understanding the mechanism of action of serotonin on the heart, investigating if there may be different 5-HT receptors on heart muscle fibers acting through multiple signaling mechanisms needs to be determined. The known fly 5-HT receptors are all G-protein coupled receptors: 5-HT1ADro and 1BDro inhibit adenylyl cyclase through activation of G $\alpha_i$ ; 5-HT7Dro activates adenylyl cyclase through G $\alpha_s$  activation; 5-HT2Dro signaling pathway has not been identified yet (Blenau and Baumann, 2001; Obosi et al., 1996; Saudou et al., 1992). A remaining possibility for

the positive chronotropic effect of 5-HT could be that 5-HT<sub>2Dro</sub> receptors are mediating HR. 5-HT<sub>2</sub> receptors couple with G<sub>αq</sub> and activation of phospholipase C (PLC). PLC cleaves PIP<sub>2</sub> to IP<sub>3</sub> and diacylglycerol (DAG). IP<sub>3</sub> leads to increases in intracellular Ca<sup>2+</sup> (Fukami et al., 2010), which could lead to contraction. However, we previously demonstrated that mutation or misexpression of the 5-HT<sub>2Dro</sub> receptor does not have a dramatic effect on *Drosophila* larval HR (Dasari et al., 2009). A second 5-HT<sub>2Dro</sub> receptor has recently been reported (Gasque et al., 2013), and it may be this receptor mediating the effects if G<sub>αq</sub> pathways are involved. Recently, a pharmacological study has shown that activation of 5-HT<sub>2</sub> receptor by 5-HT<sub>2</sub> agonist increases HR and ketanserin, which is 5-HT<sub>2</sub> antagonist, markedly decreases the action of 5-HT on HR in *Drosophila* larvae (Majeed et al., 2014). Therefore, we speculate that a PLC pathway might mediate the positive chronotropic effect of 5-HT. However, to confirm this suspicion, various pharmacological agents should be used to manipulate the PLC pathway to observe how it effects on 5-HT action related to HR. Another possibility is that 5-HT<sub>1A</sub>, 1B, and 7Dro receptors are mediating HR through coupling to other effector pathways than adenylyl cyclase. GPCRs couple to a wide range of effectors, and certain drugs and natural ligands can activate these pathways differentially through functional selectivity (Urban et al., 2007). In this scenario the 5-HT<sub>1ADro</sub> or 5-HT<sub>7Dro</sub> receptors could mediate HR through non-canonical pathways yet to be determined. In summary, we present evidence here that the positive chronotropic effect of 5-HT in *Drosophila* larva HR is not governed by the adenylyl cyclase signaling pathway, and that the negative chronotropic effects of hM4Di activation are likely due to electrical silencing rather than negative modulation of AC.

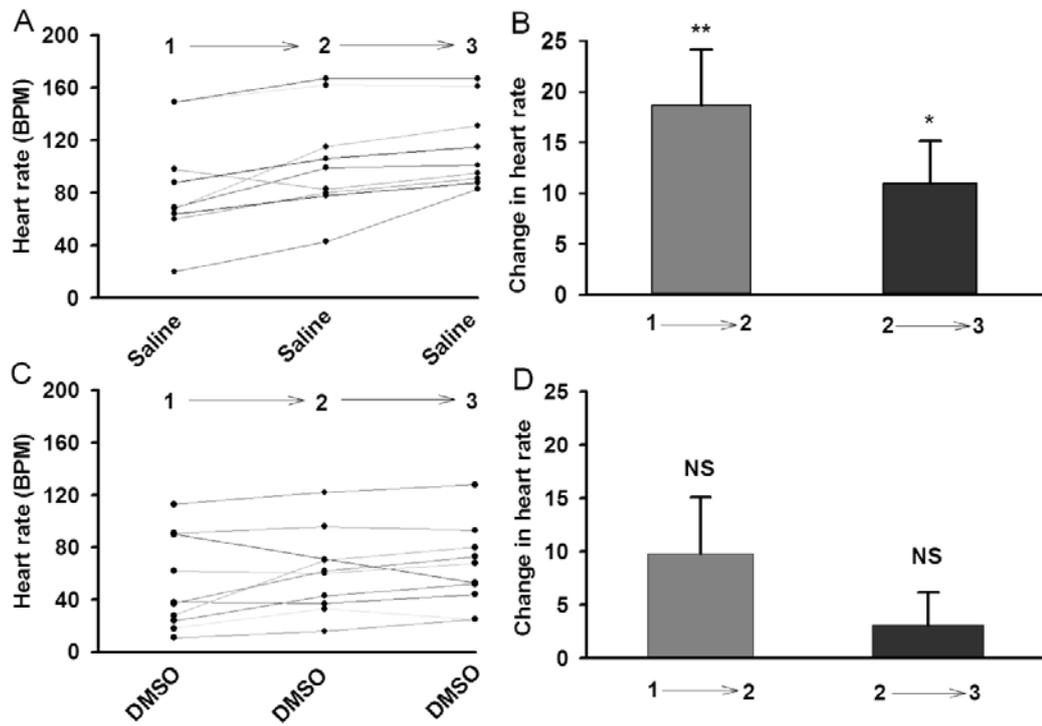


Figure 2.1: (A) The effect of changing saline with saline on larval heart rate in UAS-M4D X 24B-GFP flies, n=9. (B) Shows the difference in heart rate before and after changing solution (C) and (D) The effect of changing saline containing DMSO with saline containing DMSO on larval heart rate in UAS-M4D X 24B-GFP flies, n=10.  $P \leq 0.05$  (\*),  $P \leq 0.02$  (\*\*).

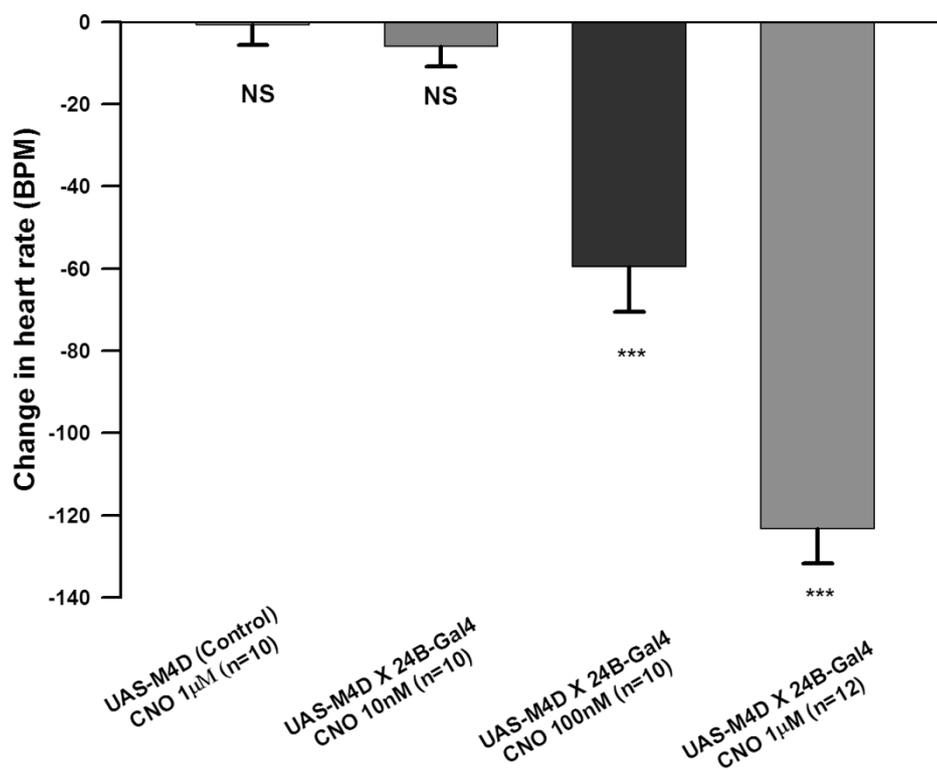


Figure 2.2: Concentration response curve for CNO at hM4Di receptors on the larval heart rate. The absolute change in rate (beats per minute) is shown  $P \leq 0.001$  (\*\*\*).

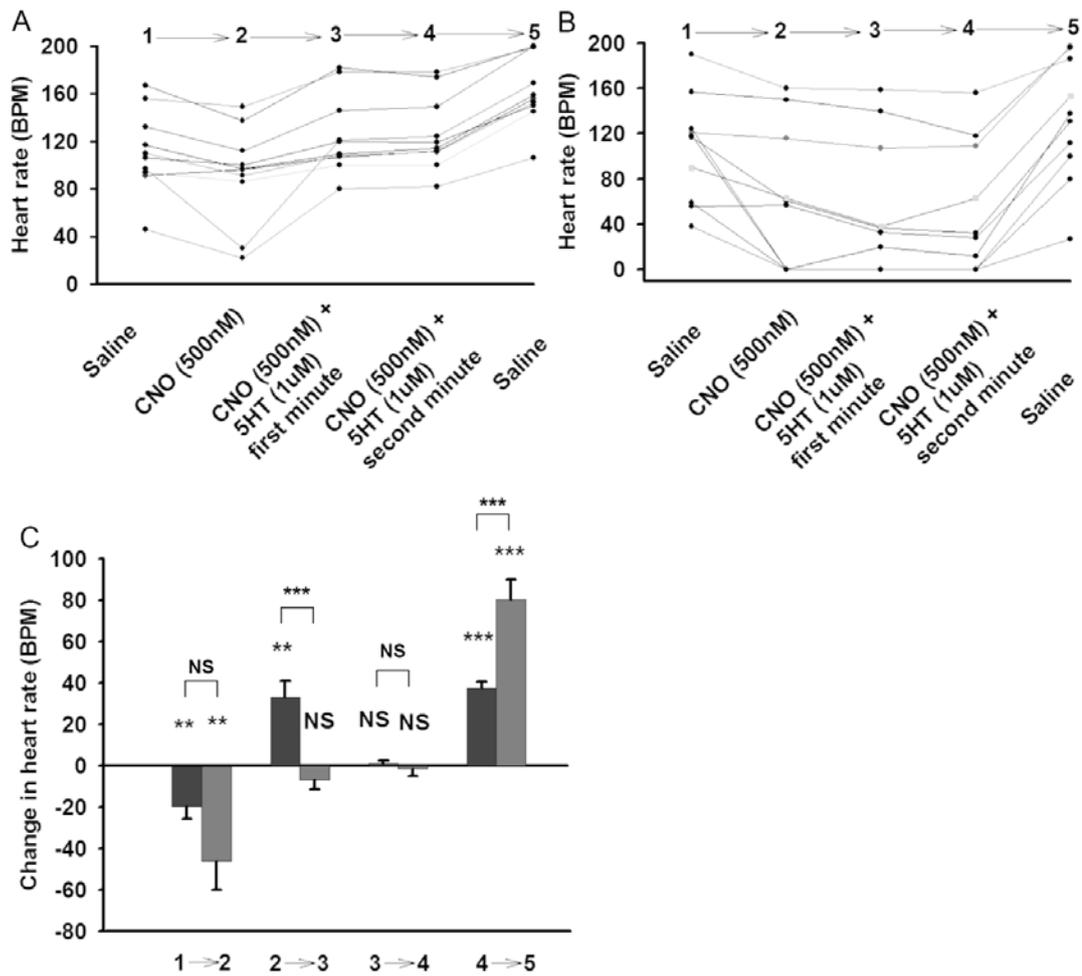


Figure 2.3: Effect of activation of hM4Di receptors on the action of 5-HT in larval heart. (A) Background UAS-M4D, n=10 (B) UAS-M4D X 24B-GFP, n=10 (C) Comparison between background (dark grey) and M4D expressing group (light grey). The differences between genotypes are represented by the NS (not significant) or asterisk(s) above the lines. The differences, which are due to the changing one solution with another one in the same genotypes, are represented by NS or asterisk(s) above the bars.  $P \leq 0.05$  (\*),  $P \leq 0.02$  (\*\*),  $P \leq 0.001$  (\*\*\*)

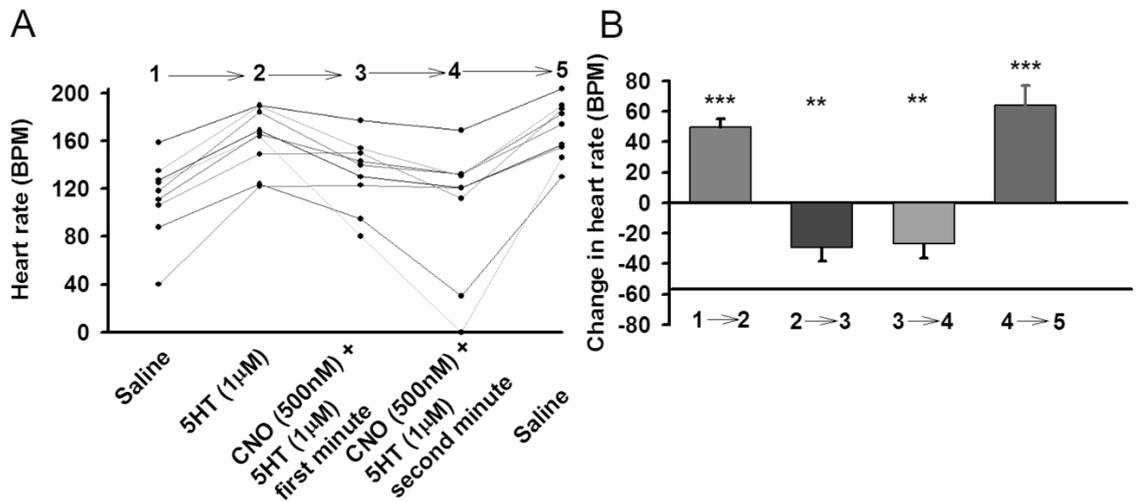


Figure 2.4: (A) Effect of 5-HT application plus hM4Di activation on larval heart rate (UAS-M4D X 24B-GFP).  $n=9$ . (B) The difference in heart rate before and after change of solution.  $P \leq 0.02$  (\*\*),  $P \leq 0.001$  (\*\*\*)

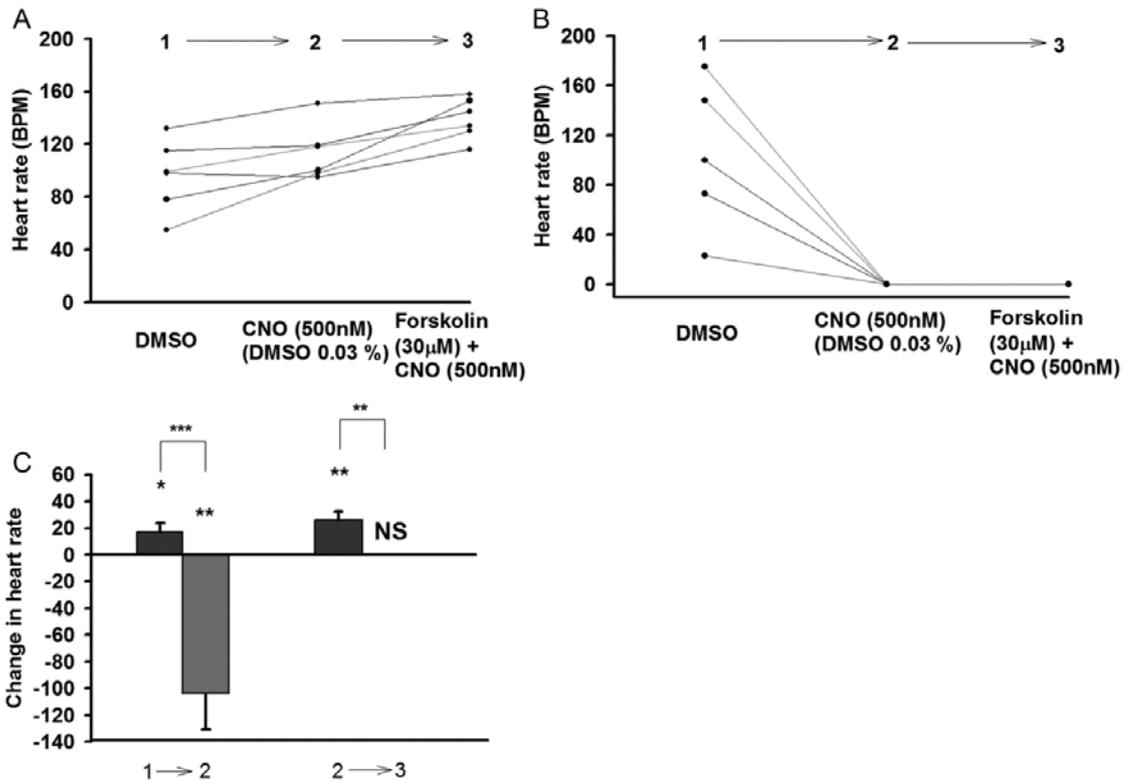


Figure 2.5: Effect of hM4Di activation plus forskolin application on larval heart rate. (A) Background strain UAS-M4D and heart rate with solution changing n=6 (B) Change of heart rate in UAS-M4D X 24B-GFP, n=5 with the same solutions as in A. (C) Comparison between background (dark grey) and M4D expressing group on the effect of CNO and forskolin (light grey). The differences between genotypes are represented by NS (not significant) or asterisk(s) above the lines. The differences, which are due to the changing one solution with another one in the same genotypes, are represented by NS or asterisk(s) above the bars.  $P \leq 0.05$  (\*),  $P \leq 0.02$  (\*\*),  $P \leq 0.001$  (\*\*\*)

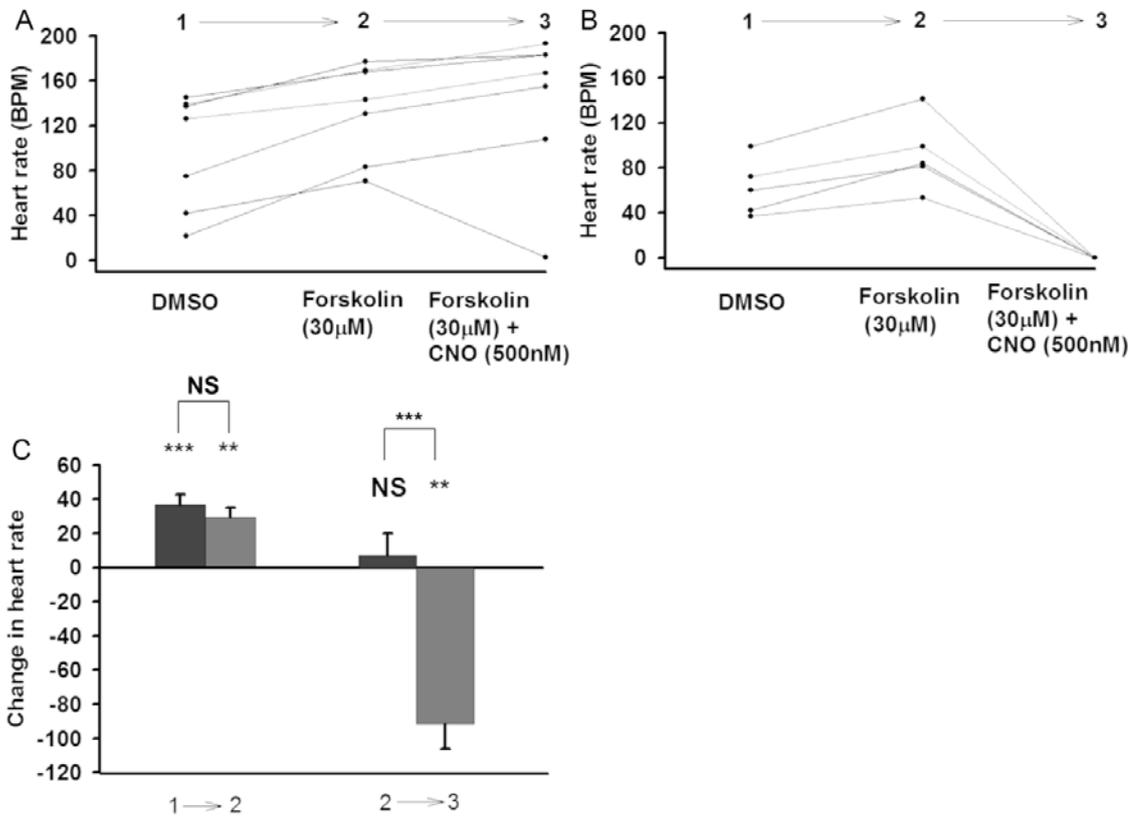


Figure 2.6: Effect of forskolin plus hM4Di activation on larval heart rate. (A) Background, UAS-M4D, n=7 (B) UAS-M4D X 24B-GFP, n=5 (C) Comparison between background (dark grey) and M4D expressing group (light grey). The differences between genotypes are represented by the NS (not significant) or asterisk(s) above the lines. The differences, which are due to the changing one solution with another one in the same genotypes, are represented by NS or asterisk(s) above the bars.  $P \leq 0.02$  (\*\*),  $P \leq 0.001$  (\*\*\*).

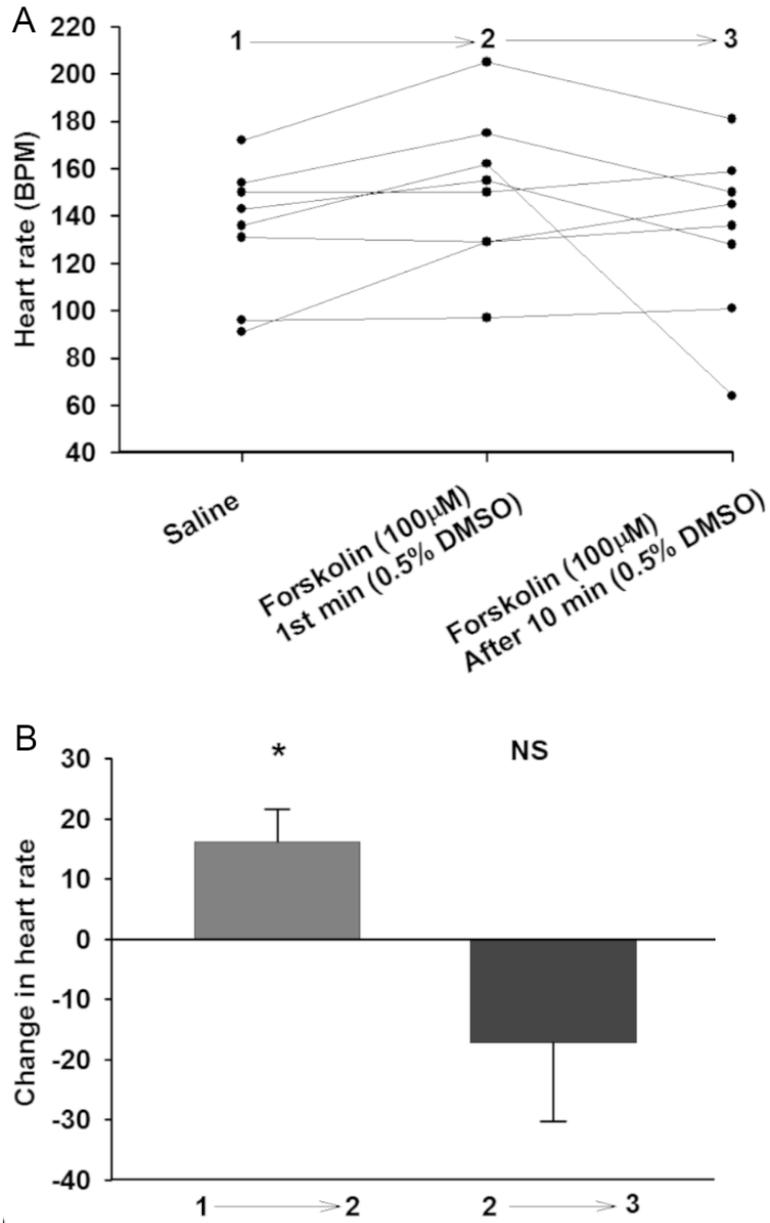


Figure 2.7: Effect of 100  $\mu$ M forskolin on larval heart rate. (A) Background, UAS-M4D,  $n=8$ , heart rate of individual preparations. (B) Background, UAS-M4D,  $n=8$ , shows the change in heart rate after application of forskolin.  $P \leq 0.02$  (\*\*),  $P \leq 0.001$  (\*\*\*)

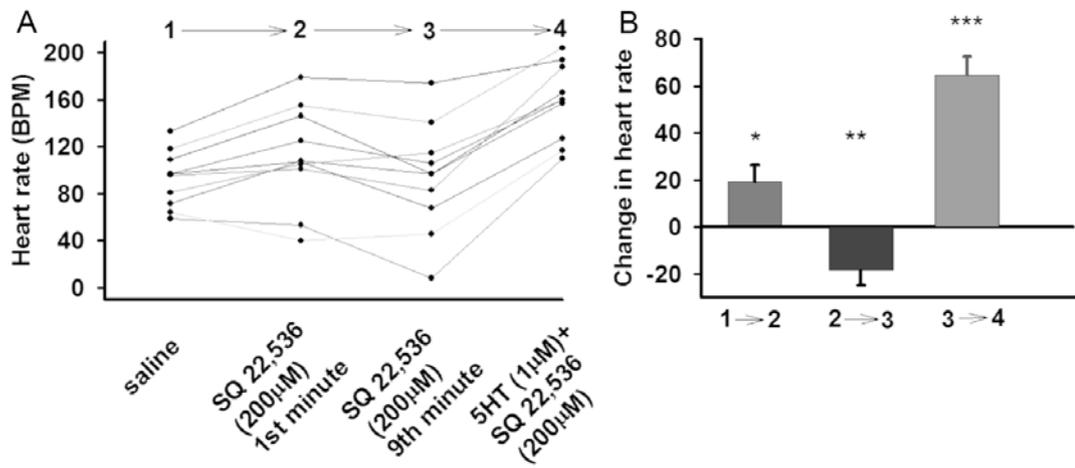


Figure 2.8: (A) and (B) Effect of SQ 22,536 and SQ + 5-HT combination on larval heart rate. (UAS-M4D X 24B-GFP). (n=10).  $P \leq 0.05$  (\*),  $P \leq 0.02$  (\*\*),  $P \leq 0.001$  (\*\*\*)

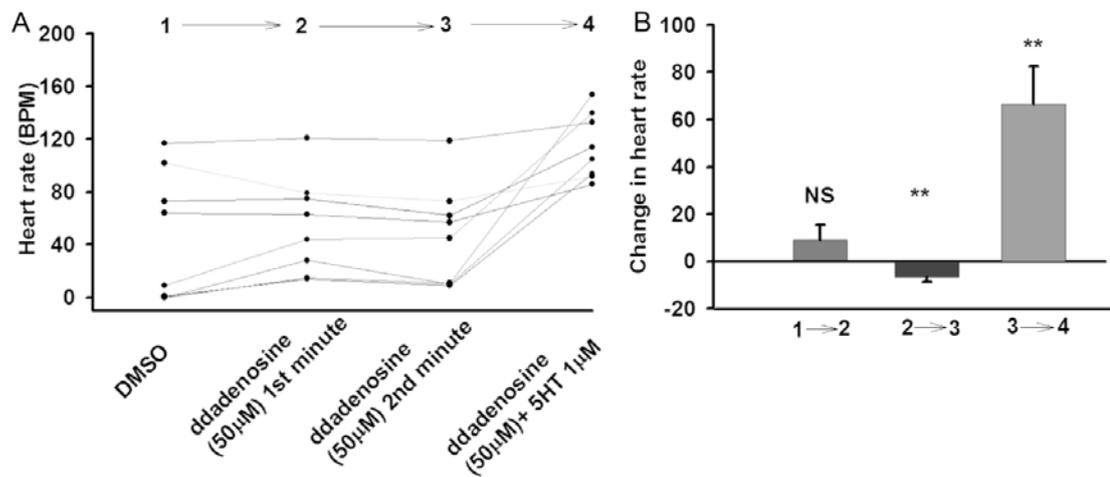


Figure 2.9: Effect of ddadenosine and ddadenosine + 5-HT combination on larval heart rate. (UAS-M4D X 24B-GFP). (n=8)  $P \leq 0.02$  (\*\*),  $P \leq 0.001$  (\*\*\*)

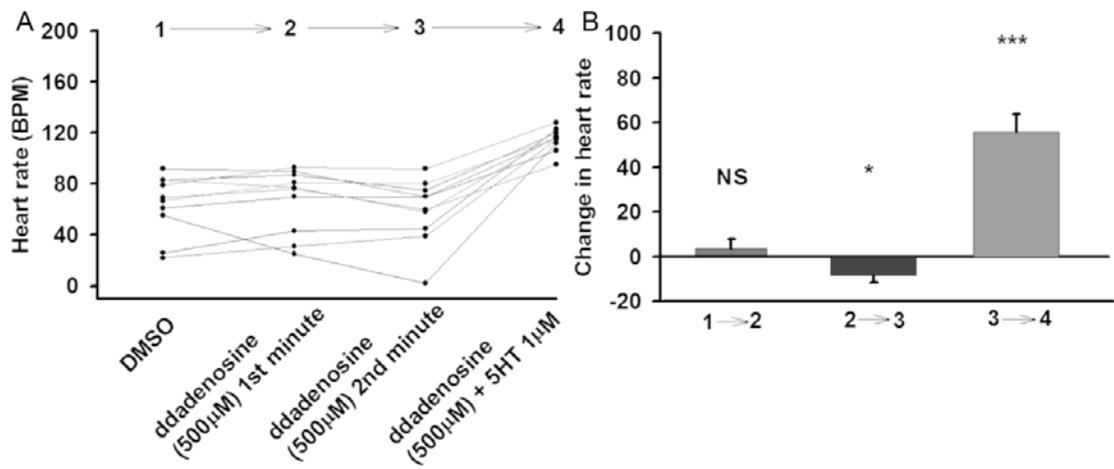


Figure 2.10: Effect of a high concentration of ddadenosine and ddadenosine+ 5-HT combination on larval heart rate. (UAS-M4D X 24B-GFP). (n=10),  $P \leq 0.05$  (\*),  $P \leq 0.001$  (\*\*\*).

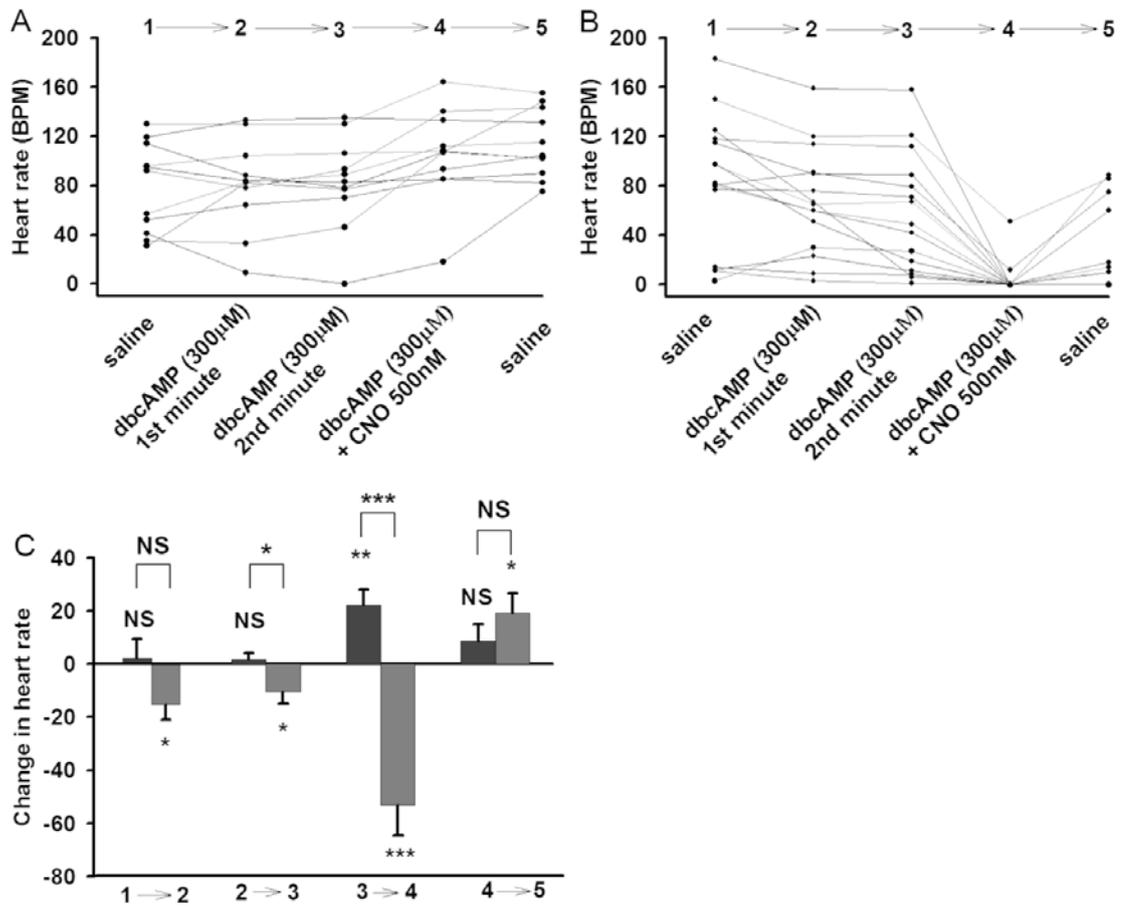


Figure 2.11: Effect of dbcAMP and dbcAMP+CNO combination on larval heart rate. (A) Background UAS-M4D, n=11 (B) UAS-M4D X 24B-GFP, n=15 (C) Comparison between background (dark grey) and M4D expressing group (light grey). The differences between genotypes are represented by the NS (not significant) or asterisk(s) above the line. The differences, which are due to the changing one solution with another one in the same genotypes, are represented by NS or asterisk(s) above the bars.  $P \leq 0.05$  (\*),  $P \leq 0.02$  (\*\*),  $P \leq 0.001$  (\*\*\*)

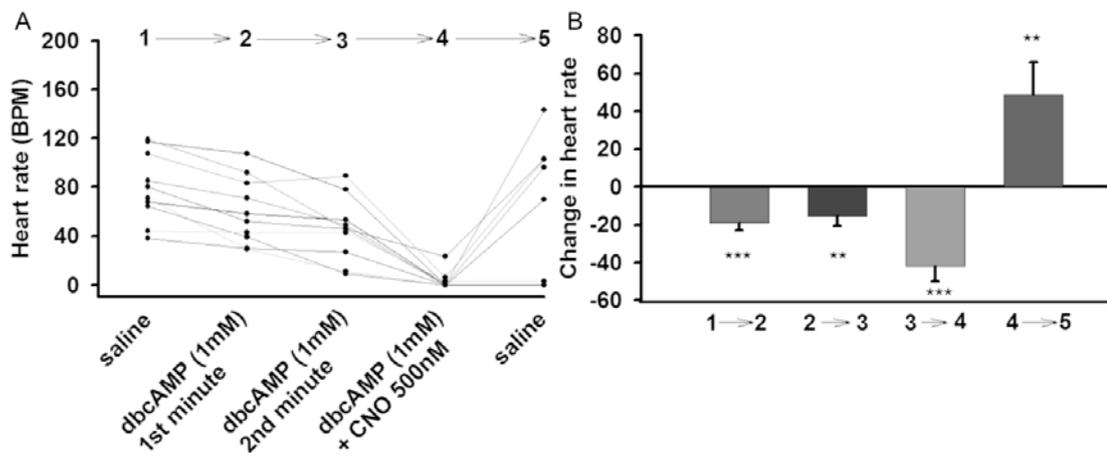


Figure 2.12: Effect of dbcAMP (1mM) and dbcAMP + CNO combination on larval heart rate. UAS-M4D X 24B-GFP (n=10).  $P \leq 0.05$  (\*),  $P \leq 0.02$  (\*\*),  $P \leq 0.001$  (\*\*\*)

## CHAPTER THREE

### Pharmacological and genetic identification of serotonin receptor subtypes on *Drosophila* larval heart and aorta

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**Author contributions:** Zana R. Majeed and Audra Stacy collected data; Zana R. Majeed analyzed data; Zana R. Majeed and Robin L. Cooper interpreted results of experiments; Zana R. Majeed and Audra Stacy prepared figures; Zana R. Majeed and Robin L. Cooper wrote, edited and revised the manuscript.

#### Abstract

Serotonin, 5-HT, plays various roles in the fruit fly, *Drosophila melanogaster*. Previous studies have shown that 5-HT modulates the heart rate in third instar larvae. However, the receptor subtypes that mediate 5-HT action in larval cardiac tissue had yet to be determined. In this study, various 5-HT agonists and antagonists were employed to determine which 5-HT receptor subtypes are responsible for the positive chronotropic effect by 5-HT. The pharmacological results demonstrate that a 5-HT2B agonist significantly increases the heart rate; however, 5-HT1A, 5-HT1B, and 5-HT7 agonists do not have a significant effect on the heart rate. Furthermore, 5-HT2 antagonist, ketanserin, markedly reduces the positive chronotropic effect of 5-HT in a dose-response manner. Furthermore, we employed genetic approaches to confirm the pharmacological results. For this propose, we used RNA interference (RNAi) line to knock down 5-HT2ADro and also used 5-HT2ADro and 5-HT2BDro insertional mutation lines. The results show that 5-HT2ADro or 5-HT2BDro receptor mutations reduce the response of the heart to 5-HT. Given these results, we conclude these 5-HT2 receptor subtypes are involved in the action of 5-HT on the heart rate in the larval stage.

**Keywords:** 5-HT, 5-HT2ADro and 5-HT2BDro receptors mutations, 5-HT agonists and antagonists, heart rate.

## Introduction

Several recent studies have demonstrated that serotonin (5-hydroxytryptamine, 5-HT) plays many versatile roles in insects, for instance, in blowfly salivary gland secretion (Röser et al., 2012) and locust swarming behavior (Anstey et al., 2009). Furthermore, many studies have shown that 5-HT is involved in many physiological and behavioral aspects in the fruit fly, *Drosophila melanogaster*, such as, modulation of the heart rate (Dasari and Cooper 2006) and neural circuit (Dasari and Cooper 2004), as well as behaviors associated with sleep regulation (Yuan et al. 2006), circadian patterns (Nichols 2007), courtship behavior (Becnel et al. 2011), learning and memory (Johnson et al. 2011) and feeding behavior (Gasque et al. 2013). Various 5-HT receptor subtypes have been cloned and characterized in *Drosophila* (Tierney, 2001): 5-HT1Adro, 5-HT1Bdro which are negatively coupled to adenylyl cyclase (AC) (Saudou et al. 1992); 5-HT2Adro (Colas et al. 1995), the intracellular signaling pathway has not yet been investigated for 5-HT2 receptor in *Drosophila*; 5-HT2B receptor was recently identified in *Drosophila* (Gasque et al. 2013); 5-HT7 is positively coupled to AC (Witz et al. 1990). It has been already shown that application of exogenous 5-HT to semi-intact larvae increases the heart rate in *Drosophila* larvae (Dasari and Cooper 2006); however, the subtype of 5-HT receptor that mediates 5-HT action in larval heart has not yet been studied pharmacologically. Interestingly, 5-HT modulates the heart rate in other invertebrates, such as, hard clam *Mercenaria mercenaria*, garden snail *Helix aspersa*, *Aplysia* (Villalón and Centurión 2007), crayfish (Listerman et al. 2000), tobacco hornworm *Manduca sexta* (Platt and Reynolds 1986), and cockroach *Periplaneta americana* (Collins and Miller 1977).

*Drosophila* has an open cardiovascular system which consists of a simple dorsal vessel, anterior aorta and posterior heart. *Drosophila* larval heart is myogenic and hemolymph borne modulators can have direct effect on cardiac tissue. The larval *Drosophila* heart has been used to investigate the electrophysiological properties of cardiomyocytes in normal and mutant larvae by recording cardiomyocyte action

potentials (Lalevée et al. 2006; Desai-Shah et al. 2010) and it is proven to be a good model to study the role of ions in generation of heart beat (Johnson et al. 1998; Desai-Shah et al. 2010). Recently, the detailed structure of *Drosophila* heart has been studied (which opens up avenues to further investigate the physiology of *Drosophila* heart). *Drosophila* heart has also been used to investigate the role of genes which are important for mammalian cardiac physiology (Johnson et al. 2001) and development since they share similar crucial transcription factors during development (Olson 2006). The morphology of heart in *Drosophila* and vertebrates is different; even though, the molecular mechanisms that underlie heart development in *Drosophila* and vertebrates share a large degree of similarity (Bodmer et al. 1998). Moreover, *Drosophila* and vertebrate hearts are functionally indexed by similar physiological measurements, such as, cardiac output, rate and time in systole or diastole (Choma et al. 2011). Various studies have demonstrated that 5-HT receptors are important for cardiac physiology and development in mammals. The 5-HT<sub>1A</sub> receptor was shown, in 5-HT<sub>1A</sub> knockout mice, to be crucial to resist the stress-induced cardiac problems (Carnevali et al. 2012). Also, it has been revealed that loss of function of 5-HT<sub>2B</sub> receptor is lethal due to cardiac abnormalities during development (Nebigil et al. 2000). Moreover, increasing 5-HT<sub>2A</sub> receptor activity has shown to be related to cardiac hypertrophy in mice (Lairez et al. 2013).

In this study, we used pharmacological approaches to characterize the 5-HT receptor subtypes that mediate the positive chronotropic effect of 5-HT on the larval heart. Many signaling pathways might be involved in the modulation of the heart rate in *Drosophila*; thus various 5-HT receptor subtypes could account for the action. We have shown that activation of 5-HT<sub>2</sub> receptor is the primary subtype to account for the increase in heart rate. To corroborate the pharmacological results, we used genetic approaches. We have demonstrated that alterations in 5-HT<sub>2</sub> receptor subtypes reduce the responsiveness of the heart to 5-HT. These results indicate that 5-HT<sub>2</sub>

receptors are implicated in the action of 5-HT; however, we cannot rule out the involvement of the other 5-HT receptor subtypes having some role.

## **Materials and Methods**

### **Fly rearing and culturing**

In this study, canton-S (CS), *w*<sup>1118</sup>, PBac-5-HT2A, Mi-5-HT2B, UAS-RNAi-5-HT2A, and 5-HT2A-Gal4 lines were used. The PBac-5-HT2A (stock #19367) and Mi-5-HT2B (stock #40810), which are 5-HT2ADro or 5-HT2BDro insertional mutant lines, respectively, 5-HT2A-Gal4 (stock #49574), and UAS-RNAi-5-HT2A (stock #31882) were obtained from the Bloomington Drosophila Stock center. The flies were reared at room temperature (22-23°C), unless otherwise stated, in vials containing cornmeal-agar-dextrose-yeast medium.

### **Visual heart rate measurement**

Third instar larvae were washed with distilled water to remove the food on the larvae. The larvae were dissected in the ventral side up position. The internal organs, intestine, and fat bodies were removed to observe the heart in modified HL3 saline (NaCl 70mM, KCl 5mM, MgCl<sub>2</sub>.6H<sub>2</sub>O 20mM, NaHCO<sub>3</sub> 10mM, trehalose 5mM, sucrose 115mM, CaCl<sub>2</sub>.2H<sub>2</sub>O 1mM, and BES 50mM and pH 7.0 (pharmacology study) or Trizma acid 25mM and pH 7.1 (genetic study). We modified the type and concentration of buffer in HL3 saline since the heart rate is very sensitive to pH change and the HL3 with BES5mM does not have a stable pH (deCastro et al., 2013). The detailed protocol for the larvae dissection and heart visualization is explained in Cooper et al., (2009). The heart rate was measured by directly counting heart beats on the television screen. The heart beats were counted over one minute at various times to obtain beats per minute (BPM) at given time periods. Various agonists and antagonists were applied. The preparation was left for one minute in saline after dissection, and then the rate was obtained for the following minute (this is considered as a first minute in the

experiments). The saline was exchanged with saline containing agonist or antagonist. Preparations were left for one minute with exposure to compounds and then the rates were obtained for the following minute. The preparations were also left for ten minutes then the rates were obtained again for one minute. When an antagonist was employed, the preparation was incubated inside an antagonist for 10 minutes then the saline-containing an antagonist was exchanged with saline-containing 5-HT and antagonist altogether. Afterwards, a percentage change of heart rate was determined.

### **Chemicals**

8-Hydroxy-DPAT hydrobromide (5-HT<sub>1A</sub> agonist), CP 93129 dihydrochloride (5-HT<sub>1B</sub> agonist),  $\alpha$ -methyl-5-hydroxytryptamine maleate (5-HT<sub>2</sub> agonist), TCB-2 (5-HT<sub>2</sub> agonist), AS19 (5-HT<sub>7</sub> agonist), WAY 100635 (5-HT<sub>1A</sub> antagonist), GR 55562 (5-HT<sub>1B</sub> antagonist), ketanserin tartrate (5-HT<sub>2</sub> antagonist), SB 258719 (5-HT<sub>7</sub> antagonist) were purchased from Tocris Bioscience (Bristol, UK). 1-(3-chlorophenyl) piperazine 2HCl (5-HT<sub>2C</sub> agonist) was purchased from Research biochemical international (RBI) (MA, USA). ( $\pm$ )-DOI hydrochloride was purchased from Sigma (St. Louis, MO, USA). Serotonin (5-HT) hydrochloride was purchased from Sigma (Steinheim, Germany). All chemicals were dissolved in fly saline but AS19 was dissolved inside dimethyl sulfoxide (DMSO). 1mM stock solution was made. Then a fresh solution of specific concentrations was made from the stock solution every time before starting the experiment. The DMSO concentration in AS19 100 $\mu$ M, which is the highest concentration used, was about 0.03%.

### **Statistical analysis**

All data are expressed as mean  $\pm$  SEM. SigmaPlot (version 12.0) was employed for statistical analysis. ANOVA test was used for multiple comparison among treatments. Bonferroni t-test was used as a post hoc test to compare the percentage change of treatments heart rate with control heart rate. Student's t-test (paired) was

used in some experiments to compare the change in heart rate before and after adding compounds. Also, Student's t-test (unpaired) was used to compare the treatments with control in case when there were two groups.  $P \leq 0.05$  is considered as statistically significant.

## **Results**

### **Heart rate variation over time**

Control experiments were carried out to show the effect of the incubation time on heart rate. When saline is exchanged with fresh saline, heart rate increased by a small percentage (Figure 3.1 A1 and A2). These results suggest that the heart is sensitive to the mechanical disturbance. After a 10 minute incubation period, the heart rate will slightly decrease. The heart rate stabilizes and remains relatively constant for the duration of these experiments. We used these results as a control for the rest of the experiments unless otherwise stated. We performed control experiments over the same length of times for the various experimental paradigms. 5-HT significantly increases the heart rate and the heart rate does not noticeably change over the subsequent 10 minutes (Figure 3.1 B1 and B2).

### **Distribution of *in situ* basal heart rates**

There is a wide variation in the initial heart rates of preparations. The distribution from 50 to greater than 200BPM is quite remarkable. There are few occurrences of preparations with low initial heart rates (50-59). The majority of rates were between 60-170 (Figure 3.2 A). This variation in rate is noted even with using the same saline within one experimental setting and these experiments were repeated over several different experimental days. Each day a fresh saline was used but wide variations were still observed. Exposure to 5-HT (100nM) ( $P < 0.001$ ) increases heart rate (Figure 3.2B and C). This is consistent with a previous study (Dasari and Cooper, 2006).

### **5-HT dose-response relationship**

5-HT concentrations (1nM, 10nM, 100nM, 1 $\mu$ M, 10  $\mu$ M) in saline were used to apply directly to the exposed heart. 1nM and 10nM 5-HT did not significantly increase the heart rate in comparison to the control exposure of saline; however, 100nM, 1 $\mu$ M, and 10 $\mu$ M 5-HT all significantly increased heart rate (Figure 3.3 A,B). After exposure to 5-HT, the increased rate is relatively consistent for up to 10 minutes (Figure 3.3 A). Dose-response relation was obtained by plotting the data acquired from the percentage change in the rate before and during exposure to 5-HT. The control percentage change (exchanging saline with saline) value was subtracted from the percentage change of 5-HT action on the larval heart rate. These adjusted values are depicted by open circles (Figure 3.3 C).

A simple regression analysis was carried out to determine if there is a relationship between initial heart rate and percentage change of the heart rate. The results generally indicate that there is a correlation between initial heart rate and percentage change with low initial heart rates having a higher percentage change (Figure 3.3 D).

### **Action of 5-HT agonists**

Various 5-HT agonists (5-HT1A, 5-HT1B, 5-HT2, and 5-HT7) were investigated for their effect on altering heart rate. 5-HT2 agonist  $\alpha$ -methyl-5-HT (100 $\mu$ M) markedly increased the heart rate upon exposure. The preparations were left for 10min to observe any further effects of agonists. No further significant changes were observed after the ten minute period. This indicates that the heart rate does not decline after the initial effect within the first few minutes (Figure 3.4A and B).

The average of heart rates with different treatments is shown for the entire experimental paradigms (Figure 3.5A). Various 5-HT2 agonists were employed to observe if they could modulate the heart rate. The 5-HT2 agonist  $\alpha$ -methyl-5-HT (100 $\mu$ M) markedly increased the heart rate within the initial exposure to levels similar

to 5-HT 100nM exposure. The preparations were also left for 10min. No further significant changes were observed over the ten minute period. This indicates that the heart rate remains stable to the effect of 5-HT or is not altered by a prolonged exposure. Screening of these agonists indicated that TCB-2 (100nM) and 1(3-CP) piperazine (100 $\mu$ M) produced an increase in rate (Figure 3.5 B).

### **Action of $\alpha$ -methyl-5-HT maleate**

The trend in the rates is shown for the various concentrations of  $\alpha$ -methyl-5-HT maleate (Figure 3.6 A). The concentrations of 100nM, 1 $\mu$ M, 10 $\mu$ M, 100 $\mu$ M and 1000 $\mu$ M were used. The preparations were left inside saline for one minute and then counted for the next minute (solution 1). Afterwards the saline was exchanged with saline containing one of the  $\alpha$ -methyl-5-HT concentrations indicated. The preparation was left for an additional minute during the exposure and the rate counted the following minute (solution 2). The percentage change (first block of columns) was calculated from saline to saline containing agonist. The preparation was left exposed to agonist for 10min and the heart rate counted in the following minute. The percentage change was calculated from saline containing agonist (first minute) to saline containing agonist (after 10min).  $\alpha$ -methyl-5-HT maleate 100 $\mu$ M and 1000 $\mu$ M significantly increased the heart rate. The preparations were left inside agonists for 10min. After that the heart rate did not decrease (Figure 3.6 B). The  $\alpha$ -methyl-5-HT-maleate at 100 $\mu$ M or 1000 $\mu$ M significantly increased the heart rate. However, 100nM  $\alpha$ -methyl-5-HT-maleate did not have a significant effect on the heart rate. The 1 $\mu$ M and 10 $\mu$ M  $\alpha$ -methyl-5-HT-maleate exposure slightly increased the heart rate. The changes measured for exchanging saline with saline as a control are subtracted from the responses and the results are shown as open circles (Figure 3.6 C).

### **Action of 5-HT antagonists**

Several 5-HT antagonists (5-HT1A, 5-HT1B, 5-HT2, and 5-HT7) were employed to determine if they blocked the action of 5-HT. The 5-HT7 antagonist (100 $\mu$ M, SB 258719) significantly decreased heart rate even in the absence of 5-HT (gray bar in first group of histograms, Figure 3.7A, B). Whereas the 5-HT2 antagonist (100 $\mu$ M, ketanserin) completely blocked the action of 5-HT on heart rate, but had no effect on its own in the absence of 5-HT within the first minute. However, 5-HT7 antagonist did not block the action of 5-HT on heart rate (third group of histograms, light blue and gray bars, Figure 3.7). 5-HT1A antagonist (100 $\mu$ M, WAY 100635) and 5-HT1B agonist (100 $\mu$ M, GR 55562) reduced the action of 5-HT on larval heart rate (Figure 3.7B).

### **Action of ketanserin**

Ketanserin 100nM, 1 $\mu$ M, 10 $\mu$ M, and 100 $\mu$ M were used to address the antagonist action to 5-HT. The trend to heart rates over the duration of the experimental paradigm was examined (Figure 3.8A). Ketanserin completely blocked the action of 5-HT (100nM) in a dose dependent manner (Figure 3.8B). When the preparations were incubated in ketanserin (100 $\mu$ M) alone for 10min, the heart rate significantly decreased ( $P=0.029$ ) (Figure 3.8B). However, exposure to 5-HT with low levels of ketanserin resulted in an increase in heart rate but the higher concentrations (1 $\mu$ M, 10 $\mu$ M, and 100 $\mu$ M) blocked the 5-HT induced increase. Ketanserin (5-HT2) antagonist decreased the heart rate in a dose-response manner and as noted 100 $\mu$ M completely blocked the action of 5-HT (Figure 3.8C).

### **Effect of 5-HT and 5-HT2 agonist and antagonist on aorta rate**

The effect of 5-HT, 5-HT2 agonist ( $\alpha$ -mthyl-5-HT) and 5-HT antagonist (ketanserin) was examined on heart and aorta rates concurrently. The results demonstrate that aorta responds to 5-HT, 5-HT2 agonist as well as 5-HT2 antagonist.

5-HT significantly increases the aorta rate (Figure 3.9A1 and A2). 5-HT<sub>2</sub> agonist ( $\alpha$ -methyl 5-HT) markedly increases the aorta rate (Figure 3.9B1 and B2). Moreover, 5-HT<sub>2</sub> antagonist (ketanserin) blocks the action of positive chronotropic effect of 5-HT on heart and aorta rates (Figure 3.9C1 and C2).

### **5-HT action in 5-HT<sub>2</sub> receptor mutant larvae**

A dose-response relationship for 5-HT in *w*<sup>1118</sup> (control) larvae was performed. In the *w*<sup>1118</sup> line 5-HT increases the heart rate in a dose-response manner (Figure 3.10 A1, A2 and A3). 5-HT<sub>2A</sub> receptor insertional mutant larvae were used to observe how mutation in 5-HT<sub>2A</sub> affects on 5-HT action on heart rate. 5-HT<sub>2</sub> (100nM) does not significantly increase the heart rate (Figure 3.10 B1, B2 and B3); however, 5-HT (1 $\mu$ M and 10 $\mu$ M) increased the heart rate. The 5-HT (10 $\mu$ M) action was lower than the 5-HT (1 $\mu$ M) action.

The RNA interference approach was used to knock down 5-HT<sub>2ADro</sub> receptor expression. The results demonstrate that 5-HT<sub>2A</sub> reduces the action of 5-HT; however, it is not statistically significant (Figure 3.11A1 and A2). The flies were raised at 30°C to increase the efficiency of RNAi-mediated knockdown since Gal4 activity is temperature dependent (Duffy, 2002). A 5-HT<sub>2BDro</sub> mutant line was used to probe the role of 5-HT<sub>2BDro</sub> in the action of 5-HT. The results show that the 5-HT<sub>2BDro</sub> mutant flies were less responsive to 5-HT in comparison to *w*<sup>1118</sup> flies (Figure 3.11B1 and B2).

### **Discussion**

The *Drosophila* heart is used as a model to obtain insights into the underlying molecular mechanisms of heart diseases in other organisms (Piazza and Wessells 2011). One of the advantages of *Drosophila* is its genetic amenability. Genetic screens in *Drosophila* can be easily carried out to find genes that might be culprits and implicated in cardiac dysfunctions (Bier and Bodmer 2004; Wolf et al. 2006). The *Drosophila* larval heart is modulated by neurotransmitters and neuromodulators (5-HT,

dopamine, octopamine) (Johnson et al. 1997; Dasari and Cooper 2006; Titlow et al. 2013). Serotonergic neurons in larval CNS synthesize 5-HT and they release 5-HT into the hemolymph when the larvae are under the stress. This results in the heart rate acceleration. In this study, the 5-HT receptor that mediates the positive chronotropic action of 5-HT was investigated by using a pharmacological approach. In this study, various 5-HT agonists and antagonists were used to investigate the 5-HT receptor subtypes in *Drosophila* larval heart. This study broadens our understanding regarding the action of 5-HT in the heart. 5-HT also modulates heart rate in mammals; however, the effect of 5-HT in mammalian heart is biphasic, which causes tachycardia and bradycardia, due to the presence of different 5-HT receptors in cardiovascular system (Villalón and Centurión 2007).

### **5-HT dose-response relationship**

5-HT is a monoamine neurotransmitter as well as neuromodulator (Coleman and Neckameyer 2005) and is synthesized from tryptophan. There are two enzymes that catalyze the biosynthesis of 5-HT, DTRH, a rate limiting enzyme of 5-HT synthesis in serotonergic neurons, and DTPHu which mediates peripheral synthesis (Neckameyer et al. 2007). As we established, the *Drosophila* larval heart is very sensitive to 5-HT with even a 100nM significantly accelerating heart rate. We also investigated varied concentrations to determine if a similar biphasic response might be present as that in mammals. It was observed that high 5-HT concentration (10 $\mu$ M) remarkably elevates the heart rate and the heart rate remains high even after 10min of exposure. 5-HT increases the heart rate in a dose-response manner and even 10 $\mu$ M might not have resulted in a plateau in the increase in heart rate. Interestingly, 5-HT also modulates the heart rate in other invertebrates (Collins and Miller 1977; Platt and Reynolds 1986). Moreover, mammalian heart is modulated by 5-HT. This modulation can even occur in a fetal heart from a disturbance in physiological 5-HT levels in the

mother. Such fetal alterations will alter the physiology of the heart in offspring (Fligny et al. 2008).

### **5-HT<sub>2</sub> agonists actions on heart rate**

In examining the 5-HT agonists we noted  $\alpha$ -methyl 5-HT maleate significantly increased the heart rate at 100 $\mu$ M and 1000 $\mu$ M (Figures 3.4, 3.5, and 3.6). TCB-2 and 1(3-CP) piperazine 2HCl had effect on heart rate; however, the action of TCB-2 and 1(3-CP) piperazine 2HCl were not statistically significant (Figure 3.5). The affinity of  $\alpha$ -methyl 5-HT maleate might be weaker than 5-HT to the 5-HT<sub>2</sub> receptor since 5-HT noticeably increases the heart rate at 100nM and  $\alpha$ -methyl 5-HT maleate markedly increases the heart rate at 100 $\mu$ M. A study has shown that  $\alpha$ -methyl-5-HT has some potency for 5-HT<sub>2A</sub>Dro (pK<sub>i</sub>=6.8) but 5-HT is more potent for 5-HT<sub>2A</sub>Dro (pK<sub>i</sub>=7.8) (Colas et al., 1995). Using heterologous expression system, it has been shown that K<sub>i</sub> of 5-HT in a COS-1 cell line for 5-HT<sub>2D</sub>ro is 200 $\pm$ 16nM, notwithstanding the K<sub>i</sub> for  $\alpha$ -methyl 5-HT is 420 $\pm$ 23nM (Schaerlinger et al. 2007). So the affinity of 5-HT to 5-HT<sub>2D</sub>ro is higher than  $\alpha$ -methyl 5-HT in these conditions. Recently, a study has revealed in *Manduca sexta* that 5-HT can activate 5-HT receptor at 100nM; however, 5-HT<sub>2</sub> agonist (DOI) weakly activates 5-HT<sub>2</sub> receptor at 10 $\mu$ M and produces a drastic activation of 5-HT<sub>2</sub> receptor at 100 $\mu$ M (Dacks et al., 2013). These data show that the invertebrate 5-HT receptor responses to vertebrate 5-HT agonist and antagonists are different. It has been demonstrated that (R)-DOI is effective in *Drosophila* (Johnson et al. 2009); even though, surprisingly, ( $\pm$ )-DOI did not increase larval heart rate (Figure 3.4). We observed that TCB-2 also increases the heart rate at low concentration but it does not increase the heart rate at higher concentration. TCB-2 might desensitize 5-HT receptors at high concentration or it might not have a high affinity for the *Drosophila* 5-HT receptors.

An insertional mutation of *Drosophila* DTRHn significantly decreases the heart rate in white prepupal stage (WPP) in *Drosophila* also indicating the 5-HT receptors

play a role in modulating heart rate (Neckameyer et al. 2007). Also the *Drosophila* pupa shows and increase in heart rate when  $\alpha$ -methyl 5-HT was injected into P1 pupal stage (Johnson et al. 2002). The  $\alpha$ -methyl 5-HT maleate agonist appears to function like 5-HT across species so this agonist may likely be viable to use in comparative studies of 5-HT receptor subtypes. Apparently,  $\alpha$ -methyl 5-HT injection into crustacean (crayfish) raises the hemolymph glucose level just as an injection of 5-HT (Lee et al. 2000) and application of  $\alpha$ -methyl 5-HT enhances synaptic transmission at crayfish neuromuscular junction (Tabor and Cooper 2002). The main 5-HT receptor subtype at the neuromuscular junction is also likely a 5-HT<sub>2</sub> form. Even in the crayfish preparations  $\alpha$ -methyl 5-HT maleate does not fully reproduce the effect of 5-HT at the same concentration likely due to the presence of other 5-HT receptor subtypes or differences in binding affinity to the 5-HT<sub>2</sub> receptor subtype. In mammals, the 5-HT<sub>2B</sub> signaling pathway is indispensable for heart development and normal physiology of adult heart (Nebigil and Maroteaux 2001). So it is likely that 5-HT<sub>2B</sub> has an evolutionarily conserved function in physiological regulation of heart function.

### **5-HT antagonists actions on heart rate**

Screening the various 5-HT receptor antagonists revealed that ketanserin (a 5-HT<sub>2</sub> antagonist) and GR 55562 (5-HT<sub>1B</sub> antagonist) substantially block the 5-HT action on larval heart rate at 100 $\mu$ M (Figure 3.7). However, after incubation of the preparation inside a combination of antagonist and 5-HT, the heart rate in GR 55562(100 $\mu$ M)-incubated preparations significantly increased; whereas, the heart rate of ketanserin (100 $\mu$ M)-incubated preparations strikingly decreased. The result of ketanserin blocking the 5-HT action on larval heart and that the heart rate decreases when incubated in ketanserin for ten minutes suggests it might work as an inverse agonist on heart 5-HT receptors. This is feasible if the 5-HT<sub>2</sub> receptor has a constitutive activity (Nitanda et al. 2005) since ketanserin further reduces the heart rate after 10 minutes incubation. This is also consistent with the data that show ketanserin

prominently inhibited cardioacceleratory action of 5-HT when it was injected into P1 pupal stage with 5-HT in *Drosophila melanogaster* (Johnson et al. 2002). Also, it has been shown that ketanserin blocks the 5-HT action on synaptic transmission facilitation in crayfish (Tabor and Cooper 2002) which again indicates across species receptor homology in pharmacological function.

### **5-HT and 5-HT<sub>2</sub> agonist and antagonist actions on aorta rate**

The aorta was separated from the heart and afterwards the various compounds were applied to the preparations. The aorta and heart rates were counted simultaneously in the same preparations which demonstrated the intrinsic aorta rate is lower than the heart rate. 5-HT, 5-HT<sub>2</sub> agonist ( $\alpha$ -methyl-5-HT) noticeably increase the heart and aorta rates. Moreover, the 5-HT<sub>2</sub> antagonist (ketanserin) significantly blocked the positive chronotropic action of 5-HT on aorta rate as well as heart rate. These results indicate that 5-HT receptors are activated in the aorta. The density of the 5-HT receptors in the aorta might be less than the receptors on the heart. Also, the ionic channel composition of pacemaker cells of the aorta might be different than for the heart.

### **5-HT<sub>2</sub> receptors mutation and 5-HT action**

It has been shown that 5-HT<sub>2A</sub>Dro receptor mutant line, which is used in this study, has reduced 5-HT<sub>2A</sub> receptor expression. However, this insertional mutation is not a null mutation it is a hypomorphic mutation since there is still low percentage rate of 5-HT<sub>2A</sub> mRNA expression (Nichols, 2007). Here we used the same 5-HT<sub>2A</sub>Dro insertional mutant line to observe the action of 5-HT. 5-HT (100nM) cannot increase the heart rate dramatically; even though, the 5-HT (1 $\mu$ M and 10 $\mu$ M) significantly increase the heart rate. We observed that the 5-HT (10 $\mu$ M) action was lower than 5-HT (1 $\mu$ M). These results suggest that 5-HT<sub>2A</sub> receptor mutation reduces the action of 5-HT and high concentration of 5-HT (10 $\mu$ M) might desensitize 5-HT receptors in 5-

HT2A mutant larvae. We speculate that both 5-HT2ADro and 5-HT2BDro might be expressed in larvae cardiac tissue. It has been shown that the EC50 of 5-HT for 5-HT2ADro is lower (99nM) compared to EC50 of 5-HT for 5-HT2BDro (293nM) (Gasque et al., 2013). We employed 5-HT2BDro receptor mutant line to further our understanding of the 5-HT receptors that might be involved in positive chronotropic action of 5-HT. The 5-HT can increase the heart rate in 5-HT2BDro mutant larvae; although, the response of 5-HT2BDro mutant larvae to 5-HT was lower in comparison to the response of w<sup>1118</sup> larvae to 5-HT. These results suggest that 5-HT2BDro might be another 5-HT receptor which could modulate heart rate.

5-HT receptors are known to be G-protein coupled receptors (GPCR) in *Drosophila* (Yuan et al. 2006). In our study, the results show that 5-HT2 agonist increases the heart rate and 5-HT2 antagonist decreases the heart rate and can block 5-HT action on the larval heart. Also, the results indicate that the 5-HT2ADro or 5-HT2BDro mutation decreases the responsiveness of the heart to 5-HT. The intracellular signaling pathway of 5-HT2Dro has not been completely identified. By using various compounds that activate or block PLC-PKC pathways and release of intracellular Ca<sup>2+</sup> release, we would be able to obtain a better understanding of 5-HT2 signaling pathway in larval heart. 5-HT2 receptor is coupled to Gαq protein in mammals. When 5-HT or 5-HT agonist binds to 5-HT2 receptor, Gαq will be activated and released from heterotrimeric G-protein complex. Gαq can activate phospholipase Cβ (PLC). This can result in the lipid membrane phosphatidylinositol 4,5-bisphosphate (PIP2) being cleaved by active PLC into two second messengers, inositol triphosphate (IP3) and diacylglycerol (DAG). The rise in DAG can activate protein kinase C (PKC); whereas, IP3 binding to IP3 receptors on endoplasmic reticulum (ER) will cause Ca<sup>2+</sup> release into cytosol. Elevated Ca<sup>2+</sup> level in cytosol can lead to the activation of calcium/calmodulin dependent kinase, subsequently this can activate calmodulin and lead to blocking of K<sup>+</sup> channels (Nichols and Sanders-Bush 2001). Active PKC is able to modulate L-type calcium channels in cardiac cells (Kamp and Hell 2000). We have

already shown that inhibition of AC does not have effect on the 5-HT action on the heart rate (Majeed et al. 2013). Therefore, we suggest that activation of PLC-IP3-PKC pathway might be responsible for the 5-HT action through activation of 5-HT<sub>2</sub> receptor in *Drosophila* larval heart. It has been shown that KEN-93, which is a CaM kinase II inhibitor, inhibits the 5-HT action on the heart in P1 pupal stage of *Drosophila*. CaM kinase II has the ability to modulate ion channels, such as, Ca<sup>2+</sup> and K<sup>+</sup> channels (Johnson et al. 2002).

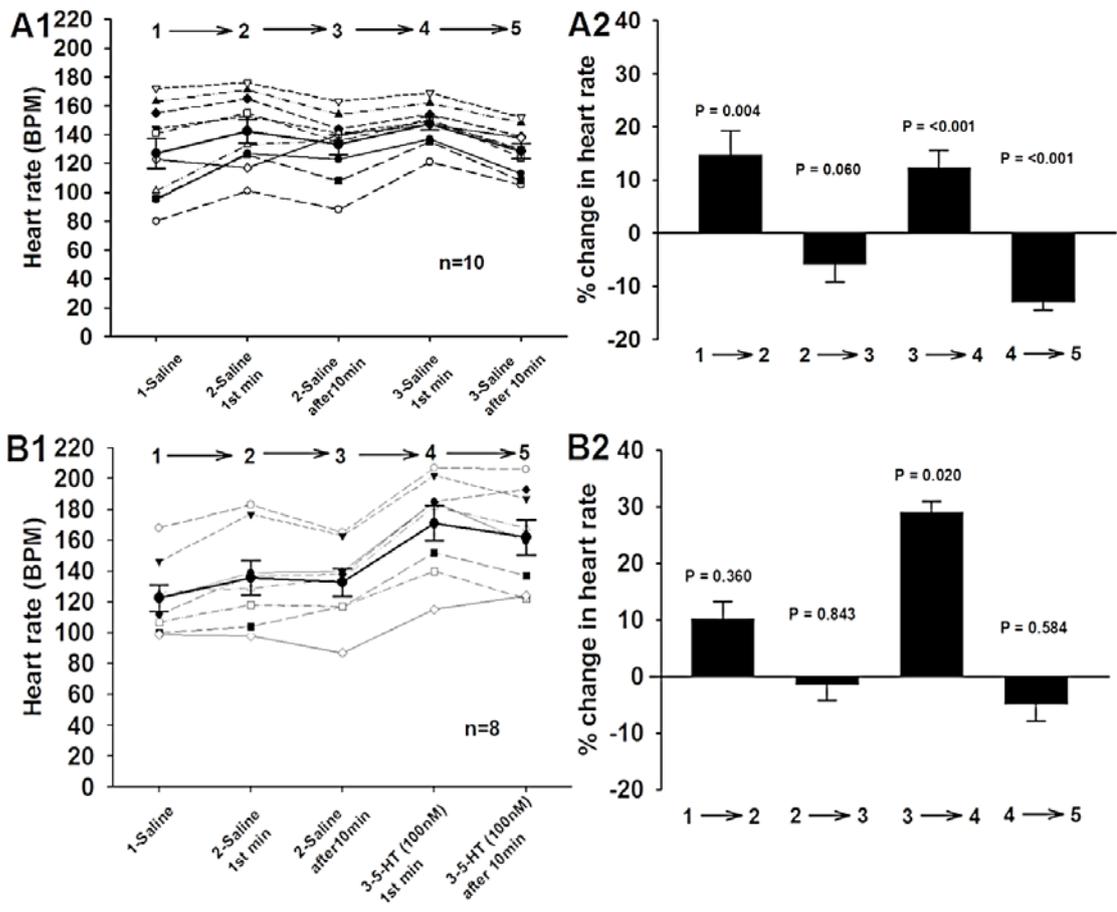


Figure 3.1: Change in heart rate for control trails and exposure to 5-HT. (A1) The raw data of the heart rate of individual preparations (n=10). (A2) The percentage change of heart rate before and after changing saline with saline as a control. (B1) Changes in heart rate during the entire experiment before and after application of 5-HT. (B2) 5-HT 100nM significantly increases the heart rate (note the third change in bathing solution). The preparation was left inside saline for one minute and then the rate was obtained for the following minute. Saline (1-Saline) was exchanged with saline (2-Saline). The preparation was left for one minute and subsequently rate was obtained over the next minute. The preparation was left for 10min then the heart rate was counted for one minute. Saline (2-Saline) was exchanged with saline (3-Saline), the preparation was left for one minute before counting the rate in the next minute. The preparations were left for 10min then the heart rate was obtained for one minute. Data is presented as mean±SEM.

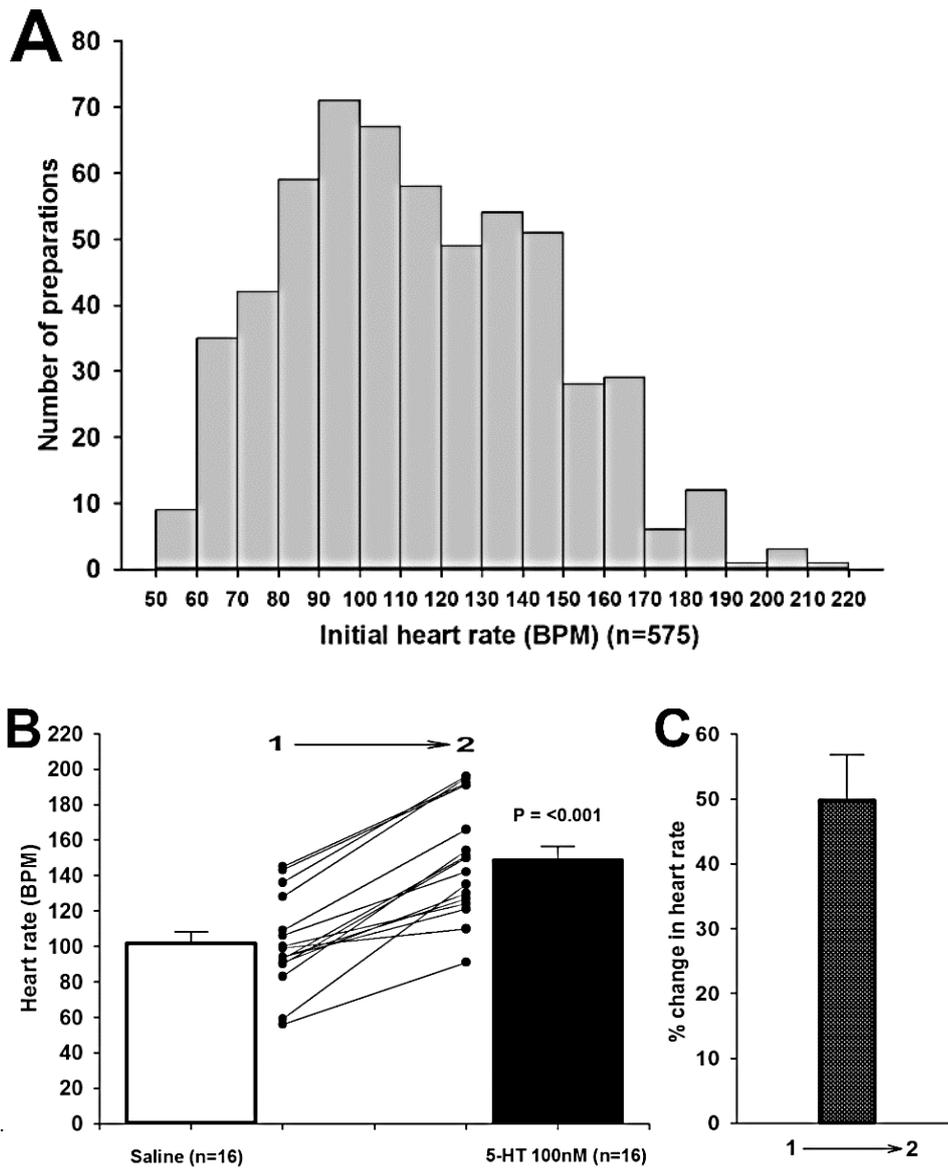


Figure 3.2: (A) Distribution of the initial heart rates of preparations used in this study (n=575). (B) There is a positive chronotropic effect of 100nM 5-HT on heart rate (paired t-test). (C) The percent change on heart rate when saline was exchanged with 5-HT (100nM). All data presented as mean±SEM.

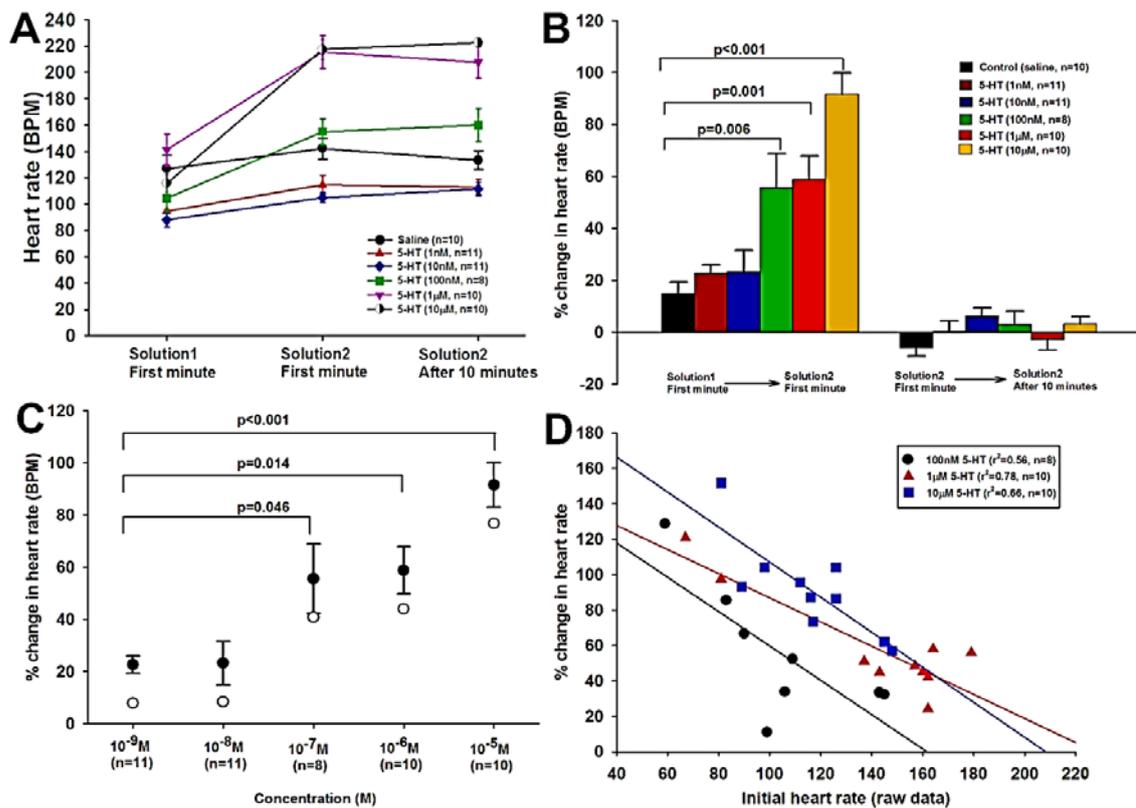


Figure 3.3: Effect of various concentrations of 5-HT on larval heart rate. (A) The trend of heart rate at various 5-HT solution concentrations. (B) At first minute (first block of columns) of 5-HT application, heart rate markedly increased for 100nM, 1µM, and 10µM concentrations in comparison to control. After 10 minutes (second block of columns), the heart rate stayed at the same level as no further % change was noted. (C) Dose-response relation of 5-HT action on larval heart rate. Open circles represents the subtraction of control saline exchanges from various concentrations of 5-HT action (Bonferroni's t-test was used for comparison). (D) Simple linear regression analysis between initial heart rate and percentage change of heart rate. There is generally a correlation between initial heart rate and percentage change in heart rate. Data presented as mean±SEM.

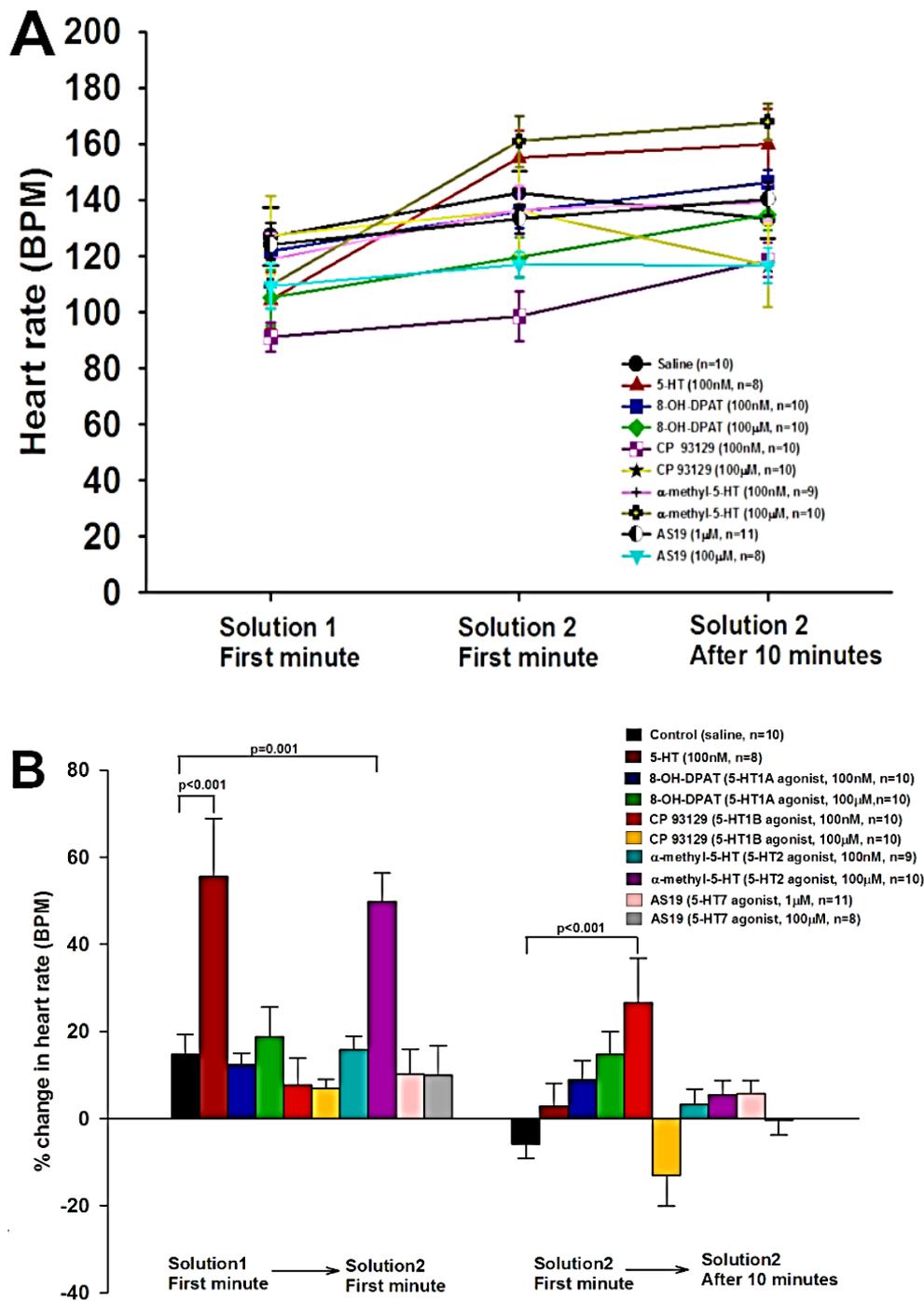


Figure 3.4: Effect of various 5-HT agonists on heart rate. (A) The raw data of heart rate throughout the experiment. (B) 5-HT (100nM) and  $\alpha$ -methyl-5-HT (100  $\mu$ M) significantly increased the heart rate (Bonferroni's t-test was used to compare the rest of the treatments to control). Data presented as mean $\pm$ SEM.

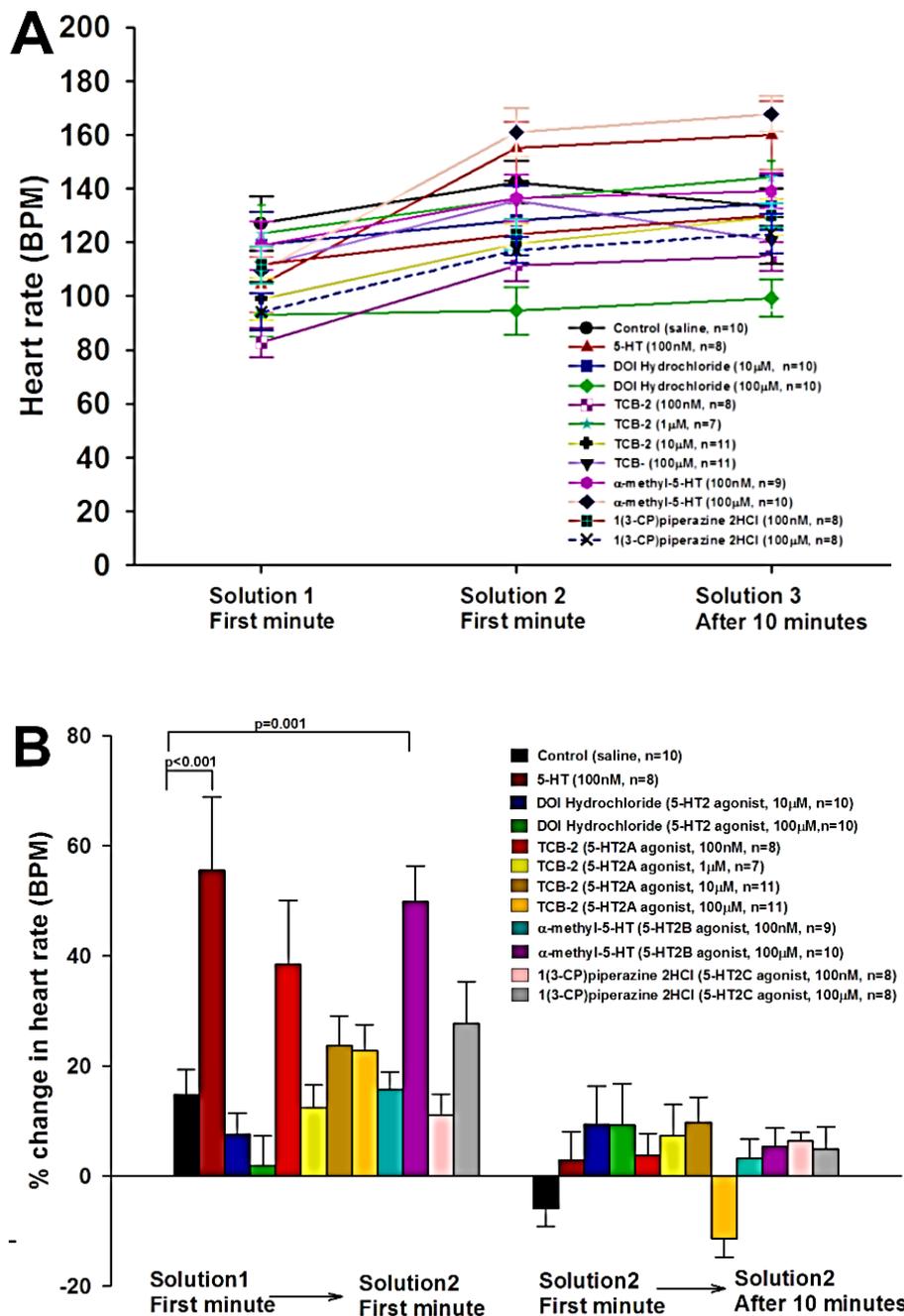
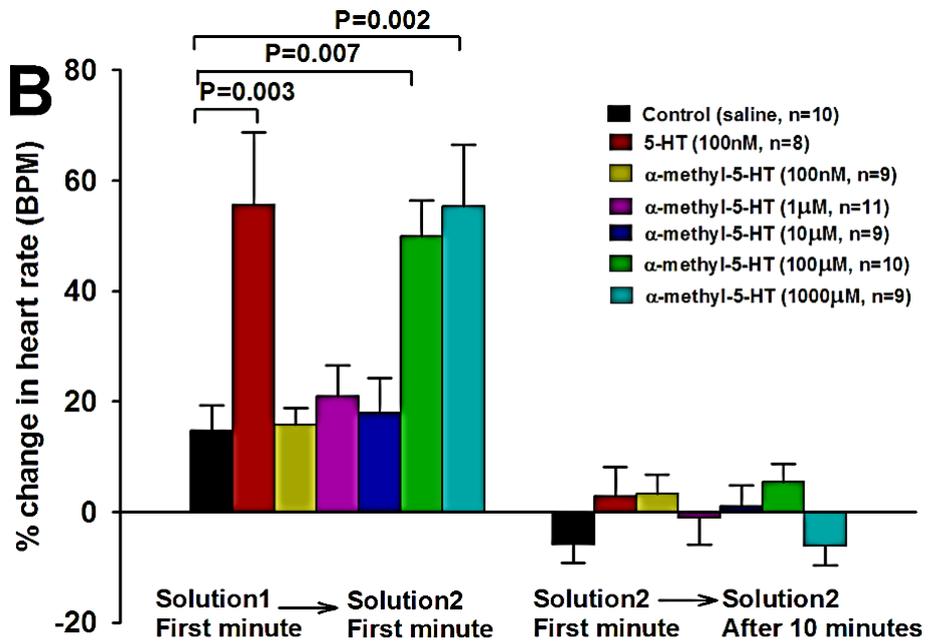
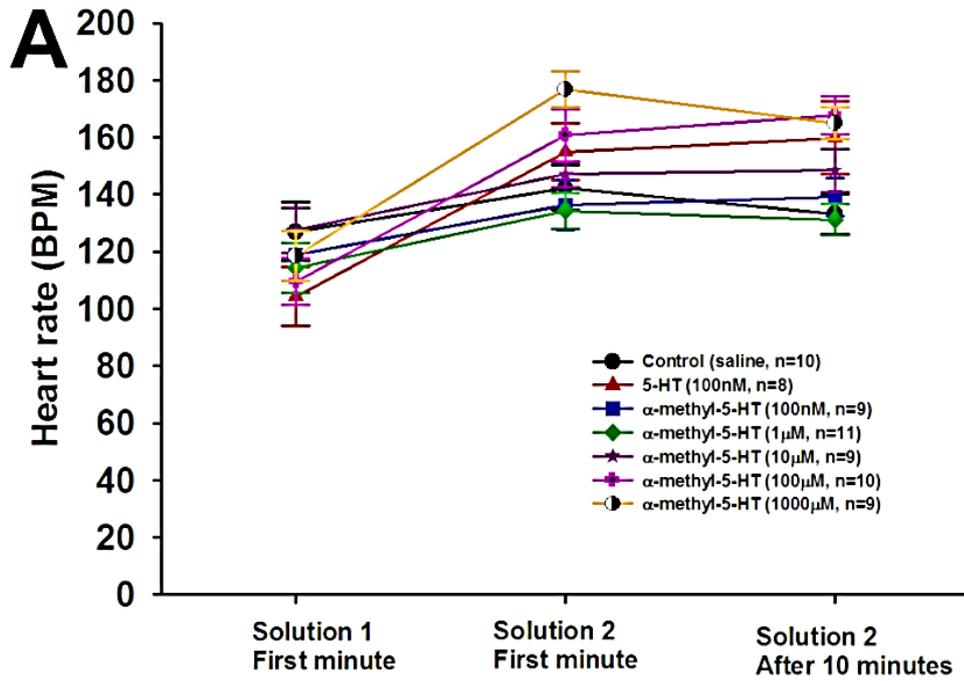


Figure 3.5: The effect of various 5-HT<sub>2</sub> receptor agonists on larval heart rate. (A) The initial long term effect of the agonists on heart rate. (B) 5-HT (100nM) markedly increases the heart rate. Also, α-methyl-5-HT significantly increases the heart rate. TCB-2 and 1(3-CP) piperazine show a slight trend of increasing the rate but is not statistically significant at  $p < 0.05$ . The rates were obtained inside saline (solution1). The saline was exchanged with saline-containing an agonist (solution 2). The percentage change in heart rate was measured from solution 1 to solution 2 (first block of columns) and from solution 2 (first minute) to solution 2 after 10min (second block of columns). Bonferroni's t-test to compare the rest of the treatments with control. Data presented as mean±SEM.



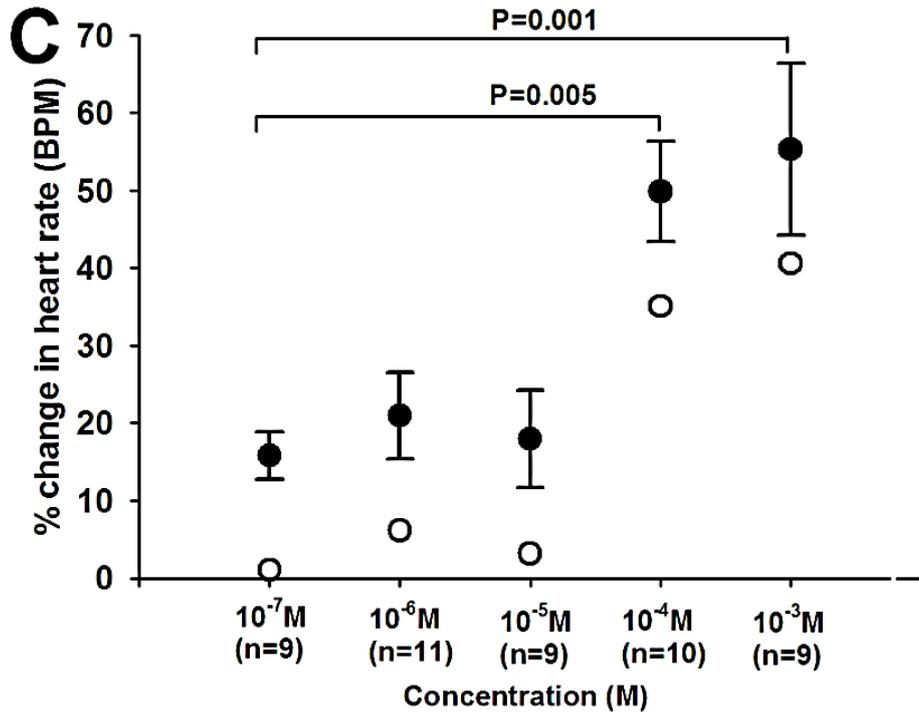


Figure 3.6: (A) Heart rate during exposure of  $\alpha$ -methyl-5-HT maleate. (B) The effect of various concentrations of  $\alpha$ -methyl-5-HT maleate (5-HT<sub>2B</sub> receptor agonist) on heart rate.  $\alpha$ -methyl-5-HT maleate (100 $\mu$ M) significantly accelerates the heart rate (Bonferroni's t-test was used to compare the effect of various concentrations of  $\alpha$ -methyl-5-HT maleate to control). (C) Dose-response relation of  $\alpha$ -methyl-5-HT-maleate (5-HT<sub>2</sub> agonist). The control (saline) value was subtracted from the values of  $\alpha$ -methyl-5-HT-maleate action on heart rate (open circles) (Bonferroni's t-test was used to compare the effect of various concentrations of  $\alpha$ -methyl-5-HT maleate to 100nM  $\alpha$ -methyl-5-HT maleate). Data presented as mean $\pm$ SEM.

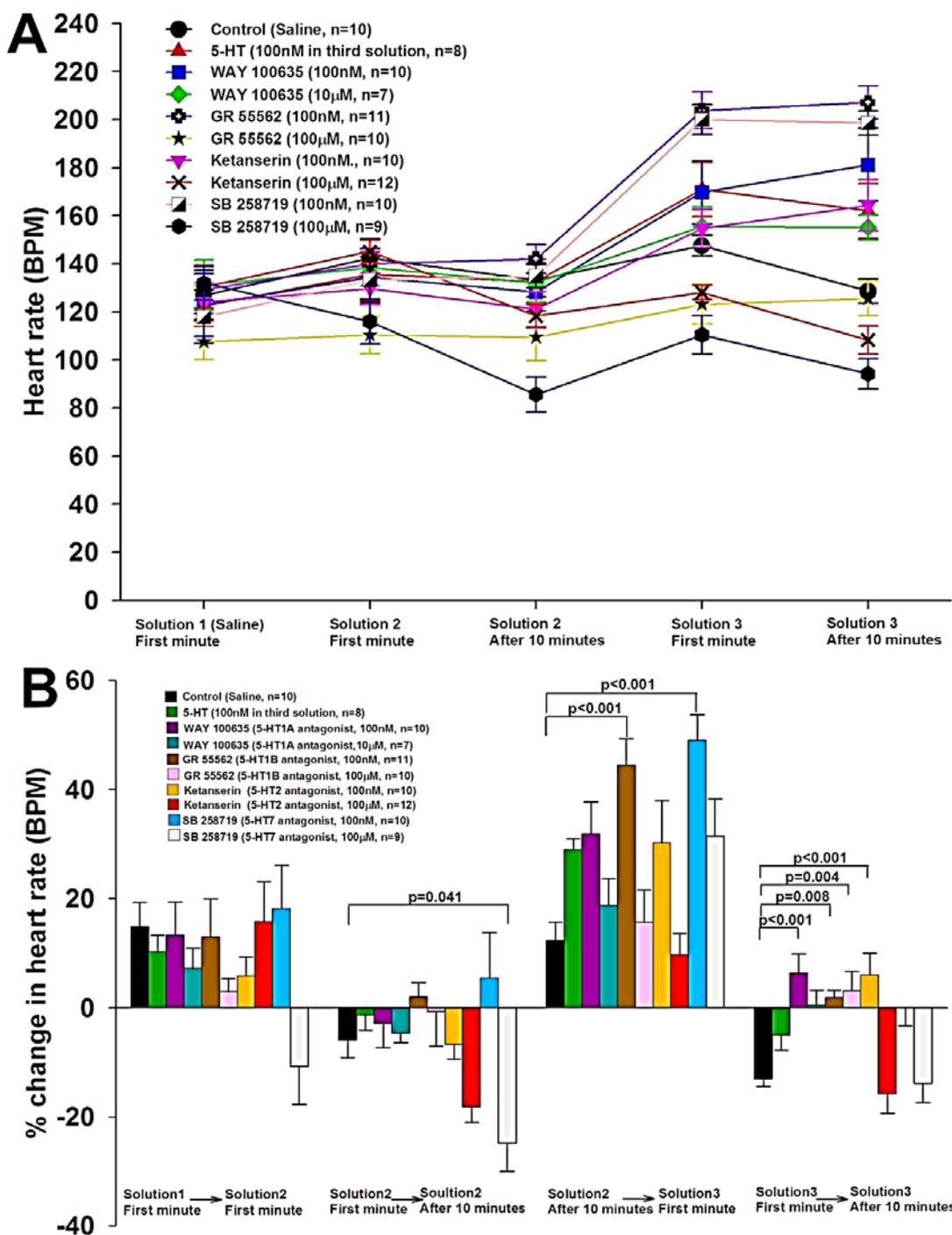
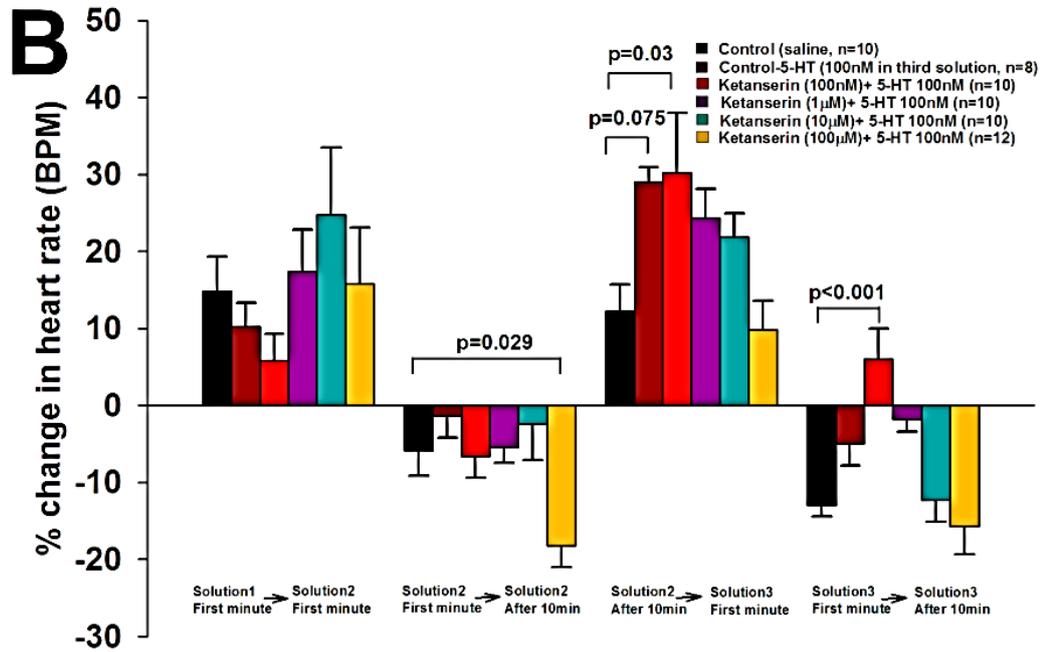
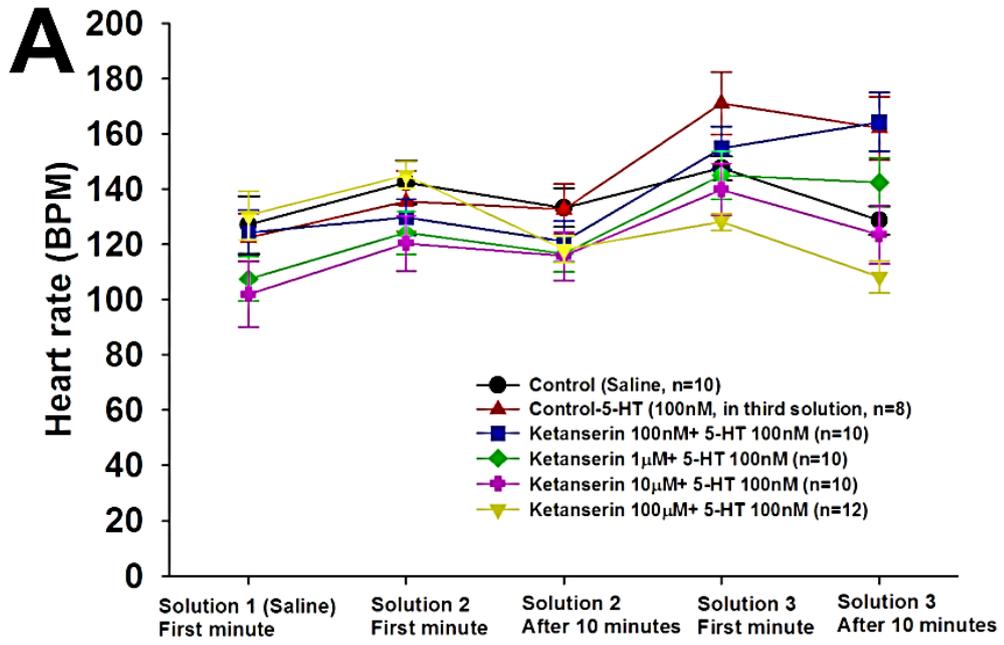


Figure 3.7: Effect of various 5-HT receptor antagonists on the action of 5-HT on heart rate. (A) The trend of heart rates during the period of the experimental paradigm. (B) Preparations were left inside saline for one minute and the rate was counted over the next minute (BPM) (solution 1). The saline was exchanged with saline containing one of the various antagonists. The preparations were left for one minute, and the heart rate was counted over the following minute (solution 2). The percentage change (first group of histogram) was calculated from saline to saline containing antagonist. The preparations were left inside antagonist for 10min and the rate counted. The percentage change was calculated from saline containing antagonist (first minute) to

saline containing antagonist (after 10min). Afterwards, the saline containing the antagonist was exchanged with saline containing a combination of 5-HT (100nM) and the same antagonists. These preparations were left for one minute and the rate was counted (solution 3). The percentage change was calculated from saline containing antagonist to saline containing 5-HT and antagonist. A percentage change was calculated from saline containing 5-HT and antagonist (first minute) to saline containing 5-HT and antagonist (after 10min). Ketanserin (100 $\mu$ M) blocks the positive chronotropic action of 5-HT in larval heart in comparison to control larvae. 5-HT7 antagonist (SB 258719) also reduced the heart rate in the absence of the 5-HT; however, 5-HT7 antagonist (SB 258719) did not block the 5-HT action (Bonferroni's t-test was used to compare the treatments with control). Data presented as mean $\pm$ SEM.



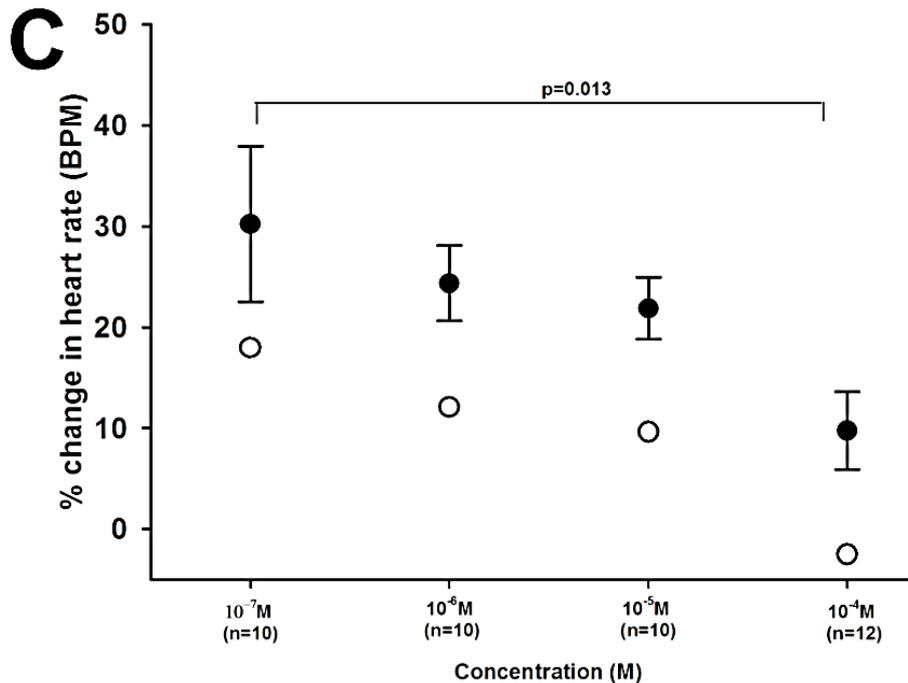


Figure 3.8: Effect of various concentrations of ketanserin on the action of 5-HT on heart rate. (A) Heart rate over the period of experimentation. (B) The preparations were left inside saline for one minute and the rate was counted over the next minute (BPM) (solution 1). Saline was exchanged with saline containing one of the ketanserin concentrations. The preparations were left for one minute, and the heart rate was counted over the next minute (solution 2). The percentage change was calculated from saline to saline containing ketanserin. The preparations were left inside ketanserin for 10min and the rate was counted for the next minute. The percentage change was calculated from saline containing ketanserin (first minute) to saline containing ketanserin (after 10min). Afterwards, saline containing ketanserin was exchanged with saline containing a combination of 5-HT (100nM) and ketanserin. The preparations were left for one minute, and the heart rate was counted over the following minute (solution 3). The percentage change was calculated from saline containing ketanserin to saline containing 5-HT and ketanserin. The preparations were left inside 5-HT and ketanserin for 10min. The heart rate was counted for one minute after 10min. The percentage change was calculated from saline containing 5-HT and antagonist (first minute) to saline containing 5-HT and antagonist (after 10min). Ketanserin (100 $\mu$ M) noticeably blocks the action of 5-HT on heart rate. However, ketanserin (100  $\mu$ M) decreases the heart rate in the absence of 5-HT. (Bonferroni's t-test was used to compare the treatments with control). (C) Dose-response relationship of ketanserin (5-HT<sub>2</sub> antagonist). The control (saline) value was subtracted from the values of ketanserin's action on heart rate (open circles) (Bonferroni's t-test was used to compare the effect of various concentrations of ketanserin to 100nM ketanserin). Data presented as mean $\pm$ SEM.

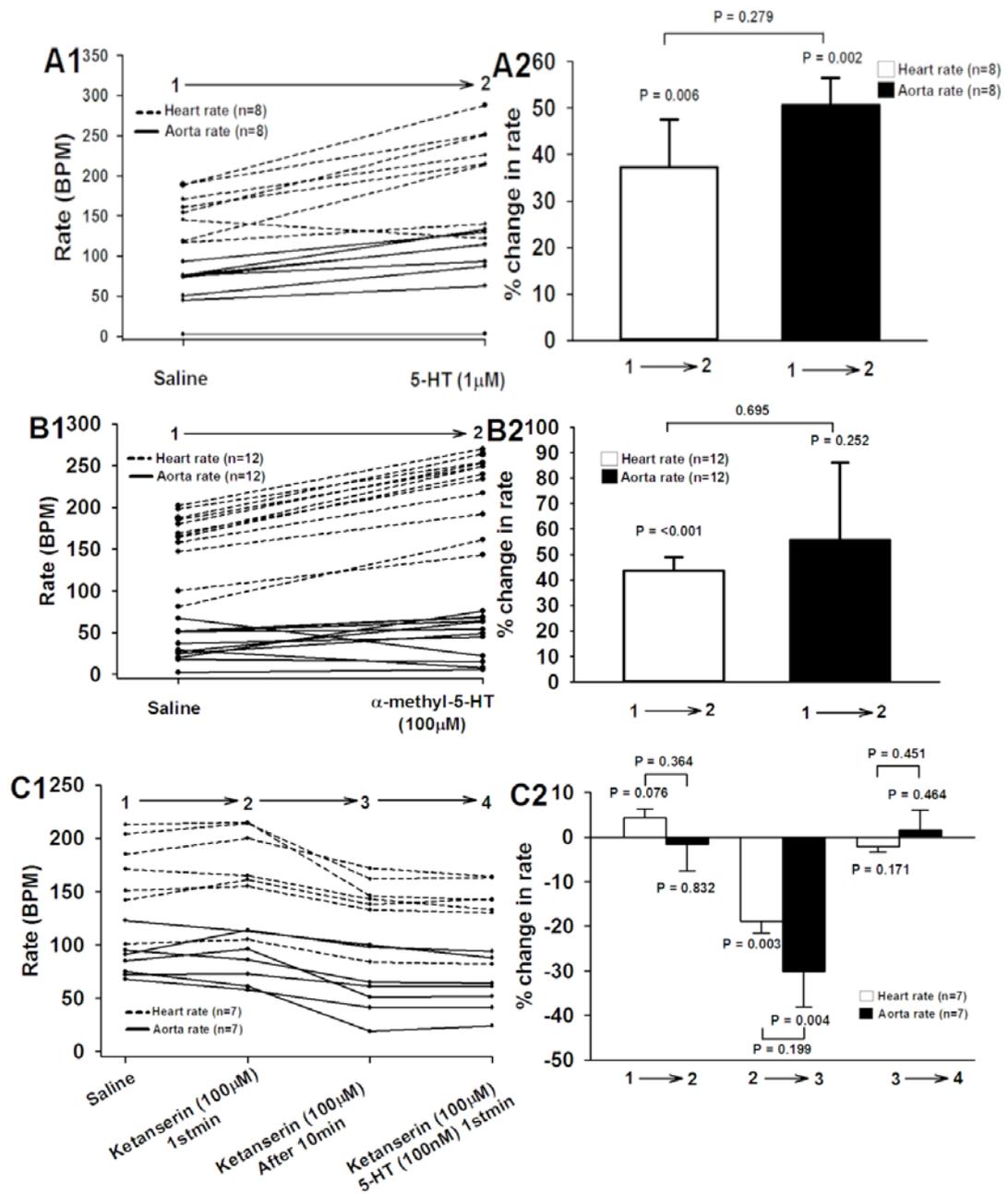


Figure 3.9: Action of 5-HT on heart and aorta independently. (A1) Heart and aortic rates before and during 5-HT application. The preparations were left for one minute then the heart and aorta rates were counted simultaneously for one minute. (A2) The effect of 5-HT (1 μM) on heart and aorta. 5-HT (1 μM) significantly increases the aorta rate as well as heart rate. The aorta rate is lower than the heart rate; although, there was no significant difference between the heart and aorta rates (Paired t-test was used to compare the heart rate before and after 5-HT application). (Student's t-test (non-paired) was used to compare between heart and aorta percentage change). (B1) Heart and aortic rates before and during 5-HT2 agonist (α-methyl-5-HT) application. (B2) 5-HT2 agonist (α-methyl-5-HT) (100 μM) application markedly increases both heart and

aorta rates. (C1) The trend of heart rate before and during 5-HT<sub>2</sub> antagonist (ketanserin) application. (C2) The effect of 5-HT<sub>2</sub> antagonist (ketanserin) on heart and aorta rates. 5-HT<sub>2</sub> antagonist (ketanserin) (100 $\mu$ M) significantly blocks the action of 5-HT on the heart and aorta. Hearts were separated from aortas prior to the chemicals being applied. The preparations were left for one minute and then the heart and aorta rates were counted for one minute simultaneously in the same preparation. All the chemicals have effect on aorta rate as well as on heart rate. Data presented as mean $\pm$ SEM.

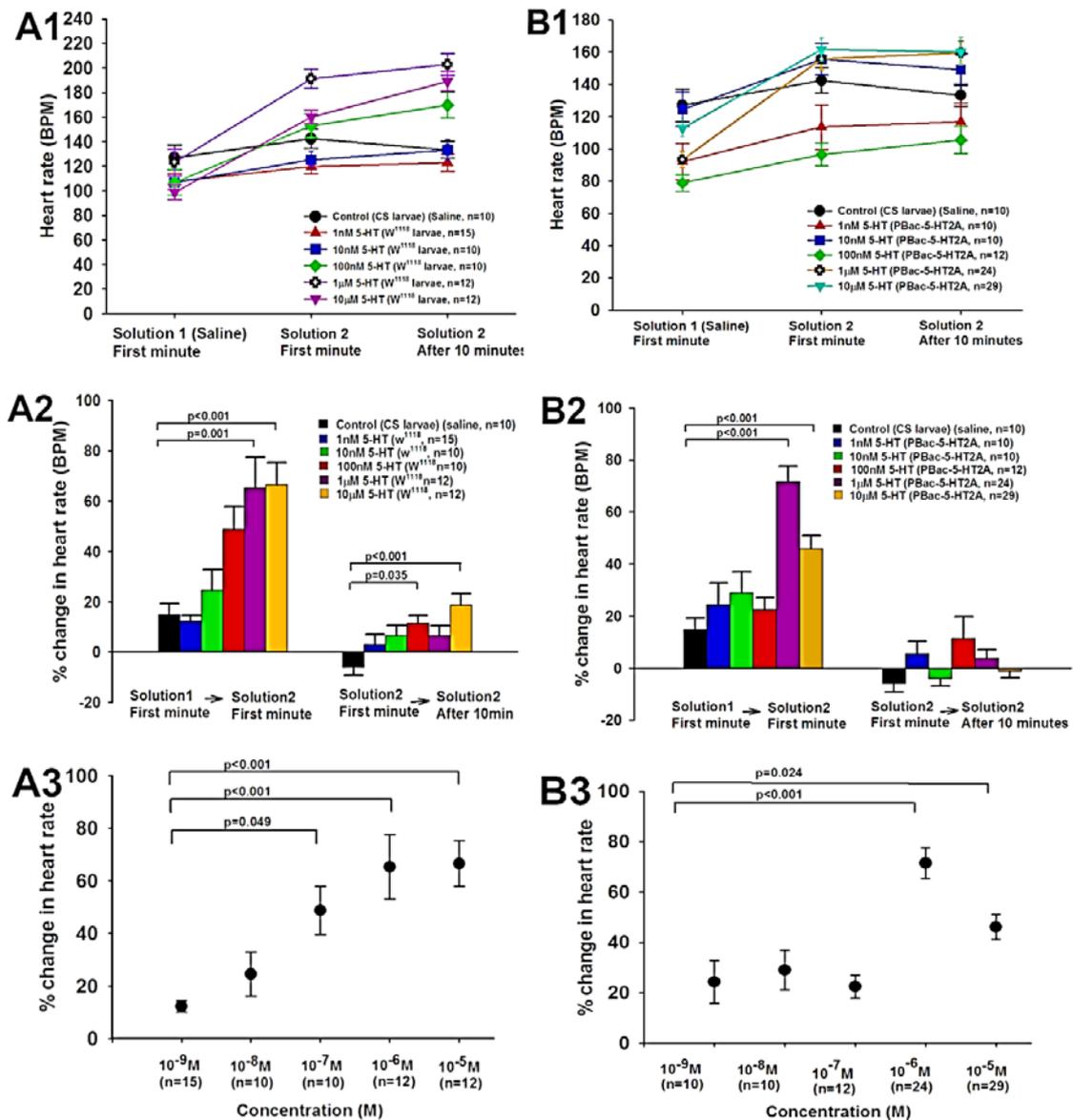


Figure 3.10: 5-HT action on heart in 5-HT2 receptor mutant larvae. (A1) The trend of heart rate for the same period of time as the experimental paradigm. (A2) The effect of various 5-HT concentrations on heart rate in *w<sup>1118</sup>* larvae. The heart rates of *w<sup>1118</sup>* larvae are compared to canton-S (CS) larvae (control). The preparations were left inside saline for one minute and afterwards the heart rate was counted for one minute. The preparations were left for 10min, and then the heart rate was counted for one minute. (A3) Dose-response-relationship of 5-HT in *w<sup>1118</sup>* larvae. (B1) Heart rate for the duration of the experimental protocol. (B2) The effect of various 5-HT concentrations on heart rate in 5-HT2ADro insertional mutant larvae. 5-HT (100nM) does not have a marked effect on heart rate. 5-HT (1μM and 10μM) significantly increased the heart rate. However, the action of 5-HT (100μM) is less than the action of 5-HT(10μM). (B3) Dose-response relationship of 5-HT in 5-HT2ADro receptor insertional mutant larvae.

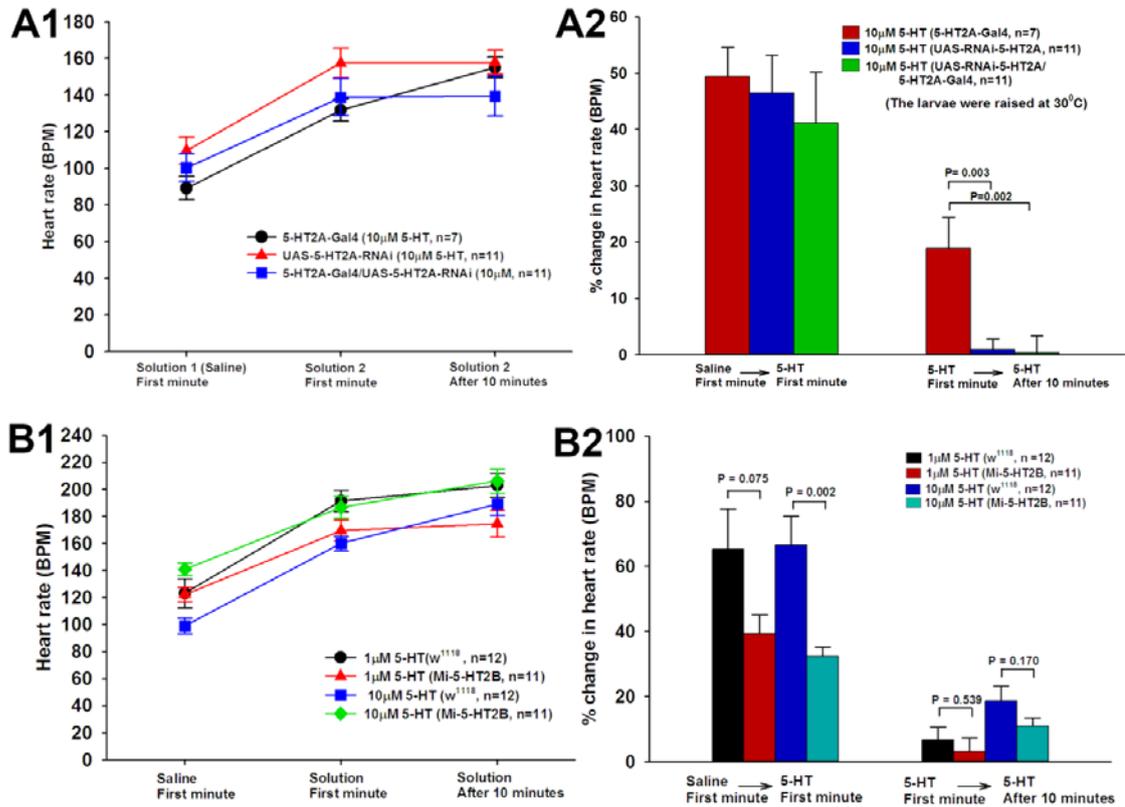


Figure 3.11: (A1) The trend in heart rate for the duration of the experimental time frame. (A2) 5-HT action in 5-HT2A knockdown larvae. The flies were raised at 30°C to increase the activity of Gal4 transcriptional activator. 5-HT (10 $\mu$ M) markedly increased the heart rate in all fly lines. The 5-HT2A receptor knockdown tends to reduce the effect of 5-HT action on heart rate but the reduction is not statistically significant. When the preparation was left inside the 5-HT solution for 10 minutes, the heart rate noticeably increased in 5-HT2A-Gal4 driver line; however, this increment was not observed in UAS-RNAi-5-HT2A and UAS-RNAi-5-HT2A/5-HT2A-Gal4 lines (Bonferonni test was used for this comparison). (B1) The trend of heart rate during the experimental conditions. (B2) 5-HT action in 5-HT2DroB receptor mutant larvae. There is a marked difference between w<sup>1118</sup> (control) 5-HT2B receptor mutant larvae regarding response to 5-HT. The response of 5-HT2B receptor mutant larvae hearts to 5-HT is weaker compared to w<sup>1118</sup> larvae hearts (unpaired t-test was used for this comparison).

## CHAPTER FOUR

### **Modulatory action by the serotonergic system: Behavior and neurophysiology in *Drosophila melanogaster***

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**Author contributions:** Zana R. Majeed, Esraa Abdeljaber, Robin Soveland, Kristin Cornwell, Aubrey Bankemper, Felicitas Koch (they were students worked in the lab) collected data; Zana R. Majeed analyzed data; Zana R. Majeed and Robin L. Cooper interpreted results of experiments; Zana R. Majeed prepared figures; Zana R. Majeed and Robin L. Cooper wrote and revised the manuscript.

#### **Abstract**

Serotonin modulates various physiological processes and behaviors. This study investigates the role of the 5-HT in locomotion and feeding behaviors as well as in modulation of sensory-motor circuits. The 5-HT biosynthesis was dysregulated by feeding *Drosophila* larvae 5-HT, a 5-HT precursor or an inhibitor of tryptophan hydroxylase during early stages of development. The effects of feeding fluoxetine, a selective serotonin reuptake inhibitor, during early second instars was also examined. 5-HT receptor subtypes were manipulated using RNA interference mediated knockdown and 5-HT receptor insertional mutations. Moreover, synaptic transmission at 5-HT neurons was blocked or enhanced in both larvae and adult flies. The results demonstrate that disruption of components within the 5-HT system significantly impairs locomotion and feeding behaviors in larvae. Acute activation of 5-HT neurons disrupts normal locomotion activity in adult flies. To determine which 5-HT receptor subtypes modulates the evoked sensory-motor activity pharmacological agents were used. In addition, the activity of 5-HT neurons were enhanced by expressing and activating TrpA1 channels or *channelrhodopsin2* while recording the evoked excitatory post-synaptic potentials (EPSPs) in muscle fibers. 5-HT<sub>2</sub> receptor activation mediates a modulatory role in a sensory-motor circuit, and the activation of 5-HT neurons can suppress the neural circuit activity, while fluoxetine can significantly decrease the sensory-motor activity.

## Introduction

The fundamental mechanisms of modulating neural circuits in relation to specific physiological actions and behaviors in organisms is an area of current interest for basic research as well as clinical treatments (Marder, 2012). Neural circuits change in activity by modulators such as serotonin (5-hydroxytryptamine, 5-HT) in altering sensitization in the gill-withdrawal reflex in *Aplysia* (Mayford et al., 2012). Biogenic amines can act as neurotransmitters and neuromodulators and are indispensable for the normal development of neural connections in organisms (Liu and Deneris, 2011; Lesch and Waider, 2012). Interestingly, the role of 5-HT as a modulator is evolutionarily conserved among organisms (Turlejski, 1996). The serotonergic (5-HTergic) system plays many roles in *Drosophila* and other insects which are crucial for the animal survival and locomotion (Silva et al., 2014), feeding behaviors (French et al. 2014), learning and memory, aggression, circadian rhythms, sleep, heart rate, salivary secretion, insulin signaling and synaptic transmission (Becnel et al., 2011; Gasque et al., 2013; Luo et al., 2012; Johnson et al. 2009, 2011; Pooryasin and Fiala, 2015; Neckameyer, 2010; Röser et al., 2012; Wu and Cooper, 2012; Yuan et al. 2006). Being able to manipulate subsets of specific neurons in *Drosophila melanogaster* allows one to address the role of neurons associated with particular neuromodulators in altering specific behaviors (del Valle et al., 2011; Jones, 2009; Yoshihara and Ito, 2012). In addition, the fruit fly nervous system is an attractive model to decode the neural circuits and interrogate the function of genes of interest in animal behavior (Dasari et al., 2009; Venken et al., 2011). Also, *Drosophila* have a relatively simple 5-HTergic system that consists of 84 5-HTergic neurons in the larval central nervous system (CNS) (Vallés and White, 1988; Huser et al., 2012) and 106 5-HTergic neurons in adult CNS (Vallés and White, 1988).

Serotonin is biosynthesized from an essential amino acid tryptophan, which is catalyzed by two enzymes, tryptophan hydroxylase (TRH) and aromatic-L-amino acid decarboxylase. After release of 5-HT into the synaptic cleft at nerve terminals by

synaptic vesicles fusion, the action of 5-HT is terminated by its uptake into the nerve terminals by the serotonin transporter (SERT) (Sanders-Bush and Nichols, 2012). SERT is an important target for many anti-depressant agents (Rodríguez et al., 2003). *Drosophila* genome contains a gene that encodes dSERT, which is homologous to the vertebrate human and rodent SERT (hSERT and rSERT). It has been shown that dSERT can be inhibited by a variety of chemicals, such as, fluoxetine, which is a potent inhibitor of the SERT and used as an antidepressant drug (Corey et al. 1994; Demchyshyn et al., 1994). The *Drosophila* genome harbors five different genes for the 5-HT receptors (5-HT1ADro, 5-HT1BDro, 5-HT2ADro, 5-HT7Dro; Johnson et al. 2009), and the recently cloned 5-HT2BDro (Gasque et al. 2013) receptor subtype. 5-HT receptors in *Drosophila* are G-protein coupled receptors (GPCR) (Obosi et al., 1996), which are expressed in various neurons and selective regions of the nervous system which correlate with the 5-HT localization (Blenau and Thamm, 2011; Pooryasin and Fiala, 2015).

Serotonin modulates locomotion activity in diverse groups of animals across the animal kingdom; for instance, in *C. elegans* (Gürel et al., 2012; Flavell et al., 2013) and mammals (Dunbar et al. 2010). Recently, it has been shown that various 5-HT receptors play a role in locomotive behavior in *Drosophila* (Silva et al., 2014). Our results concur with this recently published study. In addition, we demonstrated that mutations in 5-HT receptor subtypes significantly reduce larval locomotion behavior. Moreover, we show overactivation of 5-HT neurons in freely moving larvae and adults significantly compromises locomotor activity. At the cellular level, it was already shown that serotonin increases evoked motor unit activity which innervates body wall muscle fibers (Dasari and Cooper, 2004).

In this current study, we further investigated the 5-HT receptor subtypes that mediate the modulatory action of 5-HT in a sensory-CNS-motor circuit. 5-HT modulates the feeding behavior in mammals (Donovan and Tecott, 2013) as well as in insects. In honeybees, 5-HT increases the motility of the gut; however, it decreases

feeding behavior drive in the CNS (French et al., 2014; Neckameyer, 2010; Pooryasin and Fiala, 2015). This indicates that 5-HT differentially changes feeding behavior. Also, it has been shown that if 5-HT<sub>2A</sub> receptor subtype is blocked, there is a decrease in feeding behavior in *D. melanogaster* (Gasque et al., 2013). 5-HT also decreases the feeding behavior in the ant *Camponotus mus* (Falibene et al. 2012). In this study, we address if 5-HT alters mouth hook movements (MHMs), which is an assay for feeding behavior. This study demonstrates the role of 5-HT receptor subtypes and function of 5-HT releasing neurons in various behaviors within the ever increasingly invertebrate model *D. melanogaster*.

## Materials and Methods

### Fly rearing and stocks

Canton-S (CS) and *w*<sup>1118</sup> flies were used as controls. Trh-Gal4 (II) (stock#38388), Trh-Gal4 (III) (stock#38389), D42-Gal4 (III) (stock#8816), 5-HT1A-Gal4 (stock#49583), 5-HT1B-Gal4 (stock#46023), 5-HT2A-Gal4 (stock#49574), 5-HT7-Gal4 (stock#49414), UAS-5-HT1B-RNAi (stock#33418), UAS-5-HT2A-RNAi (stock#31882), UAS-5-HT7-RNAi (stock#27273), UAS-TrpA1 (stock#26263), UAS-Trh (stock#27638), Mi-5-HT1B (*w*<sup>1118</sup>; Mi{ET1}5-HT1B<sup>MB05181</sup>) (stock#24240), PBac-5-HT2A (*w*<sup>\*</sup>; P{FRT(*w*<sup>hs</sup>)}2A P{neoFRT}82B PBac{GAL4D,EYFP}5-HT2A<sup>PL00052</sup>) (stock#19367), Mi-5-HT2B (line2) (*w*<sup>1118</sup>; Mi{ET1}5-HT2B<sup>MB11858</sup>) (stock#29257), Mi-5-HT2B (line1) (*y*<sup>1</sup> *w*<sup>\*</sup>; Mi{MIC}5-HT2B<sup>MI06500</sup>) (stock#40810), PBac-5-HT7 (*w*<sup>1118</sup>; PBac{WH}5-HT7<sup>I05214</sup>) (stock#18848), PBac-Trh (*w*<sup>1118</sup>; PBac{PB}Trh<sup>C01440</sup>) (stock#10531) were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN, USA). UAS-shi<sup>ts1</sup> was kindly provided by Toshihiro Kitamoto (Kitamoto, 2001). The flies were raised at room temperature (22-23°C), unless otherwise stated in figure legends, in vials containing cornmeal-agar-dextrose-yeast medium. Embryos or various staged larvae were collected for the experiments. The

overexpressing and knockdown strains of the various proteins were not confirmed in this study with quantitative measures such as RT-PCR.

## **Chemicals**

The agonists and antagonists used are defined for mammalian preparations and details of binding affinity and efficacy of the various *Drosophila* 5-HT receptor subtypes are not established. 8-Hydroxy-DPAT hydrobromide (5-HT1A agonist), CP 93129 dihydrochloride (5-HT1B agonist),  $\alpha$ -methyl-5-hydroxytryptamine maleate (5-HT2 agonist), AS19 (5-HT7 agonist), were purchased from Tocris Bioscience (Bristol, UK). 5-hydroxytryptamine (5-HT), 5-hydroxytryptophan (5-HTP), fluoxetine hydrochloride, *para*-chlorophenyl alanine (PCPA), 5-Carboxamidotryptamine maleate salt, 5-Methoxytryptamine, BW 723C86 (5-HT2B agonist), R(-)-DOI hydrochloride, ( $\pm$ )-DOI hydrochloride, 1-(3-Chlorophenyl)piperazine hydrochloride (m-CPP), LP44 (5-HT7 agonist) were purchased from Sigma (St. Louis, MO, USA). All chemicals were dissolved in fly saline. However, AS19 and LP44 were dissolved in dimethyl sulfoxide (DMSO). Fresh solutions of specific concentrations were made from stock solutions each time before starting an experiment. The DMSO concentration in AS19 was less than 0.01%. The DMSO concentration in LP44 10 $\mu$ M, which is the highest concentration employed, was 0.1%.

## **Feeding of compounds**

Adult males and females were put inside a container with an apple-juice agar plate at the bottom. Specific drugs were dissolved in one milliliter (ml) of distilled water and mixed with 2 grams of cornmeal-agar-dextrose-yeast medium (shown as millimolar (mM) in the figure legends). Approximately fifteen (15) embryos were transferred from the apple juice container to the drug mixture. The embryos were left to develop to the third instar larval stage which were raised at room temperature (22-23°C), unless otherwise stated in figure legends. The same protocol was used for the

control animals except no drug was added to the food. It is known how much endogenous 5-HT concentrations are altered by feeding some of these compounds (50mM PCPA, Dasari et al., 2007); however, it is not known for other compounds we used such as the receptor agonists and antagonist. Such measures for each dosage and each compound is beyond the scope of these initial studies. Our approach was to provide varying dosages as a means to assess the potential varied concentrations on the effect of animal behaviors.

### ***Behavioral assays***

#### **Locomotion behavior in larvae**

Individual third instar larvae were placed on apple juice agar (1% agar) Petri dishes (about 8.5-9 cm diameter). The larvae were left inside the dish for one minute to acclimate to the environment; unless otherwise stated in figure legends. The body wall contractions (BWCs) were counted for one minute in a lightly illuminated environment at room temperature (22-23°C).

#### **Dominant negative *sh<sup>ts1</sup>* and larval locomotion behavior**

The flies were raised at 23°C 12:12LD cycle. To obtain the locomotion behavior at room temperature (22-23°C) for control and *shibire<sup>ts1</sup>* expressing larvae, individual third instar larvae were placed on the apple juice agar. The larvae were left for 1min to acclimate the new environment, and body wall contractions were counted for the following 1min. To obtain BWCs at restrictive temperature (37°C) for control and *shibire<sup>ts1</sup>* expressing larvae, larvae were placed inside a mixture of 0.5gm fly food plus 0.5ml water in a tube: 9.4cm height, 2.4cm diameter (top), 2.25cm diameter (bottom). The temperature range used was as previously used by Song et al. (2007) for *shibire<sup>ts1</sup>*. The larvae were incubated at 37°C for 20min in the water bath. Individual larvae were placed on pre-warmed apple juice agar on a hot plate (30-32°C). The larvae were left for 1min to acclimate, and the BWCs were counted for the following 1min.

### **TrpA1 channels and larval locomotion behavior**

The flies were raised at 23°C, 12:12LD cycle. To obtain BWCs at room temperature (22-23°C), individual larvae were placed on apple juice agar at room temperature, and left for 2min to acclimate the new environment. The BWCs were counted for 1min in a lightly illuminated environment. To obtain locomotion behavior at restrictive temperature (32°C), individual larvae were placed on a pre-warmed apple juice agar plate on a hot plate (30-32°C). The larvae were left for 2min. Afterward BWCs were counted for 1min in a lightly illuminated environment. TrpA1 channels become activated > 28°C (Hamada et al., 2008; Pulver et al., 2009).

### **Larval feeding behavior**

Individual larvae were placed inside a small Petri dish (5.5cm diameter) that contained yeast solution (a few dried yeast granules were mixed with water). The larvae were left for one minute, and then the mouth hook movements (MHMs) were counted for one minute.

### **Climbing assay in adults**

The flies were raised at 23°C 12:12LD cycle. The adult flies were anesthetized by exposing them to CO<sub>2</sub>, and then males and females sorted out one day prior to performing the behavioral assay. 6-14 adult flies (2-6 days old), males and females were tested separately. They were placed inside a tube: about 9.4 height, 2.4 cm diameter (top), 2.25cm diameter (bottom). During the experiment, the cotton plug was removed and another similar tube was placed over the top of the first tube. The bottom tube was marked at 8-cm height. The tube was tapped until all the flies fell into the bottom of the tube. A 10sec period was set to observe how many flies would cross the 8-cm line in this period. This procedure was repeated 10 times, and there was one minute interval between each trial in order for the flies to recover from first tapping. The climbing rate was calculated by combining all the fly cohorts that were tested at

room temperature (modified from Ali et al. 2011). The same fly cohorts, which were used at room temperature climbing assay, were transferred into the water bath (37°C), and they were left for 10min. After 10min incubation, the flies were returned back to room temperature (22-23°C) to repeat the climbing assay. This procedure was repeated for 10 times and with a one minute interval between each trial.

### **Locomotion activity in adult flies**

Flies were raised at 23°C, 12:12LD cycle. The adult flies, females or males, were anesthetized by exposing them to CO<sub>2</sub>. The flies were left to recover for at least 16 hours. Then the flies (less than 1 to 6 days old) were anesthetized by cold. Adult flies were transferred into empty vials, and they were placed inside an ice bucket for 20-30 sec. The flies (4-5) were transferred into a plastic Petri dish (9cm diameter, and 0.8cm height), and a glass cover was put over it. The flies were left for 10-13 min to recover from cold anesthesia at room temperature. Afterwards, the flies were placed in a 37°C room incubator. The fly locomotion activities were recorded with a webcam (WEBCAM HD4110, Hewlett-Packard Company, Palo Alto, CA), which was connected to a computer, and the activity was recorded at 5 frames per second (5fps) for 10min using VirtualDub-1.10.4 software (<http://www.virtualdub.org>). The locomotion activity was analyzed for two time points, for 10 sec at the 5<sup>th</sup> min and for 10sec at 10min using manual tracking (<http://rsbweb.nih.gov/ij/plugins/track/track.html>) in Image J software (1.47v) (<http://rsbweb.nih.gov/ij/download.html>) (Berni et al., 2010).

### **Optogenetics experiments**

#### **Fly lines**

We used a recently created *channelrhodopsin* (ChR2) line which is very sensitive to light *y1<sup>w<sup>1118</sup></sup>*; PBac{UAS-ChR2.XXL}VK00018 (BDSC stock # 58374) (Dawydow et al., 2014). Virgin females from UAS-ChR2.XXL were crossed with males of Trh-Gal4 (BDSC stock#38389) line to express ChR2-XXL variant in serotonergic

neurons. We also used UAS-H134R-ChR2;Trh-Gal4 (III) homozygous line, which is kindly provided by Dr. Andreas Schoofs (Schoofs et al., 2014), for the electrophysiological study since the ChR2-XXL was too sensitive for the electrophysiology experiments.

### **All-trans retinal (ATR) preparation**

All-trans retinal (500 mg; Sigma-Aldrich, St. Louis, MO, USA), was dissolved in 17.6 ml absolute ethanol to make a 100 mM stock solution. 100  $\mu$ l of 100 mM stock solution was transferred to small tubes and wrapped in aluminum foil, to prevent being exposed to light, and kept in -20°C freezer.

### **Preparation of fly food supplemented with ATR**

In order to prepare fly food supplemented with 1 mM ATR, 10 mL fly food was dissolved in microwave. After the food to cooled, a 100  $\mu$ l of 100 mM ATR was mixed well with fly food or 100  $\mu$ l of absolute ethanol (vehicle) was mixed with food as a control. The food vial was wrapped in aluminum foil until it was solidified. The flies were transferred to a vial containing ATR and kept in a dark place (to keep the ATR from degradation) at room temperature 22-23°C.

### **Larval locomotion behavior**

Locomotion behavior was assessed by placing a single larva on an apple-juice agar plate. The larva was left for one minute to acclimate to the new environment. The body wall contractions were being counted for one minute (BWCs/min) while the larva was being exposed to a dim white light. Also, body wall contractions were counted while the larva was exposed to focused focal blue light (a focused light through a microscope eyepiece with a mounted LED, see Titlow et al., 2014).

### **Negative geotaxic assay**

The adult flies aged 2-8 days were anesthetized with CO<sub>2</sub>. The males and females are to be sorted out and transferred into separate vials in cohorts of 10-14 flies. The flies were left to recover for 24 hours before running the experiments. A plastic vial (Drosophila culture cylindrical vial 1-1/4" diameter x 4" tall) was marked at 8cm length, and the 10-14 cohort flies were transferred to that empty marked vial. Another plastic vial was placed on top of the marked one (modified from Ali et al. 2011). The flies were left for one minute. The vials were tapped to knock down the flies to the bottom of the tube. Then number of flies which climbed across the 8 cm mark in 10 sec was recorded. This procedure was repeated before and after exposure to blue light.

### **Recording evoked sensory-motor circuit activity**

Third instar larva were placed in the dissecting dish and pinned out on anterior and posterior regions with the dorsal side up. A modified HL3 saline (NaCl 70mM, KCl 5mM, MgCl<sub>2</sub>.6H<sub>2</sub>O 20mM, NaHCO<sub>3</sub> 10mM, Trehalose 5mM, sucrose 115mM, Trizma acid 25mM or BES 25mM, and CaCl<sub>2</sub>.2H<sub>2</sub>O 1mM, the pH 7.1) was used as a physiological saline (de Castro et al., 2014). The dissection procedures have been previously described (Dasari and Cooper, 2004). The two last segmental nerves, which innervate last body segment, were cut close to the posterior end. Sharp microelectrodes (3M KCl) were used for monitoring muscle fibers 6 or 7. The segmental nerves were stimulated at 40Hz, 10pulses (S88 Stimulator, Astro-Med, Inc, GRASS Co., USA). There was a 10sec delay from first stimulation to the next stimulation train. The excitatory postsynaptic potentials (EPSPs) were recorded with an AxoClamp 2B (Axon instruments, USA), converted with a PowerLab, 4SP (ADInstruments, USA) and analyzed with LabChart 7.0 (ADInstruments, USA). The traces were measured by averaging the responses in 8 stimulations trains made with normal saline and 8 stimulations trains after exchanging saline with various

compounds; unless otherwise stated in figure legends. The suction electrodes which were used to stimulate the segmental nerves in each preparation was slightly different; therefore, a range of stimulation voltages were used. Depending on how tight the seal is with the suction electrode and the nerve the voltage must be adjusted to evoked action potentials in the sensory nerves. The current is not directly varied but is a reflection of the voltage and the seal resistance for each preparation.

### **Statistical analysis**

All data are represented as mean  $\pm$  SEM. One way ANOVA test was carried out for multiple comparisons among treatments. Bonferroni t-test was performed to obtain significant levels (P-values) of various groups of flies (SigmaPlot version 12.0). Paired t-test was employed to compare the number of EPSPs between the saline and agonist or saline-containing compound. Student's t-test was used to compare the treatment and control groups. The level of significance (P-value) less than or equal to 0.05 is considered statistically significant.

## **Results**

### **Oral 5-HT administration: locomotion and feeding behaviors**

The rationale for feeding 5-HT as compared to injections is to reduce the handling stress associated with systemic injections. 5-HT was orally administered to the larvae at various concentrations 1mM, 10mM, 50mM, and 100mM. The embryos were collected and were placed inside a mixture of 0.5gm fly food and 0.5ml water (control) or 0.5gm food and 0.5ml water plus various 5-HT concentrations. 5-HT significantly reduced the body wall contractions (BWCs) and mouth hook movements (MHMs) at 100 mM high dose (Figure 4.1 A and B). 1mM did not significantly alter mouth hook movements (MHMs); however, 100mM significantly reduced the MHMs (Figure 4.1 B). Also 5-HT altered the developmental size of larvae. A low level of 5-HT (1mM, 10mM) resulted in significant increases in body length; however, at high

concentrations (50mM and 100mM), the body length is significantly reduced compared to controls not feed 5-HT (Figure 4.1 C).

### **Oral 5-HT precursor (5-HTP) administration: locomotion and feeding behaviors**

Another approach to perturb the 5-HT level in neurons and systemically is by providing more of the precursor to synthesize 5-HT. We predicted a similar result with feeding the precursor as to feeding 5-HT itself to the larvae. The 5-HT precursor 5-HTP was orally administered during early stages of development. The embryos were collected and were placed inside a mixture of 0.5gm standard fly food and 0.5ml water (control) or 0.5gm food and 0.5ml water in addition to various concentrations of 5-HTP. 5-HTP at 5mM and 25mM reduced the BWCs in third instar larvae (Figure 4.2 A). On the other hand, there was no statistically significant reduction in MHMs (Figure 4.2 B). Body length did not change for 5-HTP at 5mM, but the lengths did decrease at 25mM as compared to controls and ones fed 5mM (Figure 4.2 C).

### **Oral PCPA administration: Inhibition of tryptophan hydroxylase (Trh)**

The rate-limiting enzyme of 5-HT synthesis was blocked by *para*-chlorophenylalanine (PCPA) administration. So instead of increasing 5-HT, a decrease level in the CNS, as well as systemic levels in 5-HT occur. A similar feeding regimen was used as in earlier studies which measured 5-HT with HPLC and showed a significant reduction in level of 5-HT (Dasari et al., 2007). The embryos were collected and were placed inside a mixture of 0.5gm standard fly food and 0.5 ml water (control CS) or 0.5gm food and 0.5ml water plus PCPA. Feeding PCPA 50mM significantly reduced the BWCs and MHMs (Figure 4.3 A and B respectively).

### **Genetic manipulation of Trh expression**

Tryptophan hydroxylase is the enzyme which promotes the synthesis of 5-HT. Thus, overexpression of *Trh* was predicted to result in similar alterations as to feeding

5-HT or the 5-HTP. *Trh* was selectively overexpressed in serotonergic neurons for comparisons. The flies were raised at 23°C or 29°C to increase the expression level of Gal4 transcriptional activator. The proposed *Trh* overexpression significantly reduced the BWCs and MHMs in third instar larvae (Figure 4.4 A and B, respectively). The 5-HT levels were not measured within the hemolymph for this strain; however, we assume the strain works as well as it has in previous studies. In addition, one of the parental lines (*Trh-Gal4(II)*) was used as a control.

### **Serotonin reuptake blocker and behaviors**

An additional approach to raise 5-HT at the synaptic cleft is to block the reuptake of 5-HT back into the presynaptic nerve terminal with selective 5-HT reuptake blockers. Fluoxetine was shown to block reuptake for the 5-HT transporter in *Drosophila* (Corey et al. 1994; Demchyshyn et al. 1994). Early second instar larvae were collected then 0.5gm fly food was mixed with 0.5ml water in case of control. Fluoxetine 10mM was dissolved in 0.5ml water, and then it was mixed with 0.5gm food. After 48 hours the behavioral assays were carried out. Since the fluoxetine is light sensitive; therefore, the larvae were placed in an incubator without light. Controls were treated similarly with light exposure. Fluoxetine 10mM significantly reduced the BWCs and MHMs in *w<sup>1118</sup>* larvae and *Trh* insertional mutant larvae compared to control groups (Figure 4.5 A and B). Moreover, fluoxetine 10mM in *Trh* insertional mutant larvae significantly reduced BWCs and MHMs in comparison to fluoxetine-fed *w<sup>1118</sup>* larvae (Figure 4.5 A and B).

### **Activation of 5-HT neurons and knockdown of 5-HT receptors: Effects on behavior**

Chronic activation of the 5-HT neurons through activating TrpA1 channels selectively expressed in 5-HT neurons significantly increased BWCs and MHMs (Figure 4.6 A and B). In order to show 5-HT receptor subtypes that are implicated in

the action of 5-HT in the nervous system, various genetic tools were used. 5-HT1B knockdown in 5-HT1B-expressing neurons during development significantly reduced the BWCs and MHMs (Figure 4.6 A and B). However, 5-HT2A and 5-HT7 receptor knockdowns in 5-HT2A and 5-HT7 receptor-expressing neurons did not markedly influence the BWCs but did decrease MHMs (Figure 4.6 A and B). All the flies were raised at 30°C to increase the Gal4 transcriptional activator activity. Similar results presented later in which the 5-HT containing neurons were stimulated with light, through expression of channel rhodopsin, complement these findings. In addition, the use of one of the parental strain (Gal4) as a control support the differential findings for the F1 strains.

#### **Manipulation of 5-HT synthesis and receptors: Effects on behavior**

In determining which receptor subtypes may play a larger role in altering the larval behaviors, strains carrying insertional mutations in 5-HT receptors were examined. In addition, a pBac-Trh strain was examined which would perturb the synthesis of 5-HT. The insertional mutation in 5-HT2A (homozygous mutant) and 5-HT7 (homozygous mutant) receptors significantly reduced the BWCs and MHMs in comparison to  $w^{1118}$  larvae (Figure 4.7 A and B); however, Trh mutant larvae did not show a noticeable change in BWCs and MHMs compared to  $w^{1118}$  larvae (Figure 4.7 A and B). Figure (4.8 A and B) shows that the fly lines were significantly reduced in BWCs and MHMs in comparison to control CS larvae. Furthermore, 5-HT2B mutant (line 2) larvae significantly decreased BWCs in comparison to  $w^{1118}$  larvae; although, there was not a marked change in MHMs between  $w^{1118}$  and 5-HT2B mutant (line 2) larvae (Figure 4.8 A and B). The 5-HT2B mutant (line 2) had a larger decrease in BWC (Figure 4.8B) than 5-HT2B mutant (line 2). However, there was no difference between these strains in MHMs. These results indicate that various 5-HT receptor subtypes play role in locomotor activity and feeding behavior.

### **Manipulation of synaptic activity in 5-HT neurons within larvae**

The dominant negative allele *sh<sup>ts1</sup>* was expressed in serotonergic neurons to block synaptic transmission. *sh<sup>ts1</sup>* is temperature sensitive which functions well at permissive temperature (22-23°C); however, it does not function at restrictive temperature (37°C). Thus, the synaptic vesicles within the presynaptic nerve terminals are not able to recycle. So an overall reduction in 5-HT being released in evoked and spontaneous vesicular events. The flies were raised at room temperature, and the third instar larvae were selected and incubated at 37°C for 20min. It was shown that expressing *sh<sup>ts1</sup>* in sensory neurons significantly reduces locomotion behavior when they are incubated at 37°C for 20min (Song et al. 2007). In this study, larvae which were expressing *sh<sup>ts1</sup>* in 5-HT neurons did not markedly alter the rate in the body wall contractions compared to control (UAS- *sh<sup>ts1</sup>*) (Figure 4.9 A). The activity of 5-HT neurons was increased by expressing temperature sensitive channel, TrpA1. It has been demonstrated that TrpA1 does not become activated at room temperature (22-23°C) but when it is exposed to >28°C, the TrpA1 channels become activated (Pulver et al. 2009). The larvae that were expressing TrpA1 channels were put on a pre-warmed (32°C) agar plate while the body wall contractions were being counted. The data indicate that acute activation of 5-HT neurons in third instar larvae significantly decreased BWCs at 32°C compared to control group (UAS-TrpA1) (Figure 4.9 B). One of the parental lines (UAS-TrpA1) was used for comparisons. The locomotion activity in third instar larvae was also decreased when 5-HT neurons were activated by light, through expression of channelrhodopsin2 (ChR2), which complements these findings (see optogenetics results).

### **Manipulation of activity in 5-HT neurons within adults**

Flies were raised at room temperature (22-23°C), which were expressing a temperature sensitive dominant negative *sh<sup>ts1</sup>* allele in 5-HT neurons. The climbing assay (i.e. negative geotaxis assay) was carried out to observe the effect of acute

activity in 5-HT neurons on locomotion behavior in adult flies. The *sh1<sup>ts1</sup>* flies were crossed with motor neuron specific Gal4 (D42-Gal4) and this cross was used as a positive control. When the flies were placed at 37°C for 10min, locomotion activity significantly decreased. Furthermore, *sh1<sup>ts1</sup>* flies were crossed with 5-HT neurons specific Gal4 driver (Trh-Gal4 (III)). When these flies were incubated at 37°C for 10min in the water bath, no significant changes were observed in locomotion behavior in both males and females in comparison to control flies (Figure 4.10 A). The climbing assay, which was performed in *sh1<sup>ts1</sup>* fly groups, was not a suitable assay for the locomotion behavior of TrpA1 expressing flies since when they were placed at 37°C. The control flies were very active but they stayed at the bottom while the climbing assay was being performed (i.e. they did not cross the 8-cm line at the 10sec time period); therefore, we decided to use another assay to record locomotion activity as depicted in (Figure 4.10 B1). Adult flies (4-5 flies in each group, males and females separately) were placed in a Petri dish which allowed the adults to walk around horizontally but not fly. Both controls (UAS-TrpA1) and TrpA1 expressing flies were transferred to a 37°C room. The locomotor activity was recorded for 10min, and the activity was analyzed for 10sec at 5<sup>th</sup> and 10sec at 10<sup>th</sup> min. Acute activation of TrpA1 channels in 5-HT neurons significantly reduced the locomotor activity in male and female TrpA1 expressing flies at 5<sup>th</sup> and 10<sup>th</sup>min compared to male and female control groups (UAS-TrpA1). Note that the females had a decreased locomotor activity compared to male flies (Figure 4.10 B2).

The locomotor activity in adult flies was also decreased when 5-HT neurons were activated by the *channelrhodopsin* ChR2-XXL (Figure 4.17 C). These results complement the results obtained from TrpA1 experiments. The other less sensitive ChR2 variants was not used in this experiments since the adult flies have a thick cuticle which reduces the level blue light penetrance through the cuticle; therefore, blue light cannot activate enough ChR2 to observe a change in the behavior. Also, we showed that blue light exposure is effective in flies that were not fed ATR. It has been

shown previously that ChR2-XXL can be activated in the absence of ATR diet supplementation (Dawydow et al., 2014).

### **Sensory-motor circuit activity and 5-HT**

Sensory-motor circuit activity was used to examine the influence of 5-HT on the circuit activity in dissected third instar larvae. The rationale of conducting these acute studies in dissected preparations is to directly examine the action of 5-HT on a neural circuit in which one could control the electrical activity of sensory neurons and examine a defined motor output on a body wall muscle. The body wall muscle m6 is used in the intact larvae for movements measured in BWM behavioral assay. Therefore, application of compounds which alter synaptic 5-HT in the excised preparation can be correlated to the approaches used to manipulate 5-HT concentration through diet, pharmacology, and genetics.

Sensory neurons of the last segmental nerves in early 3<sup>rd</sup> instars were stimulated at 40Hz, 10pulses (Figure 4.11 A). The segmental nerves were stimulated for 8 stimulation trains in the saline and 8 stimulation trains or more after exchanging saline with saline containing 5-HT, 5-HT agonists, a selective serotonin re-uptake inhibitor (SSRI, fluoxetine), or pre-warmed saline, which was used in TrpA1 expressing larvae. When saline was exchanged with saline-containing 5-HT, the number of evoked excitatory postsynaptic potentials (EPSPs) significantly increased (Figure 4.11 B).

### **Pharmacological study of 5-HT receptor subtypes**

Application of 5-HT at 100nM to the exposed larval brains significantly increased the number of EPSPs. When higher concentrations of 5-HT (1 $\mu$ M, 10 $\mu$ M) were applied, the number of EPSPs was also significantly increased; however, the percentage change at 100nM was higher than the percentage change for higher concentrations (Figure 4.12 A). Various 5-HT agonists were used to observe their

actions on the sensorimotor activity. This demonstrates that 5-HT7 agonist, AS19, and 5-HT2 agonist ( $\pm$ )-DOI HCl noticeably increased the number of EPSPs; however, no significant changes were observed after application of 8-OH-DPAT, CP9312, and  $\alpha$ -methyl-5-HT application (Figure 4.12 B). There is one study which demonstrated DOI appears to function at 5-HT2 receptors in *Drosophila* (Colas et al. 1995).

Other 5-HT agonists were used for further confirmation of the 5-HT receptor subtypes that mediate modulatory role of 5-HT in sensory-motor circuit activity. 5-MeOHT (1  $\mu$ M and 10 $\mu$ M), 5-CAT (100nM and 10 $\mu$ M), m-CPP (100nM and 10 $\mu$ M), LP44 (100nM and 10 $\mu$ M) were applied to dissected third instar larvae. There was no significant changes in EPSPs after application of these agonists (Figure 4.13 A). Moreover, different 5-HT2 agonists were employed for further confirmation. 5-HT2 agonists (BW723C86) (100nM and 10 $\mu$ M) and R(-)-DOI HCl (1 $\mu$ M) significantly increased the number of EPSPs (Figure 4.13 B). As far as we know only R(-)-DOI HCl was examined for binding to *Drosophila* 5-HT receptors; however, to the best of our knowledge, binding studies of BW723C86 have yet to be performed on *Drosophila* specific 5-HT receptors.

### **Genetic study of 5-HT receptor subtypes**

5-HT receptor subtype mutant lines mentioned previously were used for electrophysiological studies to corroborate the pharmacological results. 5-HT significantly increased the number of EPSPs in Trh mutant larvae. Also, the response of Trh mutant larvae to 5-HT was higher than control flies (CS) (Figure 4.14). In addition, the exogenous application of 5-HT in dissected third instar larvae significantly increased the sensorimotor activity in 5-HT2A receptor mutant flies; although, the response of 5-HT2A receptor flies to 5-HT was lower than the control flies (CS). In 5-HT7 mutant larvae, 5-HT application slightly increased the sensory-motor activity but at a  $P > 0.1$  for group comparisons from controls due to the high variation in this line (Figure 4.14).

### **Neural circuit activity and acute 5-HTergic neurons activation**

To examine hyperexcitability of the 5-HTergic neurons, the same sensory-CNS-motor paradigm was used in third instar larvae (UAS-TrpA1) at room temperature (22-23°C) (Figure 4.15 A1) and when switched with pre-warmed saline (37°C). When the saline was applied to the preparation it was 32-34°C.

The number of EPSPs of 7-8 stimulation trains bathed in saline and 7-8 stimulation trains inside pre-warmed saline were counted (Figure 4.15 A1 and A2). 5-HTergic neurons were activated while the motor output was being recorded from muscle fiber 6 or 7 in TrpA1 expressing larvae in saline (Figure 4.15 B1) and warmed saline (Figure 4.15 B2). The results indicate that acute activation of 5-HTergic neurons by TrpA1 cation channels (Trh-Gal4 (III)>UAS-TrpA1) showed a decrease in activity when warmed at a 92.4% significance ( $P=0.076$ ) and in comparing this line to the changes in the parental line (UAS-TrpA1) only a change at a 91% significance ( $P<0.09$ ) (Figure 4.15 C). We choose to use another approach which has recently become popular due to the specific activation of known neurons. We used the optogenetic approach to selectively activate 5-HTergic neurons while the sensorimotor neuron activity was being recorded. In dissected third-instar larvae, blue light exposure did not suppress sensorimotor activity in UAS-ChR2H134R-mcherry;Trh-Gal4 (homozygous for both constructs) larvae which were not fed ATR (Figure 4.16 B1). But blue light exposure, suppressed sensorimotor activity in UAS-ChR2H134R-mcherry;Trh-Gal4 (homozygous for both constructs) larvae which were fed food supplemented with ATR (1mM) (Figure 4.16 B2).

### **Fluoxetine and sensory-motor activity**

The sensory-motor circuit was stimulated at 40Hz, 10 stimuli, and the EPSPs were recorded in saline (control) (Figure 4.17 A). Afterwards, fluoxetine 10µM or 100µM was applied to observe how it influenced the sensorimotor circuit physiology. Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) which would potentially

cause a rise in the 5-HT within the synaptic cleft. Application of fluoxetine 100 $\mu$ M significantly reduced the number of EPSPs (Figure 4.17 B). After 10min incubation time, fluoxetine nearly completely suppressed the sensorimotor activity. However, no significant reduction was observed when fluoxetine 10 $\mu$ M was applied to dissected third instar larvae (Figure 4.17 C).

## Discussion

In the current study, we investigated the role of the 5-HTergic system in locomotion and feeding behaviors as well as activity in a sensorimotor circuit. 5-HT alone can enhance the evoked activity; however, 5-HT likely interacts with other known neuromodulators such as, dopamine and octopamine, to fulfill specific physiological or behavioral functions (Chen et al. 2013). It has been demonstrated that dysregulation in the 5-HTergic system is related to various psychological conditions such as depression, anxiety, learning and memory impairment, schizophrenia and autism in humans (Sodhi and Sanders-Bush, 2004). The disruption of physiological serotonin level in critical periods during mammalian development is postulated to abnormal development of neural circuitry that consequently results in the long-lasting psychological disorders (Nordquist and Oreland, 2010). Many studies have already shown that 5-HT plays miscellaneous physiological and behavioral roles in *Drosophila*. Also, it is known 5-HT levels play a role in development of the 5-HTergic neurons in the central nervous system (CNS) of *Drosophila* (Sykes and Condrón, 2005). A previous study has demonstrated that 5-HT<sub>2</sub> receptors are expressed in *Drosophila* during the embryonic stage (Colas et al. 1995), and its physiological level of expression is essential for the normal development and survival (Schaerlinger et al. 2007). However, the physiological role of 5-HT receptor subtypes in various neural circuit activities had yet to be further investigated.

## 5-HT action and locomotion behavior

Neuromodulators, such as dopamine and serotonin, play a role in changing the activity of locomotor neural networks (Sharples et al. 2014). Many studies have shown that 5-HT is implicated in locomotion behavior in diverse animals and relatively simple organisms such as *C. elegans* (Vidal-Gadea et al. 2011; Flavell et al. 2013). It has been shown that the sensory input is indispensable for the locomotion activity of *Drosophila* larvae (Song et al. 2007). The rapid central drive in locomotion is rapidly altered when an organism confronts eminent danger, which may well be influenced by neuromodulators. Neuromodulators are found in *Drosophila* which function to modulate specific behaviors (Chen et al. 2013) and thus maybe even survival in the outdoor environment under predatory stress. It was also shown recently that various 5-HT receptors have a role in *Drosophila* larvae locomotion (Silva et al. 2014) and that 5-HT is involved in larval turning behavior (Okusawa et al. 2014). In the study herein, the 5-HT system was manipulated by using both pharmacological and genetic approaches and complements some aspects of the results reported in Silva et al. (2014). We showed that feeding larvae high levels of 5-HT or 5-HTP significantly reduced body wall contractions as well as reduced the body length. It is likely that 5-HTP also leads to an increase in 5-HT synthesis but this does not necessarily mean more 5-HT is released with synaptic transmission. It was shown that administration of 5-HTP increases the level of 5-HT in adult flies (Dierick and Greenspan, 2007). 5-HT could alter *Drosophila* development by acting on insulin producing cells (IPCs) in the brain (Kaplan et al. 2008; Ruaud and Thummel, 2008; Luo et al. 2012; Nässel et al. 2013). This could prepare the organism for increased metabolic activity which can also be induced by promoting locomotive activity. The mechanism for the retarded growth with very high levels of 5-HT are only speculative at this time. Additionally, the Trh blocker, PCPA, significantly reduced locomotion behavior and it was demonstrated that PCPA administration results in the reduction of 5-HT in larval CNS (Dasari et al. 2007). These results demonstrate that dysregulation of 5-HT with either too much or

too little 5-HT influences locomotion behavior. We can infer that 5-HT biosynthesis homeostasis is essential during development for normal behavior in later stages in life. We further investigated the effect of 5-HT biosynthesis disturbance on locomotive behavior by over expression of the Trh gene specifically in 5-HTergic neurons which also slowed larval locomotion.

In order to determine if 5-HT was increased in the synaptic cleft of 5-HT releasing neurons we used an SSRI (fluoxetine). Such potential increase in synaptic 5-HT also decreased locomotion behavior. The effect was even more substantial in the Trh mutant larvae. It has been confirmed that in Trh mutant larvae, the 5-HT level is reduced in 5-HT neurons. Possible residual 5-HT in neurons might be due to the 5-HT uptake by dSERT from the hemolymph, which is made by the peripheral DTHPu. This may explain why administration of fluoxetine (5mg/ml) further reduces 5-HT level in Trh mutant 5-HT neurons (Neckameyer et al., 2007). The action of fluoxetine may indeed have other effects and side effects in invertebrates. The affinity of fluoxetine for *Drosophila* SERT was previously determined to be lower ( $K_i=72$  nM) than for mammals (Demshyshyn et al., 1994). High concentrations of fluoxetine are toxic to larval and adult *Drosophila* (Majeed, et al., 2015) so we kept with a 10 mM concentration for this study.

### **Genetic manipulation of 5-HTergic system**

Increasing the activity of 5-HT neurons during early stages of development through activation of the TrpA1 channels, selectively targeted these neurons and increased the locomotion behavior in third instar larvae. This is in contrast to the other manipulations in the levels of 5-HT which all decreased locomotion. Potentially over a long period of heightened neural activity there might be some compensation by possibly downregulating the expression of 5-HT receptors or possibly desensitization of the receptors. Since we do not know if these neurons may have fatigued in the ATP production required for vesicular fusion and recycling or possibly depleted the release

of 5-HT with the electrical activity more definitive studies are needed to answer these questions. Further investigations with periodic heat pulses would be interesting to pursue. Various 5-HT receptor subtypes (5-HT1A, 5-HT1B, 5-HT2A, 5-HT7; Blenau and Thamm, 2011; 5-HT2B, Gasque et al. 2013) are expressed in *Drosophila*; thus, our approach with RNAi in 5-HT1B knockdown, in 5-HT1B expressing neurons could specifically examine this receptors role in locomotion. It is interesting to note the 5-HT1B knockdown significantly reduced the locomotion but 5-HT2A and 5-HT7 receptor knockdown did not have an effect. It is known that 5-HT acts on serotonergic neurons through activation of 5-HT autoreceptors to orchestrate the serotonergic neuron branch density (Daubert and Condrón, 2010; Sykes and Condrón, 2005). Thus, the knockdown of 5-HT1B during early stages of development might increase the 5-HT neurons branching pattern which might result in increasing 5-HT concentration thereby changing the development of the neural circuitry. This is another interesting avenue to follow up in the possible anatomical restructuring.

In a recent study, it has been shown that 5-HT receptor subtypes (5-HT1A, 5-HT1B, 5-HT2 or 5-HT7) knockdown in the nervous system increases locomotion activity when the flies were raised at 19°C until the day before examining the behavior (Silva et al., 2014). However, in our study, we raised the flies at 30°C during early development. The paradoxical results might be due to the effect of 5-HT1B on larval development since the Gal4 activity would be high at 30°C compared to 19°C (Duffy, 2002). In addition, the higher temperature might increase the efficiency of the knockdown. The efficiency of the 5-HT2 and 5-HT7 RNAi lines to knock down 5-HT2 and 5-HT7 could be low since we did not observe a significant change in locomotion behavior. 5-HT receptors insertional mutant lines were also used and the results indicate that 5-HT2A and 5-HT7 insertional mutations significantly decreased locomotion behavior. However, Trh mutant larvae did not show a significant change in body wall contractions. Our results are consistent with Silva et al., (2014) which demonstrated that 5-HT7 mutation leads to the reduction in locomotion activity;

however, contradictory to Silva et al., (2014) where the Trh mutation resulted in the an increase in locomotive activity. In support to our findings for the Trh mutant, the results are consistent with Neckameyer et al., (2007), where they demonstrated no significant difference between  $w^{1118}$  and Pbac-Trh larvae body wall contractions. The contradictory results might be due to the using different approaches to measure the locomotion activity in various studies. We wanted to address the effect of Trh overexpression on larval locomotion and feeding behaviors as well since previous studies have shown that Trh overexpression, which increases the 5-HT level, would change various fly behaviors such as sleep (Yuan et al., 2006), and aggression (Dierick and Greenspan 2007). We observed that Trh overexpression significantly reduced locomotion and feeding behaviors (Figure 4.4 A and B), which corroborates the pharmacological results (Figure 4.2). In the study herein, the 5-HT<sub>2B</sub> receptor mutation, also, decreased body wall contractions in third instar larvae (Figure 4.8 A). Further support in 5-HT receptors being altered in their expression levels and that the Pbac-Trh larvae decreased 5-HT synthesis which could led to up regulation of the receptors is that these larvae show an enhanced response to exogenous application of 5-HT on the exposed sensory-CNS-motor circuit. Together these results demonstrate that dysfunction of 5-HT receptors during development negatively influence locomotion behavior. Additionally, we selectively activated 5-HT neurons with the use of optogenetics which demonstrated an effect on the sensory-CNS-motor circuit.

### **Acute activation of 5-HTergic neurons and behavioral modulation**

Since an alteration in the 5-HTergic system during development might have effect on neural circuit development, it is difficult to pinpoint specific mechanisms to account for the behavioral changes. Therefore, we also used genetic tools to acutely manipulate 5-HT neurons in freely moving animals. The synaptic transmission of 5-HT neurons was blocked by expressing *shi<sup>ts1</sup>* allele in 5-HTergic neurons. Blockage of 5-

HT neurons did not have an acute effect on the locomotion activity. However, enhancing 5-HT release by increasing the activity of the 5-HTergic neurons, through TrpA1 channel activation, significantly reduced the body wall contractions in larvae, which corroborates the recently published findings of Pooryasin and Fiala, (2015). This could suggest that 5-HT spillover from 5-HT neurons in freely moving larvae has a negative effect on locomotion behavior. In a recent study, it was stated that activation of TrpA1 channels negatively impacts locomotion activity in *Drosophila* larvae (Okusawa et al., 2014). Furthermore, our results also show that suppression of synaptic transmission in 5-HT neurons did not cause a significant change in climbing behavior (Figure 4.10 A); however, acute activation of 5-HT by TrpA1 significantly reduced the locomotion activities in adult male and female flies (Figure 4.10 B1 and B2). It was previously shown that adult *Drosophila* expressing *sh<sup>ts1</sup>* allele in 5-HT neurons and control group would increase the locomotion activity when they are exposed to high temperature (30°C) compared to low temperature (19°C). This effect is related to the temperature change and not blocking the transmitter release by the neurons (Alekseyenko et al. 2010).

Since the temperature likely has an effect on *Drosophila* behavior per se we used an optogenetic approach to activate 5-HTergic neurons in freely moving larvae and adults. Our results show that activation of 5-HTergic neurons reduces locomotion behavior in third instar larvae and adult flies (Figure 4.16)

### **Modulatory role of 5-HT in feeding behavior**

The 5-HTergic system, also, plays a role in feeding behavior in invertebrates (French et al. 2014; Schoofs et al., 2014), and vertebrates (Donovan and Tecott, 2013). For the most part, increasing or decreasing 5-HT altered the feeding behavior. In a previous study, it was demonstrated that 5-HTP administration decreases feeding behavior in larvae; however, reduction of 5-HT level through RNAi-mediated Trh knockdown increases feeding behavior. On the other hand, increasing Trh level

decreases feeding. Also, the mutation in Trh gene increases the branching pattern of serotonergic neurons in proventriculus of the digestive system (Neckameyer, 2010). In our study, chronic activation of 5-HT neurons by TrpA1 channels increased feeding behavior. However, 5-HT1B and 5-HT2A receptor knockdowns significantly decreased mouth hook movements, and 5-HT7 receptor knockdown did not produce significant change in feeding behavior. It was demonstrated previously that blocking 5-HT2A receptors result in a reduction of feeding behavior (Gasque et al., 2013) which concurs with our studies. 5-HT1B receptor knockdown might increase the branching pattern of the 5-HT neurons to locate a target which can provide feedback regulation thereby increasing 5-HT release as volume transmission and suppressing feeding behavior. 5-HT2A and 5-HT7 mutant larvae significantly decreased mouth hook movements. However, Trh mutant larvae did not show a significant reduction in feeding behavior. The reduction in 5-HT7 mutant feeding behavior might be due to the weak musculature structure because the larvae also had a significant reduction in body wall contractions. The reduction of feeding behavior in 5-HT2A mutant larvae confirms the results of 5-HT2A knockdown larvae feeding behavior. 5-HT2B mutant larvae did not show a significant change in feeding behavior compared to *w<sup>1118</sup>* larvae. Taken together, our data demonstrate that 5-HT receptors play a role in feeding behavior of *Drosophila* larvae.

### **Sensorimotor neural circuit modulation by 5-HT**

It is already known that 5-HT application to the dissected third instar larvae increases the evoked sensorimotor circuit activity (Dasari and Cooper, 2004; Dasari et al., 2007); even though, the 5-HT receptor subtypes that mediate modulatory action of 5-HT had yet to be investigated. In the herein study, the results show that 5-HT increased sensorimotor circuit activity at low (100nM) concentrations but decreased the effect at higher concentrations. This might be due to the desensitization of 5-HT receptors. In attempts to understand which 5-HT receptor subtypes are acting within

the neural circuit the various agonists were applied. For dopamine it was recently shown that administration of a high concentration itself is toxic to *Drosophila* (Cassar et al., 2015). The mechanism of this toxicity has yet to be determined. Thus, caution should be considered when exposing the *Drosophila* larvae or adults to high concentration of any biogenic amines.

Since some of the 5-HT agonist responded in a similar fashion as 5-HT, it appears that 5-HT<sub>2</sub> and 5-HT<sub>7</sub> mediate the modulatory action. Genetic approaches were used to confirm the pharmacological results. 5-HT<sub>2A</sub> mutant larvae (homozygotes) were less responsive at 100nM concentration compared to control group; however, 5-HT<sub>7</sub> mutant larvae (heterozygote mutants +/-) were responded well compared to control group. 5-HT<sub>7</sub> mutant larvae (heterozygote mutant +/-) might have enough 5-HT<sub>7</sub> receptors to respond well to 5-HT or 5-HT<sub>7</sub> receptors might work cooperatively with 5-HT<sub>2</sub> receptors to increase the circuit activity. Also, the Trh mutant larvae were very sensitive to exogenous 5-HT application. Since Trh mutant 5-HT neurons have less 5-HT (Neckameyer et al., 2007); they might compensate for that by increasing sensitivity of the 5-HT receptor expressing neurons to 5-HT. In future studies, it would be good to try some additional agonists and antagonists, such as clozapine, which are known to have a function in other species of insects (Röser et al., 2012).

We have shown that acute activation of 5-HT neurons by TrpA1 channels decreased locomotion behavior in larvae. However, we do not know the underlying cellular mechanism behind this phenomenon. Therefore, 5-HT neurons were activated by TrpA1 channels while the evoked sensorimotor activity was being recorded. We noted that reduction in locomotion activity is due to the reduction in motor output (Figure 4.15). Activation of TrpA1 channels in motor neurons, also, resulted in the reduction in locomotive activity but this reduction is due to the spastic contraction of the animals. We have demonstrated that fluoxetine administration reduced locomotion behavior; therefore, we applied fluoxetine to dissected third instar larvae while evoked

sensorimotor activities were being recorded. Fluoxetine at 100 $\mu$ M significantly reduced sensorimotor activity; however, at a lower concentration, it did not have an effect. A recent study has demonstrated that application of 100 $\mu$ M of fluoxetine while stimulating channelrhodopsin-2 expressing 5-HT neurons results in blockage of 5-HT reuptake in *Drosophila* larval ventral nerve cord (Xiao et al., 2014). It has been shown that fluoxetine can block ion channels (Hahn et al., 1999; Traboulsie et al., 2006); therefore, the interpretation of these results is difficult since we have to ensure that the observed effect of fluoxetine is due to the dysregulation of 5-HT level and no other possible non-selective side effects (see Majeed et al., 2015). This opens new avenues for the future studies on the mechanism of action of fluoxetine in altering the development in neural circuitry. In order to confirm the effect of 5-HTergic neurons activation by TrpA1 channels, we also used channelrhodopsin-2 to remotely modulate the excitability of 5-HTergic neurons. The results show that 5-HTergic neuron activation, in ChR2-expressing larvae and adults, by blue light exposure significantly decreased locomotion activity in both third instar larvae and adult flies (Figure 4.16).

As to why Canton-S (CS) and  $w^{1118}$  controls flies shown differences in body wall contractions is not known but has been reported previously (Titlow et al., 2014). However, it is known the white gene product is part of tryptophan transporter (Mackenzie et al., 1999), and tryptophan is the precursor of serotonin. It has been shown that the white gene mutation results in the reduction of 5-HT, dopamine and histamine content of synaptic vesicles (Borycz et al., 2008); therefore, the reduction in these biogenic amine levels in  $w^{1118}$  larvae might account for the slow locomotion activity compared to CS larvae.

### **5-HT action in sensory-motor circuit activity: Hypothetical models**

The paradoxical action of exogenous 5-HT application in dissected third instar larvae and acute activation of 5-HTergic neurons on locomotion activity might be due to the activation of different 5-HT receptors and the degree of 5-HTergic neuron

activity. In mammalian system, the action of 5-HT also has shown various responses on spinal motor neuron activity. Low activity of 5-HTergic neurons activate 5-HT<sub>2</sub> receptors at the 5-HT-motor neuron synapse which in turn increases the excitability of motor neurons. This results in a higher frequency of motor neuron activity compared to the absence of activity in the 5-HTergic neurons. However, when the 5-HTergic neurons release a large amount of 5-HT, the 5-HT can reach 5-HT<sub>1A</sub> receptors outside the synapse which its activation reduces the activity of sodium channels, and in turn the frequency of action potentials decreases in motor neurons (Perrier and Cotel, 2015). As previously mentioned, the *Drosophila* genome has five known 5-HT receptor genes. 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> are coupled with G $\alpha$  inhibitory (G $\alpha$ i) (Saudou et al. 1992). Activation of 5-HT<sub>1A</sub> or 5-HT<sub>1B</sub> receptor subtypes leads to the reduction of cytosolic cAMP level due to the suppression of adenylate cyclase (AC) enzyme activity. 5-HT<sub>7</sub> is coupled with G $\alpha$  stimulatory (G $\alpha$ s) (Witz et al., 1990). When 5-HT<sub>7</sub> is activated, it will activate AC that leads to the increasing of cytosolic cAMP. In mammalian systems 5-HT<sub>2</sub> is coupled with the G $\alpha$ q protein and has also been shown for the fly *Calliphora vicina* (Röser et al., 2012) and other insects (e.g., honeybees, Thamm et al., 2013). When 5-HT binds to 5-HT<sub>2</sub> receptors, it will activate phospholipase C (PLC) enzyme (Nichols and Sanders-Bush, 2001). These receptors are expressed in various neurons in multiple regions of the nervous system in *Drosophila* (Johnson et al., 2011). It has been demonstrated that the sensory neurons of the gill withdrawal reflex in *Aplysia* can be sensitized by the action of 5-HT, which is released from interneurons. 5-HT binds with various 5-HT receptors which are coupled with either G $\alpha$ s or G $\alpha$ q. Active G $\alpha$ s protein would synthesize cAMP from ATP which in turn activates PKA. In some cells PKA can lead to blockage of K<sup>+</sup> channels by phosphorylation. However, active G $\alpha$ q can activate PLC which in turn activates PKC, leading to phosphorylation of synaptic vesicle proteins (Chi et al., 2003). We present a hypothetical model to explain the molecular mechanism of modulatory action of 5-HT in sensorimotor physiology (Figure 4.18). The action of 5-HT on neuronal activity is well studied in *Aplysia* model system

(Kandel et al., 2000). Here we hypothesized that 5-HT binds with 5-HT<sub>2</sub> receptor on pre-synaptic terminals activating G $\alpha$ q and consequently phospholipase C (PLC), thereby enhancing protein kinase C (PKC). Active PKC can then lead to the established phosphorylation of synaptic vesicle proteins to increase docking of synaptic vesicles. 5-HT might also activate 5-HT<sub>7</sub> receptor which activates G $\alpha$ s protein, which activates protein kinase A (PKA). Active PKA can also phosphorylate various synaptic vesicle proteins also enhancing the number of docked vesicles and probability of evoked release. Local messenger RNA (mRNA) translation to make proteins, which is important for modulation of synaptic strength, might be another possible mechanisms for the modulatory action by 5-HT. It was demonstrated that during long-term facilitation in an *Aplysia* sensorimotor synapse, mRNAs were being translated at the synaptic site (Wang et al., 2009).

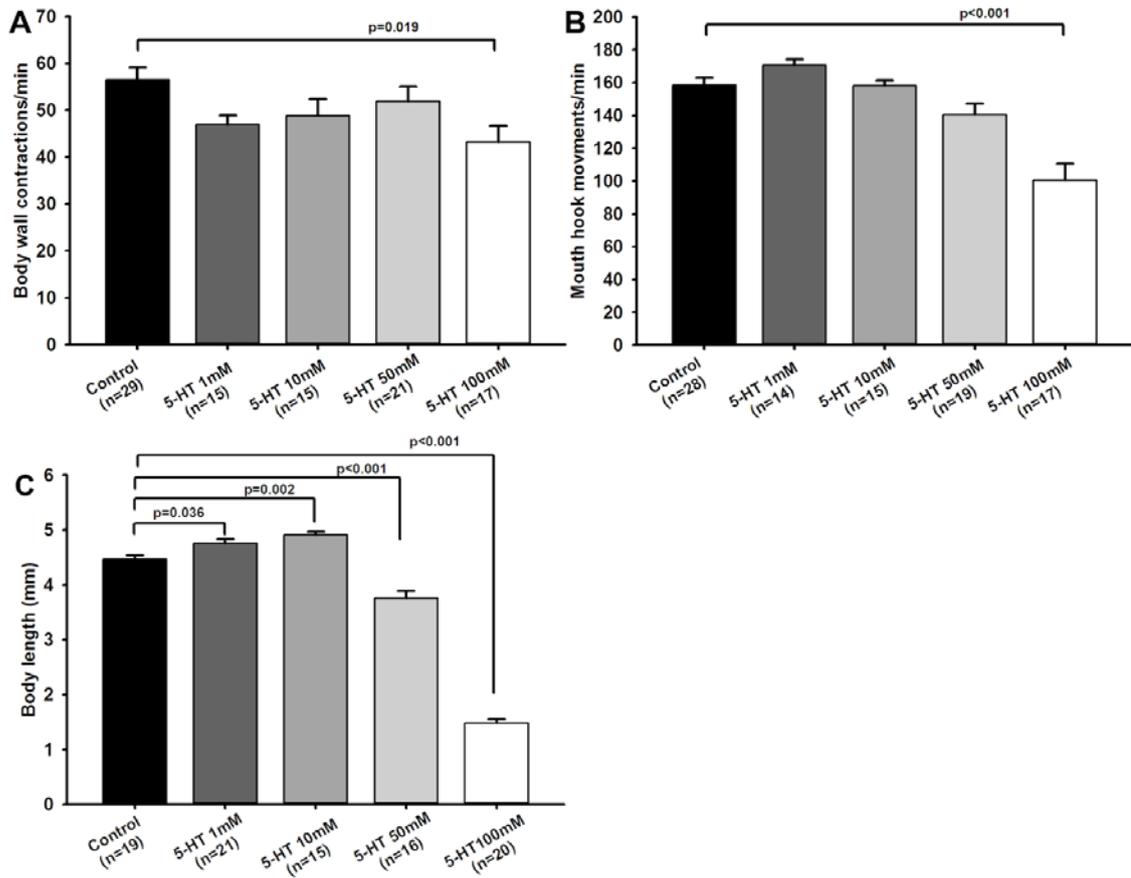


Figure 4.1: Serotonin modulatory action in feeding and locomotion behavior. (A) Effect of 5-HT on larval locomotion behavior. Canton-S (CS) larvae fed food containing 5-HT at 100mM had significantly reduced body wall contractions. (B) 5-HT at 100mM significantly reduced mouth hook movements. (C) 5-HT 1mM and 10mM significantly increased body length. However, 5-HT at 50mM and 100mM significantly reduced body length in comparison to control animals (CS). One way ANOVA was used for multiple comparisons, and Bonferroni t-test was employed to compare treatments with control group. Data presented as mean $\pm$ SEM. P-value < 0.05 are significant.

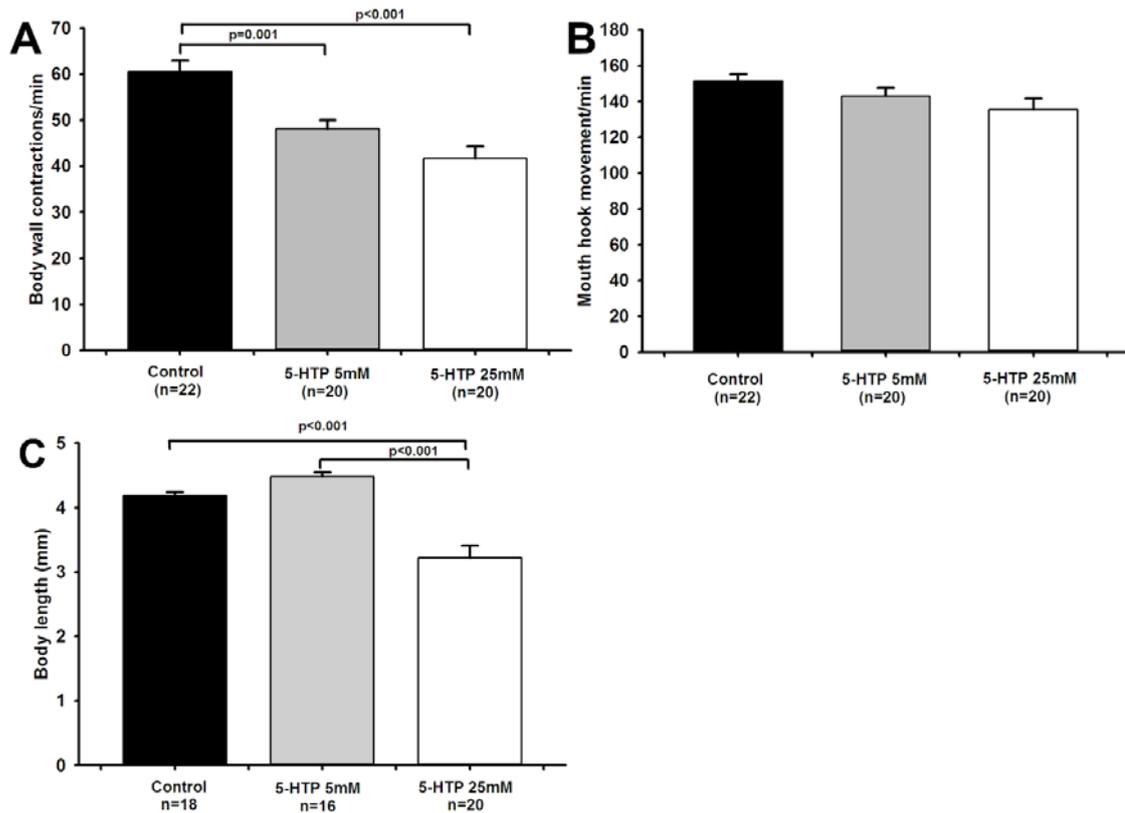


Figure 4.2: The effect of 5-hydroxytryptophan (5-HTP), a 5-HT precursor on feeding and locomotion behaviors. (A) 5-HTP (5mM and 25mM) significantly reduced the body wall contractions in larvae. (B) When the larvae were fed on food that mixed with 5-HTP, no significant difference was observed in feeding behavior in comparison to control animals (CS). (C) Body length significantly decreased at 25mM 5-HTP. One way ANOVA was used for comparison, and Bonferroni t-test was employed to compare treatments with control group. Data represented as mean $\pm$ SEM. P-value<0.05 is considered significant.

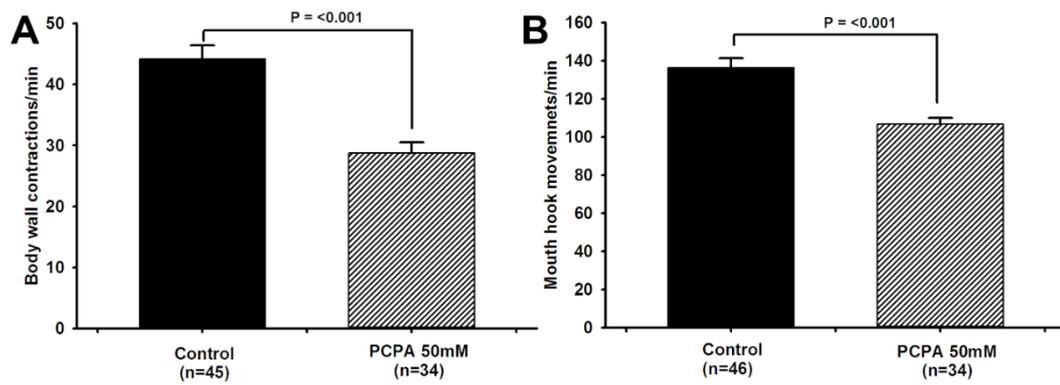


Figure 4.3: The behavioral consequences of blocking 5-HT synthesis. (A) Effect of tryptophan hydroxylase inhibitor, para-chlorophenylalanine (PCPA) on locomotion behavior. PCPA administration significantly decreased body wall contractions. (B) PCPA 50mM significantly reduced feeding behavior. Student's t-test was performed to compare between groups. Data presented as mean $\pm$ SEM.

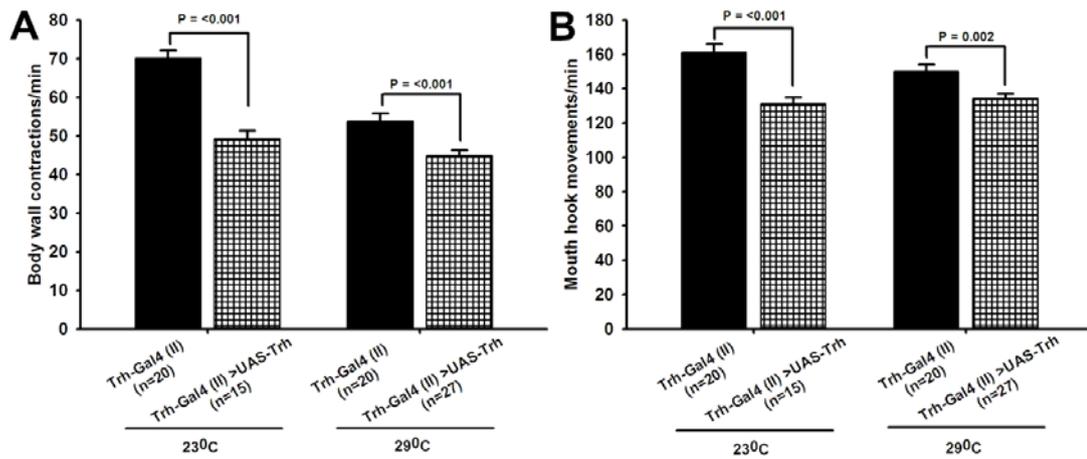


Figure 4.4: Overexpression of 5-HT biosynthesis rate-limiting enzyme, tryptophan hydroxylase (Trh) in serotonergic neurons. (A) Trh overexpression reduces body wall contractions. (B) Trh overexpression significantly decreased mouth hook movements as an indicator for feeding behavior. The flies were raised at 23°C as well as at 29°C to manipulate the expression level of transcriptional activator Gal4. Student's t-test was performed to compare between groups.

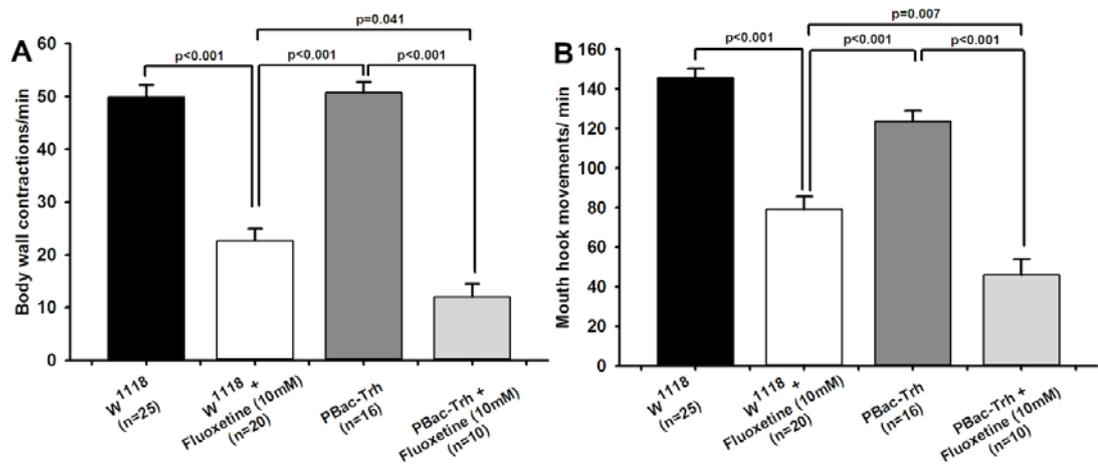


Figure 4.5: Serotonin reuptake inhibitor (SSRI), fluoxetine influences both locomotion and feeding behaviors. (A) Fluoxetine significantly decreased body wall contractions. Interestingly, fluoxetine had a greater effect on tryptophan hydroxylase (Trh) mutant animals than  $w^{1118}$  animals. (B) Fluoxetine significantly reduced mouth hook movements. Fluoxetine, also, had a greater effect on tryptophan hydroxylase (Trh) insertional mutant (homozygous) animals than  $w^{1118}$  animals. One way ANOVA was used for comparison, and Bonferroni t-test was employed to compare treatments. Data represented as mean $\pm$ SEM.

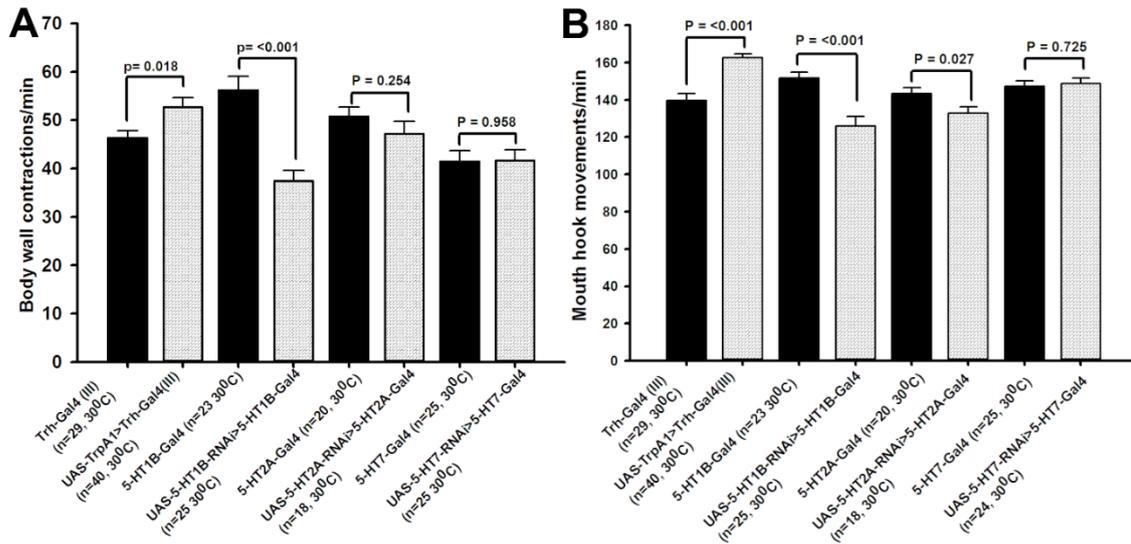


Figure 4.6: Manipulation of the 5-HTergic system. (A) Activation of serotonergic neurons, by using thermogenetic approach (TrpA1 channels), during early stages of development modulates locomotion behavior in later stage of development. RNA interference (RNAi)-directed 5-HT1B receptor knockdown during early stage of development significantly reduced the locomotion behavior in third instar larvae. (B) Activation of serotonergic neurons increased the feeding behavior. RNAi-directed 5-HT1B and 5-HT2A receptors significantly reduced the feeding behavior in third instar larvae. Student's t-test was performed to compare between groups. Data presented as mean $\pm$ SEM.

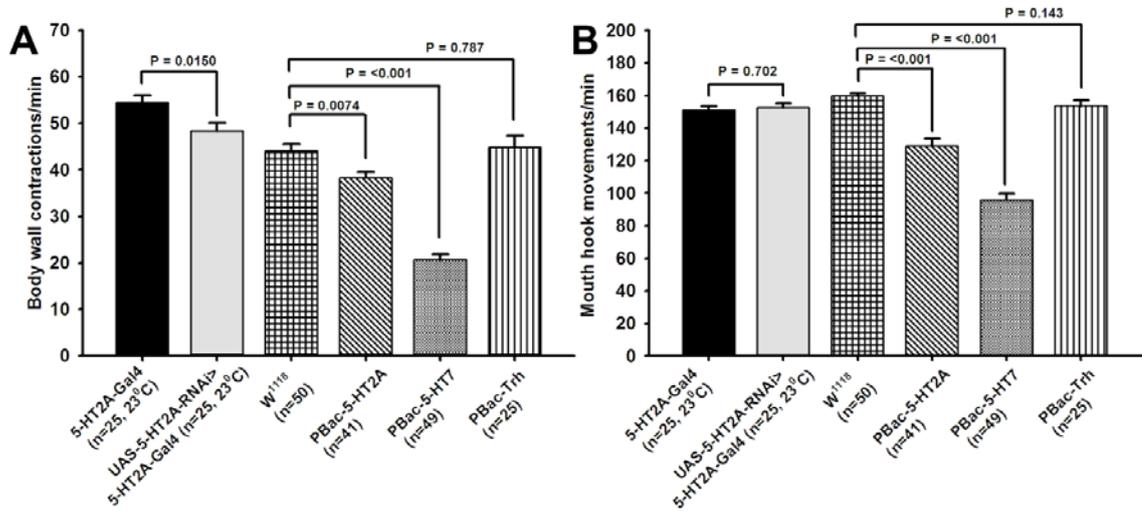


Figure 4.7: Serotonergic system dysregulation and behaviors. (A) Homozygous insertional mutations in 5-HT2A and 5-HT7 receptors significantly reduced body wall contractions in third instar larvae. However, homozygous insertional mutation in 5-HT biosynthesis rate limiting enzyme, tryptophan hydroxylase (Trh) did not have a significant effect on body wall contraction in comparison to *w<sup>1118</sup>* group. (B) Homozygous insertional mutation in 5-HT2A and 5-HT7 receptors significantly reduced mouth hook movements in third instar larvae. Even though, 5-HT2A receptor knockdown and homozygous Trh mutant larvae did not show noticeable effect on mouth hook movements. Student's t-test was performed to compare between groups. Data presented as mean $\pm$ SEM.

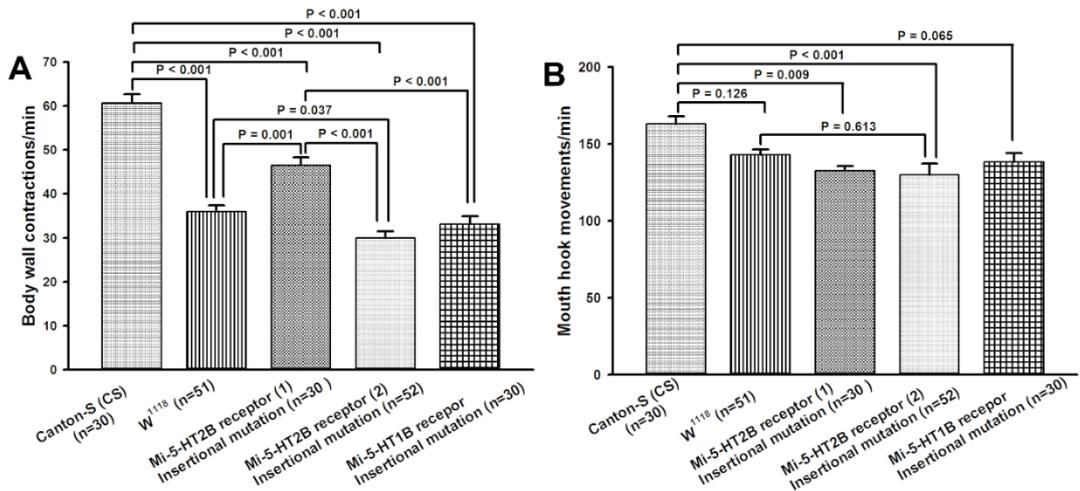


Figure 4.8: Manipulation in expression of mutated 5-HT receptors. (A) In all mutant lines, body wall contractions were significantly reduced in comparison to CS flies. However, 5-HT2B receptor (line2) insertional mutant larvae significantly reduced body wall contractions in comparison to *w<sup>1118</sup>* larvae. (B) Mouth hook movements were significantly affected in 5-HT2B receptor mutations in comparison to CS larvae; although, mouth hook movements in 5-HT2B receptor (line2) insertional mutant larvae were not significantly reduced compared to *w<sup>1118</sup>* larvae. One way ANOVA was used for comparison, and Bonferroni t-test was employed to compare treatments. Data represented as mean $\pm$ SEM. P-value<0.05 considered significant.

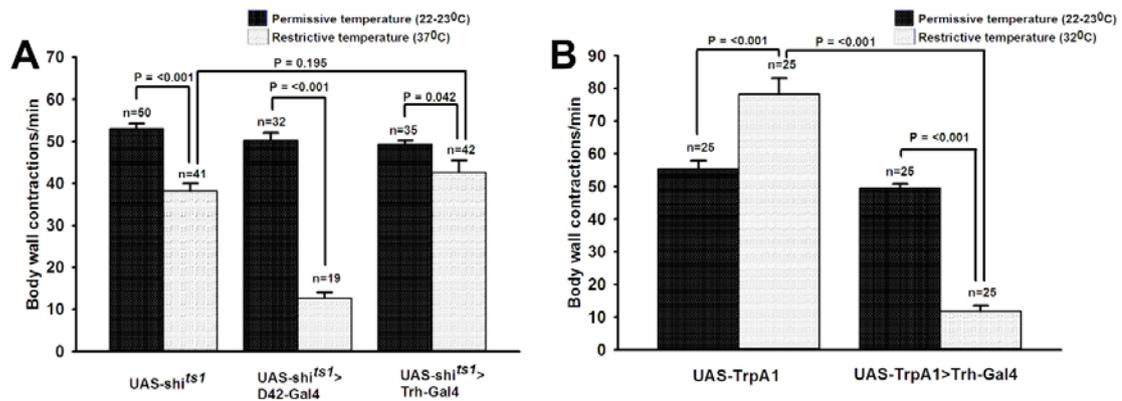


Figure 4.9: Acute manipulation of 5-HTergic neurons activity by *shi<sup>ts1</sup>* and TrpA1. (A) Synaptic transmission was blocked in the 5-HTergic neurons by expressing dominant negative temperature sensitive *shibire<sup>ts1</sup>* allele (*shi<sup>ts1</sup>*). Suppression of synaptic transmission in motor neurons (UAS-*shi<sup>ts1</sup>*>D42-Gal4) significantly reduced body wall contractions; however, suppression of synaptic transmission in 5-HT neurons (UAS-*shi<sup>ts1</sup>*>Trh-Gal4 (III)) did not significantly influence body wall contractions. The flies were raised at 23°C 12:12LD cycle. To obtain the locomotion behavior at room temperature for control and *shibire<sup>ts1</sup>* expressing larvae, an individual third instar larva was placed on the apple juice agar. The larva was left for 1min to acclimate, and body wall contractions were counted for the following 1min. To obtain body wall contractions at restrictive temperature for control and *shibire<sup>ts1</sup>* expressing larvae. The larvae were incubated at 37°C for 20min. The individual larvae were placed on pre-warmed apple juice agar on hot plate 30-32°C. The larva was left for 1min to acclimate, and the body wall contractions were counted for the following 1min. (B) Thermogenetic approach was used to activate 5-HTergic neurons. When TrpA1 channels are exposed to high temperature (32°C), they become activated which leads to inward current flux of cations. Activation of 5-HTergic neurons (UAS-TrpA1>Trh-Gal4 (III)) significantly decreases BWCs compared to control larvae (UAS-TrpA1). To obtain BWCs at room temperature, larvae were placed on apple juice agar dish at room temperature, and left for 2min to acclimate. Afterwards, BWCs were counted for 1min. The locomotor behavior at restrictive temperature (32°C) was obtained by placing one larva on a pre-warmed apple juice agar dish on a hot plate (32°C). The larvae were left for 2min, then the BWCs were counted for 1min. Student's t-test was performed to compare between groups. Data presented as mean±SEM. P < 0.05 is considered significant.

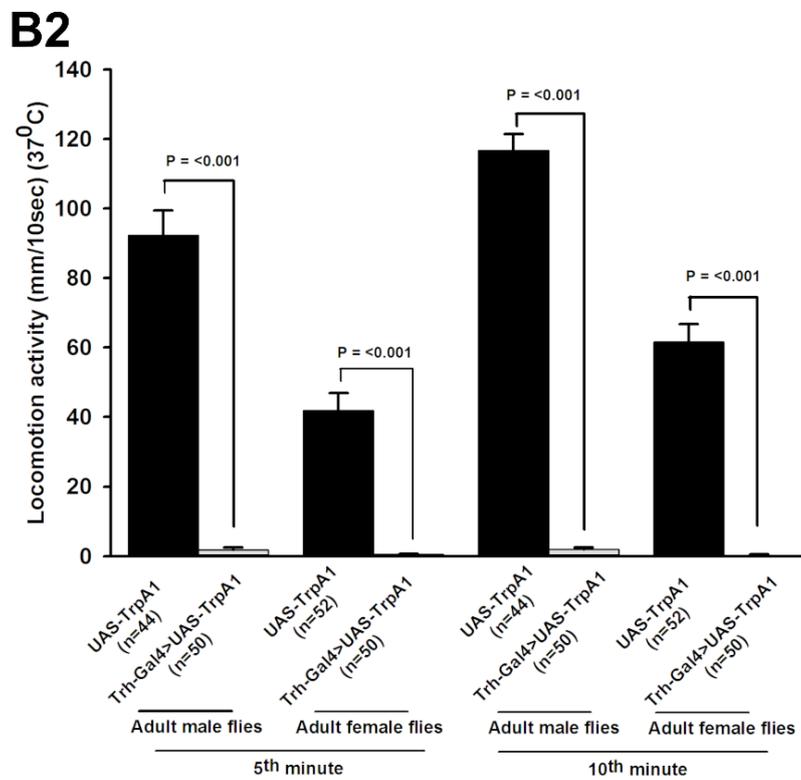
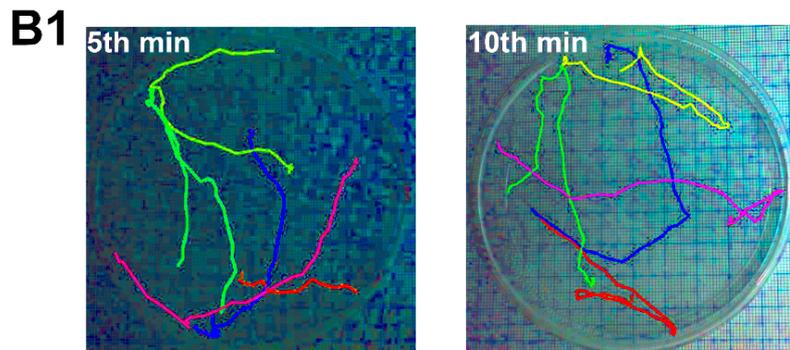
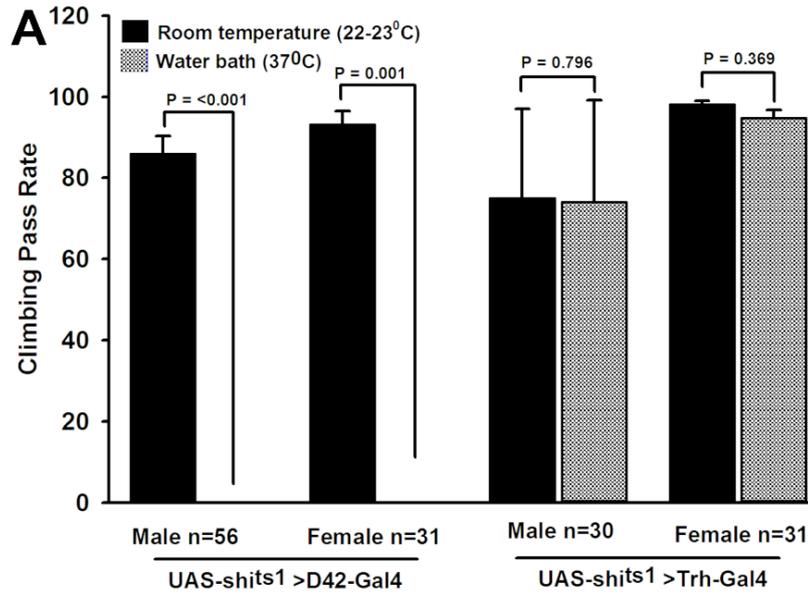
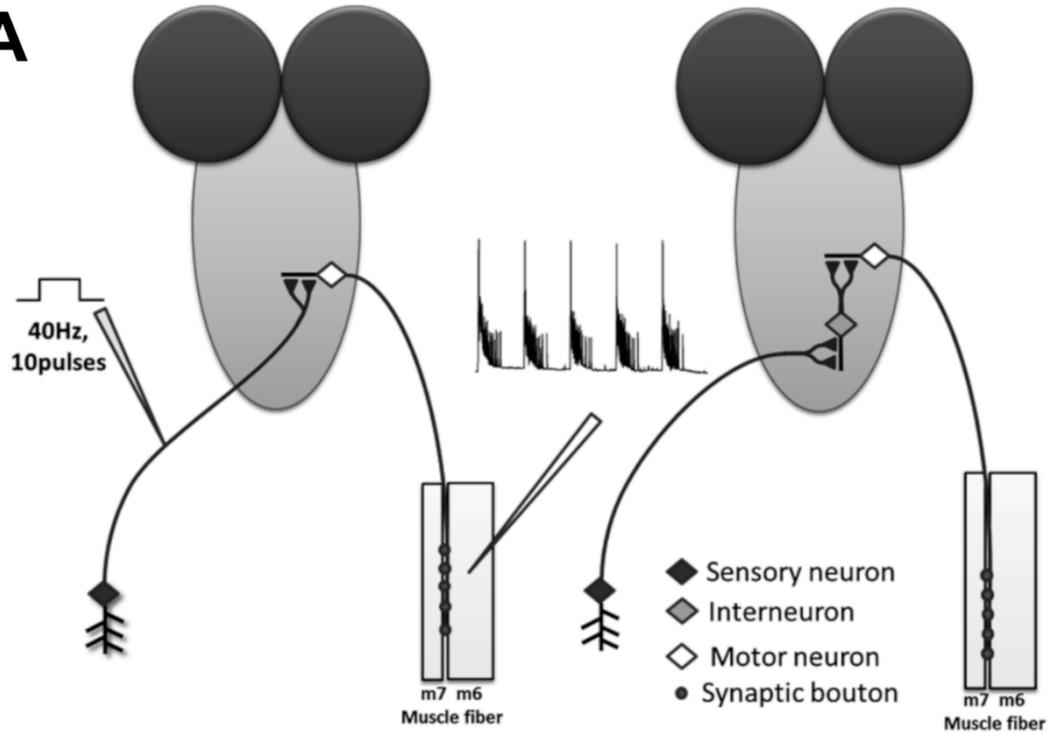
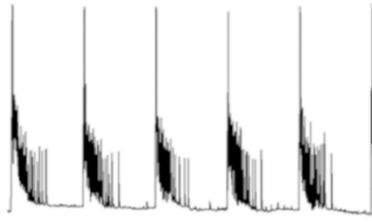
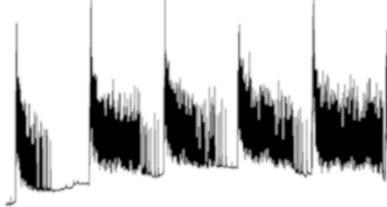


Figure 4.10: Manipulation of activity in 5-HTergic neurons within adult flies. (A) Acute inhibition of neuromuscular synapse neurotransmission by expressing *sh<sup>ts1</sup>* in motor neurons (UAS-*sh<sup>ts1</sup>*>D42-Gal4) significantly reduced climbing ability in adult female and male flies at restrictive temperature (37°C). This assay served as a proof of concept in the genetic crosses. However, acute blockage of serotonergic synaptic transmission (UAS-*sh<sup>ts1</sup>*>Trh-Gal4 (III)) did not influence climbing ability in adult flies. Student *t-test* was used to compare the climbing ability of the flies at room temperature (22-23°C) with the same flies after incubation in the water bath (37°C). (B1) Locomotion activity was recorded for 10 minutes in UAS-TrpA1 (control) and Trh-Gal4 (III)>UAS-TrpA1, which expressed TrpA1 in 5-HTergic neurons, flies at 37°C. The activity was analyzed for 10 seconds at the 5<sup>th</sup> minute and 10 seconds at the 10<sup>th</sup> minute. These two pictures show the locomotion activity of adult male flies (controls), 10sec of 5<sup>th</sup> and 10sec of 10<sup>th</sup> minute; respectively. (B2) Acute activation of 5-HTergic neurons in freely moving adults significantly reduced the locomotion activity in adult male and female flies. Mann-Whitney Rank sum test was used for comparison between the groups. Data represented as mean±SEM.

**A****B** saline5-HT (10 $\mu$ M)

saline

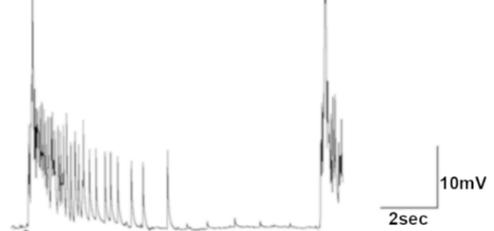
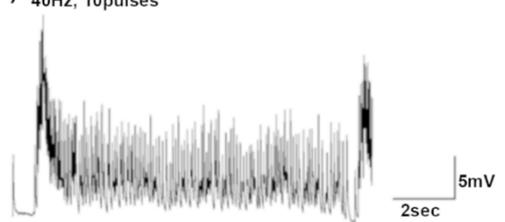
5-HT (10 $\mu$ M)

Figure 4.11: The effect of 5-HT on a CNS circuit. (A) Shows two hypothetical models for the sensory-motor circuit. The first model is a monosynaptic model (left side) with sensory neurons directly making synaptic connections with motor neurons. The second model is a multisynaptic one in which the sensory neurons make synaptic connections with interneurons then the interneurons make connections with the motor neurons. The two segmental nerves that innervate the last segment were cut and pulled into a suction electrode. The segmental nerves were stimulated at 40Hz, 10pulses, and the excitatory postsynaptic potential (EPSPs) were being recorded from body wall muscle fiber 6 or 7. (B) The modulatory action of 5-HT on sensory-motor function in third instar larvae. Application of 5-HT noticeably increased the frequency of evoked EPSPs. The far right traces are enlarge depictions of one evoked burst shown to the left.

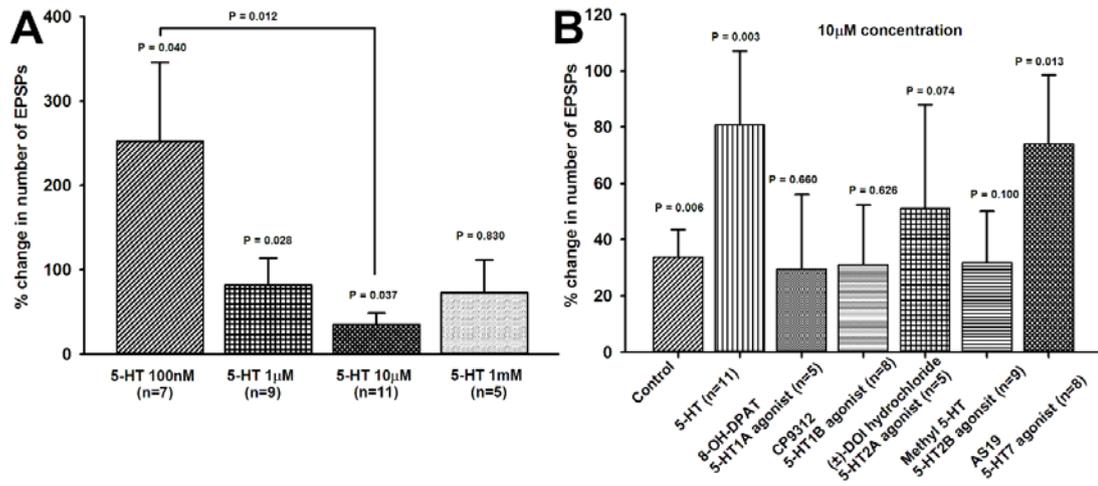


Figure 4.12: 5-HT and sensory-motor function modulation in third instar larvae. (A) Dose-response relationship of 5-HT in sensory-motor physiology. 5-HT significantly increased the frequency of EPSPs. The EPSPs of 8 stimulations were recorded for each saline and after exchanging saline with various 5-HT concentrations. (B) Effect of various 5-HT receptor agonists on the frequency of EPSPs in third instar larvae. Application of 5-HT and 5-HT<sub>7</sub> agonist significantly increased the number of EPSPs. The segmental nerve was stimulated at 40Hz, 10 pulses, and the EPSPs were recorded from muscle fiber 6 or 7. 7-10 stimulations were analyzed in each saline and after exchanging saline with a saline that contained agonist or in case of the controls fresh saline was used. Paired t-test was used to compare the number of EPSPs in saline and saline that contained an agonist. Data represented as mean±SEM. P < 0.05 is considered significant.

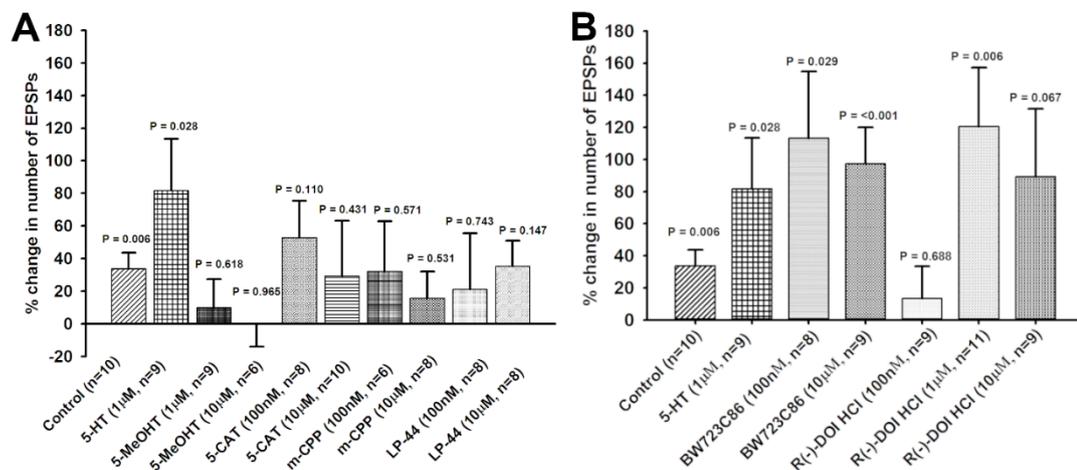


Figure 4.13: Modulation of motor-sensory function by 5-HT agonists. (A) Effect of various 5-HT agonists on the number of EPSPs in third instar larvae. The control group was obtained by changing saline with fresh saline to observe the effect of the saline switching process on EPSPs frequency. The results show that 5-HT significantly increased the number of EPSPs; however, 5-methoxytryptamine (5-MeOHT), 5-Carboxamidotryptamine maleate salt (5-CAT), 1-(3-Chlorophenyl)piperazine hydrochloride (m-CPP), and LP-44 (5-HT7 agonist) did not produce significant changes. (B) 5-HT2 agonists, BW723C86 (100nM and 10 $\mu$ M) (5-HT2B receptor agonist) and R(-)-DOI HCl (1 $\mu$ M) (5-HT2 receptor agonist) significantly increased the motor-sensory circuit activity. Paired t-test was used to compare the number of EPSPs in saline with saline that contained an agonist. Data represented as mean $\pm$ SEM.  $P < 0.05$  is significant.

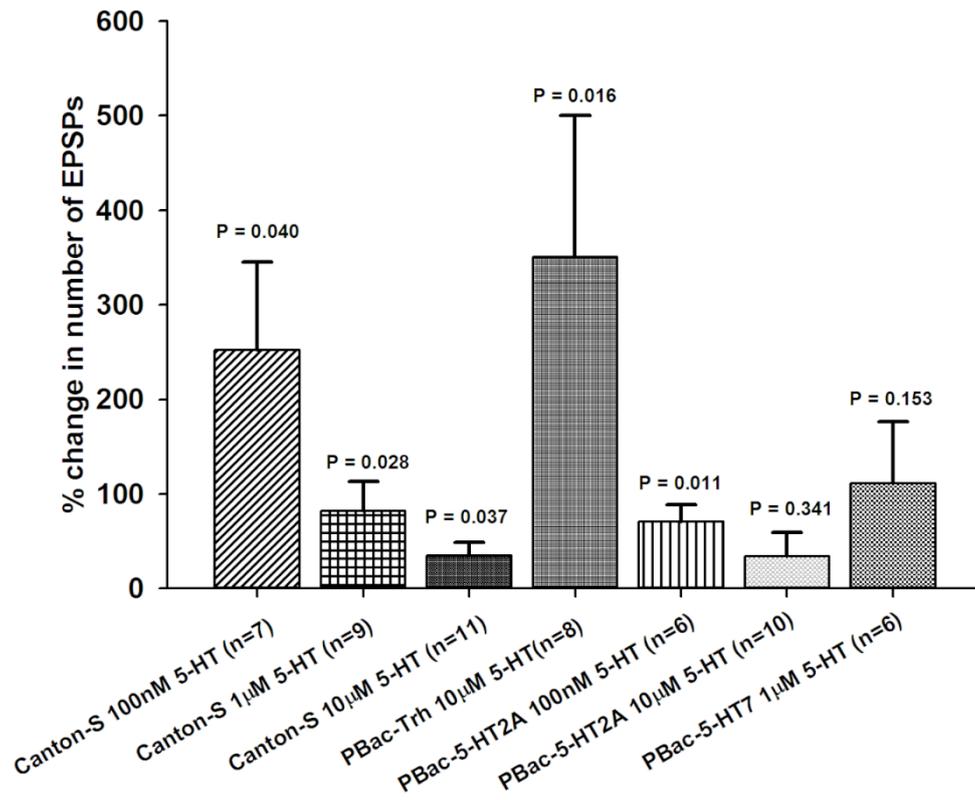


Figure 4.14: Effect in sensitivity to 5-HT modulation in lines with disrupted aspects within the 5-HTergic system. 5-HT application on tryptophan hydroxylase (Trh) mutant third instar larvae markedly increased sensory-motor circuit physiology. 5-HT at 10μM exposure in CS third instar larvae seems to desensitize 5-HT receptors since it does not markedly modulate neural circuitry in comparison to 5-HT 100nM. 5-HT2A receptor mutation significantly reduced the modulatory action of 5-HT. However, mutation in 5-HT7 receptor noticeably responds to 5-HT at 1μM. Paired t-test was used to compare the number of EPSPs in saline with saline that contained 5-HT. Data represented as mean±SEM. P < 0.05 is considered significant.

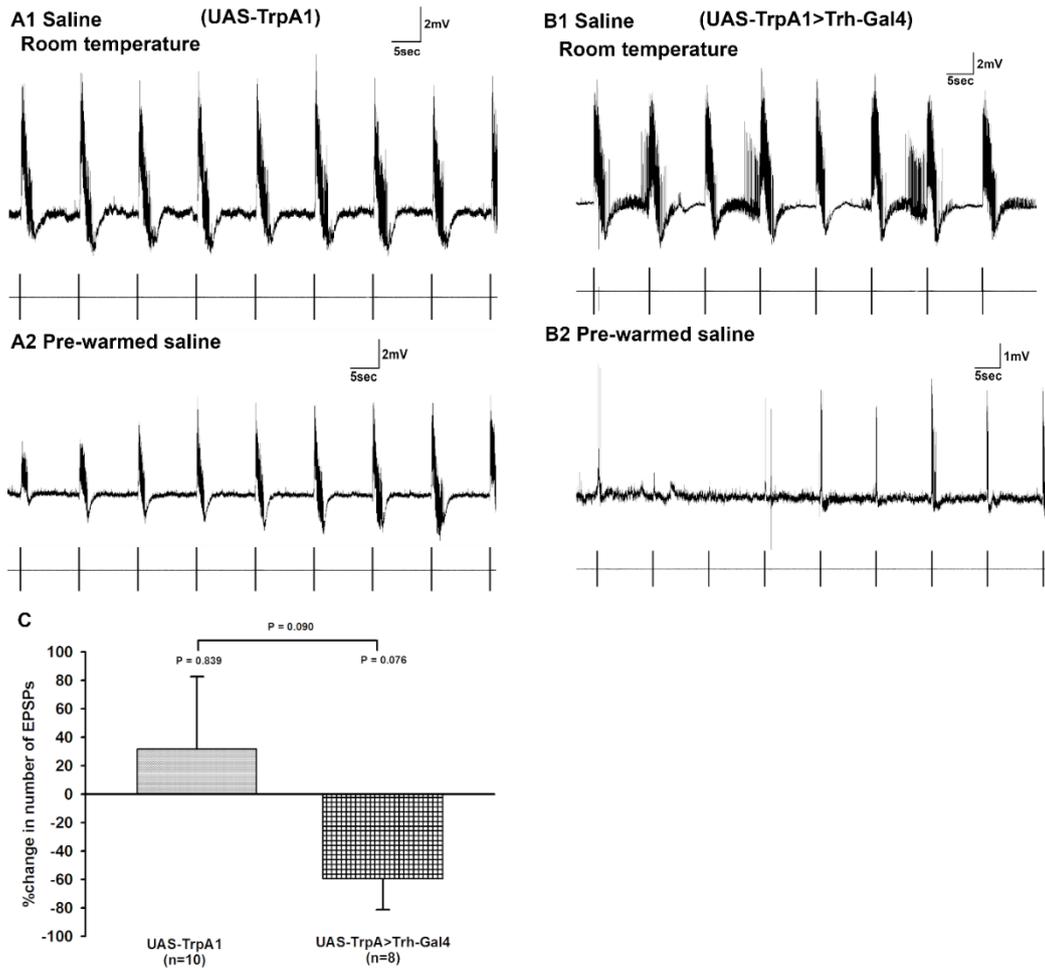
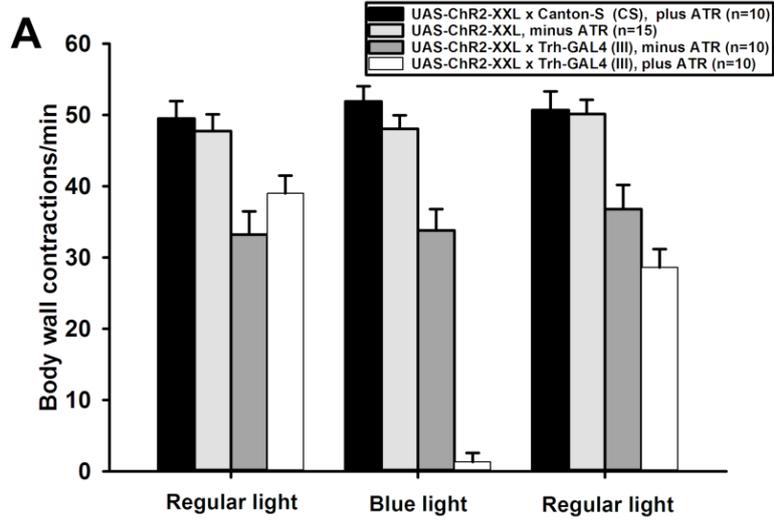
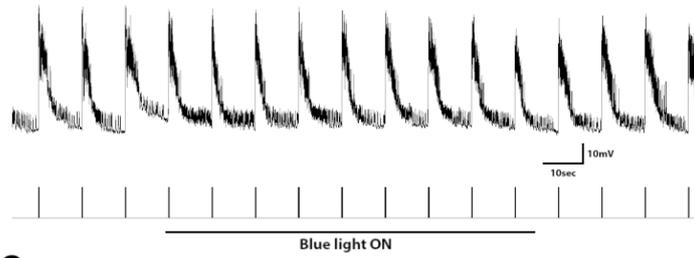


Figure 4.15: Induced activity in 5-HTergic neurons and sensory-motor physiology in third instar larvae. (A1) The segmental nerves of dissected control (UAS-TrpA1) third instar larvae were stimulated at 40Hz, 10pulses in saline at room temperature. (A2) The saline was exchanged with pre-warmed saline in a water bath (37°C) and then applied to the preparation at 32-34°C. (B1) 5-HTergic neurons expressing temperature sensitive cation channel, TrpA1 (Trh-Gal4 (III)>UAS-TrpA1). The evoked activity (EPSPs) of sensory-motor neural circuit was recorded at room temperature (22-23°C) saline. (B2) Saline was switched with pre-warmed saline in a water bath (37°C) when the evoked activity was being recorded. Activation of 5-HTergic neurons significantly reduced evoked neural activity. The pre-warmed saline was transferred to the dissection dish for both control and TrpA1 expressing third instar larvae. c Activation of 5-HT neurons markedly reduced the number of EPSPs compared to control larvae. Paired t-test was used to compare the frequency of EPSPs inside room temperature saline and pre-warmed saline. Student's t-test was used to compare different treatments. Data represented as mean±SEM.



**B1** UAS-ChR2H134R-mCherry;Trh-Gal4 (Homozygous) minus ATR (Vehicle, EtOH) (CNS intact) M6 fiber, n=8



**B2** UAS-ChR2H134R-mCherry;Trh-Gal4 (Homozygous) plus ATR 1mM (CNS intact) M6 fiber, n=7

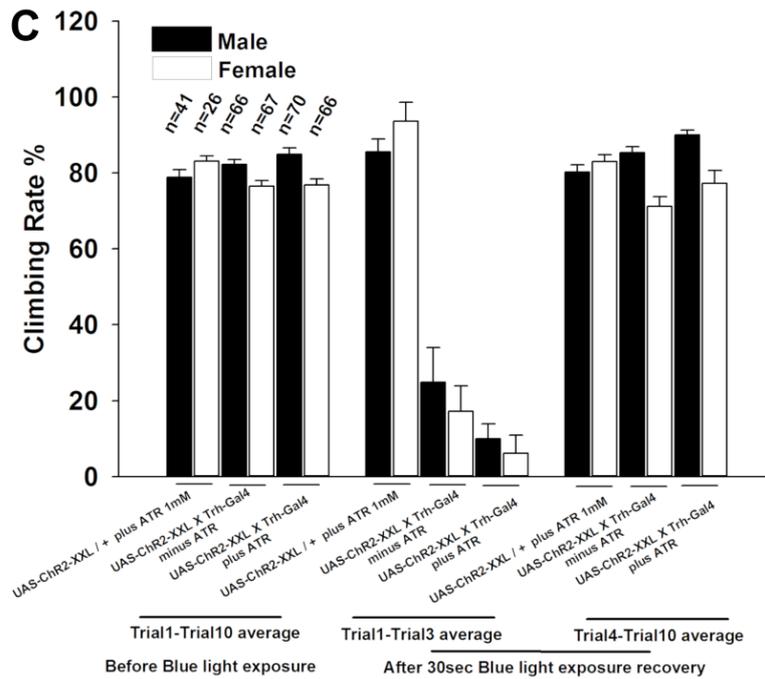
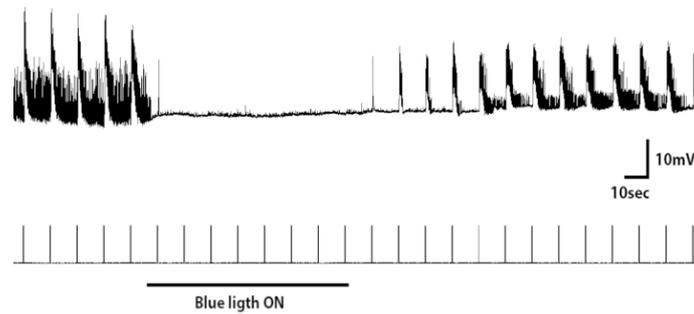


Figure 4.16: Serotonergic neuron activation modulates locomotor behavior in third instar larvae and adult flies. (A) To change serotonergic neuron activity, CHR2 was expressed in serotonergic neurons (UAS-ChR2-XXL/+;Trh-Gal4/+). The body wall contractions were counted in third instar larvae fed on food supplemented with ATR 1mM or ethanol (vehicle). When the larvae, which were fed on ATR 1mM, were exposed to blue light, the locomotor activity significantly compromised. However, when the larvae fed on a food without ATR supplementation were exposed to blue light, the locomotor activity were not significantly affected. (B1) Fly food not supplemented with ATR and exposed to blue light exposure did not suppress sensorimotor activity in dissected third instar larvae; however, (B2) serotonergic neuron activation by exposing the third instar larvae, which were fed food supplemented with ATR (1mM), to blue light shut down sensory-motor circuit activity. The sensory motor circuit was being activated by trains of stimulations at 40Hz, 10pulses while the motor output was being recorded in abdominal muscle 6 (M6). (C) Climbing assay in adult flies were used to test the locomotive ability of the flies. Activation of serotonergic neurons in adult flies decreases climbing ability. The excitability of serotonergic neurons is increased by expressing and activating ChR2 by blue light. When the adult flies were being exposed to blue light, the climbing ability significantly reduced. Both flies groups (UAS-ChR2-XXL/+;Trh-Gal4/+) which were fed supplemented with ATR 1mM or ethanol (vehicle) were affected by the blue light exposure. However, blue light did not have effect on the control lines (UAS-ChR2-XXL X Canton-S, shown as UAS-ChR2-XXL/+). The climbing assay was repeated 10 times and the average (Trial1-Trial10 average) was taken. There was a 1min rest interval between two trials before blue light exposure. Moreover, after exposing the flies to blue light for 30sec, the climbing assay was repeated for 10 trials. The average was taken for Trial1-Trial3 since the locomotion activity was decreased during this period. The average was taken for Trial4-Trial10, which the flies started recovering after blue light exposure.

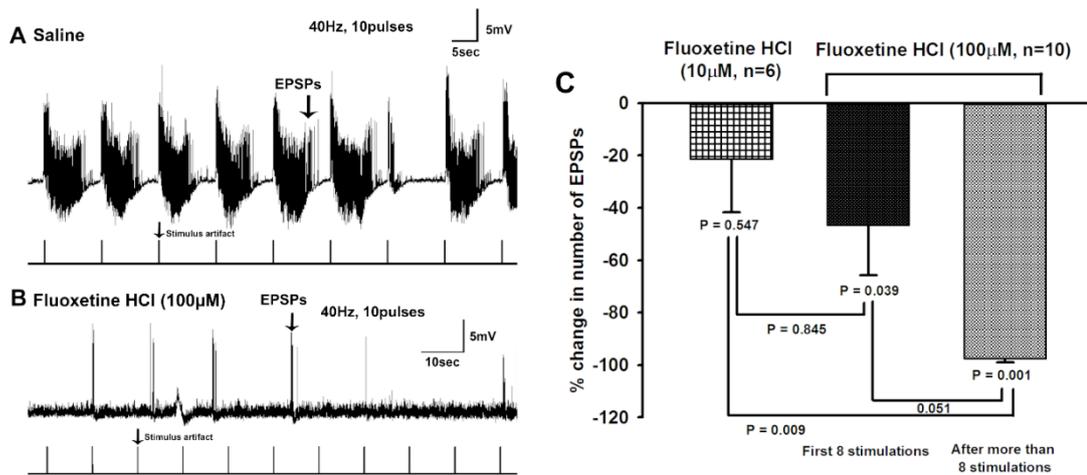


Figure 4.17: Motor-sensory function and action of a selective serotonin reuptake inhibitor (SSRI). (A) Evoked sensory-motor neural activity (EPSPs) was recorded from body wall muscle fiber 6 or 7 of third instar larvae in saline. (B) Application of fluoxetine (100µM) suppresses the evoked activity of the neural circuitry. (C) Fluoxetine at 10 µM did not significantly decrease activity but there is a decreasing trend in the mean change in the sensory-motor function. Paired t-test was used to compare the frequency of EPSPs inside saline and fluoxetine. One way ANOVA was used to compare different treatments. Bonferroni t-test was employed to obtain significant results. Data represented as mean±SEM.  $P < 0.05$  is considered as significant.

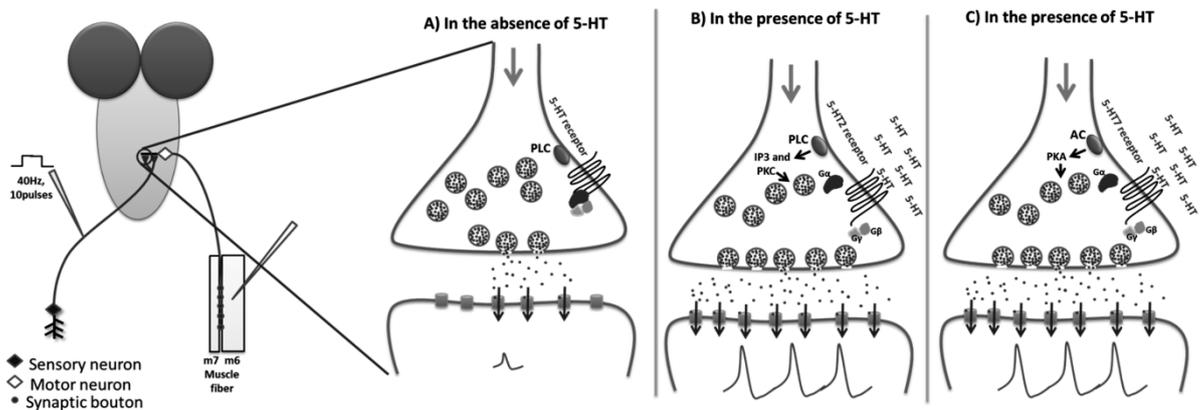


Figure 4.18: Hypothetical model for the modulatory action of 5-HT in sensory-CNS-motor neural circuitry in *Drosophila* larvae. (A) 5-HT<sub>2</sub> receptors are located in the membrane of the presynaptic terminal. In the absence of 5-HT the strength of synaptic transmission is weak. (B) 5-HT application results in the activation of 5-HT<sub>2</sub> receptors which enhances the synaptic transmission due to increment in probability of vesicles release. (C) The activation of 5-HT<sub>7</sub> receptors by the 5-HT or 5-HT agonist might activate adenylyl cyclase (AC) starting a cascade of cellular events.

## CHAPTER FIVE

### **New insights into the acute actions from a high dosage of fluoxetine on neuronal and cardiac function: *Drosophila*, crayfish and rodent models.**

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#### **Abstract**

The commonly used mood altering drug fluoxetine (Prozac) in humans has a low occurrence in reports of harmful effects from overdose; however, individuals with altered metabolism of the drug and accidental overdose have led to critical conditions and even death. We addressed direct actions of high concentrations on synaptic transmission at neuromuscular junctions (NMJs), neural properties, and cardiac function unrelated to fluoxetine's action as a selective 5-HT reuptake inhibitor. There appears to be action in blocking action potentials in crayfish axons, enhanced occurrences of spontaneous synaptic vesicle fusion events in the presynaptic terminals at NMJs of both *Drosophila* and crayfish. In rodent neurons, cytoplasmic Ca<sup>2+</sup> rises by fluoxetine (100µM) application. The *Drosophila* larval heart showed a dose dependent effect in cardiac arrest. Acute paralytic behavior in crayfish occurred at a systemic concentration of 2 mM. A high percentage of death as well as slowed developed occurred in *Drosophila* larvae consuming food containing 100µM fluoxetine. The release of Ca<sup>2+</sup> from the endoplasmic reticulum in neurons and the cardiac tissue as well as blockage of voltage-gated Na<sup>+</sup> channels in neurons could explain the effects on the whole animal as well as the isolated tissues. The use of various animal models in demonstrating the potential mechanisms for the toxic effects with high doses of

fluoxetine maybe beneficial for acute treatments in humans. Future studies in determining how fluoxetine is internalized in cells and if there are subtle effects of these mentioned mechanisms presented with chronic therapeutic doses is of general interest.

## **Introduction**

The serotonergic system is involved in many physiological and behavioral aspects of animals; therefore any dysregulation may lead to a wide range of disorders. Various medications, such as fluoxetine, have been developed that target the serotonergic system to ameliorate psychopathological symptoms (e.g. depression, obsessive-compulsive disorders, appetite, learning, and cognition). Fluoxetine (Prozac) is a commonly used medication for altering mood and the therapeutic action is mediated by blocking the reuptake of serotonin (5-hydroxytryptamine or 5-HT) into the presynaptic nerve terminals. Thus, fluoxetine is classified as a selective 5-HT reuptake inhibitor (SSRI); although, there are other mechanisms which contribute to the therapeutic effects of fluoxetine such as changes in microRNA expression which can result in the down regulation of SERT (Baudry et al., 2010). Moreover, it has been shown that fluoxetine can exert effects not related to the reuptake process (Donard et al., 2014). Usually the administration of fluoxetine does not result in severe side effects which explains its high use in prescriptions by physicians; however, high levels of fluoxetine in the body attained either by accumulation or augmented initial uptake can lead to serious health problems to even mortality. A case study documented that a child with cytochrome-P 450 2D6 genetic deficiency died because of an acute intoxication to fluoxetine (Sallee et al., 2000). The reduced metabolism caused the accumulation of the drug in the body resulting in the death of the patient. In 1999 five percent of acute intoxications lead to a fatal outcome; moreover, the treatment of ill patients (such as pulmonary disease or atrial arrhythmia) was even more severe

resulting in death within ten days after the beginning of the treatment (Harris and Heil, 2013).

Further study is needed to obtain a better understanding of mechanisms associated with the acute effects of fluoxetine; this might change the effectiveness of treatments to counteract the resulting pathological conditions of an overdose. There are various reports that fluoxetine can block ion channels in neurons within a range of 10-100  $\mu\text{M}$  (voltage-gated  $\text{Na}^+$ , Pancrazio et al., 1998;  $\text{K}^+$  channels, Tytgat et al., 1997;  $\text{Cl}^-$  channels, Maertens et al., 1999) and  $\text{K}^+$  channels in smooth muscle cells but this was varied depending on concentration (Farrugia, 1996). Deak et al., (2000) provided evidence of inhibition of T-, N- and L-type voltage-gated calcium channels by fluoxetine at a range of 1-10  $\mu\text{M}$  which is within the therapeutic dosages. In examining the potential actions of fluoxetine in altering neuronal circuitry, during development within the larval *Drosophila* CNS, we noted acute actions on neural responses with high doses. In this current study, we pursued this observation to determine why a model neuronal circuit being used was altered rapidly with a high (100  $\mu\text{M}$ ) exposure.

*Drosophila melanogaster* is not only a genetically tractable organisms but many of the physiological functions at a cellular level are conserved and have been shown to be similar to those found in mammals (Strausfeld and Hirth, 2013). In addition, the serotonergic system is known to alter the CNS activity in larval *Drosophila* (Dasari and Cooper 2004) which likely accounts for the altered functions in sleep and circadian cycles (Nichols 2007; Yuan et al. 2006), mating (Becnel et al. 2011) and eating (Gasque et al. 2013) behaviors as well as forms of memory (Johnson et al. 2011). We did expect to observe some changes in developmental timing with feeding fluoxetine to larvae since 5-HT does have an effect on exposed larvae (Dasari and Cooper 2004). In order to examine if the cellular mechanisms of action by fluoxetine are common in another invertebrate model, the crayfish animal model was used. The crayfish is commonly used as a model for synaptic transmission and neurophysiology (Katz and Kuffler 1946; Wiese, 2002; Cooper and Cooper, 2009). The motor unit associated with

opening the chelae on the first walking legs is well characterized (Cooper and Cooper, 2009). In this preparation the axon close to the terminals is large enough in diameter to obtain intracellular recordings; this allows one to assess actions of compounds on ion channels that shape the action potential (He et al., 1999; Sparks et al., 2003). The motor axons in *Drosophila* and in mammalian preparations are too narrow for readily obtaining intracellular recordings.

As with neurons, the regulation associated for developmental genes and ionic channels of the cardiac pacemaker as well as cardiac function is similar in some regards from *Drosophila* to humans (Bodmer, 1995; Bodmer and Venkatesh, 1998; Choma et al. 2011; Majeed et al., 2014; Titlow et al., 2013). During this investigation into the mechanisms of fluoxetine altering synaptic transmission it became apparent there might be an action on the release of  $\text{Ca}^{2+}$  from internal stores within the nerve terminal. So we used the *Drosophila* larval heart as another assay for perturbations in  $\text{Ca}^{2+}$  dynamics since previously it was demonstrated that this preparation is very sensitive to alterations in internal  $\text{Ca}^{2+}$  which is regulated by the plasma membrane pump (PMCA), the sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) and  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) (Desai-Shah et al., 2010).

Since this preliminary study focused on the fundamental mechanisms in the actions of high doses of fluoxetine, as noted with an overdose, we used mammalian glutamatergic neurons in culture for drawing parallels to commonalities in the acute effects noted for the *Drosophila* and crayfish preparations. Imaging of calcium flux within neurons was critical in differentiating extracellular and intracellular calcium dynamics in response to exposure of fluoxetine.

## **Materials and Methods**

Canton S (CS) *Drosophila melanogaster*, crayfish (*Procambarus clarkii*) and isolated brainstem neurons from mice were used in these experiments. The *Drosophila* were maintained on a 12/12 light dark cycle except when exposed to fluoxetine due to

the light sensitivity of the compound. Controls for the experimental conditions were treated in the same manner except the food was not tainted with fluoxetine. *Drosophila* were raised on a mixture of cornmeal-agar-dextrose. The crayfish were obtained from Atchafalaya Biological Supply (Raceland, LA, USA). Crayfish were housed in an aquatic facility and fed dried fish food weekly. The crayfish and *Drosophila* saline is defined in previous publications (Cooper and Cooper, 2009; Desai-Shah et al., 2010). All compounds for the salines were obtained from Sigma-Aldrich as well as fluoxetine-HCl.

### **Behavioral studies: *Drosophila***

The developmental and survival assays were carried out by selecting late 1<sup>st</sup> instars and placing them in food containing fluoxetine and allowing them to develop at 21°C. The vials were checked every 12 hrs and each individual pupa was marked on the side of the vials. The time to pupation and the time spent as a pupa were indexed for developmental time and survival rate (Li et al., 2002).

### **Behavior-Crayfish**

Tail flip and behavioral responses were performed as previously described (Kellie et al., 2001; Pagé et al., 2007; Sparks et al., 2003). Concentrated fluoxetine solution was injected into the animal (Listerman et al., 2000). The amount of concentrated fluoxetine to inject was determined by calculating the systemic dilution which would occur after injection. The volume of hemolymph is estimated to constitute 30% of the animal's gross weight (Gleeson and Zubkoff, 1977; Pagé et al., 2007). Saline injections of equal volume per weight of animal were used as sham controls.

## **Synaptic responses**

### **Crayfish-Evoked EPSPs at NMJ**

The recording of evoked EPSPs were performed in the distal fibers of the opener muscle from the first or second walking legs. The dissection, stimulation and recording techniques for this preparation are described in Cooper and Cooper (2009), Chung et al., (2012) and Crider and Cooper (2000).

### **Drosophila-Evoked EPSP at NMJ**

Evoked EPSPs were recorded in 3<sup>rd</sup> instar larval body wall muscle fibers (m6 or m7) in abdominal segment 3. The dissection, stimulation and recording techniques for this preparation are previously described (Desai-Shah and Cooper, 2009; Lee et al., 2009) with the exception of using a modified physiological saline (De Castro et al., 2014). Briefly, a third instar larvae was dissected in a dorsal side-up position. The internal organs were removed and the segmental nerve were severed at the base of ventral nerve cord. A segmental nerve, from third segment, was pulled into the suction electrode, which was filled with fly saline. Microelectrodes of 40 megaOhm were used for intracellular recordings. Muscle fiber 6 or 7 was impaled by microelectrode and the segmental nerve was stimulated at 40 Hz for 4 pulses in each train.

### **Spontaneous quantal events at NMJs of crayfish and *Drosophila***

In order to observe the spontaneous quantal responses, the same recording techniques were used as for obtaining the evoked responses in these preparations but in the absence of stimulating the motor nerves. The gain in the recording was increased from the recordings made for evoked responses to observe the small amplitude of the single quantal events (Wu and Cooper, 2012, 2013). In order to examine if fluoxetine was enhancing a Ca<sup>2+</sup> influx through calcium channels, cadmium (1 mM) was used prior to exposure and during exposure to fluoxetine (100 µM). Nerve

stimulated EPSPs are completely blocked with CdCl<sub>2</sub> at 1 mM in saline at *Drosophila* and crayfish NMJs (Bierbower and Cooper, 2013; Kuromi et al., 2004).

### **Action potential recording within crayfish motor neurons**

Evoked action potentials were recorded within the excitatory motor neuron that innervates the opener muscle in crayfish with 3M KCl filled microelectrodes. The nerve was stimulated at 1 Hz while monitoring the amplitude of the action potential prior to and during exposure to fluoxetine (10 µM, 100 µM, 500 µM). The technique to record intracellular action potentials in this preparation is described in detail (He et al., 1999).

### ***Drosophila* Heart Rate**

The *Drosophila* larval heart rate was monitored while directly exposing the heart to saline containing 100 nM, 1 µM, 10 µM or 100 µM fluoxetine. The larval dissection and importance of carefully monitoring of the saline pH for heart rate measures is described in video format as well as in text (Cooper et al., 2009; De Castro et al., 2014). Briefly, a third instar larvae was dissected in ventral side up position. The internal organs were removed carefully without damaging the cardiac tube. The dissected larvae was left for one minute, and the heart beats were counted for one minute inside saline. Then the saline was exchanged with saline containing various concentrations of fluoxetine. The heart beats were counted for one minute. A percent change in rate was determined from saline exposure to fluoxetine.

### **Neuronal culture of mice neurons**

Brainstem neurons were dissociated and cultured, as previously described (Brailoiu et al. 2013). Briefly, the mice were euthanized by cervical dislocation, the brains were quickly removed and immersed in ice-cold Hanks balanced salt solution (Mediatech, Manassas, VA). The cerebral cortex was dissected, minced, and the cells were dissociated by enzymatic digestion with papain, followed by mechanical

trituration. After centrifugation at 1000 g, fractions enriched in neurons were collected and re-suspended in culture medium containing Neurobasal-A (Invitrogen), which promotes the survival of postnatal neurons, 1% GlutaMax (Invitrogen), 2% penicillin-streptomycin-amphotericin B solution (Invitrogen) and 20% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). Cells were plated on round, 25 mm glass coverslips previously coated with poly-L-lysine (Sigma-Aldrich) in 6-well plates. Cultures were maintained at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. After 24 hours, the cultured neurons were transferred in complete growth medium containing 10% fetal bovine serum. The mitotic inhibitor cytosine β-D-arabino furanoside (1 μM) (Sigma-Aldrich) was added to the culture to inhibit glial cell proliferation (Schoniger et al. 2001). Cells were used for imaging after 2-4 days in culture.

### **Identification of glutamatergic neurons in mice CNS**

Glutamate levels were detected using an enzymatic assay (Duarte et al., 1996; Ayoub and Dorst, 1998; Innocenti et al., 2000). In the presence of glutamate, L-glutamic dehydrogenase (GDH) reduces 13-nicotinamide adenine dinucleotide (NAD<sup>+</sup>) to NADH, a product that fluoresces when excited with UV light. Provided that GDH and NAD<sup>+</sup> are added to the saline in which neurons were bathed, any glutamate released in the medium can be detected as an increase in NADH fluorescence. Neurons on coverslips were subsequently mounted in an open bath chamber (RP-40LP, Warner Instruments, Hamden, CT) on the stage of an inverted microscope Nikon Eclipse TiE (Nikon Inc, Melville, NY). The microscope is equipped with a Perfect Focus System and a Photometrics CoolSnap HQ2 CCD camera. During the experiments, the Perfect Focus System was activated. The neurons were pre-incubated with 1 mM NADP<sup>+</sup> and 50 U of glutamate dehydrogenase for 5 min. All the experiments were performed at room temperature (20–23°C). The fluorescence (excitation- 340 nm and emission - 460 nm) was acquired and analyzed using NIS-Elements AR software (Nikon).

## **Calcium imaging of neurons from mice**

Briefly, cells were incubated with 5  $\mu\text{M}$  Fura-2 AM (Invitrogen) in HBSS at room temperature for 45 min, and washed with dye-free HBSS. Coverslips were mounted in an open bath chamber (RP-40LP, Warner Instruments, Hamden, CT) on the stage of an inverted microscope Nikon Eclipse TiE (Nikon Inc., Melville, NY), equipped with a Perfect Focus System and a Photometrics CoolSnap HQ2 CCD camera (Photometrics, Tucson, AZ). During the experiments the Perfect Focus System was activated. Fura-2 AM fluorescence (emission 510 nm), following alternate excitation at 340 and 380 nm, was acquired at a frequency of 0.25 Hz. Images were acquired/analyzed using NIS-Elements AR 3.1 software (Nikon). After calibration, the ratio of the fluorescence signals (340/380 nm) was converted to  $[\text{Ca}^{2+}]_i$  (Grynkiewicz et al., 1985). In order to deplete the ER from calcium, primary neuronal cells were incubated in thapsigargin (1  $\mu\text{M}$ ) for 1 h prior to imaging.

## **Results**

### **Evoked and spontaneous EPSPs at NMJ response to fluoxetine**

Evoked transmission at the *Drosophila* larval NMJ (Figure 5.1 A1 and A2) and the crayfish opener muscle (Figure 5.1 B1 and B2) was rapidly and completely silenced upon exposure of saline containing fluoxetine (100  $\mu\text{M}$ ). The spontaneous EPSPs responses could still be observed while evoked EPSPs were blocked. With repetitive exchanging the bath with fresh saline, the evoked responses did not recover in either preparation. The resting membrane potential did not depolarize upon exposure to fluoxetine.

With increased amplification of the recordings the spontaneous events at the NMJs are more readily observed. In normal saline, the spontaneous events usually occur as discrete events and are of relatively low frequency in occurrence. The crayfish muscle fibers are larger and have a lower membrane resistance than the small *Drosophila* larval muscles, resulting in a better signal to noise ratio for the *Drosophila*

than crayfish muscle fibers to detect spontaneous events. In both preparations, the occurrence of spontaneous events increases upon exposure to fluoxetine. In order to examine if the increase in spontaneous events occurred due to  $\text{Ca}^{2+}$  leaking across the plasma membrane from the bath, a saline containing  $\text{Cd}^{2+}$  (1 mM) was exchanged with the normal saline before exposure to saline containing fluoxetine and  $\text{Cd}^{2+}$ . The 1 mM [ $\text{Cd}^{2+}$ ] is well established to block evoked transmission in both preparations (Bierbower and Cooper, 2013). The exposure to saline containing  $\text{Cd}^{2+}$  reduces the frequency of spontaneous events in all preparations ( $P < 0.05$ , rank sum). In five out of five preparations of the larval *Drosophila* as well as the crayfish NMJ exposure to fluoxetine (100  $\mu\text{M}$ ) in the presences of  $\text{Cd}^{2+}$  substantially increased the frequency of spontaneous events ( $P < 0.05$ , rank sum). To examine if a lower concentration of fluoxetine showed the same effect, a 10  $\mu\text{M}$  was also used with a 1mM  $\text{Cd}^{2+}$  combination followed by 100  $\mu\text{M}$ . Representative traces of spontaneous quantal events recorded in the muscle fibers of *Drosophila* third instar larva for this experimental paradigm are shown in Figure 5.2 A-D. Some preparations had such an increase in the rate of spontaneous events with fluoxetine at 100  $\mu\text{M}$  the events were not able to be counted. In preparations in which the rate of spontaneous events was practical for counting, the number of occurrences for a 10 sec window for 10  $\mu\text{M}$  and 100 $\mu\text{M}$  exposure are shown in Figure 5.3 A. The 10  $\mu\text{M}$  does not appear to have any effect on the resting membrane potential or the occurrences of spontaneous events. The resting membrane potential of the muscle is more negative for the 100  $\mu\text{M}$  exposure (Figure 5.3 B). The crayfish opener preparation depicted similar effects with increase in spontaneous events for 100  $\mu\text{M}$  exposure (Figure 5.4).

### **Fluoxetine dampens axon action potentials**

Since evoked EPSP could not be induced after exposure to fluoxetine at 100  $\mu\text{M}$ , or higher concentration if a prior exposure to 10  $\mu\text{M}$  occurred, and the EPSPs did not show a gradual decrease in amplitude but a drastic cessation, we examined if the

action potential in motor neurons might decrease below the threshold of the voltage gated presynaptic calcium channels. The axons are too small for intracellular recording in the *Drosophila* larval preparations; however, the crayfish axon to the opener muscle is large enough to obtain intracellular recordings. The action potential showed a decrease in amplitude relatively quickly (~ 1 minute) to baseline (Figure 5.5; 1 to 5 in series). When exposing the axon to 10  $\mu\text{M}$  prior to 100  $\mu\text{M}$  the effect of 100  $\mu\text{M}$  was not always as prominent as exposure to 100  $\mu\text{M}$  directly (Figure 5.6). Increasing fluoxetine 500  $\mu\text{M}$  in such cases did fully block the action potential from occurring.

### **Fluoxetine causes behavioral and heart rate alterations**

In conducting behavioral assays with larval *Drosophila* being fed fluoxetine, we noticed that the hearts were decreasing in rate. Thus, we conducted a heart assay, exposing it to various concentrations of fluoxetine. At 100 nM no significant changes were noted; however, at 1  $\mu\text{M}$  2 out of the 5 preparations completed stopped beating. At 10  $\mu\text{M}$  and 100  $\mu\text{M}$  all preparations rapidly stopped beating when exposed to saline containing fluoxetine (Figure 5.7;  $P < 0.05$ , rank sum).

In order to obtain insights into the long term effect of fluoxetine during development of larvae, early 2<sup>nd</sup> instar were placed in food containing various concentrations of fluoxetine. At concentrations less than 500  $\mu\text{M}$  only slight delays in time to pupation with high concentrations (non-significant) and almost all the larvae survived to pupation. However, at 500  $\mu\text{M}$  there was a very substantial delay in development and a large number of deaths. The larvae showed a slowed development upon exposure to fluoxetine and deaths occurred throughout the time to pupation (Figure 5.8). Only 1/5 of the larvae survived to pupation.

As a comparison for the behavioral effects, we injected crayfish with fluoxetine and observed their responses to taps on the telson every 15 minutes (Figure 5.8 C). Various ranges in behavioral activity were assessed to index the responses. Controls were injected with saline of the same volume to determine if handling and injecting the

crayfish altered responses. Injections, which were estimated to 100  $\mu\text{M}$  in the circulating hemolymph, did not result in any noticeable acute effects. However, when the circulating concentration was at 2 mM the crayfish started to show altered responses to tail flip behavior. Seven out of the nine crayfish rapidly decreased responses to tail taps. One crayfish quickly became non-responsive and we almost assumed it was dead; however, after some time it regained its ability to move and respond to tail touches. Whereas another crayfish appeared to be recovering fully from the injection and then died. The black line on the graph (Figure 5.8) for the controls consists of 5 crayfish which all showed full responsiveness each time tested.

Since the NMJs exposed to  $\text{Cd}^{2+}$  and fluoxetine still showed a substantial increase in spontaneous EPSPs it was assumed that  $\text{Ca}^{2+}$  must be released from internal stores of  $\text{Ca}^{2+}$ . We examined if glutamatergic neurons in the vertebrate brain stem would substantiate the possibility since these neurons loaded well with calcium indicators. The response was rapid in the intracellular calcium response in preparations bathed in a saline containing  $\text{Ca}^{2+}$  as well as a bathing saline devoid of  $\text{Ca}^{2+}$  (Figure 5.9). Treating the preparations with thapsigargin, which blocks the SERCA uptake of calcium into the ER, demonstrated that fluoxetine's mechanism of action in increasing the internal calcium signal is due to dumping of  $\text{Ca}^{2+}$  from the internal ER store.

## **Discussion**

Fluoxetine is commonly used as an anti-depressant agent. However, the detailed actions of fluoxetine on various cellular functions has not been fully studied. In this study, it was demonstrated that a high concentration of fluoxetine, which could occur with an overdose, delivered as a systemic exposure or in direct application to cardiac tissue or neurons results in side effects associated with ion channel blockage and intracellular release of  $\text{Ca}^{2+}$ . Even though in the current study, the concentrations of fluoxetine used were greater than the therapeutic range, the observed side effects

may occur at varying degrees for individuals with altered metabolism and excretion of fluoxetine. A recent meta-analysis on pregnancy outcomes in patients taking SSRI's within a therapeutic range showed an increased risk of spontaneous abortion and major malformations during pregnancy (Nikfar et al., 2012). In addition, the use of fluoxetine to treat anxiety in patients may open a wider range therapeutic applications (Altieri et al., 2014) and potential for overdose and manifestation of subtle side effects. To treat an overdose and potential subtle side effects it is of interest to know what acute high dosages may have on physiological process.

Fluoxetine is water soluble and therefore it is therapeutically administrated as an oral medication and it is known to be readily taken up in the gastrointestinal tract of mammals (Hiemke and Härtter, 2000) as well as able to gain access to intracellular organelles (Caccia et al., 1990; Donard et al., 2014). Medications have effects on other organisms such as invertebrates (Fong and Ford, 2014) and many of the cellular process are conserved (Reaume and Sokolowski, 2011). It has been shown that fluoxetine has affinity to the *Drosophila* serotonin transporter (dSERT) (Corey et al., 1994; Demchyshyn et al., 1994). Also, a previous study has demonstrated that oral administration of fluoxetine is effective in *Drosophila* larvae (Neckameyer et al., 2007). Thus, delivering fluoxetine in the food for *Drosophila* is appropriate for administration in this model system; however, the pharmacokinetics, pharmacodynamics in *Drosophila* has not yet been elucidated. Furthermore, fluoxetine has been used to study the effect of 5-HT on behavior in crustaceans such as crayfish, (Huber et al., 1997; Huber and Delago, 1998). Since it is not feasible to feed fluoxetine to crayfish we used injections into the hemolymph directly and used sham saline injections as controls. We do not know the free circulating concentration of fluoxetine from the injection paradigm as the drug could rapidly bind to proteins and tissues once in the hemolymph. The direct application to the dissected crayfish and *Drosophila* neuromuscular junctions, the *Drosophila* heart and the rodent brain stem neurons were very likely exposed to the dissolved concentrations in the saline applied as the tissue

is minimal in comparison to the volume of bathing saline. Considering cellular metabolism and potential excretion within the fed or injected animals, the longer term effects might be due to metabolites of the fluoxetine. However, the rapid paralysis or lethargic effects of the crayfish upon injection into the hemolymph as well as the immediate responses in the heart rate, synaptic responses and calcium signaling with bath application would suggest direct actions of fluoxetine itself.

The immediate cessation of heart rate for all 12 larvae at the higher concentrations and for a few larvae at the lower concentrations would suggest a direct action on ion channels on the plasma membrane surface, potentially fluoxetine being internalized to have rapid action on either calcium dynamics or altering coupling of the electrical-mechanical action in cardiac contraction. It was shown that fluoxetine reduces calcium ion current and modifies other electrophysiological properties in mammalian cardiac tissue (Pacher et al., 2000). Therefore, we suggest that fluoxetine might block calcium channels on the plasma membrane of the *Drosophila* heart as well which causes cardiac arrest. Likewise, the rapid effect of stopping evoked synaptic transmission might be due to a reduction of the action potential amplitude. This would also suggest an effect on blocking ion channels. Given that the larval *Drosophila* heart has not been shown to express a voltage-gated sodium channel one might expect an action on another channel related to the pacemaker potential or one for contraction such as a voltage dependent calcium channel as a site of action. The effect of blocking voltage-gated calcium channels on the crayfish axon would not explain the rapid decrease in action potential amplitude; however, a blocking of the voltage-gated sodium channel would (Sparks et al., 2003). Considering the resting membrane potential did not demonstrate rapid depolarization upon exposure to fluoxetine while other effects were prevalent would be indicative of the plasma membrane being stable in the presence of fluoxetine. There was no alteration in pH of the saline by fluoxetine as compared to normal saline. This was directly examined with sensitive pH paper ( $\pm$  0.2 units; Fluka Analytical pH strips 6.0-8.1). If the fluoxetine-HCl reduced the pH from

7.4 to 7.2 in the crayfish saline or decreased the pH from 7.2 to 7.0 in *Drosophila* saline this would still not explain the results observed. The *Drosophila* heart rate increases with a decrease in pH and will continue to beat for prolonged periods of time even at pH of 5.0 (Badre et al., 2005). Also, a low pH of crayfish saline of 5.0 results in a slight depolarization of the muscle as well as in the axon but both action potentials and EPSP are still able to be measured (Bierbower and Cooper, 2010) which is not the case for fluoxetine exposure.

The increase in spontaneous quantal events in the presence of cadmium, which blocks voltage gated calcium channels, is convincing evidence that the increase in spontaneous fusion is not due to an influx of  $Ca^{2+}$  through presynaptic calcium channels. The rapid rise in the frequency of spontaneous events upon exposure to fluoxetine is striking. The degree in increased number of occurrences for the given concentration of fluoxetine was always more dramatic for the *Drosophila* larval NMJs than for the crayfish opener NMJ. This may be due to the fact the motor nerve terminals are not as embedded in the subsynaptic reticulum as for the crayfish preparation (Atwood and Cooper, 1995, 1996a,b). The pronounced effect lasted for a 2 to 5 minutes before there appeared to be run down in the high frequency of events. We did not quantify the depression in the rate of spontaneous events as it was not a focus for this study. The decrease in the resting membrane potential with fluoxetine at 100  $\mu$ M for the skeletal muscle may be indicative of blocking cationic leak channels which would then allow the membrane potential to remain closer to the lower  $E_K$  potential.

Exchanging the bathing solution several times, very vigorously, did not wash out the effect of fluoxetine at the NMJs for either the *Drosophila* larval or crayfish preparations. Also, the action potentials did not recover in the crayfish motor axons where the intracellular recordings would have detected a slight recovery even if it were below the threshold of activating the voltage-gated calcium channels in the presynaptic terminal. Whatever the mechanism of action might be on the ionic channels it must be a strong interaction. Possible in vivo experiments with fluoxetine might have a strong

association with circulating substances (proteins and lipids) to decrease the long term interaction with the ionic channels. Furthermore, fluoxetine might metabolize very quickly which could reduce its effect on ion channels. This may explain where in the one case in which the injected crayfish was paralyzed and then recovered.

The ability of fluoxetine to release  $\text{Ca}^{2+}$  from the ER was substantiated in the imaging studies with and without thapsigargin treatment. In addition, the effect was rapid upon exposure to fluoxetine indicating that penetration and/or uptake across the membrane is rapid. Since it was shown that fluoxetine has an effect in disrupting mitochondrial function (Caccia et al., 1990; Donard et al., 2014) there is a possibility to also disrupt proteins in other cytoplasmic organelles. The mechanism remains to be determined how fluoxetine causes the ER to dump calcium (Desai-Shah and Cooper, 2009), but it might be selective to interacting with ryanodine-like proteins and not passively causing the ER membrane to become leaky as the resting membrane of the skeletal muscle and the axons did not show depolarization upon exposure to fluoxetine. It has been shown in human oral cancer (OC2) cells that fluoxetine increases the intracellular calcium concentration through PLC-PKC pathway. The calcium-free medium reduces the fluoxetine action on  $[\text{Ca}^{2+}]_i$ ; however, it was shown that application of SERCA blockers in calcium-free medium blocks fluoxetine action on  $[\text{Ca}^{2+}]_i$ . In this study, the removal of extracellular calcium did not effect on fluoxetine-induced rise in  $[\text{Ca}^{2+}]_i$ . The inhibition of SERCA by thapsigargin blocked fluoxetine action on  $[\text{Ca}^{2+}]_i$ . In our preparations, PLC-PKC pathway might be activated to enhance ER  $\text{Ca}^{2+}$  release. A model representing these mechanisms is depicted in Figure 5.10.

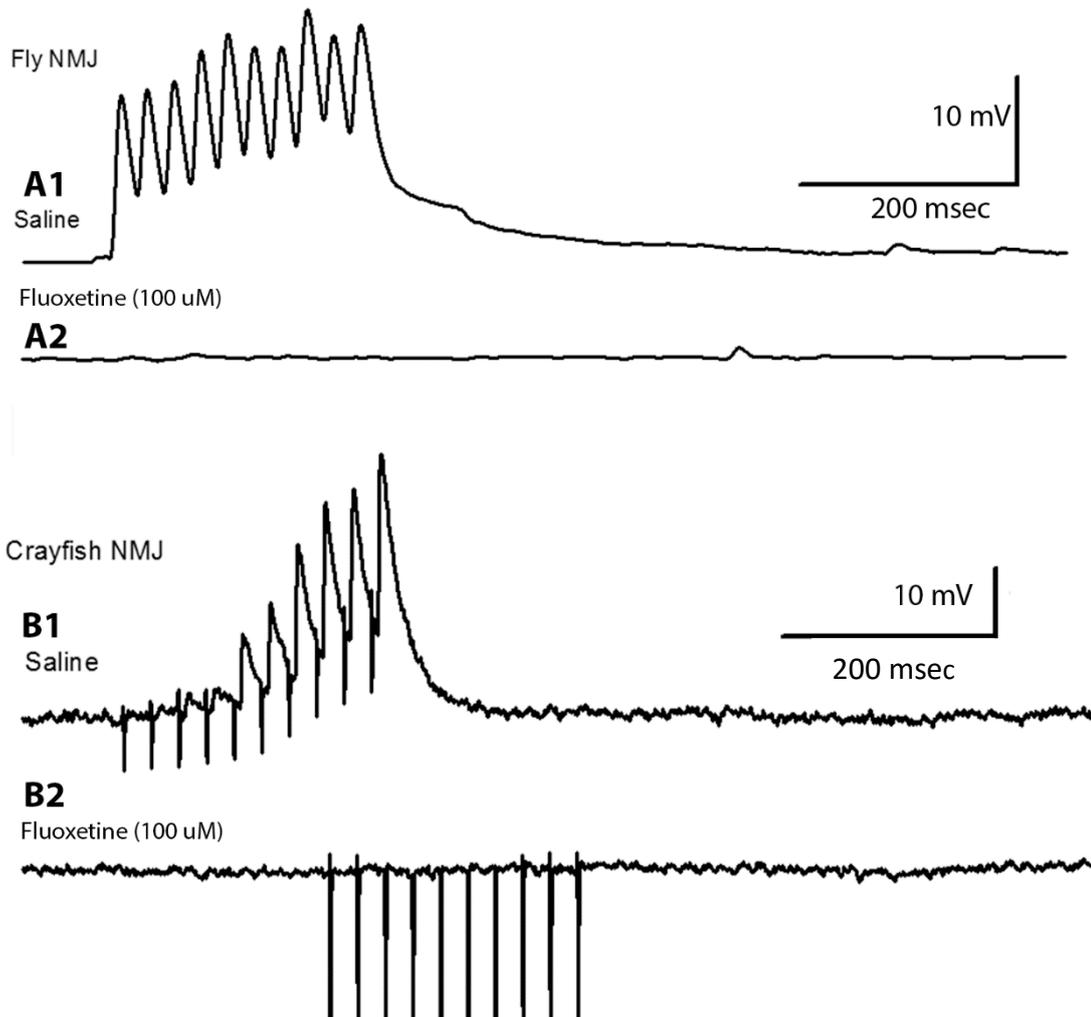


Figure 5.1: Fluoxetine blocks neurotransmission at neuromuscular junctions (NMJ). Effect of fluoxetine on evoked excitatory postsynaptic potentials (EPSPs). The *Drosophila* larval NMJ (A1) as well as the crayfish (B1) NMJ had prominent amplitudes of EPSPs with the stimulation trains but were rapidly attenuated upon exposure to saline containing fluoxetine (A2 & B2). In the recordings shown for the *Drosophila* NMJ (A1 & A2) one can also observe the spontaneous events which occur before and after exposure with fluoxetine.

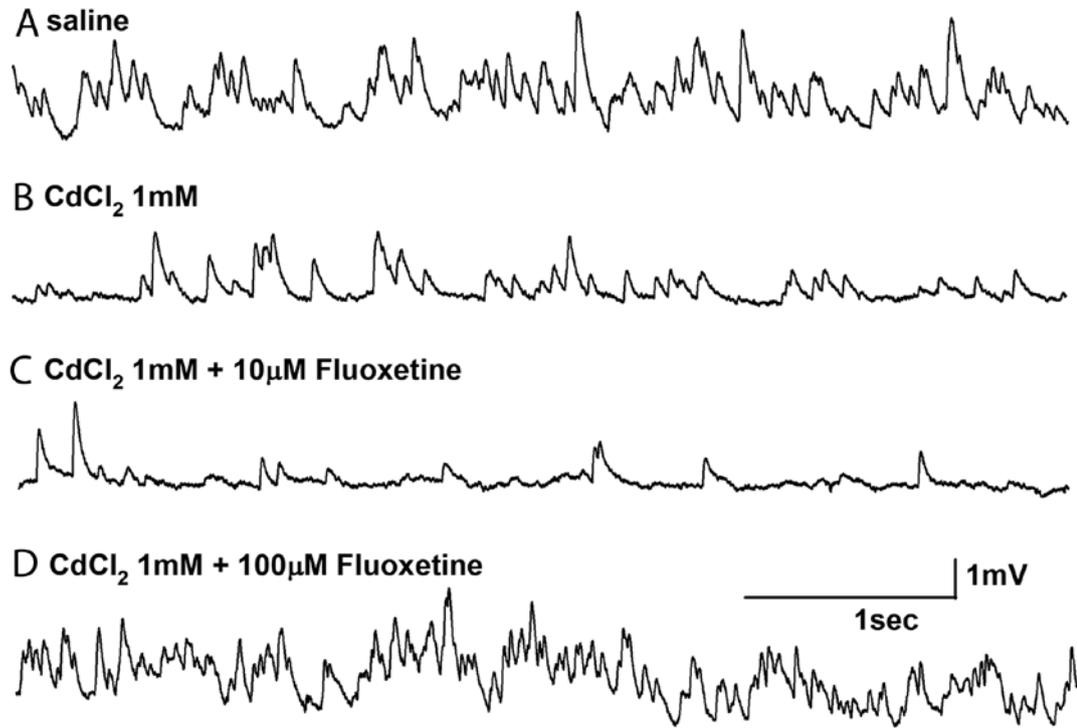


Figure 5.2: Spontaneous quantal events and effect of fluoxetine. (A) The spontaneous events in muscle 6 of the larval *Drosophila* preparation exposed to normal saline. (B) Upon exposure to saline containing 1 mM Cd<sup>2+</sup> the rate of spontaneous event decreased; however, the quantal events are still prominent in amplitude. Evoked transmission is completely blocked at this concentration of Cd<sup>2+</sup> due to blocking presynaptic voltage-gated Ca<sup>2+</sup> channels. (C) The low exposure to fluoxetine 10 µM with Cd<sup>2+</sup> did not show any increase in spontaneous events. (D) Exposure to Cd<sup>2+</sup> and fluoxetine at 100 µM promotes a high frequency of spontaneous events.

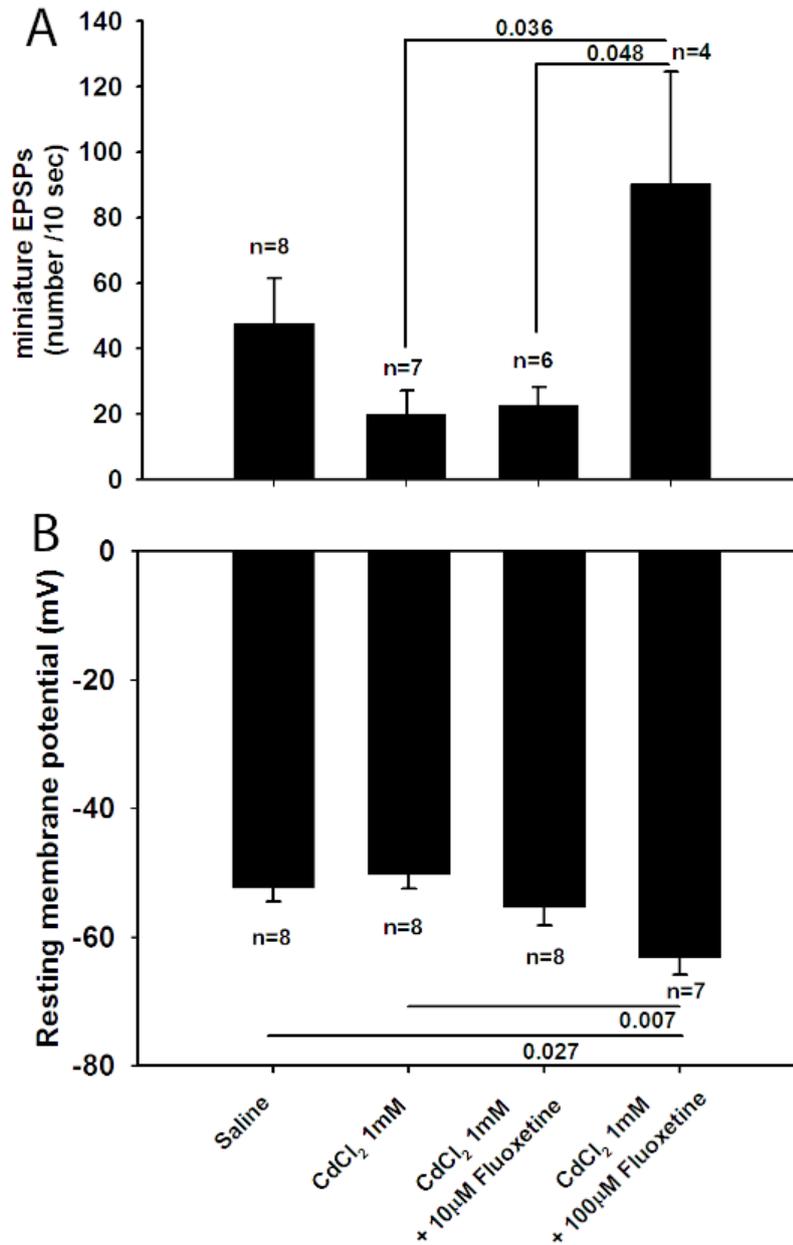


Figure 5.3: Spontaneous quantal events and resting membrane potential for the *Drosophila* larval muscle preparation. (A) Four of the preparations in which the rate of spontaneous events for 100  $\mu\text{M}$  fluoxetine was not too high to count, the number of occurrences in a 10 second period is shown in comparison to 10  $\mu\text{M}$  fluoxetine,  $\text{Cd}^{2+}$  alone and saline exposures. The rate greatly increases for 100  $\mu\text{M}$  exposure. (B) The resting membrane potential of the muscle significantly becomes more negative for the 100  $\mu\text{M}$  fluoxetine exposure.

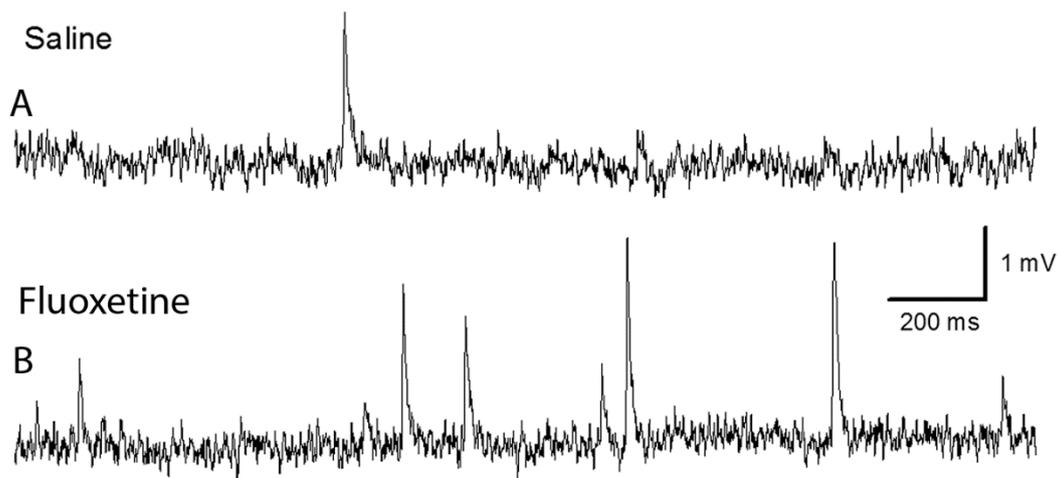


Figure 5.4: The crayfish opener neuromuscular junction show a similar phenomenon for exposure to a 100  $\mu$ M fluoxetine in increasing the occurrences of spontaneous quantal events. Saline alone has few events normally (A) and a marked increasing when exposed to fluoxetine at 100  $\mu$ M (B).

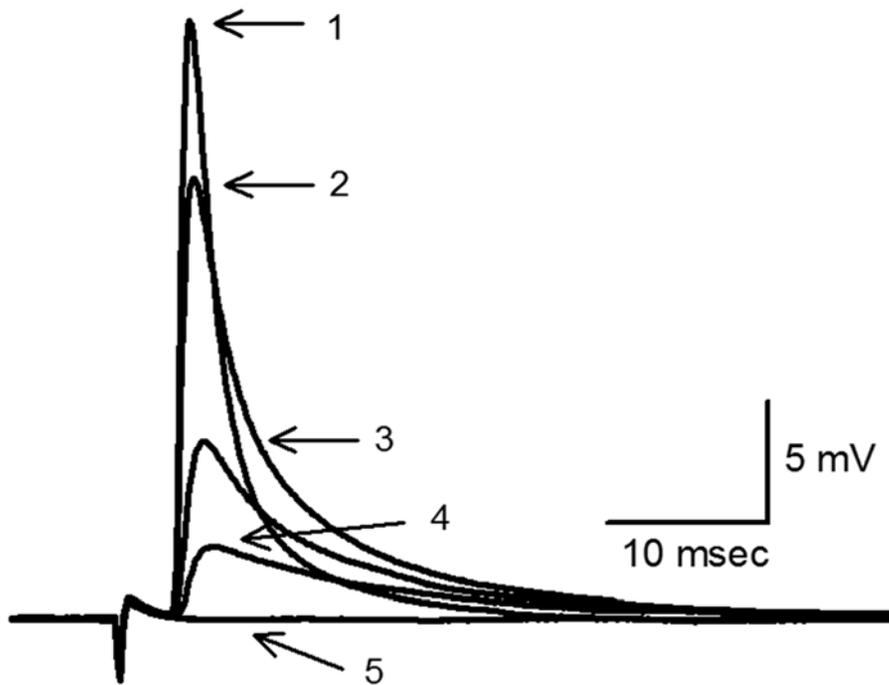


Figure 5.5: Intracellular recordings in an opener motor neuron of action potentials while exposed to 100  $\mu\text{M}$  fluoxetine over time. The initial saline (1) exposure and sequential (2 to 5) representative traces over the next few minutes of exposure to fluoxetine.

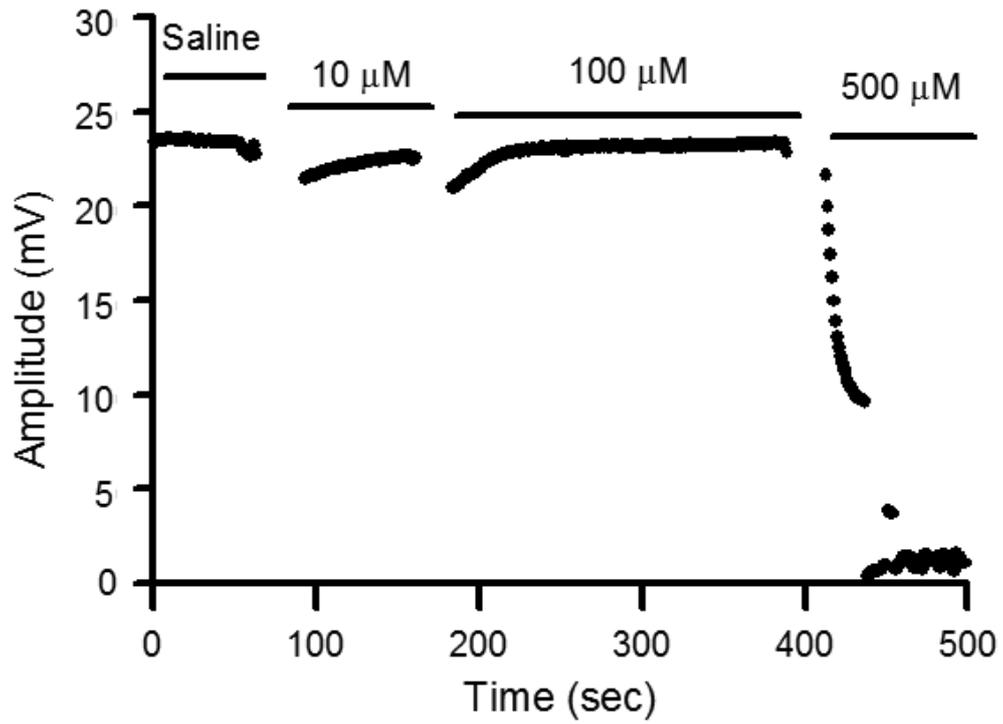


Figure 5.6: The action potential amplitudes did not decrease as much when the preparations were previously exposed to 10  $\mu\text{M}$  and then exposed to 100  $\mu\text{M}$ . However higher concentrations 500  $\mu\text{M}$  would result in a complete cessation of the action potential.

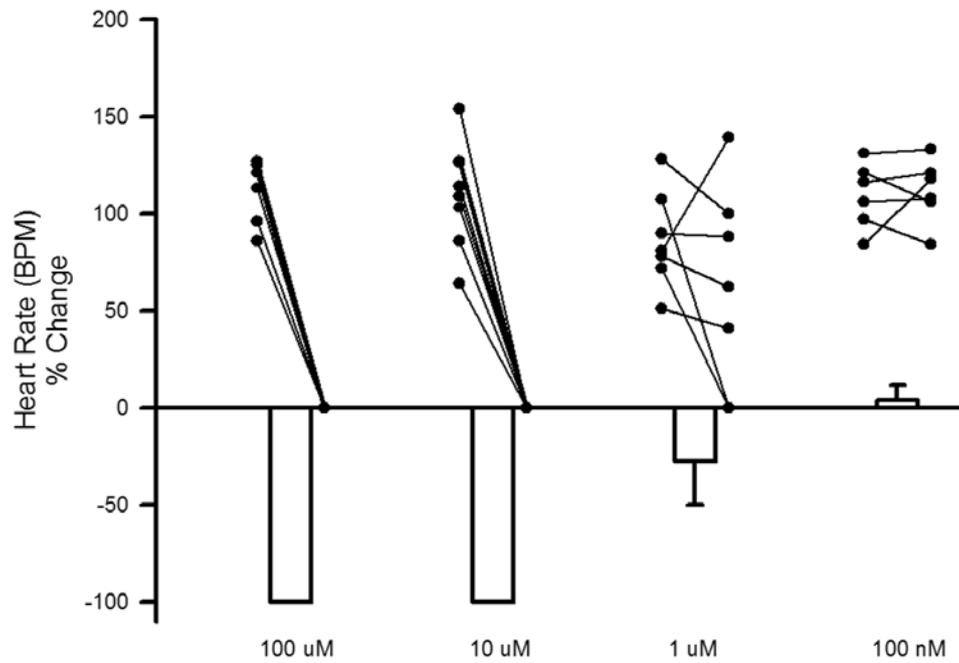


Figure 5.7: Effect of fluoxetine on heart rate in dissected larval *Drosophila*. Fluoxetine at 10  $\mu\text{M}$  and 100  $\mu\text{M}$  stops the heart rate within 1 minute; however, a low concentration of 100 nM did not show any significant effect. The 1  $\mu\text{M}$  exposure showed a general trend of decreasing heart rate in all except one preparation.

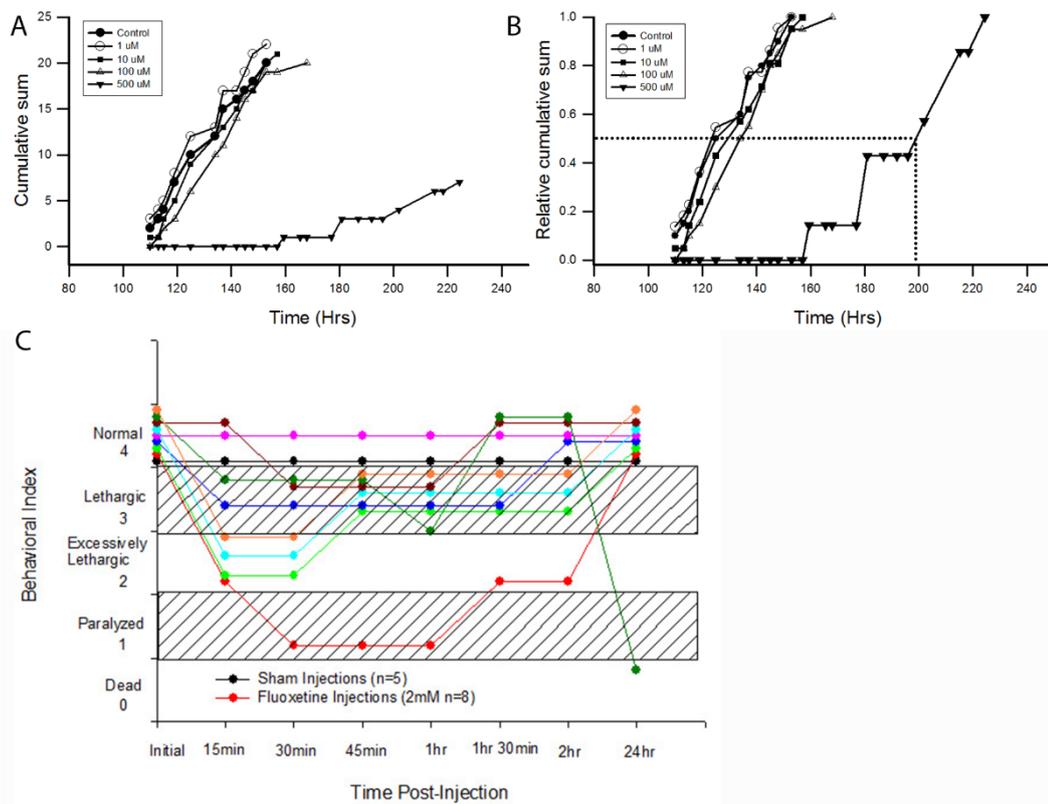


Figure 5.8: Effect of fluoxetine on development in larval *Drosophila* and behavior in crayfish. (A1) Fluoxetine at various concentrations was fed to *Drosophila* larvae from first to third instar stage. The time to pupation from eggs were calculated for each larva. Cumulative sum for time to pupation from eggs at different concentrations was converted to relative cumulative sum for easier comparison with groups containing different numbers of samples (A2). Larvae fed the highest concentration took the longest time to pupation. Note 25 larvae were used to start and that many had died at the highest concentration of fluoxetine (A1). (B) Crayfish injected with fluoxetine to a circulating concentration estimated to be 2 mM generally decreased their movements and became less responsive to taps on their telson as indexed.

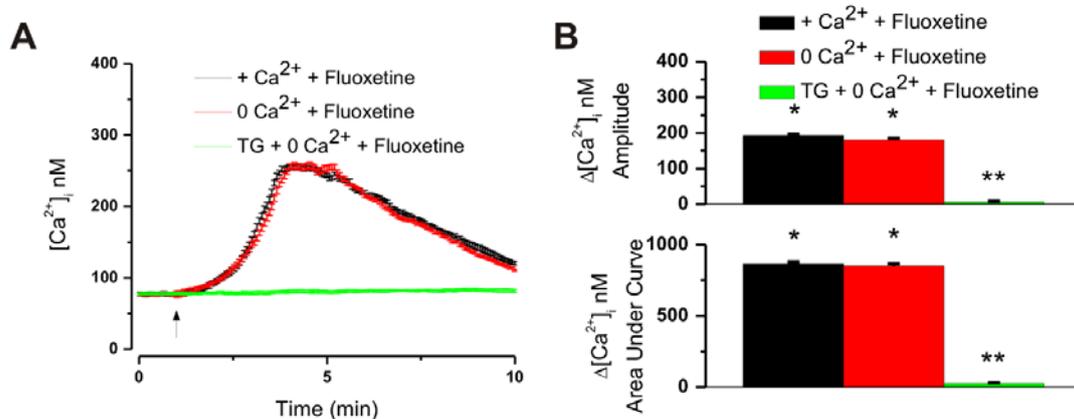


Figure 5.9: The effect of fluoxetine on intracellular  $Ca^{2+}$ . (A) Exposure of fluoxetine to the rodent brainstem neurons in culture revealed an increase in internal  $Ca^{2+}$  only when the endoplasmic reticulum (ER) is able to unload the stored  $Ca^{2+}$ . With zero  $Ca^{2+}$  in the bath and exposure to fluoxetine a  $Ca^{2+}$  signal is present. When the ER is blocked from taken up  $Ca^{2+}$  by application with thapsigargin (TG) ( $1\mu M$  for 1 h prior to imaging) then the  $Ca^{2+}$  response to fluoxetine exposure is not present. (B) The representative responses of the  $Ca^{2+}$  signal over time in the same conditions shown in (A). There is a significant decrease in the response when TG is applied and a significant increase of intracellular  $Ca^{2+}$  during the fluoxetine exposure which indicates  $Ca^{2+}$  released from the ER by fluoxetine.

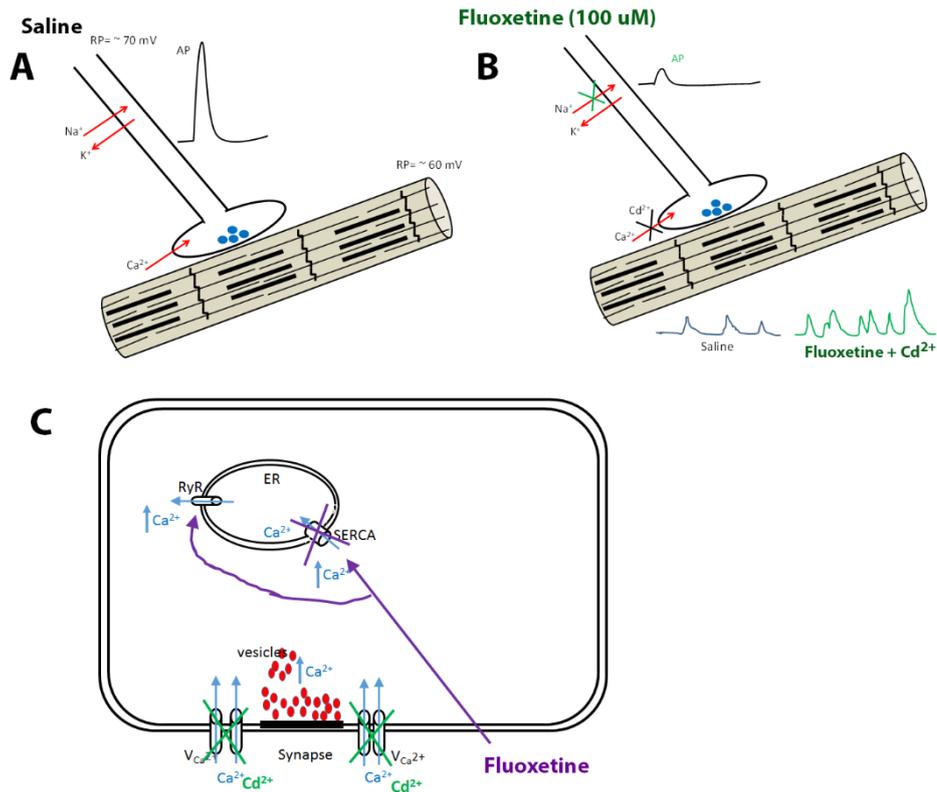


Figure 5.10: A model representing the potential mechanisms of action by fluoxetine and experimental paradigms used in this study. A neuromuscular junction (NMJ) before (A) and after (B) exposure to fluoxetine. Fluoxetine reduces the amplitude of the action potential in the axon. Thus, there is a rapid block of evoked transmission. In the presence of Cd<sup>2+</sup>, to block the presynaptic voltage-gated Ca<sup>2+</sup> channels, there is a high rate of spontaneous quantal events with exposure to fluoxetine. (C) The ability of fluoxetine to raise intracellular Ca<sup>2+</sup> likely occurs through ryanodine receptors (RyR) on the ER. If the SERCA is blocked with thapsigargin the ER will be unloaded of Ca<sup>2+</sup> then no rise in intracellular Ca<sup>2+</sup> occurs with treatment by fluoxetine. In modeling the NMJ in this regard, potentially fluoxetine maybe even block the SERCA as the occurrences of spontaneous events (vesicle fusion events) are prolonged even in the presence of Cd<sup>2+</sup> blocking the voltage-gated presynaptic Ca<sup>2+</sup> channels (V<sub>Ca<sup>2+</sup></sub>). The rapid cessation of the larval *Drosophila* heart may also be explained by the dysregulation of the intracellular Ca<sup>2+</sup> dynamics since it known to be very sensitive to intracellular Ca<sup>2+</sup> regulation.

## CHAPTER SIX

### General Discussion

#### Significance and impact

In this dissertation, I have studied the role of 5-HTergic system in heart physiology, feeding and locomotion behaviors, and sensorimotor circuit activity in the fruit fly *Drosophila melanogaster*. The findings of these studies are shown and discussed in various chapters (Chapter Two to Chapter Five). The invertebrate systems have been cornerstones in decoding the neural functions due to their small number of cells in the nervous system (crustacean ganglia) (Wiese (Ed), 2002), large-sized neurons (leech) (Muller et al. (Eds), 2010), giant axons and synapses (squid) (Hodgkin and Huxley, 1952a; Llinas, 1999), and availability of advanced genetic tools (*Drosophila*) (Venken et al., 2011) which have led to many breakthroughs and revolutionized the field of neurobiology. For instance, our understanding of cellular and molecular mechanisms of learning and memory has been advanced by using sea slug *Aplysia* (Kandel et al., 2014).

Likewise, *Drosophila* has extensively been used to study neuronal functions and provide new insights into the role of neural circuits governing various behaviors (Dubnau, 2014). In addition, the advances in genetic tools and technological innovations have made *Drosophila* a model of choice. The binary expression systems are used to drive the expression of an effector gene of interest in a subset of neurons with spatial and temporal control. Furthermore, genetically encoded calcium indicators (GCAMP) (Chen et al., 2013) and voltage indicators (GEVIs) (Cao et al., 2013) allow monitoring of the neural activity of many neurons simultaneously in behaving animals, which cannot be attained by classical electrophysiological techniques especially for the small-sized neurons in the fly brain.

The chemical substances that are used for communication between neurons are evolutionary conserved across species. Many organisms, invertebrates and

vertebrates, use 5-HT as a neurotransmitter and/or neuromodulator (Olivier, 2015; Hamilton et al., 2016). 5-HT was first discovered in the blood serum in mammals, which was found to alter the tonic properties of the smooth muscle, hence was called serotonin (Watts et al., 2012). In mammalian system, 5-HT is synthesized in the gut and nervous system by the action of rate-limiting enzyme tryptophan hydroxylase 1 (Tph1) and tryptophan hydroxylase 2 (Tph2), respectively. A high percentage of total body 5-HT is found in the gut which is synthesized by enterochromaffin cells (ECs). The released 5-HT from EC into the bloodstream can be taken up by blood platelets through serotonin transporter (SERT) (Janušonis, 2014). In the central nervous system of mammals, 5-HT is mostly synthesized by 5-HTergic neurons in the raphe nuclei, which send axon projections to multitude regions of the brain (Haase and Brown, 2015). It has been found that there are two isoforms of tryptophan hydroxylase (Trh) in *Drosophila melanogaster* as well, one for the 5-HT biosynthesis in the peripheral tissue and another isoform for the nervous system 5-HT synthesis, which are called DTPHu and DTRHn, respectively (Neckameyer et al., 2007).

The 5-HT action is mediated by 5-HT receptor subtypes, which are G-protein coupled receptors. The endogenous ligand might activate various GPCR receptor signaling pathways; therefore, I used a novel chemogenetic tool, called Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to study the signaling pathway of chronotropic action of 5-HT in *Drosophila* larval heart. The results and discussion are presented in Chapter Two. The data demonstrate for the first time that activation of DREADDs, which negatively couples to adenylate cyclase (AC), arrests the heart beat in third instar larval stage (Becnel et al., 2013; Majeed et al., 2013). Various pharmacological agents were used to target the cAMP-PKA pathway when exogenous 5-HT was applied to preparations; however, the inhibition of cAMP-PKA pathway did not block the positive chronotropic action of 5-HT in larval heart. These findings open up venues for potential therapeutic intervention for the human cardiac disorders by expressing and activating DREADDs in cardiac tissue to modulate heart

rate and control arrhythmic cardiac conditions. An inert ligand, clozapine-N-oxide (CNO) is used to activate various DREADDs in *Drosophila* and mouse models; the CNO is considered pharmacologically inactive to endogenous ligands and it specifically activates DREADDs (Urban and Roth, 2015). Using an inert molecule like CNO to activate engineering receptors provides a better understanding of fundamental principles of the biological actions of G-protein coupled receptors (GPCRs), which cannot be achieved by endogenous ligand due to their binding affinity to multiple receptor subtypes.

5-HT can modulate the heart rate in vertebrates through activation of various 5-HT receptor subtypes, which are coupled to different heterotrimeric G-proteins; the 5-HT receptor subtype that mediates 5-HT cardioacceleratory action is different in various species (Watts et al., 2012). I sought to identify 5-HT receptor subtypes that are involved in positive chronotropic action of 5-HT in larval heart using various pharmacological substances and genetic tools (Chapter Three). The data show that 5-HT<sub>2</sub> receptor subtypes mediate the action of 5-HT in third instar larval heart. These findings broaden our knowledge regarding the mode of action of 5-HT as a modulator in cardiac tissue which can be used to study the action of 5-HT in a wide array of animals across the animal kingdom to find evolutionary relationships among various species in the phyla. In addition, understanding 5-HT action on cardiovascular system is essential since in some neuropsychiatric disorders, the concentration of circulating 5-HT is high. For instance, hyperserotonemia is observed in autism spectrum disorder (ASD) (Veenstra-VanderWeele et al., 2012) which might cause dysregulation in cardiovascular system physiology. It has already been shown that low concentration of 5-HT in circulation, due to the *Tph1* gene mutation, of the pregnant parent results in dysfunction of cardiac physiology in the offspring (Fligny et al., 2008). Therefore, physiological amounts of 5-HT in the body is essential for normal development of an organism; however, dysregulation of 5-HT synthesis will lead to various developmental disorders.

The 5-HTergic system has intensively been studied in both mammalian system (Pollak et al., 2014; Okaty et al., 2015) and *Drosophila* (Huser et al., 2012); however, the function of various 5-HT neurons and 5-HT receptor subtypes in physiology and behavior has yet to be investigated well. I have examined the role of various 5-HT receptor subtypes and 5-HT neurons in feeding and locomotion behaviors as well as sensorimotor circuit activity. The results and discussion of the findings are delineated in Chapter Four. In this study, I used various pharmacological and genetic tools to manipulate 5-HT receptors and alter neural electrical excitability of 5-HT neurons to observe how each component of 5-HT system is implicated in behavior and locomotor neural circuitry. The data reveal that various components of 5-HT system are involved in feeding and locomotion as well as sensorimotor circuit activity. It has been shown that a specific behavior is driven by a specific motor system, which consists of central pattern generator (CPG) neural network in central CNS, motor neurons, and motor neuron targets, such as mouth hook and body wall muscles. The neurophysiological characteristics of feeding (Hückesfeld et al., 2015) and locomotion (Fox Let al., 2006) motor system are well established in *Drosophila melanogaster*.

In this dissertation, the results show that acute activation of 5-HT neurons in behaving animals noticeably reduces locomotor activity in both larva and adult stages. In addition, 5-HT neuron activation, by channelrhodopsin2 (ChR2) or transient receptor potential1 (TrpA1) cation channel ectopic expression and activation, blocks evoked rhythmic pattern activity of the motor output in dissected *Drosophila* larvae preparations. The underlying mechanisms governing inhibitory action of 5-HT neurons in motor neural networks have remained unknown. On the other hand, 5-HT neuron blockage, by expressing *shibire<sup>ts</sup>* gene, does not show profound abnormal phenotype in locomotor activity. These results demonstrate that 5-HT neurons are not required for the locomotor system; although, 5-HT neurons activity are sufficient to alter the speed of forward peristaltic crawling behavior in larval stage and climbing ability in

adult stage. The electrophysiological studies show that exogenous application of 5-HT in third instar larvae preparations increases sensorimotor circuit activity.

The paradoxical results of exogenous 5-HT application and 5-HT neuron activity manipulations might be due to the activation of various 5-HT receptor subtypes in each experimental method. The behavioral and sensorimotor circuit activity effects which are induced by the activation of 5-HT neurons might be more physiologically relevant compared to effects observed from exogenous application of 5-HT. It has been reported that activation of neuromodulatory 5-HT neurons in neural networks generates effects that are more physiologically relevant than the responses obtained from exogenous 5-HT application (Katz and Hooper, 2007). 5-HT neurons might communicate with their partners through synaptic transmission and/or volume transmission. 5-HT can be released from presynaptic terminals and bind with their receptors on the postsynaptic site (wiring synaptic transmission). Moreover, 5-HT can be released from extrasynaptic sites, such as dendrites and soma. In this case, released 5-HT can diffuse to a distant location from the release site to modulate the electrical properties and intracellular signaling pathways in target cells (volume transmission) (Fuxe et al., 2007; De-Miguel et al., 2015). These findings open new venues to further investigate the role of 5-HT as a neuromodulator in rhythmic pattern alteration in a neural network which, in turn, changes the motor output pattern.

The results demonstrate that the neural circuits are not hard-wired and can be changed by neuromodulators as revealed in other neural network models as well (Katz and Hooper, 2007; Miles and Sillar, 2011; Marder, 2012). While investigating the neural circuits controlling specific behaviors, searching only for the neural connectivity does not tell us much about the function of a specific neural circuitry that pertains to a specific behavior. For instance, the neuroanatomical architecture and connectivity map of round worm *Caenorhabditis elegans* whole nervous system, which consists of 302 neurons, is well established (White et al., 1986; Varshney et al., 2011), which is necessary for the study of neural circuit function. However, it does not provide holistic

view on how the neural activity output, which is manifested in behavior, can be altered by neuromodulators. The neural output has flexibility and can show a wide degree of adaptability to internal and external challenges. The malleability properties of neural networks, which are regulated by neuromodulators, in the nervous system are pivotal for animal survival.

The action of neurotransmitters and neuromodulators is terminated after their release either by hydrolysis (acetylcholine) (Dvir et al., 2010), transporting into neuroglia (glutamate) (Vandenberg and Ryan, 2013) or transporting back into presynaptic terminal (5-HT). The action of 5-HT is terminated by serotonin transporter (SERT), which transports 5-HT from extracellular milieu back into presynaptic terminal (Gabrielsen et al., 2012). SERT is a member of solute carrier protein (SLC) family. Moreover, SERT is encoded by *SLC6A4* gene in mammalian system. Various genetic polymorphisms, such as single nucleotide polymorphisms (SNPs), and variable number of tandem repeats (VNTR) polymorphism are found in *SLC6A4* gene. Most of the genetic variations that impact the transcription level and activity of SERT are located in regulatory region of *SLC6A4* gene, which is called SERT gene-linked polymorphic region (5-HTTLPR) (Murphy and Lesch, 2008).

I used fluoxetine (Prozac), which is a selective 5-HT reuptake inhibitor (SSRI) (Wong et al., 2005), as a pharmacological tool to manipulate synaptic transmission activity at the 5-HTergic synapse and to observe how that would change crawling behavior and sensorimotor circuit activity in *Drosophila* larvae. Astonishingly, I noticed that Prozac (100 $\mu$ M) suppresses synaptic transmission at the neuromuscular junction (NMJ) independently of Prozac canonical mode of action, which exerts its effect by blocking SERT. In Chapter Five, I presented direct actions of Prozac (100 $\mu$ M) on synaptic transmission, neural properties, and cardiac function. Prozac application at 100 $\mu$ M concentration blocked action potentials in crayfish axons, enhanced occurrences of spontaneous synaptic vesicle fusion events (miniature EPSPs) in the presynaptic terminals at NMJs of both *Drosophila* and crayfish. In rodent neurons,

cytoplasmic  $\text{Ca}^{2+}$  is raised by fluoxetine. These findings pave the way to better understand the mode of action of most widely used antidepressant drug, Prozac. Furthermore, it opens up new avenues for research on how one can deal with the side effects of this drug especially when high doses are taken. In some cases, patients might be more sensitive to Prozac due to the presence of genetic variations, such as mutations in enzymes that are implicated in Prozac metabolism. Prozac might be very toxic in this case due to the prolonged drug action and altered pharmacokinetics and pharmacodynamics properties of the drug.

Prozac is metabolized by P450 2D6 (CYP2D6) enzyme to norfluoxetine in liver (LLerena et al., 2004; Mandrioli et al., 2006). Also, it has been shown that Prozac blocks CYP2D6 activity which, in turn, prolongs the presence of Prozac in the body. This might have detrimental effects on the body due to its toxicity (Otton et al., 1993). There is individual variability on how one reacts to a drug in human population, which is due to the genetic variations in genes that encode for enzymes essential for drug metabolism. For instance, genetic variability and polymorphism in *CYP2D6* gene impacts the metabolic rate of those drugs that are metabolized by this enzyme. In this case, one specific dosage of a drug might not be qualified for all human populations (Ingelman-Sundberg, 2005); therefore, the amount of drug should be prescribed based on the genetic composition of patients. This individual-based modification in prescribed drug dosage is called personalized medicine in the genomic era. This approach of drug prescription increases the beneficial effects of available medications.

### **Future directions and prospects**

Novel genetic tools and technological innovations have advanced our understanding of anatomical architecture and electrophysiological properties of *Drosophila melanogaster* nervous system. Various binary expression systems (UAS/Gal4/Gal80 system; LexA/LexAop system; QF/QUAS system) and effector gene lines have been developed in *Drosophila melanogaster* (Venken et al., 2011; del Valle

Rodríguez et al., 2011). These tools can be exploited to target the expression of gene of interest to a subset of neurons and manipulate the neural electrical excitability in a controlled manner of time and space and at high resolution (Venken et al., 2011; Jenett et al., 2012; Pauls et al., 2015). Individual neurons can be labeled in a forest of neural networks of *Drosophila* nervous system by using mosaic analysis with a repressible cell marker (MARCM) system, which provides detailed information about morphological features of a specific subset of neurons in the brain (Luo, 2007). One can dissect the components of neural circuits and study the anatomical features of neurons in the brain by using a multitude color system called flybow (Hadjieconomou et al., 2011) or more recent one, MultiColor FlpOut (MCFO) technique (Nern et al., 2015). Using these tools, one can label individual cells in a population of neurons with different fluorescence colors. Furthermore, the synaptic contact between neurons can be studied by using GFP reconstitution across synaptic partners (GRASP) strategy (Feinberg et al., 2008). In recent study, GRASP technique was employed to probe the neural basis of temperature sensing machinery in *Drosophila* (Frank et al., 2015). In addition, the advent of high-resolution microscopies, such as stimulated emission depletion (STED) microscopy, has advanced our understanding of architecture of neurons at synaptic and molecular levels (Liu et al., 2011; Sigrist and Sabatini, 2012).

The *Drosophila melanogaster* nervous system contains approximately  $10^4$  neurons in larval stage (Ohyama et al., 2015), and  $10^5$  neurons in adult stage (Chiang et al., 2011); however, mammalian nervous system consists of roughly  $10^8$  neurons in mouse, and  $10^{11}$  neurons in humans (Luo, 2015). The less complex nervous system, a complex behavioral repertoire, and powerful genetic tools make *Drosophila* a successful model to study the neuronal connectivity to decode the neural basis of various behaviors. The activation of 5-HTergic neurons results in reduction of locomotor activity in *Drosophila* larvae and adults; however, the underlying neural circuit diagram of 5-HT neurons in sensorimotor neural networks has yet to be investigated. By using the above-mentioned tools in *Drosophila*, the connectivity map

of 5-HT neurons can be obtained. GRASP technique can be employed to investigate the synaptic connectivity map of 5-HT neurons in the sensorimotor neural network. Moreover, to map the connectivity between 5-HT neurons in sensorimotor neural networks, electrophysiological techniques can be an invaluable tool to define the nature of connections between two neurons. It is known that neurons are able to make contacts with their partners through chemical synapse or electrical coupling. Channelrhodopsin2 (ChR2) can be expressed in 5-HT neurons to induce electrical excitability in those neurons. On the other hand, genetically encoded voltage indicator (GEVI) can be expressed in motor neurons to monitor voltage change. ChR2 and GEVI can be simultaneously expressed in two different subsets of neurons by using UAS/Gal4 and LexA/LexAop binary expression systems to show the nature of connectivity between these two different sets of neurons.

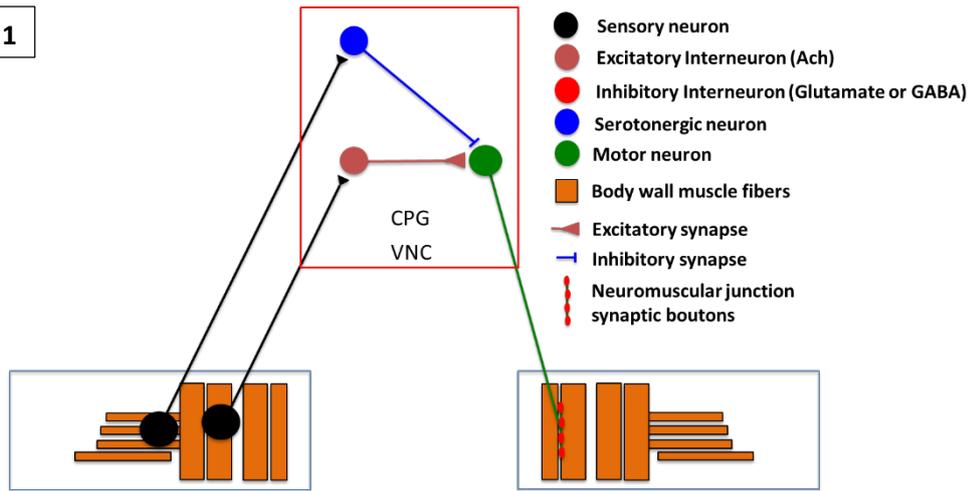
*Drosophila* has a simple 5-HTergic system that consists of 96 5-HT neurons in larval CNS, which are distributed across the CNS: brain lobes, subesophageal zone, and ventral nerve cord (Huser et al., 2012). Different sets of 5-HT neurons in various regions of the CNS might be important for various behaviors; therefore, the activity of small subsets of 5-HT neurons should be manipulated instead of targeting all the 5-HT neurons. A study has shown that activation of a subset of 5-HT neurons in the brain increases feeding behavior; although, activation of the entire 5-HT neurons in the CNS reduces feeding behavior in *Drosophila* adult stage (Albin et al., 2015). The intersectional technique can be used to manipulate a small subset of 5-HT neurons in the nervous system of *Drosophila melanogaster* (Bohm et al., 2010; Alekseyenko et al., 2014; Pooryasin and Fiala, 2015). Additionally, the available Gal4 driver resources can be screened to find those lines that their Gal4 expressions are under the control of cell specific enhancers, which are only active in a small subset of 5-HT neurons (Jenett et al., 2012; Li et al., 2014).

I have proposed a hypothetical model on how 5-HT neurons make contacts with their partners in the sensorimotor neural network. This model explains how

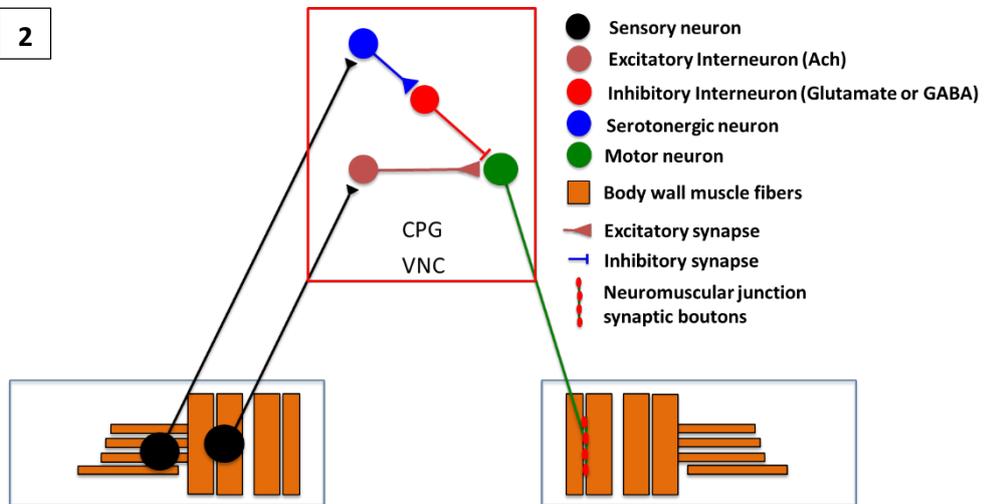
activation of 5-HTergic neurons reduces locomotor activity. (1) 5-HTergic neurons might make direct synaptic contacts with motor neurons and activation of 5-HT<sub>1A</sub> or 5-HT<sub>1B</sub> receptor might lead to inhibition of motor neuron activity. (2) 5-HTergic neurons activation might result in the activation of inhibitory interneurons (GABAergic or glutamatergic interneurons), through activation of 5-HT<sub>2</sub> or 5-HT<sub>7</sub> receptor subtype intracellular signaling pathway, that directly make synaptic contacts with motor neurons; in turn, the activity of motor neurons would be blocked. (3) The activity of excitatory interneurons that make synaptic contacts with motor neurons might be blocked by 5-HTergic neurons activity through activation of 5-HT<sub>1A</sub> or 5-HT<sub>1B</sub> receptor subtype signaling pathways; therefore, the motor neurons activity would be abolished. (4) 5-HTergic neurons might directly make inhibitory synaptic contacts with sensory neurons, which shut down sensorimotor circuit activity (Figure 6.1).

5-HT system consists of 5-HT neurons, which synthesize and release 5-HT upon activation; in addition, 5-HT can bind with and activate various 5-HT receptor subtypes to modulate a vast array of physiological actions and behaviors (Roth (Ed), 2006). A mutation in Tph2 (R439H), which causes reduction in 5-HT concentration, in mouse model results in a significant increase of 5-HT<sub>2A</sub> receptor expression level (Jacobsen et al., 2012). The overexpression of 5-HT<sub>2A</sub> receptor might be a compensatory mechanism to adapt with the reduction in 5-HT level. A comprehensive study is necessary to show how a loss of function mutation in one component of 5-HT system would change the expression level of other components of the 5-HT system. The findings of this study will broaden our knowledge in understanding the biological basis of some of the neuropsychiatric disorders such as depression, anxiety, and autism spectrum disorder.

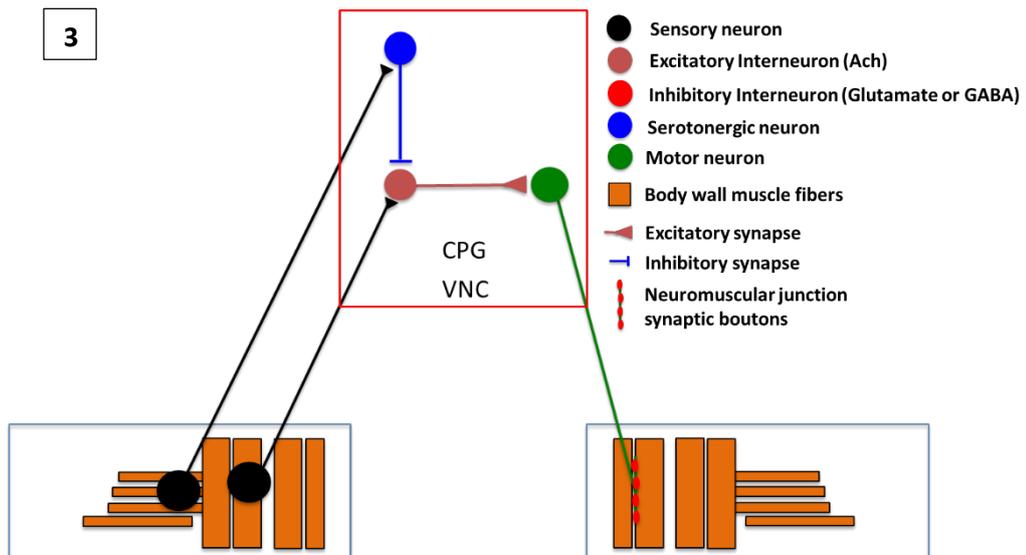
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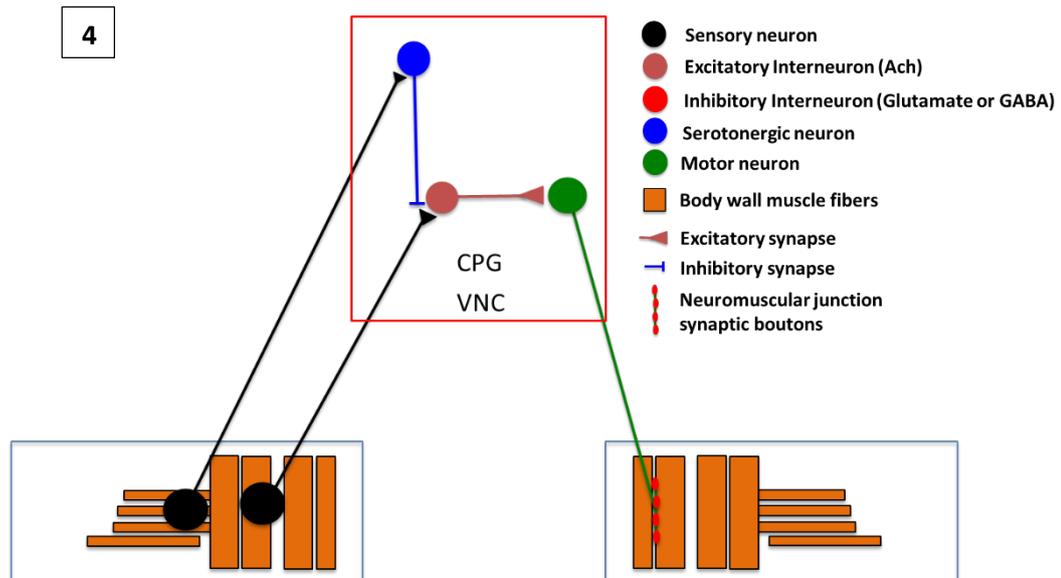


Figure 6.1: A hypothetical model of 5-HT neurons connectivity map in sensorimotor neural network. (1) 5-HTergic neurons make direct synaptic contacts with motor neurons and activation of 5-HT<sub>1A</sub> or 5-HT<sub>1B</sub> leads to inhibition of motor neuron activity. (2) 5-HTergic neurons activation results in activation of inhibitory interneurons (GABAergic or glutamatergic interneurons). This occurs through activation of 5-HT<sub>2</sub> or 5-HT<sub>7</sub> receptor subtype signaling cascades in inhibitory interneurons. In this case, inhibitory interneurons make synaptic contacts with motor neurons; in turn, the activity of motor neurons are blocked. (3) The activity of excitatory interneurons that make synaptic contacts with motor neurons is blocked by 5-HTergic neuron activity, which is mediated through activation of 5-HT<sub>1A</sub> or 5-HT<sub>1B</sub> receptor subtype signaling pathways; therefore, the motor neurons activity would be abolished. (4) 5-HTergic neurons directly make inhibitory synaptic contacts with sensory neurons; therefore, 5-HT neurons activity leads to inhibition of sensory neurons activity which, in turn, sensorimotor circuit activity will be abolished. CPG: central pattern generator neural network; VNC: ventral nerve cord.

## CHAPTER SEVEN

### Teaching related study

#### **Manipulation of various neural circuits and the effect on behavior in *Drosophila* using optogenetics: NGSS-Neurons, genetics, and selective stimulations**

\*This CHAPTER is in preparation for publication in an educational-related journal.

**Author contributions:** Zana R. Majeed, Felicitas Koch (she worked in the lab), and Robin L. Cooper collected data; Zana R. Majeed, Felicitas Koch, and Robin L. Cooper analyzed data; Zana R. Majeed and Robin L. Cooper interpreted results of experiments; Zana R. Majeed, Felicitas Koch, and Robin L. Cooper prepared figures; Zana R. Majeed and Robin L. Cooper wrote and revised the manuscript.

#### **Abstract**

The objective of this module is to explain and address principle concepts in neurobiology. The goal of neurobiology is to show how neural circuit activity controls corresponding behavior in animals. We use *Drosophila melanogaster* as a model system and powerful genetic tool to manipulate various populations of neurons: glutamatergic neurons, serotonergic neurons, GABAergic neurons as well as cholinergic neurons. The optogenetic tool, channelrhodopsin 2 (ChR2), is employed to increase the activity of each population of neurons in a spatiotemporal controlled manner in behaving larvae and adult flies. Various behavioral assays are used to observe the effect of a specific neuron population activation on crawling behavior in larvae and climbing behavior in adult flies. Students will be exposed to important aspects of neural circuits that govern the animal's behavior. By doing these activities, the students will become well acquainted with the actions of different neurotransmitters in the nervous system. A pre- and post- assessment survey on the content was provided to the high school and college students who participated in the experimentation. A large improvement in the student's understanding of content and concepts was gained by conducting this module in the high school and college classes.

## Introduction

Manipulation of inhibitory or excitatory neurons electrical activity while monitoring effects on behaviors provides insights into the functionality of these neurons in neural circuits. To do this type of manipulation, we used *Drosophila melanogaster* that is easy to rear and maintain and ideal for hands-on inquiry-based learning for high school and college courses, which emphasize life science topics. This teaching module is designed to integrate modern genetics, engineering, physics, life sciences, modeling and experimental design. Researching the primary scientific literature and the utilizing the related findings as well as postulating the outcome for newly designed experiments based on the results one collects, the students can test their own predictions and draw hypotheses. This approach provides autonomous learning within and among student groups. The measureable outcomes with obtaining quantitative data for analysis and interpretation are a valuable learning experience. Based on one's findings in the initial experiments, one can readily redesign experimental paradigms to test the formulated hypotheses utilizing one's own prior data. The integration with Arduino hardware and software opens the doors for students to a world of writing codes.

The underlying science in these modules focuses on neurobiology. The seminal discoveries by Hubel and Wiesel (1970) demonstrated that activity in sensory input is indispensable in the development and maintenance of neural circuits. This concept is also essential for development and maintaining synaptic communications at neuromuscular junctions (NMJ) of skeletal muscles (Balice-Gordon et al., 1990; Lomo, 2003). In some cases, the activity profile must occur prior to developmental time points to have plasticity before the neural circuits become more hardwired. After the critical period in synaptic formation, a circuit is not as dependent on activity for competition with other neurons in the establishment of connections. This fundamental phenomenon occurs in organisms from fruit flies to humans. It is known in mice that even after established connections are made as adults that the terminals at NMJs are not fixed to a given location on the muscle fiber. The motor nerve terminals grow out

and pull back over time while continuing to communicate with the muscle fibers (Lichtman and Sanes, 2003).

If motor neurons that are normally innervating a muscle are removed then other motor neurons will take control of the target and innervate it. Thus, motor nerves are searching out targets not already committed by other synaptic inputs (Chang and Keshishian, 1996). This was examined in embryonic and larval *Drosophila* by laser ablating various body wall muscle fibers during development. Even pharmacologically activating or silencing neural circuits during development can have long-term consequences in neural connections and overall physiological functions (Smith et al., 2015). For example, exposing rodents to nicotine during development changes the dendritic morphology within the CNS, which lasts into adulthood (McDonald et al., 2005). Even short exposures in the juvenile stages have long lasting effects in adults for these mice (Ehlinger et al., 2014). It is also established that collective synchronized synaptic activity is important for development of the neural structure (Winnubst et al., 2015). Thus, long term consequences in the established neural circuitry within the CNS and at the NMJ can occur based on neural activity when the initial circuits are being wired.

A guided self-inquiry based approach to learning science has been demonstrated to be a very effective means for student learning over the long term (Bradforth, et al., 2015; Waldrop 2015). The engineering design with the Arduino systems is a very engaging educational experience sought after in many schools within the USA and abroad. Students can design the experiments with various computer codes to control the duration of light on-off time period and frequency of stimulation to observe how activating or inhibiting specific sets of neurons can alter development and behavior of the *Drosophila* larvae or adults. The hardware for the Arduino and associated LED required hardware is relatively inexpensive <\$20 USD for an individual unit. There are dozens of demonstration videos on YouTube for a wide variety of inventions and coding using Arduino.

In this educational module, we demonstrate an approach with optogenetics to selectively activate neurons synthesizing the neurotransmitter GABA, glutamate, serotonin, or acetylcholine. The approach used to stimulate these selective neurons is to activate light sensitive channels expressed in these neurons. Different *Drosophila* lines will be used for each type of neurotransmitter. The ability to control the stimulation with light is to be managed by Arduino system that the students can program or a simplified version with an LED connected to a small battery. Since many of the experimental paradigms will be novel and many unanswered questions remain to be answered in neurobiology, students may uncover unique findings worthy of publication in scientific journals.

This educational module is also designed to embrace the Next Generation Science Standards (NGSS Lead States, 2013) through approaches scientists employ in the development of scientific knowledge. The participants for this exercise will be able to construct models in the neural circuits to explain the observed behavioral phenomenon to make sense of what they observe. The direct real life examples with how neural circuits develop in one's self as well as in other animals is of general interest but also has applied implications for medicine and health. The ability to manipulate various neurotransmitter systems and stimulation paradigms promotes experimental design and redesign based on the observed findings from each experiment. This is an integral aspect of the NGSS. This approach promotes explanations of the findings in order to set a new or altered stimulation paradigm as participants continue to study a phenomenon in different contexts. NGSS recommends that models be used in Developing, Evaluating, Using, and Revising explanations and predictions of science phenomena.

## Materials and Methods

### Fly strains and crosses

Some of the experimental procedures require being able to make selective genetic crosses of two different lines. To perform the crosses, it may be necessary to identify male and female adults and to be able to obtain virgin females (Figure 7.1). The instructors of the course can decide on their resources (dissecting microscopes and time management of students) for either performing the crosses themselves or if the students should be given the time to make the crosses. As a learning experience, the teacher could allow the students to try these procedures but have a cross already prepared for class use. A number of online resources are available to see the differences in males and female adult flies. We look for the black tuft of hairs on the forelegs of the male fly (Figure 7.1). It is good to compare the flies side by side to tell the differences.

There are some procedures where the fly lines obtained can be directly examined without having to make filial 1 ( F1) generations with selective crosses. The lines which are OK371-Gal4;UAS-ChR2H134R-mcherry ( homozygous line, there are two copies for each construct), which expresses the light-activated channelrhodopsin-2 in motor neurons. This line is made by crossing  $w^{1118};P\{GawB\}VGlut^{OK371}$  (BDSC stock # 26160) with  $w^*; P\{UAS-H134R-ChR2\}2$  (BDSC stock # 28995 Pulver et al., 2011). We used another recently created ChR2 line which is very sensitive to light called  $y^1 w^{1118}; PBac\{UAS-ChR2.XXL\}VK00018$  (BDSC stock # 58374) (Dawydow et al., 2014). Virgin females from UAS-ChR2.XXL line were crossed with males of D42-Gal4 (BDSC stock#8816), Trh-Gal4 (BDSC stock#38389), Gad1-Gal4 (BDSC stock# 51630, or ppk-Gal4(BDSC stock# 32078) line to express ChR2-XXL variant in motor neurons, serotonergic neurons, GABAergic neurons or type IV sensory neurons, respectively. We also used UAS-H134R-ChR2;Trh-Gal4 (III) homozygous line, which is kindly provided by Dr. Andreas Schoofs (Schoofs et al., 2014), to compare its behavioral effects with more sensitive ChR2 line. There is no need to make crosses as

this line is homozygous. The larvae or adults should be raised on food supplemented with all-trans retinal (ATR), which is a cofactor essential for ChR2 function, unlike mammals the flies cannot synthesize sufficient amount of ATR for ChR2 function) and a control group without ATR (use ethanol (EtOH) as a vehicle since ATR is dissolved inside absolute ethanol).

### **All-trans retinal preparation**

All-trans retinal (500 mg), which was purchased from Sigma-Aldrich (St. Louis, MO, USA), is dissolved in 17.6 ml absolute ethanol to make 100 mM stock solutions. 100  $\mu$ l of 100 mM stock solution is transferred to small tubes and wrapped in aluminum foil and kept in -20°C freezer. The ATR should be kept away from light since it is sensitive to light; therefore, it will become ineffective if it is exposed to light for a long time.

### **Preparation of fly food supplemented with ATR**

In order to prepare fly food supplemented with 1 mM ATR, 10 mL fly food is dissolved in microwave. The food is left to cool down, then 100  $\mu$ l of 100 mM ATR is mixed well with fly food or 100  $\mu$ l of absolute ethanol is mixed with food as a control. The food vial is wrapped in aluminum foil then the food is left until it was solidified well; otherwise, the flies will be stuck in the wet food. The flies are transferred from its vial to ATR containing vial and kept in a dark place (to keep the ATR from degradation) at room temperature 22-23°C.

### **Larval locomotion behavior**

Locomotion behavior is assessed by placing a single larva on an apple-juice agar plate. The larva is left for one minute to acclimate to the new environment. The body wall contractions are being counted for one minute (BWCs/min) while the larva is being exposed to regular light. Then the body wall contractions are counted for one

minute while larvae are being exposed to blue light (470nm wavelength) (a dispersed-soda-can device). In addition, body wall contractions are being counted while the larva was being exposed to focused focal blue light (a focused light through a microscope eyepiece with a mounted LED). This assay was performed for first, second and third instar larvae. The microscope eyepiece can be bought on Amazon.com as 10X eyepieces. Try to look for the wide opening type so the LED can fit down inside.

### **Rolling behavior in larvae**

The rolling behavior is performed by placing a single larva on the surface of an apple-juice agar plate. The occurrence of rolling behavior is counted for 1<sup>st</sup> and 2<sup>nd</sup> minute. The percentage of larvae that show rolling behavior should be presented in a graphical form.

### **Adult fly behavior assays**

For the adult behavior assays, one can put the left over larvae from conducting the larval behaviors back into the food vial in which they were taken for later conducting adult behaviors. One needs to save the 1<sup>st</sup> crosses and wait until they become adults. Thus, one can directly compare the differences from the larval lines and the adult lines with the same crosses. Also, if one saved the ATR food from the larval assays this can be used to feed the adults. The adults should be a few days (2-8 days) old before conducting these behavioral experiments to insure they have built back up the levels of ATR in the body as the ATR might be degraded in the pupa stage. There are a number of behavioral assays which are commonly used for adult *Drosophila* (Badre and Cooper, 2008; Nichols et al., 2012; <http://www.sdbonline.org/sites/fly/aimain/6behavior.htm>). For some of the assays, one should consider separating males and females, as there are differences in the size and weight of the adult flies. Also, as the adults age, there maybe differences in the behaviors.

The two commonly used behaviors, which are relatively easy to implement, but informative for the biological concepts are the negative geotactic and phototactic assays, which are described below. These assays can be expanded on for deeper investigations into the neurobiology. In addition, these assays allow for data gathering, redesign and vivid discussion for inquiry based labs.

### **Negative geotactic assay**

The adult flies aged 2-8 days are to be anesthetized with ice or CO<sub>2</sub>. The males and females are to be sorted out and transferred into separate vials in cohorts of 10-14 flies. The flies should be left to recover for 24h before running the experiments. A plastic vial (*Drosophila* culture cylindrical vial 1-1/4" diameter x 4" tall; <http://www.enasco.com/product/SB11136M>) is marked at 8cm length, and the 8-10 cohort flies are transferred to that empty marked vial. Another plastic vial is placed on top of the marked one (Figure 7.3) (Ali et al. 2011). The flies are left for one minute. The vials are tapped to knock down the flies to the bottom of the tube. Then number of flies which climbed across the 8 cm mark is recorded for 10 sec. (Figure 7.4). This procedure is repeated three times. The data should be graphed. An schematic example for UAS-ChR2-XXL/+;D42-Gal4/+ flies exposed to blue light and showing the recovery to crossing the 8 cm marked line see Figure (7.4).

### **Phototactic assay**

A device with a 25 cm long plastic tube and light source at one end in a dim-light room is used to assess the phototactic behavior of the adult flies. The tube is narrow enough not to allow the adults to fly but only walk along the tube. Also, the standard small LED fits in one end (Figure 7.5). The male or female flies are anesthetized by ice for 25-30 sec. Individual flies are placed in each apparatus. The flies are left to recover for at least 10 min. Each apparatus with individual fly, which is positioned horizontally or vertically, is tapped until the fly fall to the bottom of the tube,

which was closed by a rubber stopper. The time the fly crossed 10 cm line and 20 cm line is recorded. This apparatus could be positioned horizontally or vertically, but vertical placement examines both geotaxis as well as light sensitivity. The data obtained should be graphed.

### **Data collection and interpretation**

The results from the various experiments highlighted can be tabulated or graphed in various ways depending on the variables one wishes to investigate. Data which can be plotted over time, such as time for the adults to cross the 10 and 20 cm line, can be graphed with free web based graphing software “Joinpoint” which allows students to work at home or at school. Also, graphing the values for the different experimental lines of flies allows for discussion of the data in relation to the biological significance. These exercises provide an inquiry based experience relating to real life topics and can be related to investigations of practical neurobiological phenomenon in relation to human disorders (Parkinson’s, Stiff man syndrome, and epilepsy) along with medical interventions with pharmacological agents on these various neurotransmitter systems. Conducting a literature search on the transmitter systems and how sensory-motor neural circuits function can help to make predictions of the behavioral outcomes when stimulating the particular subsets of neurotransmitter systems for the larva and adults before conducting the experiments on the animals. This establishing a conceptual model of the neurotransmitter and the neural circuits related to the mammalian behavior and then testing if the model hold for the *Drosophila* is an important concept of the NGSS in the use of models and redesigning to observations (Krajcik & Merritt, 2012; NGSS Lead States, 2013).

Figures 7.6, 7.8, 7.10, 7.11 to 7.18 are examples of data collected for various experiments related to these protocols in a research lab environment with controlling as many variables as possible which can be difficult in a teaching laboratory. Ideas in

how one may graph the data can also be obtained by examining these graphs for the various experimental paradigms.

To examine learning outcomes from this laboratory-designed module a pre- and post-survey were given to volunteering high school and college level students. Institutional approval was obtained for the students' surveys. The pre-survey was given prior to telling the students anything about what they were to experience and the post-survey was given after the students were given a brief introduction and time to conduct the experimental manipulations presented. The pre- and post-surveys are shown below:

### **Pre-assessment questions**

1. What kind of neurons carry signals away from the brain to skeletal muscles ?
  - A. Interneurons
  - B. Motor neurons
  - C. Sensory neurons
  - D. Multipolar neurons
2. The part of the neuron that conducts an electrical signal to the next neuron is called the:
  - A. Soma
  - B. Axon
  - C. Dendrites
  - D. Microtubules
3. Normally the resting membrane potential of a neuron is close to:
  - A. -60
  - B. +40
  - C. 0
  - D. +10

4. If voltage gated  $K^+$  channels opened on a typical neuron,
- A.  $K^+$  ions would rush out
  - B.  $K^+$  ions would rush in
  - C.  $Na^+$  ions would rush out
  - D.  $Na^+$  ions would rush in
5. When the membrane is more positive than the resting potential it's called:
- A. Depolarization
  - B. Hyperpolarization
  - C. EPSP
  - D. IPSP
6. Neurons in general release evoked neurotransmitter when stimulated by being \_\_\_\_\_ (complete the sentence).
- A) hyperpolarized (a more negative membrane potential).
  - B) depolarized (a less negative potential ).
  - C) able to remain at a resting membrane potential.
7. Evoked neurotransmitter release is generally released from a presynaptic neuron \_\_\_\_\_ (complete the sentence).
- A) by oozing across the bi-lipid membrane.
  - B) by synaptic vesicles, which containing the neurotransmitter, to diffuse across from one cell to the target cell.
  - C) by synaptic vesicles fusing with the presynaptic membrane and releasing neurotransmitter which diffuses across from one cell to the target cell.

8. A neuron generally releases a single neurotransmitter such as GABA or glutamate or acetylcholine and can be identified by the transmitter they synthesize or contain?

A) True

B) False

9. Whole animal behaviors can be modified by selectively stimulating neurons which store primarily GABA or glutamate or acetylcholine?

A) True

B) False

10. How do neural circuits develop within an animal's brain as the animal develops from an embryo to an adult ? (open response question).

(Assessment: blank answers, answered with examples)

11. Can the development of neural circuits be altered by activity of the neurons within the circuit?

A) True

B) False

12. Please name any particular diseases or conditions within humans which are present when neural circuits do not develop correctly? (open response)

(Assessment: blank answers, answered with examples)

13. Please name any particular diseases or conditions within humans which are present when the receptors for the neurotransmitters do not function correctly?

(open response)

(Assessment: blank answers, answered with examples)

14. Do you think it is possible to take bacterial genes which make proteins for light sensitivity and import those genes into *Drosophila* (fruit fly) genes to then make the same protein as in the bacteria to be sensitive to light in the fruit fly?

A) True

B) False

15. Would you be interested in being able to selectively activate specific types of neurons in animals and see how their behavior changes?

A) yes

B) No

The post-assessment questions were similar in content and in addition questions were asked about how the lab might have helped in understanding neurobiological content.

**Post-assessment questions is as follows:**

1. Please name any particular diseases or conditions within humans which are present when neural circuits do not develop correctly? (open response)

(Assessment: blank answers, answered with examples)

2. Please name any particular diseases or conditions within humans which are present when the receptors for the neurotransmitters do not function correctly?

(open response)

(Assessment: blank answers, answered with examples)

3. How do neural circuits develop within an animal's brain as the animal develops from an embryo to an adult ? (open response question).

(Assessment: blank answers, answered with examples)

4. Would skeletal muscle develop differently or show differences if motor neurons had a lot more or a lot less activity than normal during development?

A) No as skeletal muscles develop independent of motor nerve activity

B) Yes as skeletal muscles development is dependent of motor nerve activity

5. How can exciting some neurons in the brain of the fruit fly cause motor neurons to be less active ? (open response question).

(Assessment: blank answers, answered with examples)

6. What kind of neurons carry signals away from the brain to skeletal muscles ?

A. Interneurons

B. Motor neurons

C. Sensory neurons

D. Multipolar neurons

7. The part of the neuron that conducts an electrical signal to the next neuron is called the:

A. Soma

B. Axon

C. Dendrites

D. Microtubules

8. Normally the resting membrane potential of a neuron is close to:

A. -60

B. +40

C. 0

D. +10

9. If voltage gated K<sup>+</sup> channels opened on a typical neuron,
- A. K<sup>+</sup> ions would rush out
  - B. K<sup>+</sup> ions would rush in
  - C. Na<sup>+</sup> ions would rush out
  - D. Na<sup>+</sup> ions would rush in
10. When the membrane is more positive than the resting potential it's called:
- A. Depolarization
  - B. Hyperpolarization
  - C. EPSP
  - D. IPSP
11. Neurons in general release evoked neurotransmitter when stimulated by being \_\_\_\_\_ (complete the sentence).
- A) hyperpolarized (a more negative membrane potential).
  - B) depolarized (a less negative potential ).
  - C) able to remain at a resting membrane potential.
12. Evoked neurotransmitter release is generally released from a presynaptic neuron \_\_\_\_\_ (complete the sentence).
- A) by oozing across the bi-lipid membrane.
  - B) by synaptic vesicles, which containing the neurotransmitter, to diffuse across from one cell to the target cell.
  - C) by synaptic vesicles fusing with the presynaptic membrane and releasing neurotransmitter which diffuses across from one cell to the target cell.

General understanding of the lab exercises

13. The lab exercises with optogenetics improved your understanding of selectively stimulating neural circuits within an intact animal.

Strongly agree      Agree      Neutral      Disagree      Strongly Disagree

14. These lab exercises were appropriate for the class level I am in.

Strongly agree      Agree      Neutral      Disagree      Strongly Disagree

15. I have a better appreciation after completing these exercises of how optogenetics can be utilized in potential therapy for humans.

Strongly agree      Agree      Neutral      Disagree      Strongly Disagree

The analysis of the pre- and post-survey is still in the process of being analyzed and will take some time beyond the defense of this dissertation. The intention is to publish this educational module after the student's assessment is completed.

Table 7.1: *Drosophila melanogaster* lines

<u>Line</u>	<u>Sex</u>	<u>Outcome</u>
w <sup>1118</sup> ; P{GawB}VGlut <sup>OK371</sup> Cross with line below opposite sex neurons).	Male or Female	ChR expressed in neurons which transporter for glutamate (motor neurons).
w*; P{UAS-H134R-ChR2}2	Male or Female	
y <sup>1</sup> w <sup>1118</sup> ; PBac{UAS-ChR2.XXL}VK00018 Cross with below lines		Virgin Female
D42-Gal4	Male	ChR2 expressed in motor neurons.
Trh-Gal4	Male	ChR2 expressed in serotonergic neurons
Gad1-Gal4	Male	ChR2 expressed in GABAergic neurons
ppk-Gal4	Male	ChR2 expressed in Type IV sensory neurons
w*; P{UAS-H134R-ChR2}2; Trh-Gal4	(homozygous line)	ChR2 expressed in serotonergic neurons.

The observations by the students would be filled in to the tables below:

Table 7.2: Larval behaviors is monitored while the electrical activity of various neurons, expressing different types of neurotransmitters, is being increased by ChR2 activation.

<u>Neuron type</u>	Dim or regular light	Low intensity blue light	Dim or regular light	High intensity blue light	Dim or regular light
<u>OK371-Gal4;UAS-ChR2H134R-mcherry</u>					
<b>Soda can</b>					
motor neurons	?	?	?	?	?
?					
<b>Focuses light</b>					
motor neurons	?	?	?	?	?
?					
<u>UAS-ChR2-XXL crossed with appropriate neurotransmitter line</u>					
<b>Soda can</b>					
motor neurons	?	?	?	?	?
?					
serotonergic neurons	?	?	?	?	?
?					
GABAergic neurons	?	?	?	?	?
?					
Type IV sensory neurons	?	?	?	?	?
?					
<b>Focuses light</b>					
motor neurons	?	?	?	?	?
?					
serotonergic neurons	?	?	?	?	?
?					
GABAergic neurons	?	?	?	?	?
?					
Type IV sensory neurons	?	?	?	?	?
?					
<u>UAS-ChR2H134R-mcherry;</u>					
<b>Soda can</b>					
Serotonergic neurons	?	?	?	?	?
?					
<b>Focused light</b>					
Serotonergic neurons	?	?	?	?	?
?					

Type of behavior coding used: continue crawling forward (CC), crawling backward (CB), stop (S), head casting (HW), rolling (R), keeps turning left or right while crawling (T).

Table 7.3: Representative sample data for the negative geotaxis assay in flies with various neuron population activation by light.

<u>Neuron type</u>	<u>Time taken to cross 8 cm line (10 trials before blue light exposure, 10 trials after blue light exposure)</u>
<u>OK371-Gal4;UAS-ChR2H134R-mcherry</u>	
<b>Soda can</b>	
motor neurons	?
<b>Focused light</b>	
motor neurons	?
UAS-ChR2.XXL crossed with appropriate neurotransmitter line	
<b>Soda can (low intensity)</b>	
motor neurons	?
serotonergic neurons	?
GABAergic neurons	?
Type IV sensory neurons	?
<b>Focused light (high intensity)</b>	
motor neurons	?
serotonergic neurons	?
GABAergic neurons	?
Type IV sensory neurons	?
<u><b>UAS-ChR2H134R-mcherry:</b></u>	
<b>Soda can</b>	
Serotonergic neurons	?
<b>Focused light</b>	
<u>Serotonergic neurons</u>	?

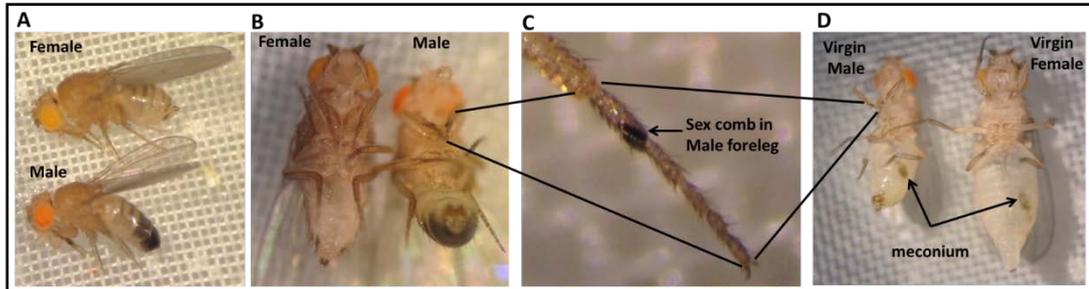


Figure 7.1: (A) (Lateral view) Morphological characteristics and sexual dimorphism of adult *Drosophila melanogaster*. Adult female fly (top) has a light colored abdomen region; however, adult male fly (bottom) has a dark posterior abdomen region. (B) (Ventral view) Shows morphological differences between male and female flies. (C) Magnified view of the male fly foreleg shows male sex comb structure. (D) Newly eclosed male and female adult flies, which have meconium. Male has a pointed brown-colored posterior region.

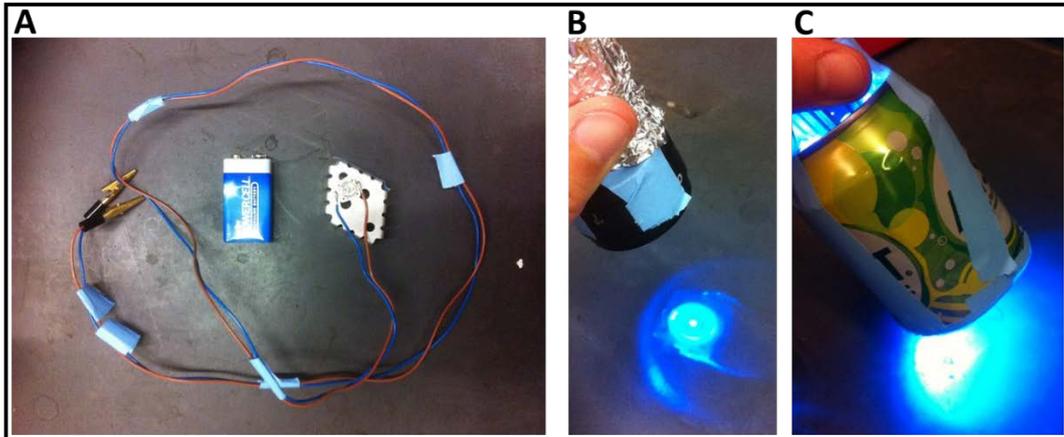


Figure 7.2: (A) The blue light emitting diode (LED) (wavelength = 470nm) is glued on a cooler plate with a temperature resistant glue. The LED light is connected to a 9V battery. Various intensity of LED light can be used by attaching the LED. (B) to microscope ocular lens, which gives off high intensity light or (C) soda can, which gives low intensity dispersed light.

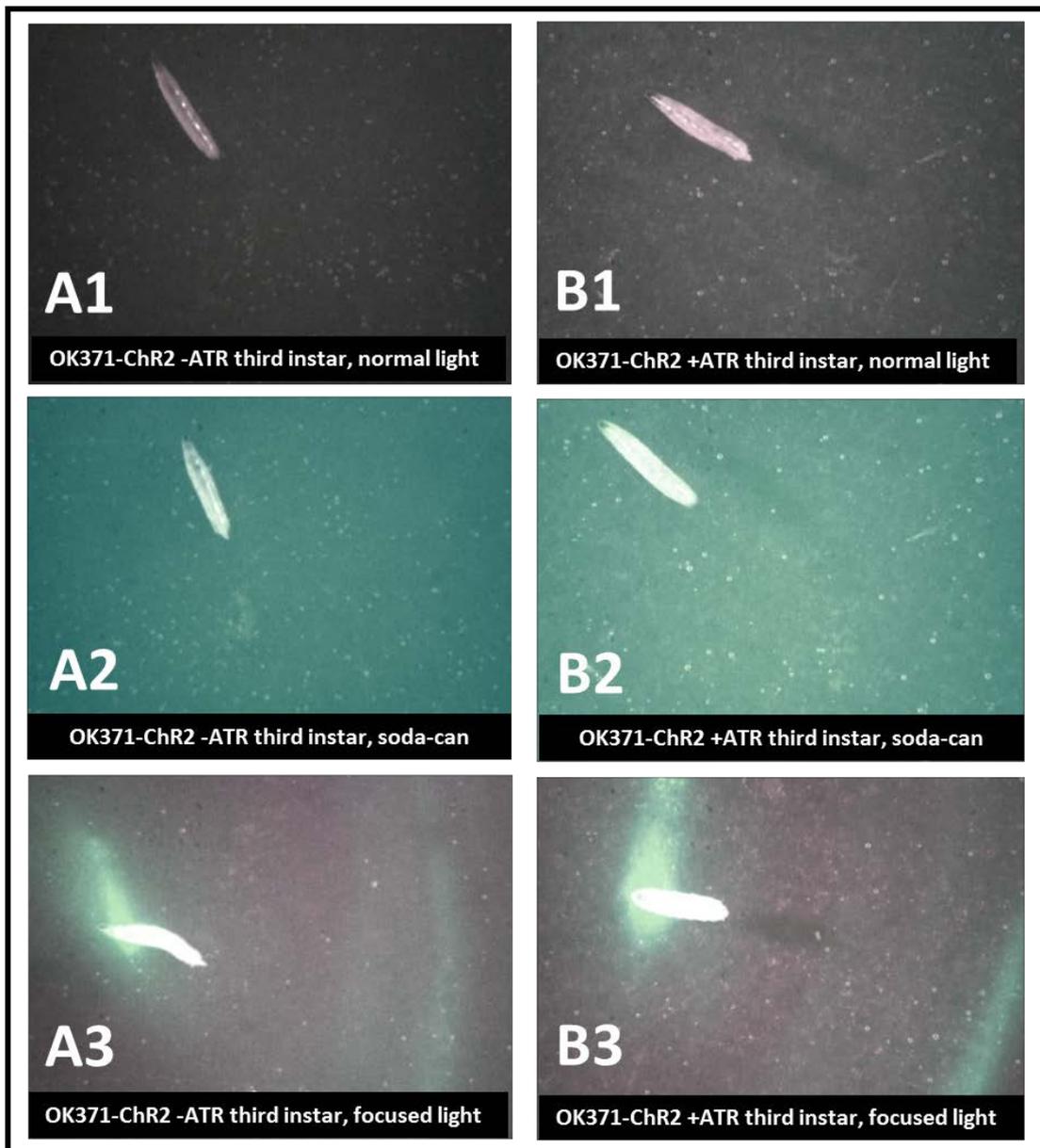


Figure 7.3: Activation of ChR2 channels in motor neurons of third instar larva. OK371-Gal4 (Gal4 driver specific for motor neurons) is crossed with UAS-ChR2H134R-mcherry line (This line is homozygous for both Gal4 and UAS constructs). The progeny is expressing ChR2 in motor neurons. (A) The larvae were raised in a fly food which was not supplemented with all-trans retinal (ATR), which is a cofactor important for ChR2 membrane integration and function. (A1) The body wall contractions are counted on an apple juice agar plate for 1min when the larva exposed to regular light. (A2) The larvae is being exposed to low intensity blue LED light (470nm) for 1min while the BWCs are being counted. (A3) The crawling behavior of larva is being observed while it is being exposed to intense blue light for 1min. (B) The larva was fed ATR (1mM), which is mixed with fly food. The body wall contractions are being counted when the larva is being exposed to regular light (B1), low intensity blue light (B2), or high intensity blue light (B3). The larva does not respond to the low intensity light although when it is being exposed to high intensity blue light, the body wall muscles contract which can be observed by shortened body length (B3).

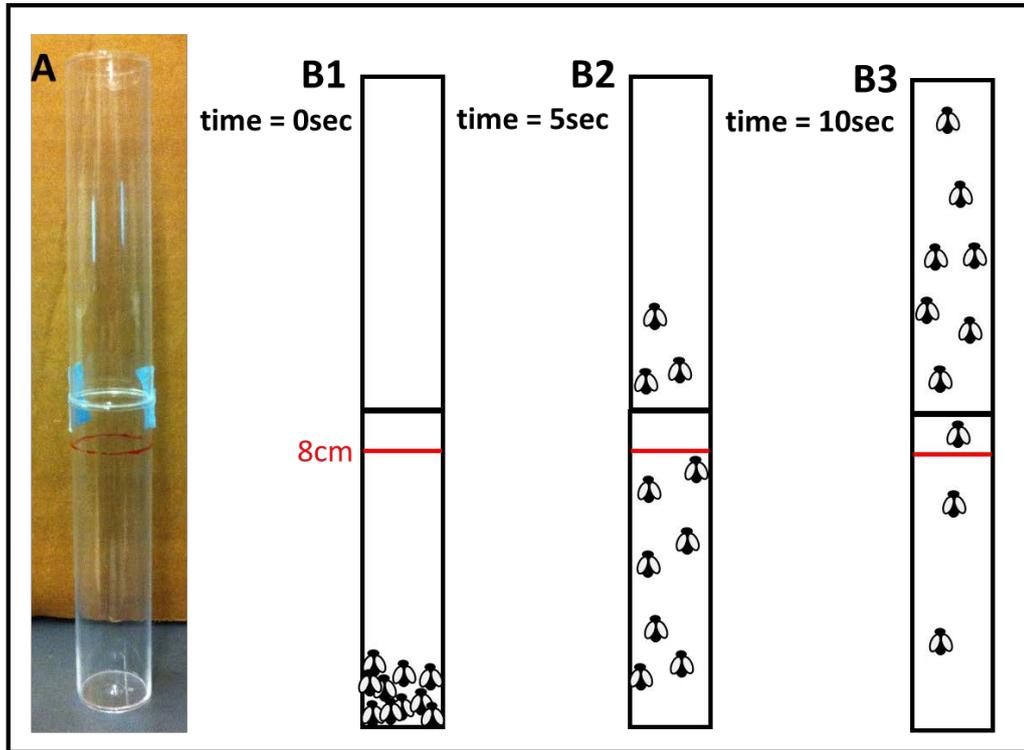


Figure 7.4: Negative geotaxis assay in adult flies. (A) Two plastic vials are put together for this assay. 10-14 adult male or female flies are transferred to an empty plastic tube which is marked at 8 cm length. Second plastic tube is put on the top of the first marked plastic tube and sealed with tape. (B1-B3) The tube is tapped until all the flies fall into the bottom of the first tube (B1). The flies start climbing up on the wall of the plastic tubes. After 10 seconds the number of the flies that cross the 8 cm red line is counted which shows the percentage of the flies that are crossed the line in 10 sec. (B1) 0 %, (B2) 30 %, (B3) 8% of the flies are crossed the red line.

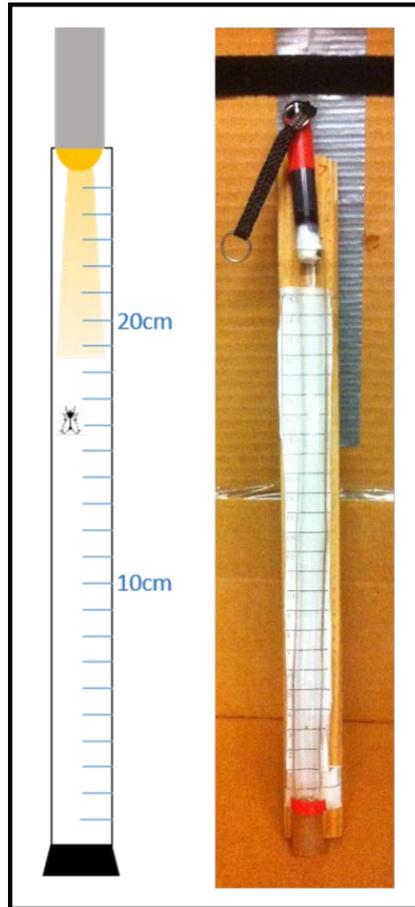


Figure 7.5: The experimental set up for adult phototactic assay in a narrow tube. A single male or female fly is transferred in the tube. The tube is tapped until the fly falls into the bottom. The time that it would take to reach 10 cm and 20 cm is recorded.

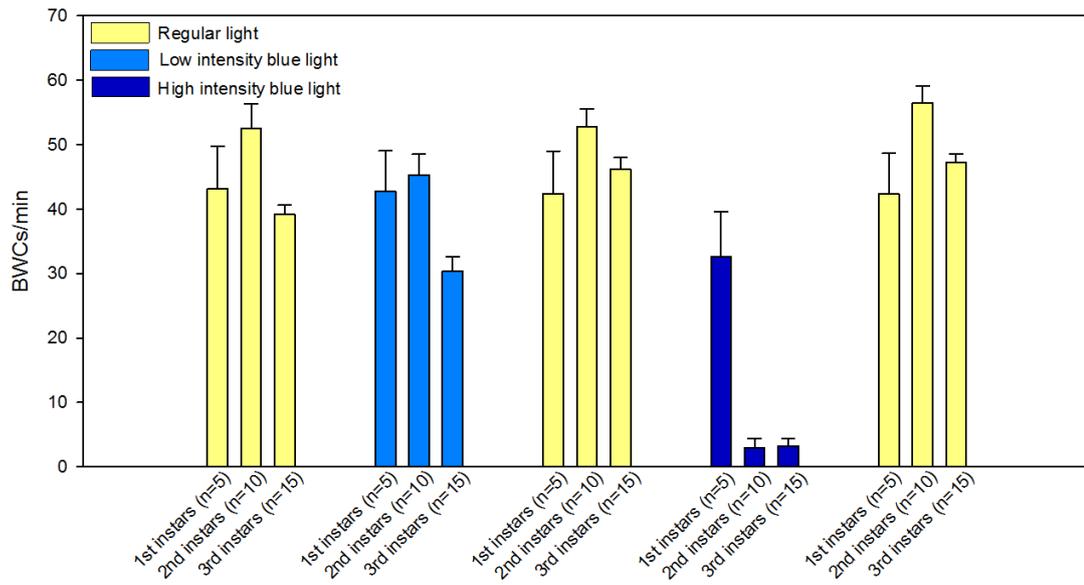


Figure 7.6: Activation of ChR2 channels in motor neurons (UAS-ChR2H134R-mcherry;OK371-Gal4) produces different responses in various instar larvae (The larvae were fed ATR 1 mM). The body wall contractions for one minute (BWCs/min) were counted while the larva was exposed to regular light, low intensity blue light or high intensity blue light. The data shows that the first instar larvae do not respond well to even high intensity blue light.

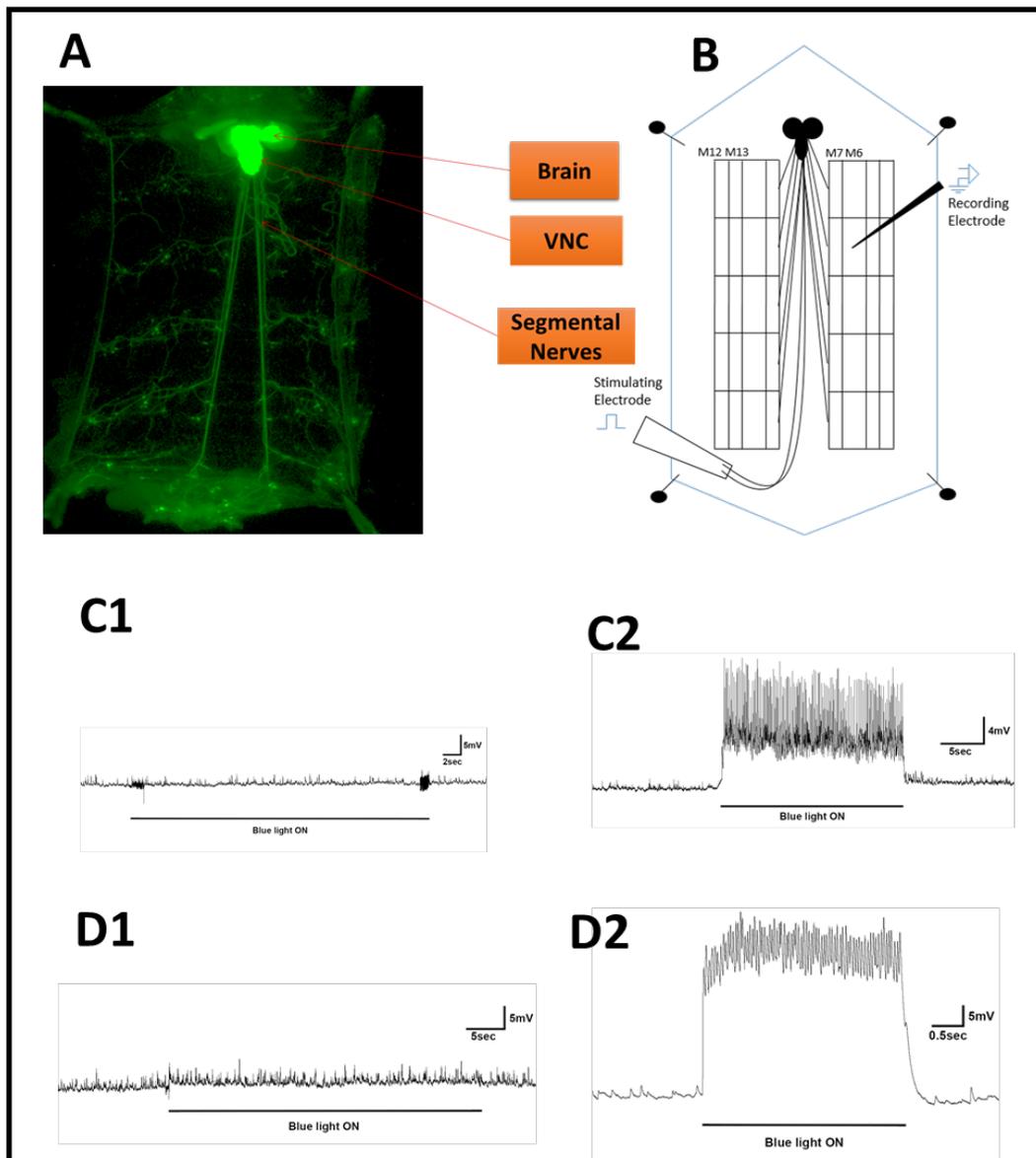


Figure 7.7: (A) The nervous system in *Drosophila melanogaster* third instar larvae expressing green fluorescent protein (GFP) in the whole nervous system. (B) Dissected third instar larvae shows m6 muscle fibers and intracellular microelectrode to record excitatory postsynaptic potentials (EPSPs) while the ChR2 in motor neurons are being activated by blue light exposure. (C1) Intracellular recording in OK371-ChR2 minus ATR (CNS intact) third instar larvae. The blue light (low intensity) exposure does not produce any postsynaptic responses in muscle fiber M6 since the larvae is not fed ATR, which is a required supplementation for the action of ChR2. (C2) Intracellular recording in OK371-ChR2 plus ATR 1 mM (CNS intact) third instar larvae. Blue light (low intensity) exposure produces responses in M6 muscle fiber which is presented as excitatory postsynaptic potentials (EPSPs). (D1) Intracellular recording from M6 fiber muscle in OK371-ChR2 minus ATR (CNS intact) third instar larva. Blue light (high intensity) exposure does not activate motor neurons. No EPSPs are seen in this trace although the miniature EPSPs are still present. (D2) The evoked response is being recorded in OK371-ChR2 plus ATR 1mM (CNS intact) third instar larvae while it is being exposed to blue light (high intensity).

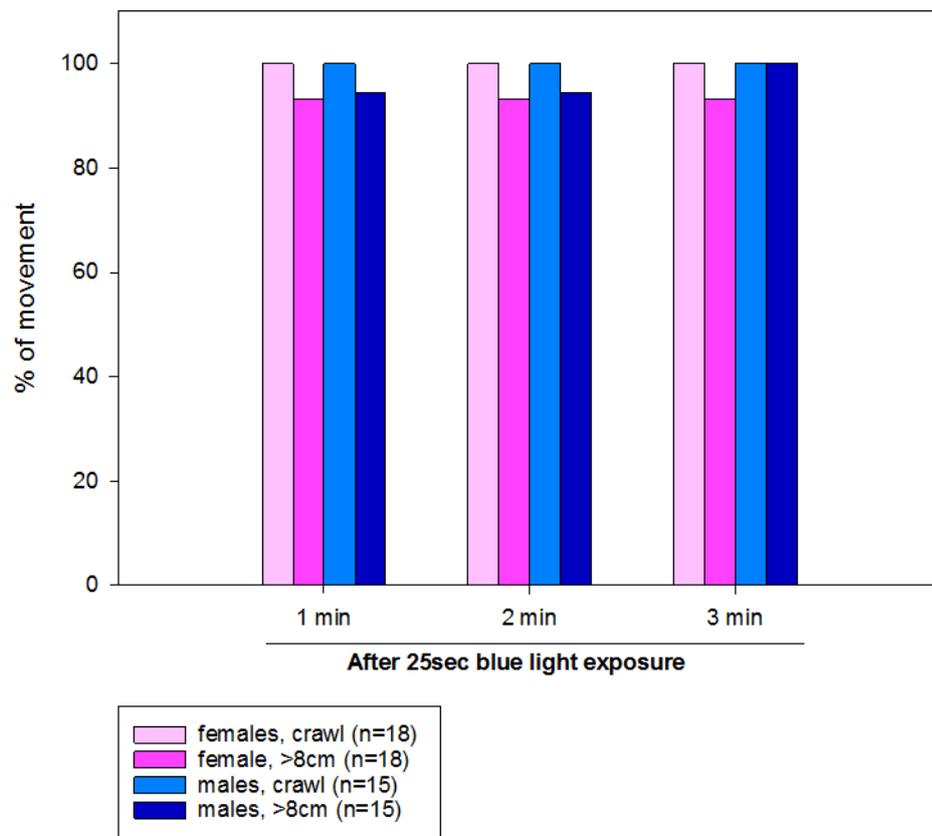


Figure 7.8: Locomotor activity in adult male and female flies after exposure to blue light. These flies are expressing ChR2 in motor neurons and are fed ATR 1 mM (UAS-CHR2H134R-mcherry;OK371-Gal4). The blue light does not exert influence on the negative geotaxis assay since the blue light cannot penetrate well the thick dark adult cuticle.

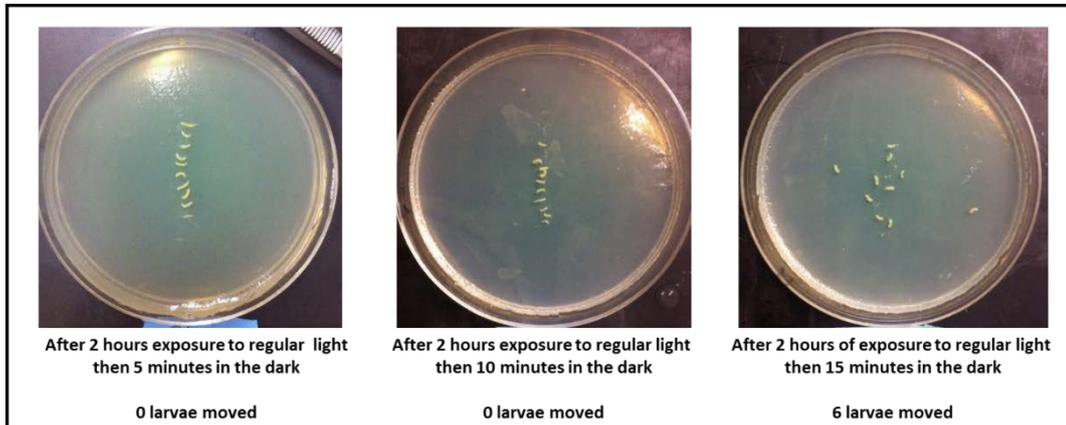


Figure 7.9: Locomotor activity in third instar larvae expressing ChR2 in motor neurons (UAS-ChR2-XXL/+;D42-Gal4/+). When the larvae were exposed to regular light, they were all contracted and did not move (n=30, 10 larvae per agar plate per condition). 10 third instar larvae were placed on an apple juice agar plate. The Larvae were exposed to regular light for 2 hrs. The larvae were stayed in their location without any movement. Three different conditions were used to show how much time it would take for the larvae to start moving again after 2 hr regular light exposure. The data shows that it takes about 15 min for the larvae to restore their locomotor activity.

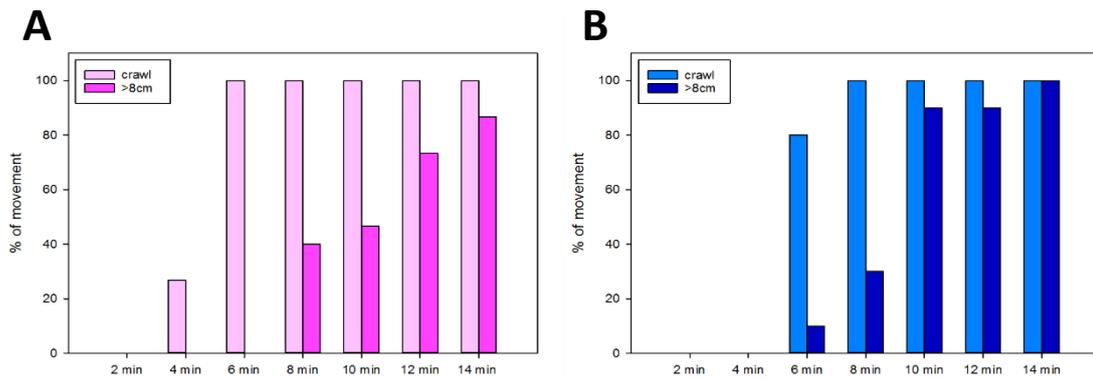


Figure 7.10: Activation of highly sensitive ChR2 in motor neurons (UAS-CHR2-XXL/+;D42-Gal4/+). (A) The crawling and negative geotactic behavior of adult female flies is decreased after 25 sec blue light (low intensity) exposure. After 14 min the flies restored their normal climbing ability. (B) The ability to crawl and climb was markedly compromised in adult male flies being exposed to blue light for 25 sec. The crawling ability restored after 6 min of paralysis although the climbing behavior went back to normal after 12 min of paralysis. These flies were raised of food supplemented with ATR 1 mM.

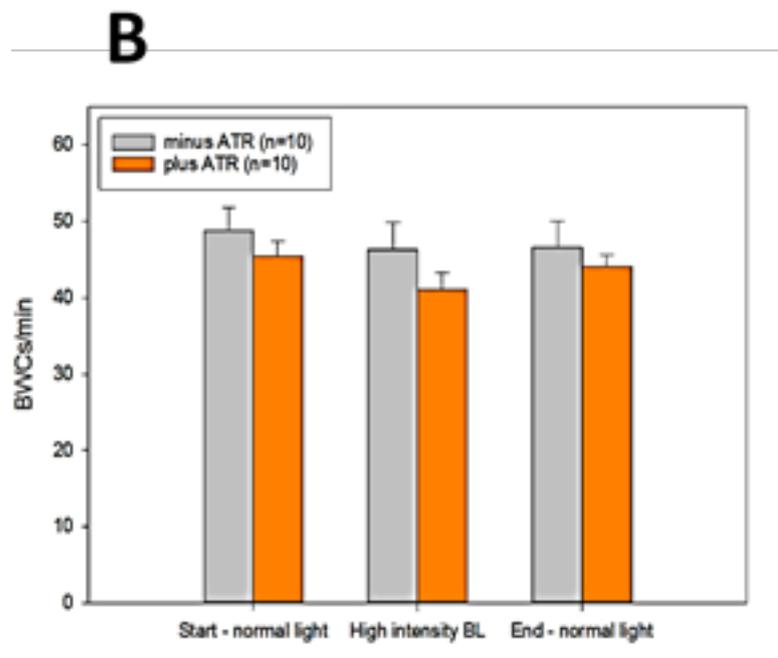
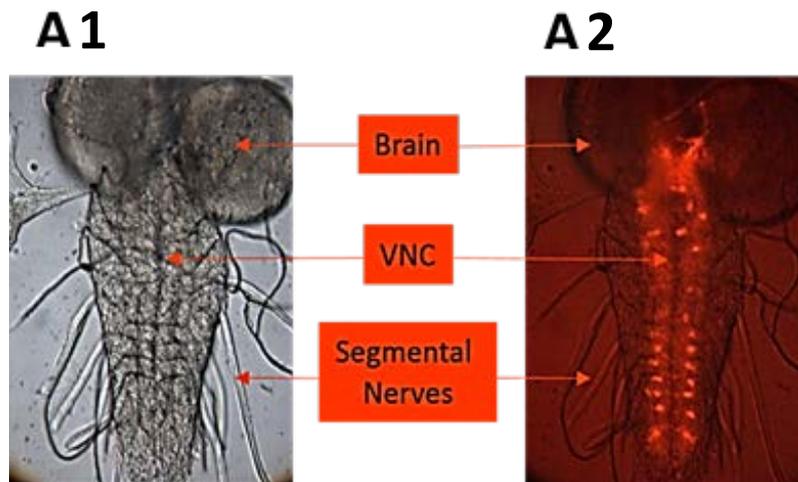


Figure 7.11: (A1) Central nervous system in third instar larva. (A2) Serotonergic neurons expressing mCherry fluorescent protein (UAS-mCherry-ChR2 H134R; Trh-GAL4, homozygous for both constructs). (B) Activation of serotonin producing neurons did not produce a significant effect on locomotor activity in third instar larvae.

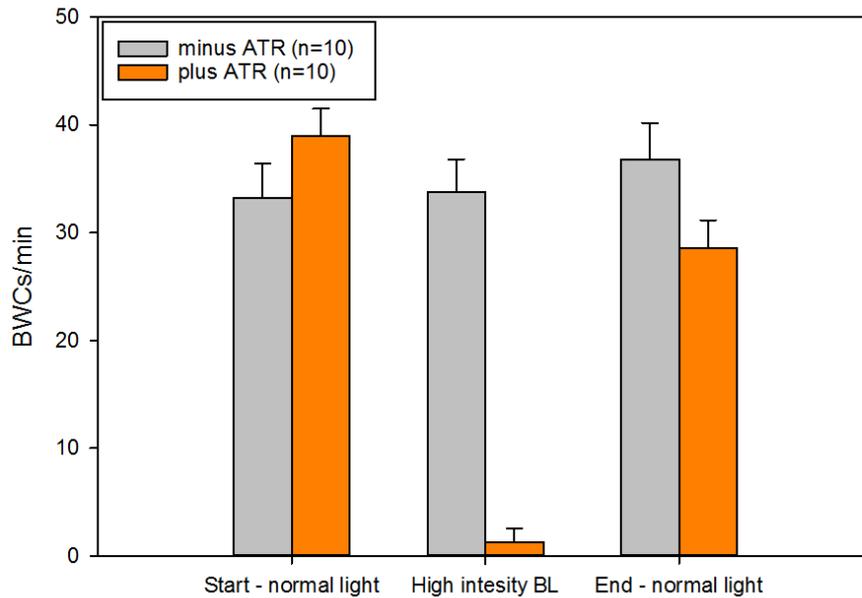


Figure 7.12: Serotonergic neuron activation modulates locomotor behavior in third instar larvae. To change serotonergic neuron activity, CHR2 were expressed in serotonergic neuron population (UAS-ChR2-XXL/+;Trh-Gal4/+). The body wall contractions were counted in third instar larvae fed on food supplemented with ATR 1 mM or ethanol (vehicle). When the larvae, which were fed on ATR 1 mM) were exposed to blue light (high intensity), the locomotor activity significantly compromised. However, when the larvae fed on a food without ATR supplementation were exposed to blue light (high intensity), the locomotor activity were not affected.

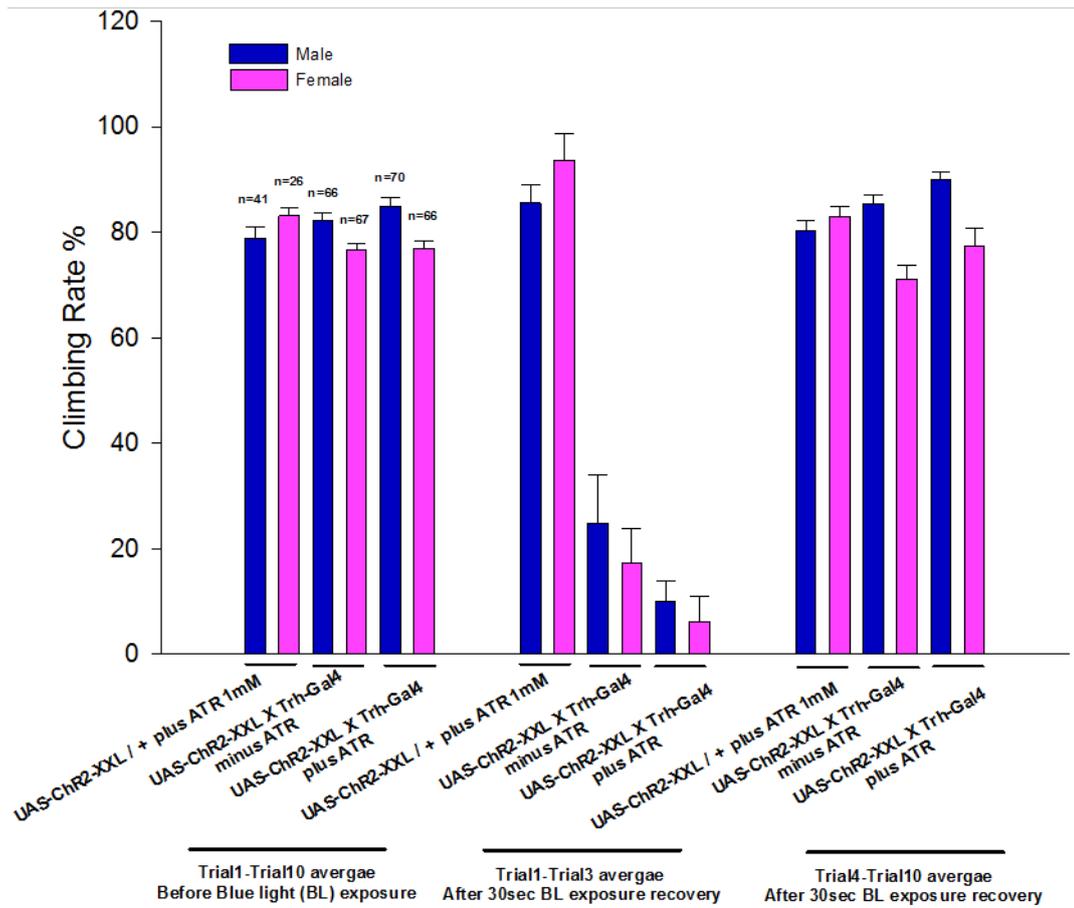


Figure 7.13: Activation of serotonergic neurons in adult flies decreases climbing ability. The electrical activity in serotonergic neurons is increased by expressing ChR2. When the adult flies were exposed to blue light (low intensity), the climbing ability significantly reduced. Both flies groups (UAS-ChR2-XXL/+;Trh-Gal4/+) which were fed supplemented with ATR 1mM or ethanol (vehicle) were affected by the blue light exposure. However, blue light did not have effect on the control lines (UAS-ChR2-XXI/+). (Majeed et al., (2016). *Neural Plasticity* 2016:7291438).

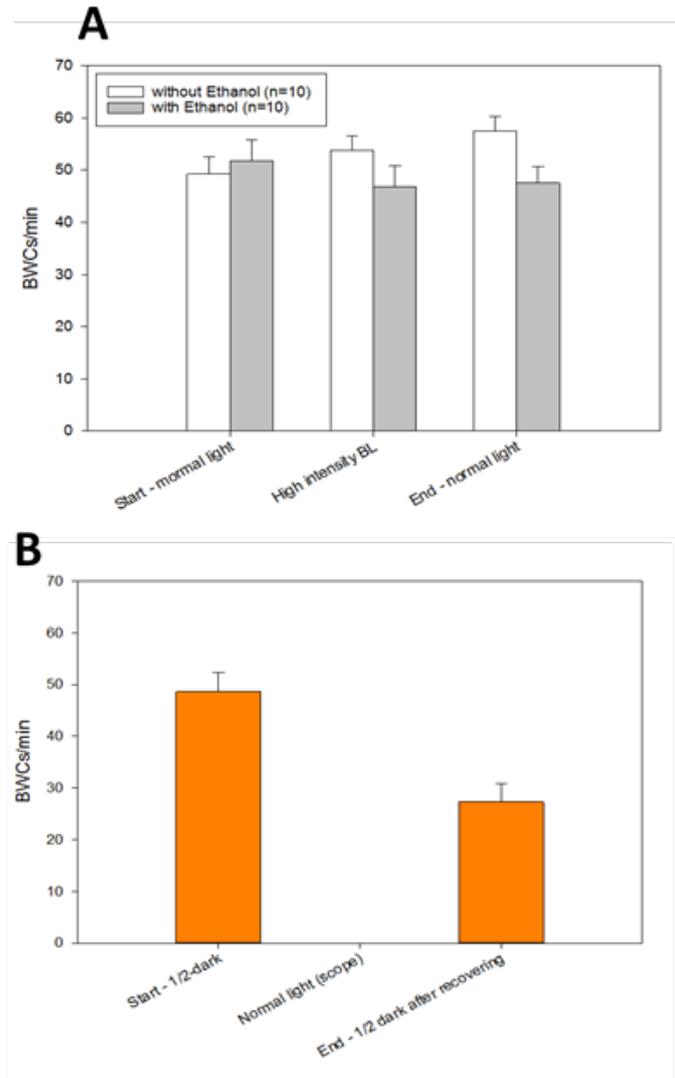


Figure 7.14: Manipulation of GABAergic neuron electrical activity diminishes locomotor activity in third instar larvae (UAS-ChR2-XXL/+;Gad1-Gal4/+). (A) The locomotor activity in larvae fed ethanol (vehicle) does not change with either normal white light or blue light (BL) exposure. (B) Although, when the larvae which were raised on food supplemented with ATR 1 mM are exposed to regular white light, the locomotor activity significantly decreased. During the exposure to white light, larvae started contracting which was followed by body muscle relaxation.

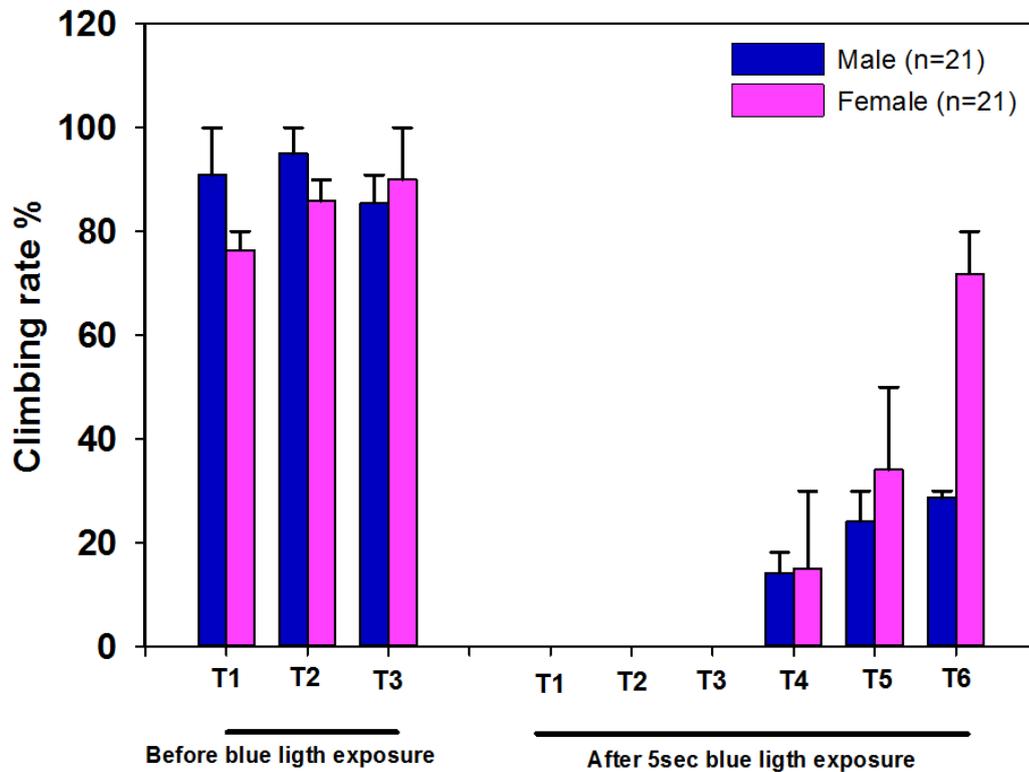


Figure 7.15: Climbing ability in adult flies expressing ChR2 in GABAergic neurons (UAS-ChR2-XXL/+; Gad1-Gal4/+) is compromised after 5 sec blue light exposure. The climbing ability was measured in three different trials before the blue light exposure. After 5 sec blue light exposure, the climbing ability was performed in six different trials. ChR2-XXL activation significantly reduced climbing ability. Flies were fed food supplemented with ATR 1 mM. The climbing assay was carried out in a dim light room since the bright light might also activate ChR2 channels which make it difficult to perform the assay.

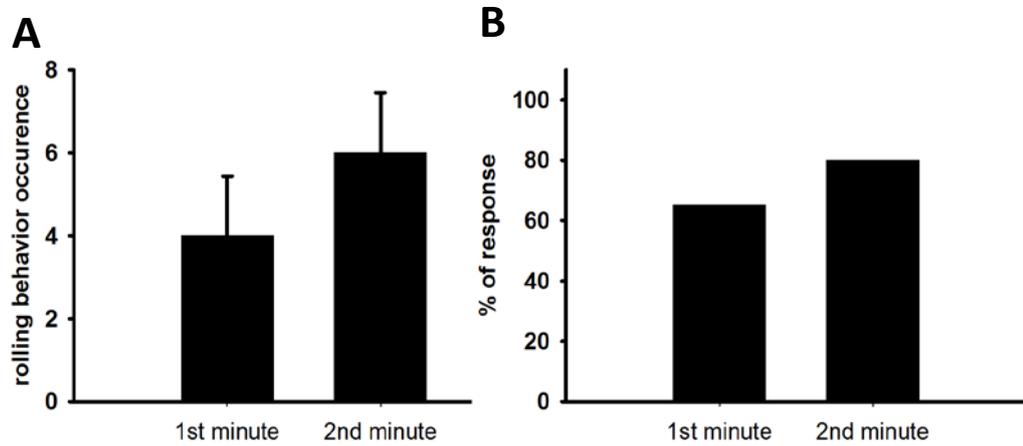


Figure 7.16: Rolling behavior is induced by activating ChR2 in type IV sensory neurons in third instar larvae (UAS-ChR2-XXL/+; ppk-Gal4/+) (n=20), food supplemented with ATR 1mM). (A) Shows the occurrence of rolling behavior in the 1<sup>st</sup> and 2<sup>nd</sup> minute of light exposure (normal scope light). (B) Most of the larvae showed rolling behavior when they are exposed to light.

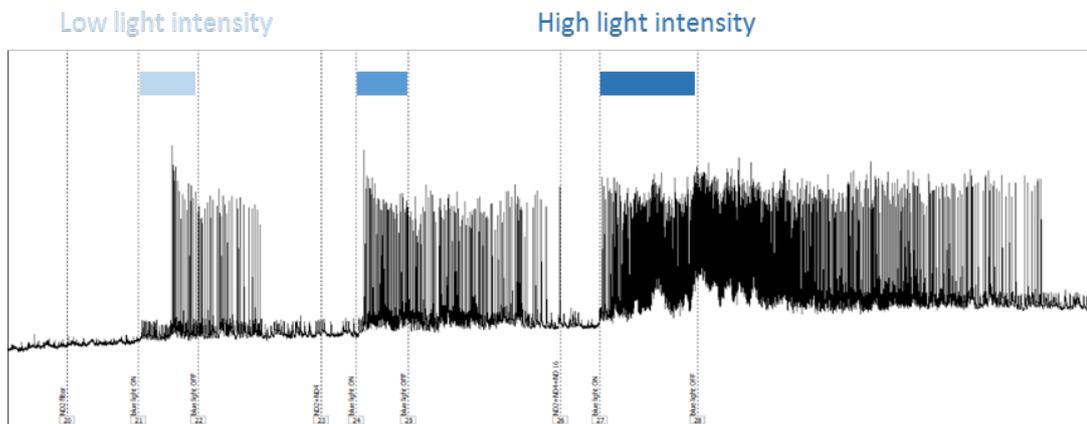


Figure 7.17: Intracellular EPSPs recording from body wall muscle fiber in third instar larva expressing ChR2 in type IV sensory neurons (UAS-ChR2-XXL/+;ppk-Gal4/+, food supplemented with ATR 1 mM). Activation of ChR2 in type IV sensory neurons makes motor neurons to fire action potentials which, in turn, depolarize muscle fibers. The motor output (ie., EPSPs traces) is being recorded while the third instar larva is being exposed to various intensity of blue light.

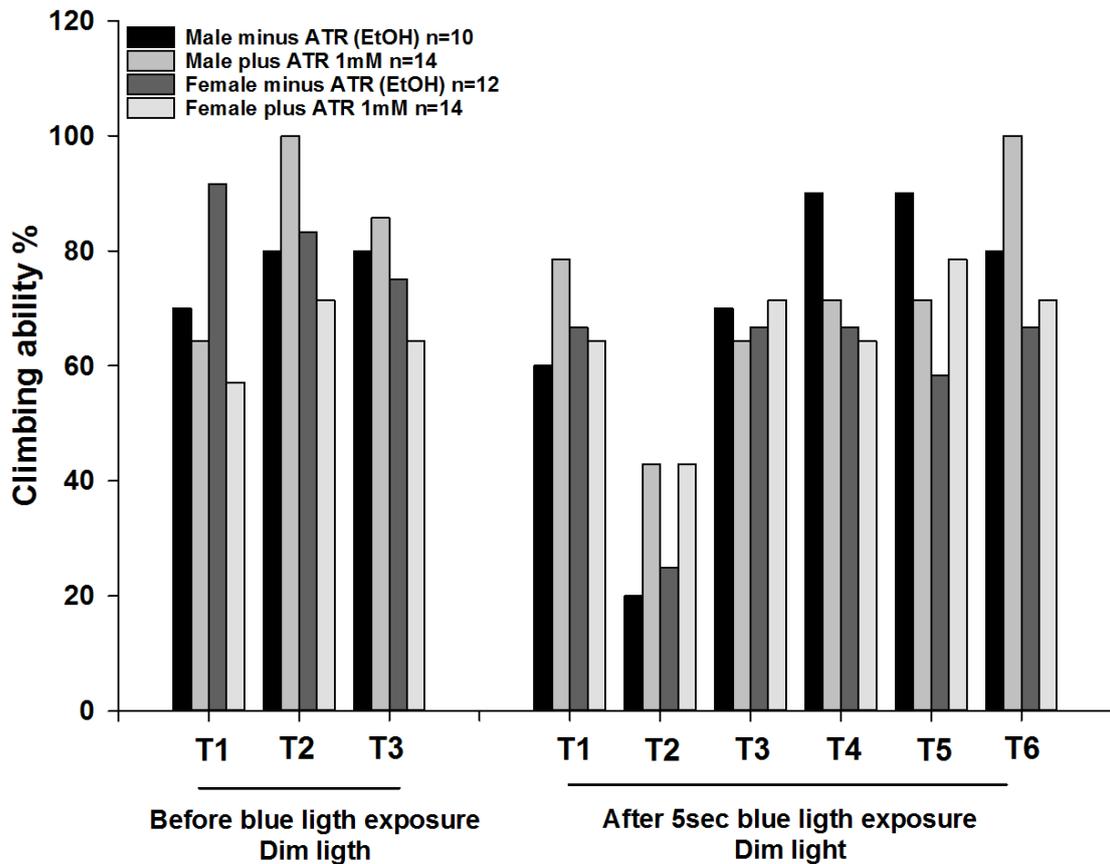


Figure 7.18: Type VI sensory neuron activity modulation affects climbing ability in flies expressing ChR2 in pickpocket neurons (UAS-ChR2-XXL/+; ppk-Gal4/+). After 5 sec blue light exposure, some of the flies were paralyzed for 1-2 seconds then they recovered well. As it is shown that the first trial (T1) after blue light exposure, the flies do well in climbing assay; although, in the second trial (T2 after blue light exposure), the flies climb the middle of the bottom tube then they stop climbing further. They recover quickly in the following trials.

## CHAPTER EIGHT

### Teaching-related study

#### Proprioception and tension receptors in crab limbs: Student laboratory exercises

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**Author contributions:** Zana R. Majeed and Robin L. Cooper performed the experiments; Zana R. Majeed and Robin L. Cooper analyzed data; Zana R. Majeed, and Robin L. Cooper interpreted results of experiments; Zana R. Majeed and Robin L. Cooper prepared figures; Robin L. Cooper drafted the manuscript; Zana R. Majeed, Josh Titlow, H. Bernard Hartman and Robin L. Cooper edited and revised the manuscript.

#### Abstract

The primary purpose of these procedures is to demonstrate for teaching and research purposes how to record the activity of living primary sensory neurons responsible for proprioception as they are detecting joint position and movement, and muscle tension. Electrical activity from crustacean proprioceptors and tension receptors is recorded by basic neurophysiological instrumentation, and a transducer is used to simultaneously measure force generated by stimulating a motor nerve. In addition, we demonstrate how to stain the neurons for a quick assessment of their anatomical arrangement or for permanent fixation. Staining reveals anatomical organization that is representative of chordotonal organs in most crustaceans. Comparing the tension nerve responses to the proprioceptive responses is an effective teaching tool in determining how these sensory neurons are defined functionally and how the anatomy is correlated to the function. Three staining techniques are presented allowing researchers and instructors to choose a method that is ideal for their laboratory.

## Introduction

Proprioception is the sensation of limb position and movement that enables coordinated motor behavior. Proprioceptors consist of position (static) and movement (kinesthetic) receptors. In insects and crustaceans, chordotonal organs are the structures that provide that information to the CNS (Whitear, 1960). Not all chordotonal organs span a joint but they can still monitor joint movements due to their attachment on the apodemes (tendon like structures) which span the joint and move in association with the skeletal muscle and joint articulation. Crab legs have six joints, each having one or two chordotonal organs (Alexandrowicz, 1972). Typically a chordotonal organ has 60-100 or more sensory neurons embedded within an elastic strand, neurons that signal static joint position, direction and speed of movement (Hartman and Boettiger, 1967; Burke, 1953). The input from chordotonal organs at each joint and leg is then centrally processed allowing coordinated movements by the animal.

The forces that leg muscles produce during isometric and isotonic contractions are detected by tension receptors associated with muscle fibers and their attachments to apodemes (Hill, 1970; Macmillan and Dando, 1972). In the crustacean walking leg protocols that follow, we present methodology for recording from primary sensory neurons that monitor proprioception, and neurons that respond to forces generated by muscle fibers. A technique for activating leg movements and quantifying force generation is also presented, as well as anatomical techniques that can be used to characterize the arrangement of these peripheral nervous system structures.

The procedures demonstrated below enable structural and functional analysis of the neurons that innervate both types of receptors relative to their location on a chordotonal elastic strand and apodeme. To illustrate, we use the propodite-dactylopodite (PD) chordotonal organ, the organ that spans the distal most segment of the crab leg (Hartman and Boettiger, 1967). Though detailed electrophysiological studies began in the 1930's and are still being carried out today, some aspects are becoming known about the segmental connections of proprioceptors in the various

joints and their roles in coordinated control of muscles (Bévengut et al., 1983; Hartman, 1985). Establishing structure-function relationship between the proprioceptive organs, muscles and the nervous system will help define these roles. Labeling the somata and distal endings of tension neurons inserted into the apodeme reveals their location relative to muscle fibers (Stuart et al., 1972; Holsinger, 2013; Whitear, 1962).

We present three staining techniques for crustacean legs that can be used in research or academic laboratories. Methylene blue staining provides suitable contrast for muscles and nerves and is recommended as a simple technique for students to learn the anatomy. Labs that have fluorescence microscopy setups can accomplish more selective neuronal staining by briefly exposing the nerves to the vital dye 4-di-2-ASP. The third alternative is CoCl<sub>2</sub> backfill, which stains and fixes the neurons, and does not require fluorescence imaging. Though it is labor and time intensive, this staining process gives high contrast and specificity for the nerves that are filled. Together these techniques can be used for comparing various chordotonal organs not only within a limb or between limbs but also among other crustacean and insect species. 20-22 Blue crabs (*C. sapidus*) used in physiological recordings and for anatomical staining are readily available all around the Southern and Southeastern border of the United States. This species serves as a representative of the chordotonal and tension nerve arrangements found in most crabs. Laboratories on the west coast will prefer to use the much larger Dungeness crab (*Cancer magister*) for these experiments.

### **Procedure:**

#### **Dissection and recording electrical activity from the propodite-dactylopodite (PD) nerve**

1. Hold the crab across the carapace from behind, and avoiding the claws, pinch the proximal part of the meropodite with forceps. The leg will autotomize to prevent the animal from bleeding to death. Use caution when handling blue

crabs as they are rather aggressive and very fast (Figure 8.1).

2. Make a cut between the propodite and carpodite. Discard the carpopodite and the attached meropodite (Figure 8.1).
3. Cut a large window in the cuticle on the pigmented (lateral) side of the propodite with a scalpel with a #11 blade (Figure 8.2 and 8.3). (Note: Do not cut deeply).
4. Remove the cuticle layer by sliding the scalpel blade beneath and parallel to the cuticle. This severs the muscle fibers attached to the cuticle.
5. Using the same technique cut a smaller window on the pigmentless (medial) side of the propodite, but leave the condyle (the socket joint or hinge between segments) attachment intact.
6. Prepare a Sylgard-lined dish containing crab saline to pin the preparation down.
7. Locate the PD organ by carefully probing with the fire-polished glass needles. The elastic strand spanning the joint has a silver appearance.
8. Remove muscle fibers that obscure your view of the organ from both sides of the tendon. Be very careful not to injure the PD organ or its nerve.
9. Once this has been accomplished, firmly re-attach the preparation to the dish with the pigment side (lateral) facing up (Figure 8.4).
10. Follow the PD organ nerve in the propodite as far proximally as possible in order to free-up a long length of nerve (1.5 cm) for recording purposes. This is best done while the PD nerve is still attached to the main leg nerve.
11. After separating the PD nerve from the main leg nerve with the aid of glass needles, sever the PD nerve proximally with the iris scissors. (Note: Do not stretch or pull on the nerve during the dissection).
12. Move the dactylopodite to an extended and fully flexed position. Take note about where the extreme flexion and extension positions are and a half-way point for later use.
13. Place a ground wire inside the saline bath.
14. Turn on the electrophysiology recording hardware/software. Our setup has

been described previously (Leksrisawat et al., 2010).

15. Position the microscope so that it is overlooking the microscope stage. Once it is placed on the stage, you will need to adjust the position of the high intensity illuminator beam to best visualize the preparation.
16. Position the micromanipulator so the attached suction electrode assembly will have easy access to the saline bath and preparation. The suction electrode is constructed as shown in an on line video (Baierlein et al., 2011).
17. To detect neural activity, draw the cut end of the PD nerve into the suction electrode.
18. Move the dactyl throughout extended and flexed positions for several cycles with the aid of a glass probe or wooden dowel.
19. Next observe activity when the dactyl is pinned in the extended, flexed and mid positions.

#### **Recording electrical activity from the tension while monitoring force generation.**

1. Determine the lateral and medial side of the leg. The medial side has a soft texture that can be felt by pinching gently in the meropodite region with a fingernail.
2. Face this soft cuticle side up in the dish. The excitor motor nerve that innervates the opener muscle also innervates the stretcher muscle in the carpopodite.
3. In order to stimulate the opener muscle the stretcher motor nerve in the carpodite region is isolated and stimulated with a suction electrode. The proximal part of the leg is removed by transecting the meropodite with scissors.
4. Remove a section of the cuticle in the carpus region on the inner side (medial side, Figure 8.5 A).
5. Cut the apodeme of the bender muscle and remove the muscle carefully as not to pull the main leg nerve out of the leg cavity (Figure 8.5 B and C- note the arrows where to bender apodeme is separated). Then the main leg nerve and

- a branch to the stretcher muscle can be observed (Figure 8.5 D).
6. Find the nerve branching from the main nerve bundle to the stretcher muscle (Figure 8.5 E, at arrow) and this can be cut close to the muscle and pulled into a suction electrode to stimulate (Figure 8.5 F and G, arrow depicts the branch).
  7. Tease out a section of the nerve that projects towards the opener without pinching the nerve. Transect the stretcher/opener motor nerve.
  8. Pull the motor nerve into the suction electrode and then stimulate it.
  9. To expose the opener muscle and tension nerve remove the closer muscle and the ventral region of the propodite. Cut off the closer muscle with a scalpel with a #11 blade in the propodite (Figure 8.6). (Note: Do not cut deeply in the proximal region because it might damage the opener motor nerve).
  10. Exchange the saline with fresh cooled saline throughout the dissection process to keep the neurons alive. For further dissection, place the preparation dish under a dissecting microscope and use fiber-optic illumination.
  11. Carefully cut the closer tendon from its attachment to the dactyl using sharp-pointed medium-size scissors.
  12. Be very careful not to disturb the branches to the opener muscle from the main leg nerve which should be clearly visible.
  13. Remove and discard the closer muscle and tendon.
  14. Make a hole in the dactyl with a dissection pin. This hole will be used later to hook the metal pin on the tension (force) transducer, but it is necessary to make it while the procedure is at this stage.
  15. Locate the nerve branch that projects to the opener muscle and apodeme in the distal region of the opener muscle. Carefully probe the nerve with the fire-polished glass tool.
  16. Observe the tension nerve that is arising from the distal end of the apodeme and proceeding to the motor nerve bundle.
  17. To detect neural activity, fill a suction electrode with crab saline and draw the

cut end of the opener tension nerve into the electrode. Ensure that the suction electrode fits tightly on the nerve.

18. Examine for the neural correlate of passive tension on the opener apodeme. While recording electrical activity from the tension nerve, rotate the dactyl rapidly into an extended joint (i.e. stretching the opener muscle).
19. Next test active force development related to stimulation frequency.
20. Firmly attach a metal hook so that the tip of the hook goes through a small hole in the dactyl.
21. Attach the other end of the metal hook to the force transducer. (NOTE: Make sure the transducer is at a 90 degree angle each time so that the pin is perpendicular to the transducer for maximal detection of the force generated.)
22. Pull the hook and dactyl so that it is about a 45 degree angle of the opener joint.
23. Now stimulate the motor nerve at 100 Hz for 250 msec and measure the force as well as the firing frequency of the tension nerve.
24. Place the joint into a fully extended position so that the opener muscle fibers are flaccid.
25. Stimulate the motor nerve at 100 Hz for 250 msec and measure the force as well as the firing frequency of the tension nerve.
26. Bend/flex the joint so that it is fully flexed (~90 degrees). In this position the muscle fibers of the opener are fully stretched. There may be some force measured by the transducer due to this passive stretch of the muscle.
27. Stimulate the motor nerve at 100 Hz for 250 msec and measure the force as well as the firing frequency of the tension nerve.
28. After measuring a force, proceed in a series of various frequencies: 20, 40, 60, 80 and 100 Hz for 8 to 10 seconds in each joint position.
29. Measure the response with a quick tension release. Arrange the preparation so that the hook on the force transducer can easily be pushed out of the hole

in the dactyl.

30. Bend/flex the joint so that it is fully flexed (~90 degrees). Now stimulate the motor nerve at 100 Hz with a continuous stimulation of 5 seconds.
31. After the first second or less, as tension has built up on the opener muscle, push the pin out of the hole in the dactyl.
32. Examine the tension nerve recording before and after release of the pin holding the dactyl.
33. Modulatory effects of neuroactive substances such as octopamine, serotonin, and proctolin, which alter the output of the tension neurons, can be determined by adding these molecules to the bathing media over the exposed opener muscle and repeating the experiments.
34. Use a pipette and drip 1-2 ml over the preparation.

### **Staining peripheral nervous system structures in crustacean walking legs.**

#### **Methylene blue technique**

Dilute one part methylene blue chloride stock solution (0.25%) with two parts of distilled water. Add this mixture to five parts of buffered saline. Thoroughly irrigate the area of interest. Dissect a leg according to the protocol in section 1. Incubate the preparation in the methylene blue solution at 12-13°C. Examine the preparation every 10-15 minutes with the dissecting microscope using low intensity illumination to follow the progress of the staining. In some cases staining will be completed in an hour, in others it may take overnight.

#### **4-di-2-ASP technique**

Fluorescent dyes can be used to back-fill the PD neuron or directly stain the preparation from the bath. However a microscope with abilities to view the fluorescent stain is required. Dissect a leg according to a protocol in section 1. Incubate the preparation in a 10 µM concentration of 4-Di-2-ASP solution and leave the preparation

in the refrigerator for 15 minutes. Use just enough solution to cover the preparation. Photograph the preparations quickly and avoid over exposure to the mercury light. This fluorescent dye fades away relatively quickly.

### **Cobalt chloride technique**

1. Expose the PD nerve according to the protocol in section 1 and keep it immersed in cold saline.
2. Make a petroleum jelly well to hold the  $\text{CoCl}_2$ . If any  $\text{CoCl}_2$  spills into the saline bath the entire preparation will stain black, and the preparation should be discarded.
3. Slip a small cut of a polystyrene sheet, to make a plastic platform, and pin it in such a manner that it will not float away or become immersed in the saline bath (Figure 8.7).
4. Eject petroleum jelly from a fine hypodermic needle fastened to a disposable syringe, then make a barrier (a circle might work well), which should be about 1-1.5 mm high except for a shallow "V" at midpoint where the nerve will be draped across, on top of the slip of polystyrene.
5. Make a small puddle of saline on either side of the barrier near the "V". Taking care not to stretch or pinch the nerve. Lift the nerve carefully from the dish and place it in the saline puddle in the petroleum jelly well.
6. Work quickly so that the section of nerve does not dry out, carefully eject petroleum jelly to cover the exposed nerve. Now test the barrier with saline and make sure it is not leaking.
7. Blot away saline on the inside of the barrier and see if it fills up when the bath saline is high around the wall of petroleum jelly to be sure that the two sides are isolated from one another (Figure 8.8).
8. Wick away the saline in the well using a piece of tissue paper. Avoid letting the paper wrap up the nerve.

9. Make a new puddle using a few small drops of distilled water, and then cut the nerve end. Be very careful not to pull the nerve cord through the barrier when making the cut. The osmotic shock of the distilled water will "balloon" the axons of the connectives.
10. Within 30 seconds add a small drop of  $\text{CoCl}_2$  to the water, soak up this solution, and then add enough  $\text{CoCl}_2$  to form a puddle over this shortened section of nerve (Figure 8.9). Preparations are best kept refrigerated at  $13^\circ\text{C}$  for 12-24 hrs.
11. Remove the humidifying puddles. Blot away the cobalt solution using a tissue and wash away the remnants of cobalt with several changes of saline.
12. Transfer the isolated preparation to a small glass Petri dish containing about 10 ml of crab saline. The neurons are washed and the following steps are performed *in situ*. Good metal tools are not to be used to handle the preparation after this step (you should use specific tools that are not to be used at a later time for physiology).
13. Add 1-2 drops of ammonium sulfide  $(\text{NH}_4)_2\text{S}$  to the saline. Cap the  $(\text{NH}_4)_2\text{S}$  bottle tightly and place back into the hood. Observe the reaction in the preparation under a dissecting microscope
14. Within a minute or two, cobalt-filled neurons and their processes should begin to appear because they will stain black. After 5-10 min, replace the development solution with fresh saline. Make certain that the development solution you have poured into the sink drain is followed by running tap water for a few minutes or in a waste bottle with a lid.
15. Pour out the saline and fix the nerve preparation for about 15 minutes with two changes of Bouin's solution fixative (Sigma). For larger tissues like the opener muscle (to stain tension neurons), increase the duration of fixation to 30 minutes.

16. Dehydrate in a series of ascending order of ethanol concentration beginning at 70% (i.e. 70%, 80%, 90%, 100%). About 10 minutes at each concentration is sufficient for small tissues.
17. After about 10-15 minutes in two changes of 100% ethanol, clear the tissue by replacing ethanol with 100% methyl salicylate. The preparation will stay in this solution permanently for repeated viewing. With time the filled cells will become more apparent because the surrounding tissue will become clearer. Intensification methods can be used to help prevent fading over time (Delaney and Gelperin, 1990).
18. Use the same process to fill the tension nerve with  $\text{CoCl}_2$ . However, change ethanol solution multiple times in each ethanol dehydration step and incubate the preparation inside alcohol for about 20 minutes for each step to thoroughly dehydrate the muscles and allow them to clear well.

### **Representative Results**

When the PD organ is stretched by fully extending the joint, activity in the PD nerve is robust during the movement as shown for the first second in Figure (8.10). Some activity remains while it is held still in the open position. This activity is from the static position sensitive neurons (second half of the recording shown in Figure (8.10). Movement evokes a response during displacement, and the firing is mostly present during the stretching of the chordotonal strand (Figure 8.11).

Further analysis of the spikes can be readily approached by sorting the relative amplitudes. This is an approach to demonstrate different populations of sensory neurons being recruited for positions or types of movements (Cooper, 2008). Typical amplitudes range from 0.25-1.5mV, but these values are dependent on the resistance (i.e., tightness) of the suction electrode seal. Frequency of spikes in the various size ranges can also be graphically represented for analysis.

Forces generated by the opener muscle with respect to the stimulation frequency can be compared by superimposing the respective voltage-time traces on top of each other (Figure 8.13 A and B). This can also be performed for each joint position at each given stimulation frequency. Activity of the tension nerve can then be correlated with the amount of relative force generated at each stimulation frequency, and for each joint position. As in the PD nerve, a variety of spike amplitudes are seen in response to contraction of the opener muscle (Figure 8.13 C).

Anatomical arrangement of neurons in the walking leg is clearly observed with methylene blue staining (Figure 8.14). Note the elastic chordotonal strand and tension neuron that is close to the apodeme. Several somata with different diameters and specific locations are also visible in this figure. The entire course of the tension nerve and stretcher motor nerve are shown in Figure (8.15). Individual neurons of the PD nerve are shown with higher contrast using 4-Di-2-ASP (Figure 16) and  $\text{CoCl}_2$  (Figure 8.17) backfill techniques. At high magnification the sensory endings can be seen inside of the supportive scolopales (Figure 8.16 B; Whitear, 1962; Whitear, 1965; Hartman and Cooper, 1994).

## **Discussion**

The purpose of this set of experiments is 1) to teach and exhibit the fundamental principles of extracellular recordings from an identifiable proprioceptive organ and tension nerve and 2) to stress the importance of anatomical mapping related to physiological function of particular sensory systems. This experimental approach and animal models utilized are inexpensive and relatively easy to conduct in neurophysiology teaching laboratories.

The neurons of chordotonal organs are of two specific functional types, those that respond to movement and those responding to static positions. Single cell recordings from a variety of chordotonal organs, no matter which joint is examined, have shown this to be the case (Hartman and Boettiger, 1967; Cooper, 2008). Indeed,

chordotonal organs associated with the antennal joints of lobsters reveal the same two sensory types and basic anatomy (Hartman and Austin, 1972). In addition to there being two neuron types (movement and position), the neurons share the same anatomical arrangement on their respective elastic strands. The large somata located proximally on the strand tend to belong to the dynamic movement sensitive neurons. Neurons that signal static positions have small somata and are located distally. These cells are tonically active. The PD joint only contains a single chordotonal organ while there are two chordotonal organs in the carpus-propodus (CP) and merus-carpus (MC) joints.

The dissection to expose proprioceptive structures in blue crabs (*C. sapidus*) for electrophysiological recording requires a strategy that allows joint movements to take place in the natural positions while recording from sensory neurons. The tension nerve for the opener muscle in the walking leg is a very fine nerve made up of several neurons. Unless precaution is taken the tension nerve, as well as the motor nerve innervating the muscle to be stimulated, can readily be damaged during this dissection. For optimal recordings the suction electrodes need to be tailored to the size of the nerve. Recordings are readily accessible in a student laboratory using a 30-40x magnification dissecting microscope and low-end micromanipulators.

Future types of experimentation that would be interesting to pursue with the joint chordotonal organs are to examine the structural and physiological profiles during leg regeneration in various species at different stages in the life cycle as a follow up to an initial study that used *Cancer magister* (Parsons, 1982). Questions remaining to be addressed are 1) does the distribution and organization of regenerated neurons depend on the age of the animal when regenerating a limb, 2) are the axonal projections to the CNS (ventral nerve cord) in a regenerating limb functional or does it take time and joint use to establish functional connections, and 3) what happens to the severed axons proximal to the autotomy plane when the limb is autotomized (Cooper, 1998)?

Crustaceans conform to environmental conditions and the surrounding temperature, but it is unclear how they maintain coordination within a neural circuit as neurons alter their activity in response to temperature changes. A slow rate of change might allow the animal some time for acclimatization whereas a rapid change may not (Chung et al., 2012; Blundon, 1989). Physiological changes in pH or osmolarity due to metabolism, behavior (Cooper et al., 2011), or environmental impact may present similar challenges to neural circuits involved in proprioception. These crustacean preparations are ideal for addressing these types of problems because their function is well characterized at a single cell level.

In this protocol we have demonstrated the physiological importance of tension neurons in monitoring force generated by the opener muscle. These tension receptors can be traced to their location within the apodeme by staining procedures. These neurons, as in mammals, detect force at various levels and recruit additional neurons as the force increases. The frequency in activity is related to the stimulation frequency of the motor neuron until saturation in reception is reached. Using a quick release protocol with the flexed dactylus joint, tension activity quickly disappears but then returns upon regaining tension in a fully extended joint. This is a classic experimental procedure to illustrate the force measured by tension receptors. Various neuromodulators can be applied to the preparation to see how it effects the development of force and neuronal response. One of the important aspects is how the neural responses are processed and integrated in the central nervous system and their impact on activity of motor neurons. The techniques we have shown allow one to start to address more information about the tension (sensory) nerve-motor neuron circuit function, i.e., signal in an intact leg to the ganglion and back to the muscle.

The staining procedures demonstrated are key to understanding the physiology of sensory neurons that innervate proprioceptive organs. Anatomical arrangement of the neurons based on function and the size of the soma are similar in the various chordotonal organs within the crab legs. It is not known if similar neuronal

arrangements are also similar in other crustacean species or insects. Combining physiological recordings from single cells and mapping the location allows direct structure function relationships. The long term preservation of the anatomical arrangement with  $\text{CoCl}_2$  staining and fixation allows one to repetitively make measures and assess the structural arrangement.

Proprioception and tension reception of skeletal muscles are sensory modalities that enable coordinated behaviors and responses to external and internal environment for articulated animals in a variety of skeletal muscle configurations. The muscle receptor organ in the abdomen of the crayfish is another well-documented preparation (see the *Crawdad Project*; <http://www.crawdad.cornell.edu/>) for teaching purposes of proprioception with only two neurons per abdominal hemi-segment (Leksrisawat et al., 2010). Being able to record from single neurons to sensory nerve bundles provides further details that aid in understanding the basic principles of sensory reception. These relatively simple crustacean preparations allow one to address fundamental aspects of proprioception and tension monitoring, with the potential to dissect the neural circuits that enable central integration of proprioceptive and other sensory inputs (Macmillan and Dando, 1972; Bévengut et al., 1983; El Manira et al., 1991; Le Bon-Jego and Cattaert, 2002; Bierbower and Cooper, 2013; Bierbower et al., 2013).

Table 8.1: Recipes for crab saline.

Saline	g/L
NaCl	27.29
KCl	0.81
MgSO <sub>4</sub> •7H <sub>2</sub> O	4.81
CaCl <sub>2</sub> •2H <sub>2</sub> O	1.85
Na <sub>2</sub> SO <sub>4</sub> •10H <sub>2</sub> O	0.97
Dextrose (D-Glucose)	1.982
HEPES acid	0.476
HEPES salt	2.08
Adjust to <a href="#">pH</a> 8.1 with NaOH or HCl	

Table 8.2: Materials

Name	Company	Catalog Number	Comments
Sylgard	Dow Corning	182 silicone kit	
NaCl	Sigma-Aldrich	S7653	
KCl	Sigma-Aldrich	P9333	
CaCl <sub>2</sub>	Sigma-Aldrich	C5670	
HEPES acid	Sigma	3375	Free acid, crystalline
HEPES base	Sigma		
D-Glucose	Sigma	G7021	
MgSO <sub>4</sub> •7H <sub>2</sub> O	Sigma	M2643	
Na <sub>2</sub> SO <sub>4</sub> •10H <sub>2</sub> O	Sigma	246980	

Bouin's solution fixative	Sigma	HT10-1-32	Caution: Hazardous material (Special shipping cost required)
CoCl <sub>2</sub>	Sigma		Caution: Hazardous material. Please follow proper disposal according to local and federal regulations.
Methylene blue chloride	Matheson Co., Inc	Basic Blue 9, C.I. 52015	
4-Di-2-ASP	Molecular Probes		4-(4-diethylaminostyryl)-N-methylpyridinium iodide
Bleach	Sigma-Aldrich		To chloride silver wire
NaOH	Sigma-Aldrich	221465	To adjust pH
HCl	Sigma-Aldrich	H1758	To adjust pH
Materials			

Dissecting tools	World Precision Instruments	assortment	
Intracellular electrode probe			
Faraday cage			
Insect Pins	Fine Science Tools, Inc	26001-60	
Dissecting microscope (100X)			
Fiber optic lamp			
Small adjustable mirror			To direct light beam toward the preparation.
Glass electrodes	Sigma-Aldrich	CLS7095B5X	Box of 200, suction electrodes
Micromanipulator	World Precision Instruments	MD4-M3-R	Can fix for base or on a metal rod
Raised preparation stand			

Silver wire (10/1,000 inch)	A-M Systems	782500	
Computer	Any company		
AC/DC differential amplifier	A-M Systems	Model 3000	
PowerLab 26T	AD Instruments	27T	
Force transducer	AD Instruments	0-50g	MLTF050/ST
Head stage	AD Instruments		Comes with AC/DC amplifier
LabChart7	AD Instruments		
Electrical leads	Any company		
Glass tools	Make yourself		For manipulating nerves
Cable and connectors	Any company		

Pipettes with bulbs	Fisher Scientific	13-711-7	Box of 500
Beakers	Any company		
Wax or modeling clay	Any company or local stores		
Stimulator	Grass Instruments	SD9 or S88	
Plastic tip for suction electrode	Local hardware store (Watt's brand)	¼ inch OD x 0.170 inch ID	Cut in small pieces. Pull out over a flame and cut back the tip to the correct size

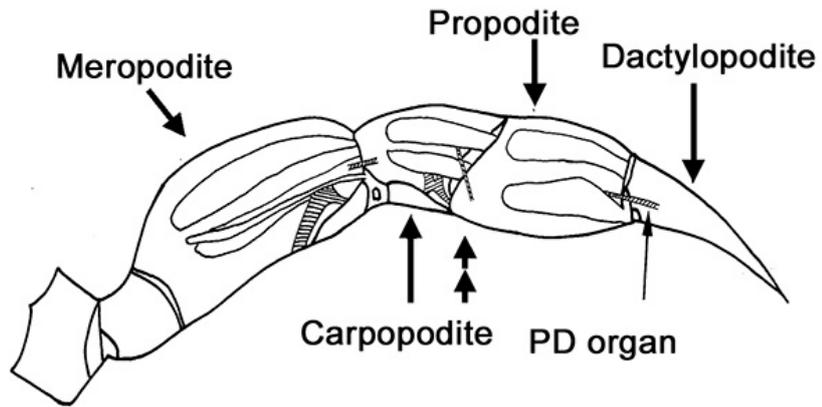


Figure 8.1: First walking leg of a crab. The anatomical location of chordotonal organs (hatched regions) are superimposed on this schematic. The double arrow head indicates where to transect the leg for the PD nerve experiments.

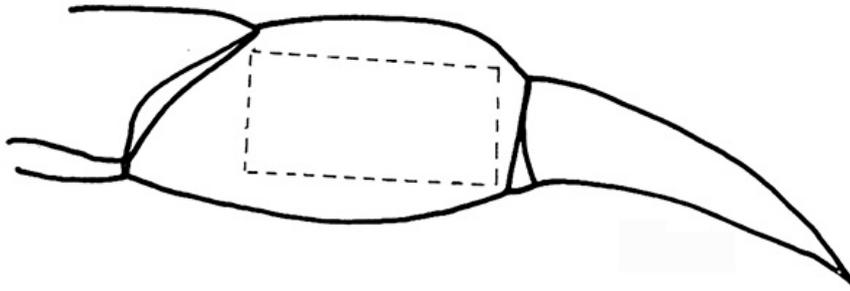


Figure 8.2: Cut along the dotted line on the propodite

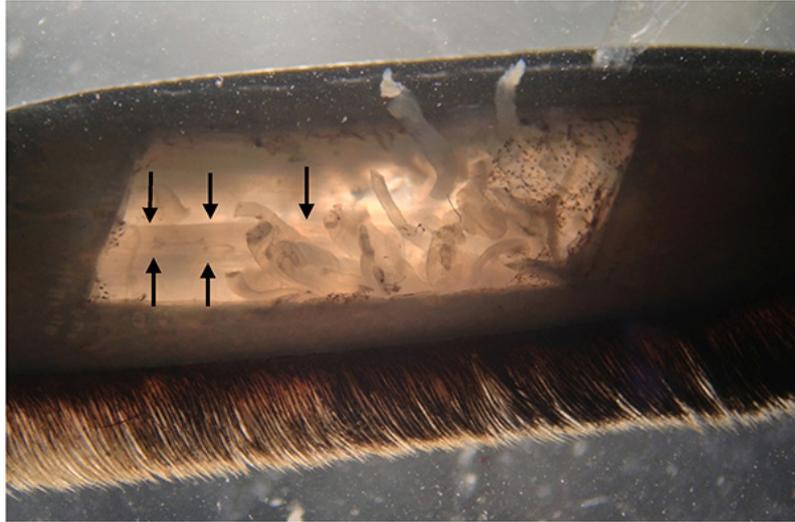


Figure 8.3: Expose the nerve in the window (the arrows outline the nerve bundle)

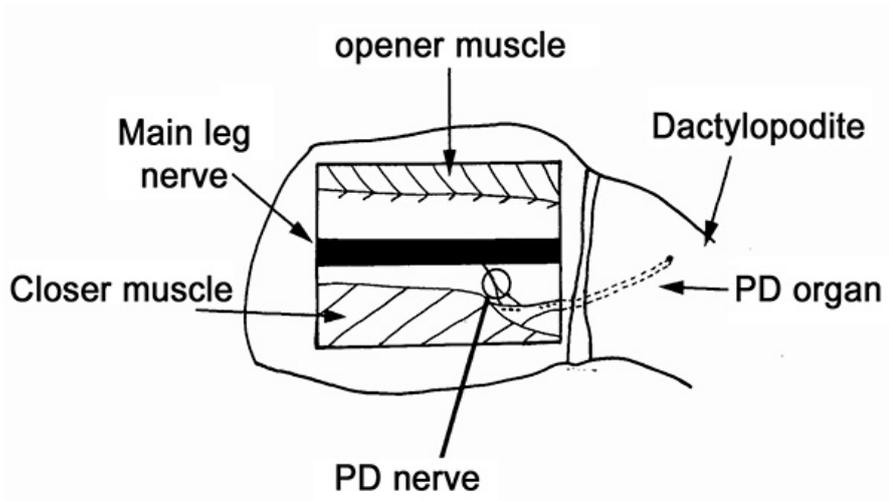


Figure 8.4: Exposed PD organ and nerve.

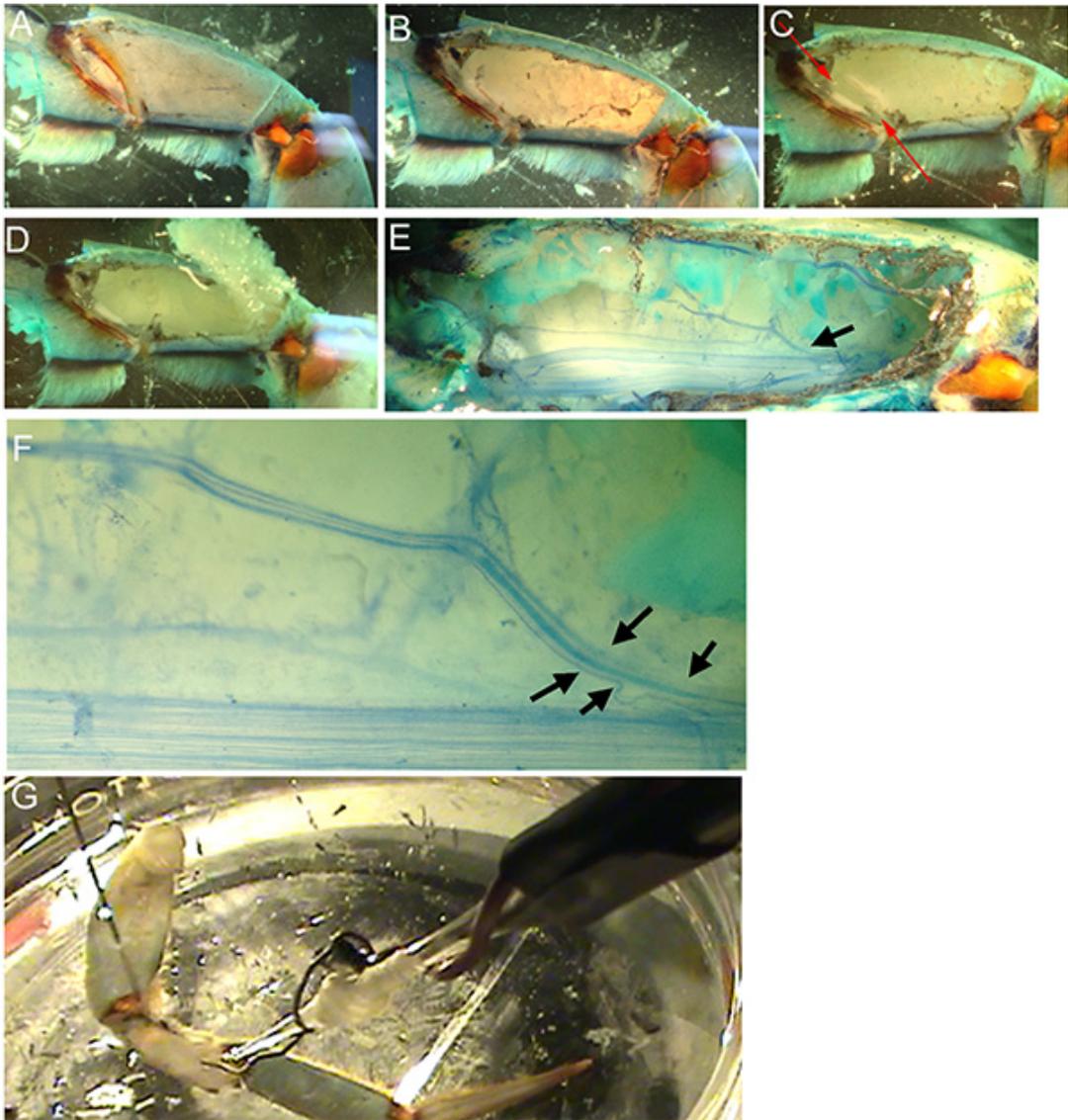


Figure 8.5: Dissection steps for exposing the motor neuron for stimulating the opener muscle.

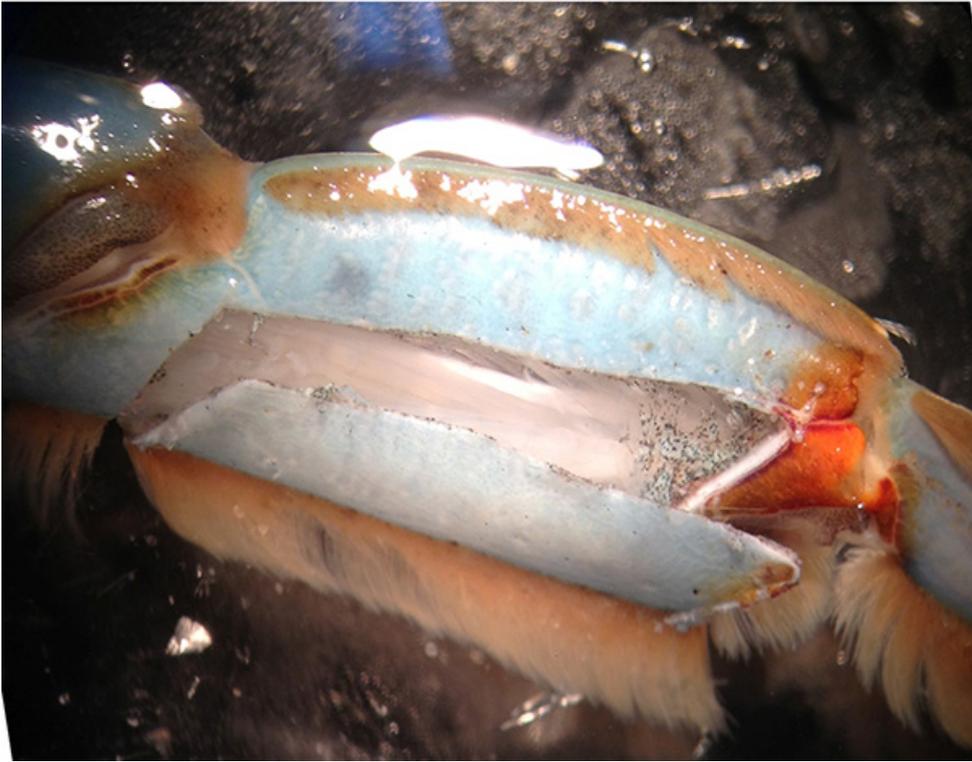


Figure 8.6: Cut the cuticle on the ventral half of the propodite to expose the closer muscle so it can be removed.

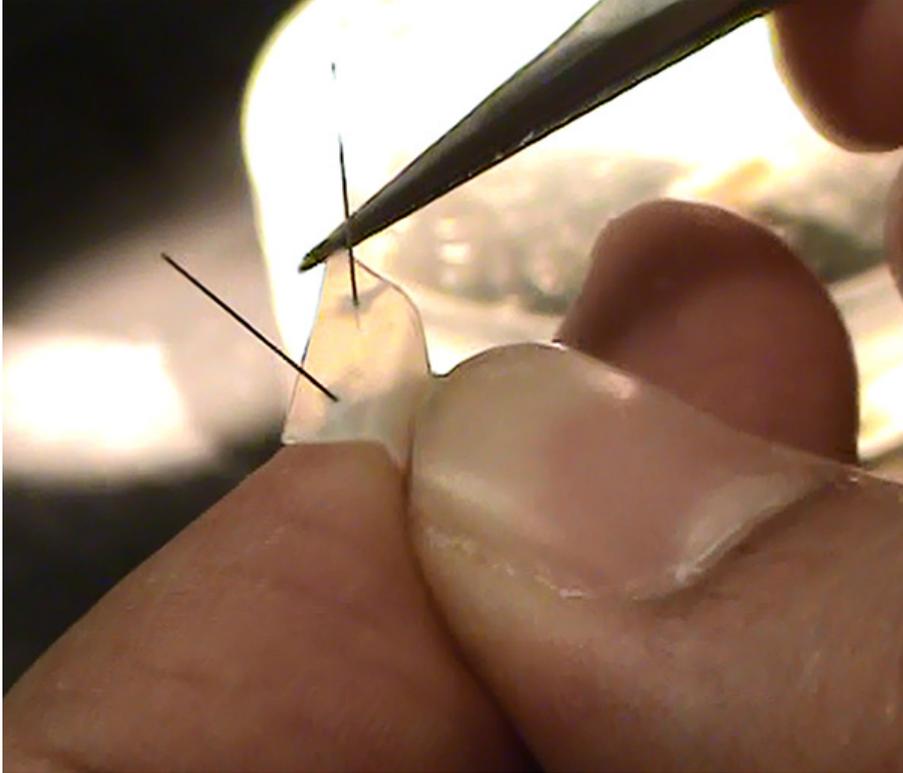


Figure 8.7: Polystyrene sheet with pins to hold in place in dish.

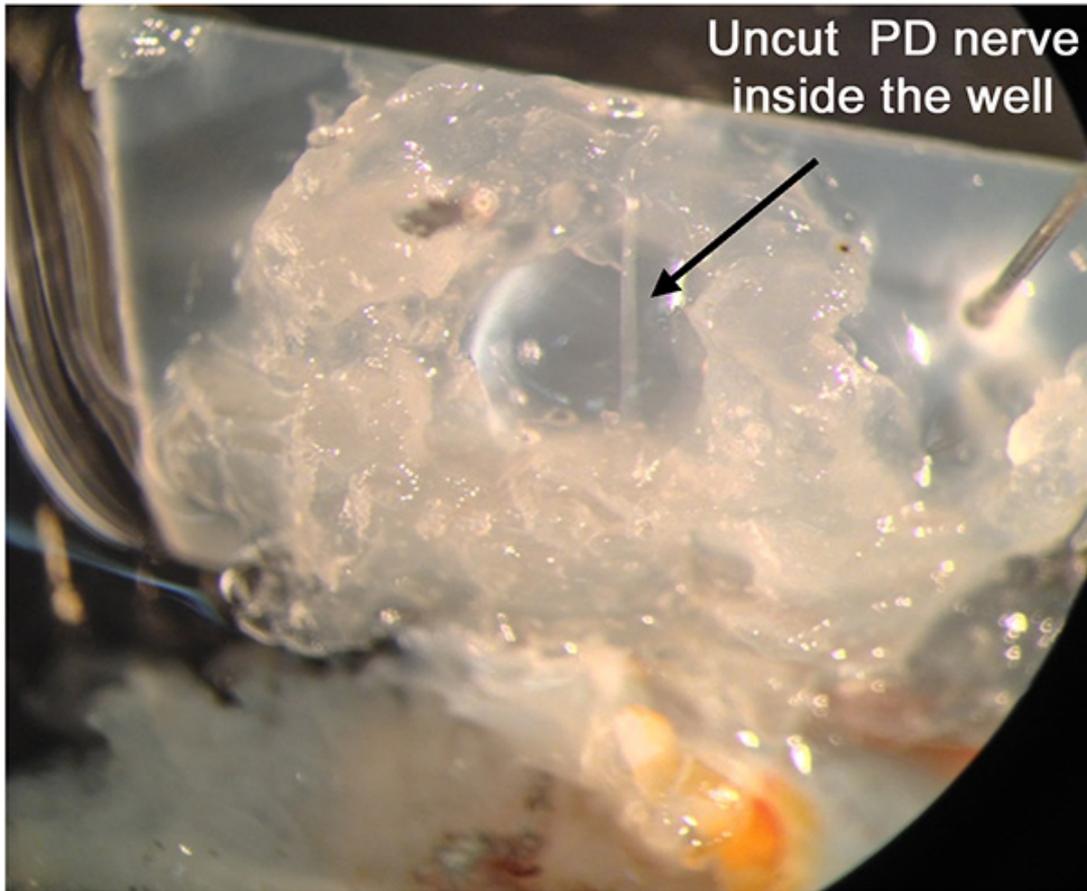


Figure 8.8: Petroleum jelly well with saline and PD nerve spanning the well. The PD nerve has not been cut yet.

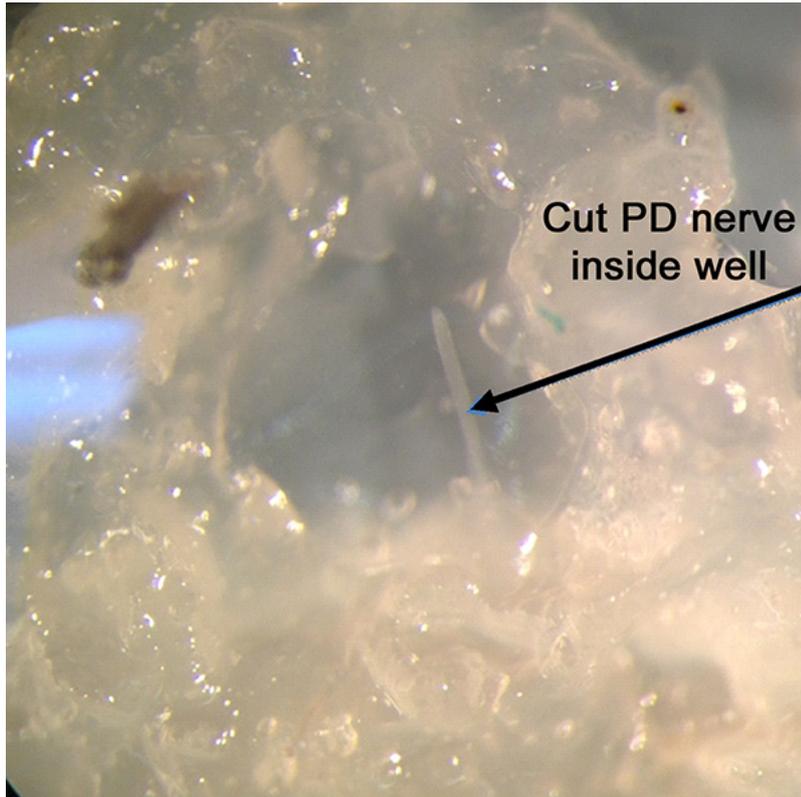


Figure 8.9: The cut PD nerve being exposed to  $\text{CoCl}_2$ . The cut nerve swells up in the presence of water that facilitates and accelerates the movement of dye molecules through the axons into the neuron cell bodies.

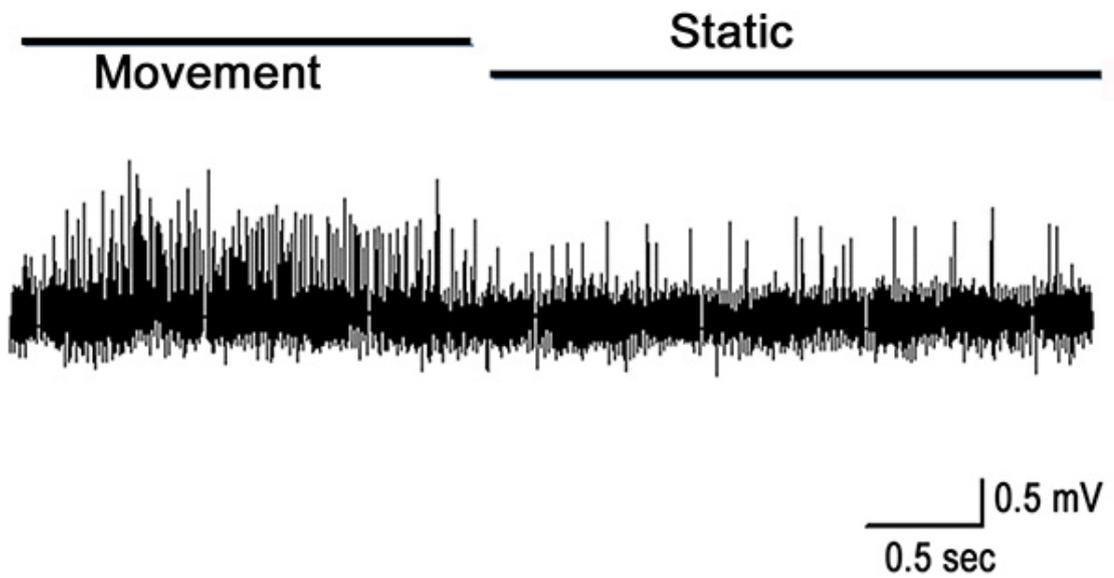


Figure 8.10: Move and hold at 0 degrees. The dynamic neurons are robust in firing during the movement and spikes from static sensitive neurons are present while the joint is held open.

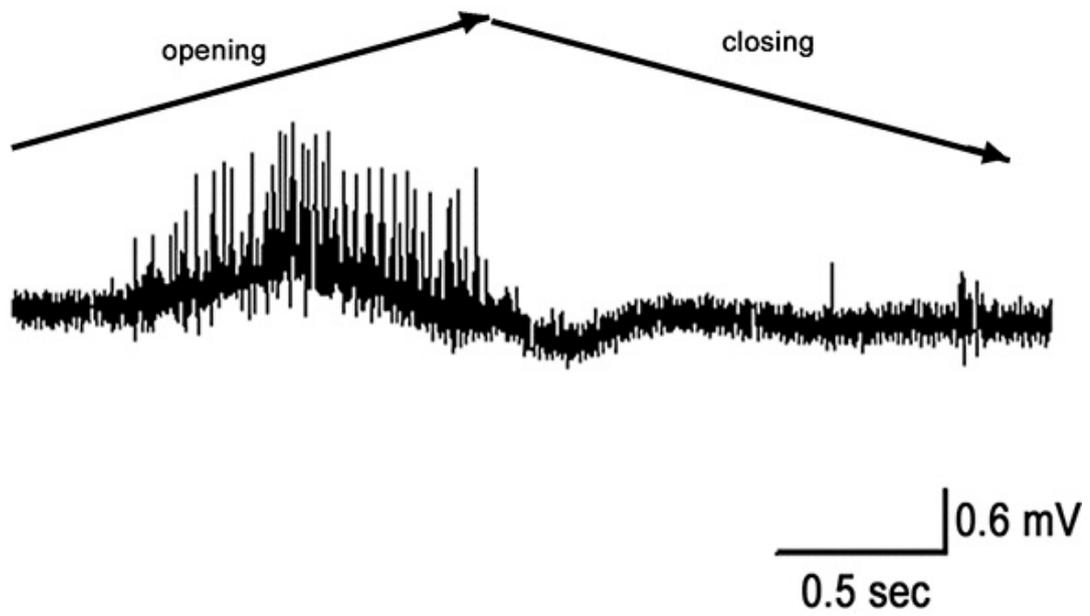


Figure 8.11: Rapid open and closing from fully flexed to extended (90 to 0 degrees) position.

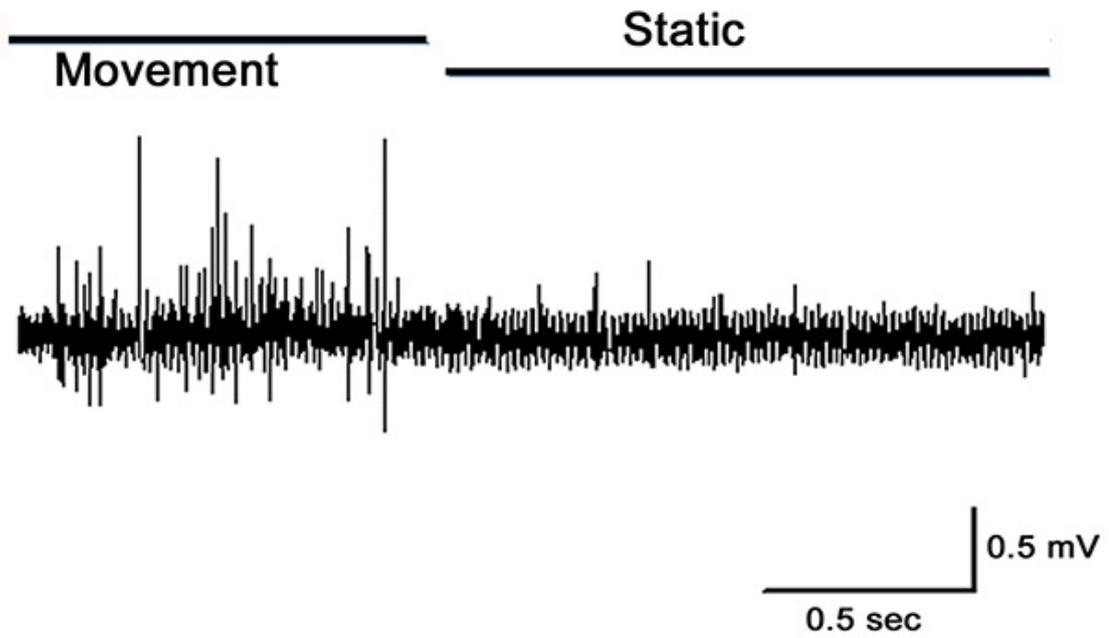


Figure 8.12: Extracellular spikes recorded from the PD nerve. The joint is fully extended and then quickly moved to a  $\frac{1}{2}$  flexed position and then held still. Notice the activity during the movement and decreased activity when static.

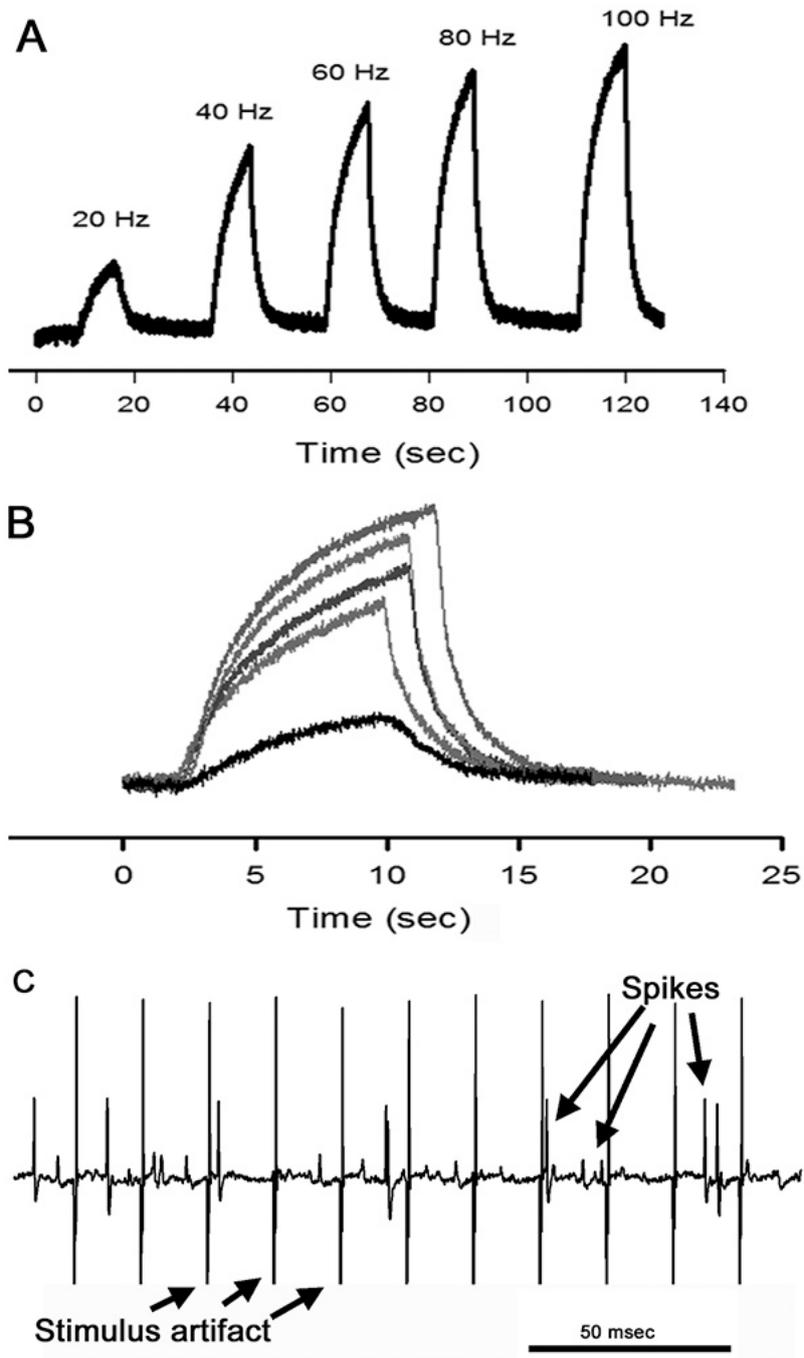


Figure 8.13: The relative forces that are developed with the joint fully flexed and stimulated at the various frequencies. (A) Voltage-time traces from the force transducer are shown with each stimulation frequency. (B) The traces in panel A are superimposed in different colors for ease in comparison. (C) Voltage-time traces of electrical activity recorded from the tension nerve when the motor nerve was stimulated at 80 Hz. Note the regular pattern of the stimulus artifacts as compared to the neural activity. Also, note the various amplitudes of the neural responses.

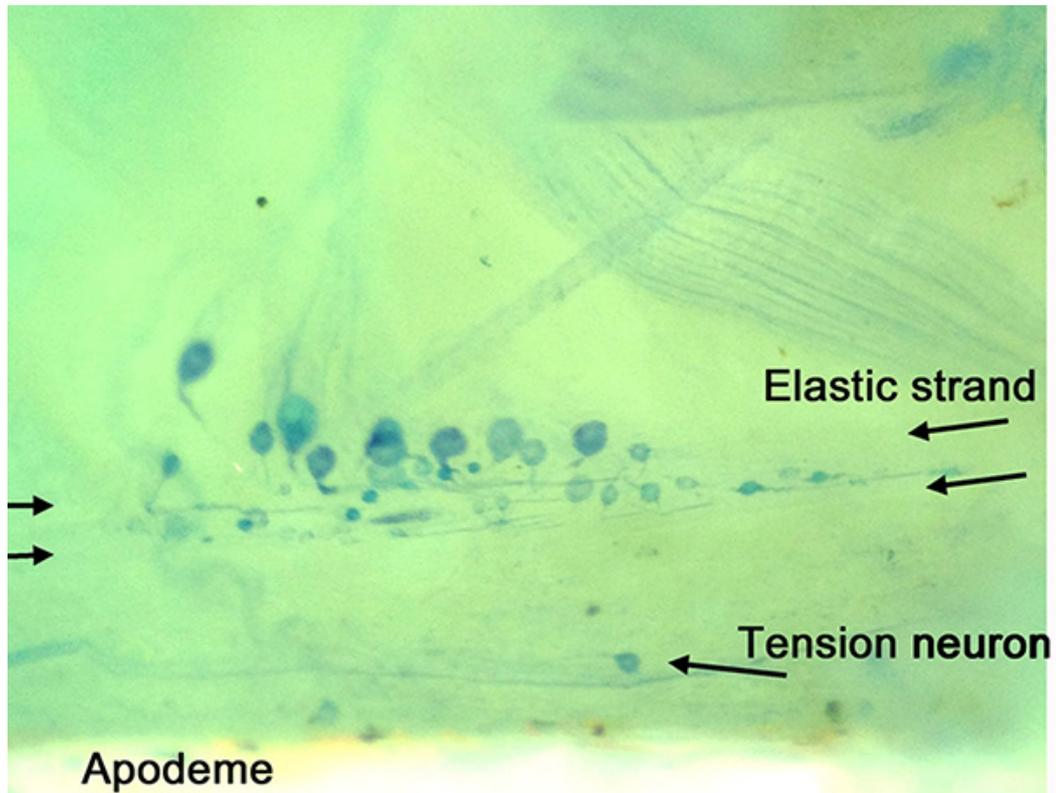


Figure 8.14: Methylene blue stain of the walking leg preparation. Individual somas are shown with their sensory endings projecting into the elastic strand. Close to the apodeme a tension neuron is shown.

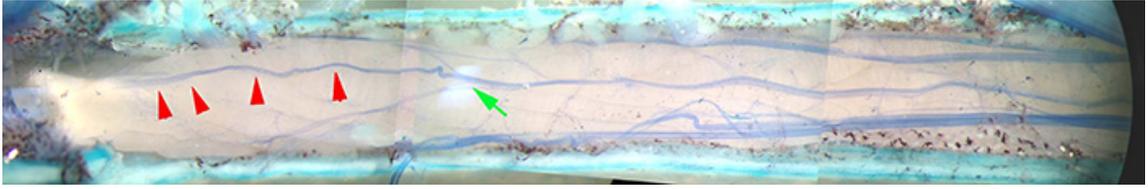


Figure 8.15: The tension nerve arising from distal end (red arrows) and joining the motor nerve (green arrow).

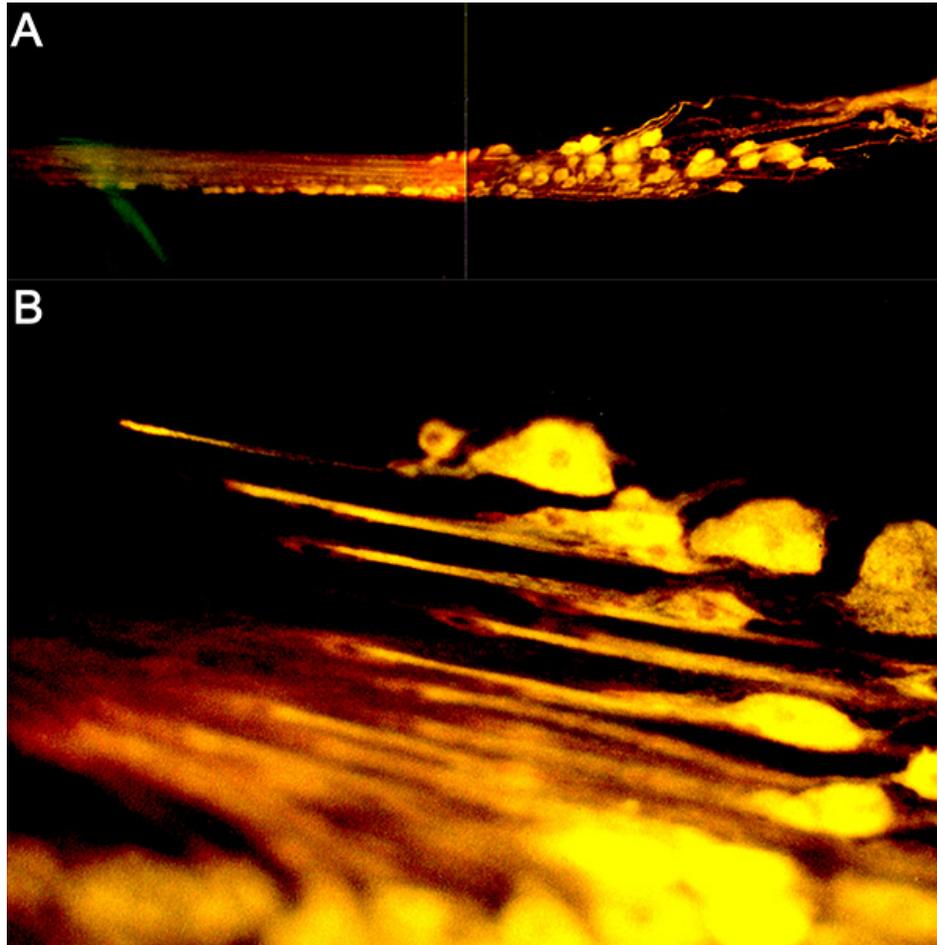


Figure 8.16: (A) A back-fill of the PD nerve in *Cancer magister* with 4-Di-2-ASP. (B) Higher magnification of sensory endings. The neurons are slightly stained with methylene blue from a blue crab. The PD nerve in *Cancer magister* was filled with 4-Di-2-ASP.

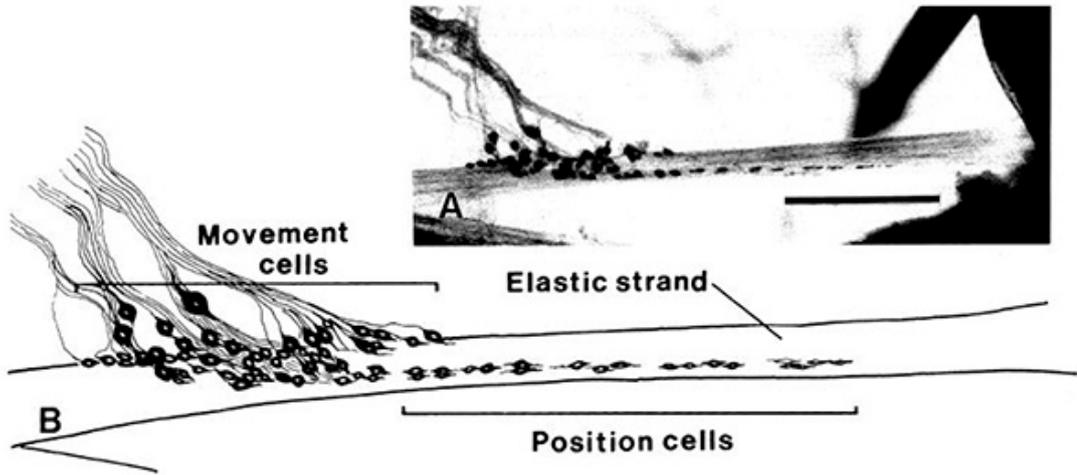


Figure 8.17: Neurons that were filled with  $\text{CoCl}_2$  and processed (A). Traced outline of the stained preparation shown (B).

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### CHAPTER ONE

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## CHAPTER FIVE

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**Vita**  
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**EDUCATION**

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**M.Sc. in Biology** November 2004 – August, 2006

**Research project:** Effects of L-Carnitine and Melatonin on Biochemical and Hematological Parameters in Male Albino Rats.

2. Department of Biology, College of Science, University of Salahaddin, Erbil, Iraq.

**B.Sc. in Biology** October 1999 – July 2003

**Research Project:** Incidences of Bacterial Urinary Tract Infection in Pregnant Women in Pediatric and Maternity Hospital, Erbil.

3. Rizgari High school, Erbil, Iraq.

**Baccalaureate degree** October 1996 – July 1999

**AWARDS**

1. Awarded the First Place Prize for oral presentation of Graduate Research Competition at the Kentucky Academy of Science meeting (2012)
2. Awarded the second Place Prize for oral presentation of Graduate Research Competition at the Kentucky Academy of Science meeting (2013)

**CERTIFICATES**

1. Certificate of English Language Course, University of Kentucky, Center for English as a Second Language, Lexington, KY.

August 2010-December 2010

2. Certificate of Computer Training Course, University of Salahaddin, Erbil, Iraq.

July, 2009-August 2009

3. Certificate of Teaching Methods, University of Salahaddin, Erbil, Iraq.

September 2008-March 2009

4. Certificate of English Language Course, University of Salahaddin, Language Center, Erbil, Iraq.

July, 2007-August 2007

5. Certificate of English Language Course, University of Salahaddin, Language Center, Erbil, Iraq.

October 2006-December 2006

### **JOB EXPERIENCES**

1. Lecturer, College of Science, University of Salahaddin, Erbil, Iraq.

October 2010-present

2. Assistant Lecturer, College of Science, University of Salahaddin, Erbil, Iraq.

November 2006- 2010

3. Biological Assistant, College of Science, University of Salahaddin, Erbil, Iraq.

October 2003- November 2004

### **TEACHING EXPERIENCES**

1. Lab-based comparative anatomy course for four years.
2. Lab-based genetics, introductory biology, and principles of experimental animal physiology.

### **PROFESSIONAL AFFILIATIONS**

1. Society for Neuroscience (2011-present)
2. American Physiological Society (2014-present)

### **SCHOLARSHIP**

1. PhD scholarship by the Higher Committee for Education Development in Iraq (HCED) (6 years).

### **PUBLIC SERVICES AND OUTREACH ACTIVITIES**

#### **A. Science Fair Judge**

- 1- Morton Middle School Science Fair, Lexington, KY (2011)
- 2- Glendover Elementary School, Lexington, KY (2012)

- B. Developed hands-on learning modules for high school and college level courses (2015).

## PEER REVIEWED PUBLICATIONS

1. **Majeed ZR**, Abdeljaber E, Soveland R, Cornwell K, Bankemper A, Koch F, Cooper RL (2016) Modulatory Action by the Serotonergic System: Behavior and Neurophysiology in *Drosophila melanogaster*. *Neural Plasticity* vol. 2016, 23 pages. <http://www.hindawi.com/journals/np/2016/7291438/>
2. Zhu YC, Uradu H, **Majeed ZR**, Cooper RL (2016) Optogenetic stimulation of *Drosophila* heart rate at different temperatures and Ca<sup>2+</sup> concentrations. *Physiol Rep*. 4(3). pii: e12695.
3. **Majeed ZR**, Ritter K, Robinson J, Blümich SL, Brailoiu E, Cooper RL (2015) New insights into the acute actions from a high dosage of fluoxetine on neuronal and cardiac function: *Drosophila*, crayfish and rodent models. *Comp Biochem Physiol C Toxicol Pharmacol*. 176-177:52-61.
4. Titlow JS, Rice J, **Majeed ZR**, Holsopple E, Biecker S, Cooper RL (2014) Anatomical and genotype-specific mechanosensory responses in *Drosophila melanogaster* larvae. *Neurosci Res*. 83:54-63.
5. **Majeed ZR**, Stacy A, Cooper RL (2014) Pharmacological and genetic identification of serotonin receptor subtypes on *Drosophila* larval heart and aorta. *J Comp Physiol B*. 184(2):205-19.
6. de Castro C, Titlow J, **Majeed ZR**, Cooper RL (2014) Analysis of various physiological salines for heart rate, CNS function, and synaptic transmission at neuromuscular junctions in *Drosophila melanogaster* larvae. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol*. 200(1):83-92. doi: 10.1007/s00359-013-0864-0. Epub 2013 Nov 5.
7. Titlow JS, **Majeed ZR**, Hartman HB, Burns E, Cooper RL (2013) Neural circuit recording from an intact cockroach nervous system. *J Vis Exp*. 81:e50584.
8. Titlow J, **Majeed ZR**, Nicholls JG, Cooper RL (2013) Intracellular recording, sensory field mapping, and culturing identified neurons in the leech, *Hirudo medicinalis*. *J Vis Exp*. (81):e50631.
9. **Majeed ZR**, Titlow J, Hartman HB, Cooper RL (2013) Proprioception and tension receptors in crab limbs: student laboratory exercises. *J Vis Exp*. 80:e51050. doi: 10.3791/51050.
10. **Majeed ZR**, Nichols CD, Cooper RL (2013) 5-HT stimulation of heart rate in *Drosophila* does not act through cAMP as revealed by pharmacogenetics. *J Appl Physiol* (1985). 115(11):1656-65.
11. Becnel J, Johnson O, **Majeed ZR**, Tran V, Yu B, Roth BL, Cooper RL, Kerut EK, Nichols CD (2013) DREADDs in *Drosophila*: a pharmacogenetic approach for controlling behavior, neuronal signaling, and physiology in the fly. *Cell Rep*. 4(5):1049-59.

## PROFESSIONAL MEETING ABSTRACTS

1. **Majeed, Z.R.**, Cooper, R.L. and Nichols, C.D. (2011) Effect of DREADD receptor activation in *Drosophila* motoneurons on synaptic transmission. Annual meeting of Society for Neuroscience. Washington, DC, USA.
2. **Majeed, Z.R.**, Nichols, C.D. and Cooper, R.L. (2012) Effect of DREADD receptor activation in *Drosophila* motoneurons on synaptic transmission.

- Spring Neuroscience Day, University of Kentucky, Lexington, Kentucky. March 29, 2012.
3. Cooper, R.L., Titlow, J. 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, Kentucky. March 29, 2012.
  4. Nichols, C.D., Becnel, J., Johnson, O., **Majeed, Z.R.**, Tran, V., Yu, B., Roth, B.L. and Cooper, R.L. (2012). DREADD receptor control of behavior, signalling, and physiology in the model organism *Drosophila melanogaster*. Meeting on: Optogenetics and Pharmacogenetics in Neuronal Function and Dysfunction. Hilton Riverside, New Orleans, LA, USA. 11-12 October 2012. Sponsored by Thorlabs (Photonics).
  5. **Majeed, Z.R.**, Cooper, R.L., and Nichols, C.D. (2012). The influence of DREAD receptors activation in the CNS of *Drosophila melanogaster*. Annual meeting of Society for Neuroscience. New Orleans, LA., USA.
  6. Cooper, R.L., Titlow, J. and **Majeed, Z.R.** (2012). Introduction of a new neurophysiology laboratory for students at the University of Kentucky. Annual meeting of Society for Neuroscience. New Orleans, LA., USA.
  7. **Majeed, Z.R.**, Nichols, C.D. and Cooper, R.L. (2012). Pharmacogenetic approach in directing inhibition of the larval heart in *Drosophila melanogaster*. The Kentucky Academy of Science annual meeting. Oct.19-20, 2012, Eastern Kentucky University, Richmond, Kentucky.
  8. Stacy, A., **Majeed, Z.R.** and Cooper, R.L. (2012). Characterization of 5-HT (serotonin) receptor subtypes in *Drosophila melanogaster* larval heart. The Kentucky Academy of Science annual meeting. Oct.19-20, 2012, Eastern Kentucky University, Richmond, Kentucky.
  9. **Majeed, Z.R.**, Nichols, C.D. and Cooper, R.L. (2012). Pharmacogenetic approach in directing inhibition of the larval heart in *Drosophila melanogaster*. The Center for Muscle Biology Univ. of KY. Oct. 25, 2012.
  10. Stacy, A., **Majeed, Z.R.** and Cooper, R.L. (2012). Characterization of 5-HT (serotonin) receptor subtypes in *Drosophila melanogaster* larval heart. The Center for Muscle Biology Univ. of KY. Oct. 25, 2012.
  11. Crosthwaite, T., **Majeed, Z.** and Cooper, R.L. (2013) Role of PLC-IP3-PKC pathway in 5-HT mediated heart rate modulation in *Drosophila* larvae. Annual meeting of the Ky chapter of the American Physiological Society, Univ of Kentucky, Lexington, Ky. March 25, 2013.
  12. Titlow, J., **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. March 25, 2013.
  13. **Majeed, Z.R.**, Titlow, J., 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. March 25, 2013.
  14. Bankemper, A., **Majeed, Z.** and Cooper, R.L. (2013) Characterization of 5-HT receptor subtype in sensory-CNS-motor circuit in *Drosophila* larvae. Spring Neuroscience Day, University of Kentucky, Lexington, Kentucky. April 8, 2013.
  15. **Majeed, Z. R.**, Swoveland, R. and Cooper, R. L. (2013) The influence of serotonin alteration on behavior and development in *Drosophila*. Spring Neuroscience Day, University of Kentucky, Lexington, Kentucky. April 8, 2013.
  16. 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. April 8, 2013.
17. 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. April 20-24, 2013. Boston, MA. USA.
  18. Stacy, A., **Majeed, Z.R.** and Cooper, R.L. (2012). Characterization of 5-HT (serotonin) receptor subtypes in *Drosophila melanogaster* larval heart. Undergraduate Showcase of Scholars, Univ of Kentucky, Lexington, Ky. April 24, 2013.
  19. Crosthwaite, T., **Majeed, Z.** and Cooper, R.L. (2013) Role of PLC-IP3-PKC pathway in 5-HT mediated heart rate modulation in *Drosophila* larvae. Undergraduate Showcase of Scholars, Univ of Kentucky, Lexington, Ky. April 24, 2013.
  20. Bankemper, A., **Majeed, Z.** and Cooper, R.L. (2013) Characterization of 5-HT receptor subtype in sensory-CNS-motor circuit in *Drosophila* larvae. Undergraduate Showcase of Scholars, Univ of Kentucky, Lexington, Ky. April 24, 2013.
  21. 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. Oct. 11, 2013
  22. 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. Oct. 11, 2013
  23. **Majeed, Z.R.**, Stacy, A., Crosthwaite, T. and Cooper, R.L. (2013) 5-HT receptor subtypes and associated intracellular signaling pathway that mediate heart rate modulation in *Drosophila* larvae. University of Kentucky Gill Heart Institute Cardiovascular Research Day. Oct. 11, 2013.
  24. 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.
  25. Dabbain, N., Graff, J., **Majeed, Z.R.**, and Cooper, R.L. (2013) The effects of GABA on development, behavior and survival in *Drosophila*. Annual meeting of the Kentucky Academy of Sciences. Nov. 8-9, 2013 at Morehead Univ. KY.
  26. 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.
  27. Crosthwaite, T., **Majeed, Z.R.**, and Cooper, R.L. (2013) The modulatory mechanism of action of serotonin in *Drosophila* larval heart. Annual meeting of the Kentucky Academy of Sciences. Nov. 8-9, 2013 at Morehead Univ. KY.
  28. Cornwell, K., **Majeed, Z.R.**, and Cooper, R.L. (2013) The effect of evolutionarily conserved neuromodulator, serotonin, on behavior in *Drosophila melanogaster*. Annual meeting of the Tennessee Academy of Sciences. Nov. 15, 2013 at Motlow State Community College in Tullahoma, TN
  29. Abdeljaber, E., **Majeed, Z.R.**, and Cooper, R.L. (2013) Role of 5-HT<sub>2B</sub> in *Drosophila* development and behavior. Annual meeting of the Kentucky Academy of Sciences. Nov. 8-9, 2013 at Morehead Univ. KY. 1st place Poster award-undergrad.

30. Stacy, A., **Majeed, Z.R.**, and Cooper, R.L. (2013) Serotonin and heart: How serotonin modulates the heart rate in *Drosophila* larvae. Annual meeting of the Kentucky Academy of Sciences. Nov. 8-9, 2013 at Morehead Univ. KY.
31. Bankemper, A., **Majeed, Z.R.**, and Cooper, R.L. (2013) Studying the role of serotonin in neural circuit modulation and behavior in *Drosophila melanogaster*. Annual meeting of the Kentucky Academy of Sciences. Nov. 8-9, 2013 at Morehead Univ. KY.
32. 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 neuromuscular junctions in *Drosophila melanogaster* larvae. Annual meeting of the Kentucky Academy of Sciences. Nov. 8-9, 2013 at Morehead Univ. KY. 2nd place Poster award-undergrad.
33. **Majeed, Z.R.**, Cornwell, K., Bankemper A., Abdeljaber, E. and Cooper, R.L. (2013). The effect of serotonergic system dysfunction on neural circuitry and behavior in *Drosophila melanogaster*. Annual meeting of the Kentucky Academy of Sciences. Nov. 8-9, 2013 at Morehead Univ. KY.
34. Cooper, R.L., **Majeed, Z.R.**, Santin, J.M. and Hartzler, L.K. (2014) Alteration in synaptic transmission by CO<sub>2</sub>: Glutamate insensitivity. Society for Integrative and Comparative Biology. Annual meeting. January 3-7, 2014, Austin, Texas.
35. **Majeed, Z.R.**, Santin, J., Hartzler, L. and Cooper, R.L. (2014) How CO<sub>2</sub> suppresses synaptic transmission: Changing in glutamate sensitivity. Bluegrass Chapter of Society for Neuroscience annual meeting. Lexington, KY. March, 27.
36. Dabbain, N., Graff, J., **Majeed, Z.R.** and Cooper, R.L (2014) The effects of GABA on sensory- motor circuit activity, behavior and development in *Drosophila*. Bluegrass Chapter of Society for Neuroscience annual meeting. Lexington, KY. March, 27.
37. Abdeljaber, E., **Majeed, Z.R.**, and Cooper, R.L. (2014) Modulatory role of serotonergic system in locomotion neural circuitry and behavior in *Drosophila melanogaster*. Bluegrass Chapter of Society for Neuroscience annual meeting. Lexington, KY. March, 27.
38. Bankemper, A., **Majeed, Z.R.** and Cooper, R.L. (2014) Studying the role of serotonin in neural circuit modulation and behavior in *Drosophila melanogaster*. Bluegrass Chapter of Society for Neuroscience annual meeting. Lexington, KY. March, 27.
39. Bankemper, A., **Majeed, Z.R.** and Cooper, R.L. (2014) Studying the role of serotonin in neural circuit modulation and behavior in *Drosophila melanogaster*. NCUR-National Council on Undergraduate Research. April 5-7. Univ of KY, Lexington, KY.
40. 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.
41. Abdeljaber, E., **Majeed, Z.R.**, and Cooper, R.L. (2014) Role of 5-HT<sub>2B</sub> in *Drosophila* development and behavior. NCUR-National Council on Undergraduate Research. April 5-7. Univ of KY, Lexington, KY.
42. Dabbain, N., Graff, J., **Majeed, Z.R.** and Cooper, R.L. (2014) The effects of GABA on development, behavior and survival in *Drosophila*. NCUR-National Council on Undergraduate Research. April 5-7. Univ of KY, Lexington, KY.
43. 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.
44. Dabbain, N., Graff, J., **Majeed, Z.R.** and Cooper, R.L. (2014). Inhibitory neurotransmitter GABA: its role in locomotive neural circuit activity and behavior in *Drosophila melanogaster*. 2nd Annual meeting of the Ky chapter of the American Physiological Society, Univ. of Louisville, Ky. March 31, 2014.
  45. Bankemper, A., **Majeed, Z.R.** and Cooper, R.L. (2014). The role of various serotonin receptor subtypes in modulation of neural circuitry and behavior in *Drosophila melanogaster*. 2nd Annual meeting of the Ky chapter of the American Physiological Society, Univ. of Louisville, Ky. March 31, 2014.
  46. Abdeljaber, E., **Majeed, Z.R.** and Cooper, R.L. (2014). Serotonin modulates locomotion neural circuitry and behavior in *Drosophila melanogaster*. 2nd Annual meeting of the Ky chapter of the American Physiological Society, Univ. of Louisville, Ky. March 31, 2014.
  47. Biecker, S., Titlow, J.S., Rice, J., **Majeed, Z.R.**, Holsopple, E. and Cooper, R.L. (2014). A novel system to investigate sensory habituation. 2nd Annual meeting of the Ky chapter of the American Physiological Society, Univ. of Louisville, Ky. March 31, 2014.
  48. **Majeed, Z.R.**, Santin, J., Hartzler, L. and Cooper, R.L. (2014). Synaptic transmission suppression by carbon dioxide: Glutamate receptor sensitivity. 2nd Annual meeting of the Ky chapter of the American Physiological Society, Univ. of Louisville, Ky. March 31, 2014.
  49. DeCastro, C., Titlow, J., **Majeed, Z.R.**, Vaughn, M., 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.
  50. Cooper, R.L., **Majeed, Z.R.**, Malloy, C., Blümich, S.L.E. and Putnam, R.W. (2014). Synaptic transmission: Effects of intracellular and intravacuolar pH. Annual meeting of Society for Neuroscience. Washington, D.C, USA.
  51. Cooper, R.L., **Majeed, Z.R.**, Malloy, C., Potts, D., Zeidler-Watters, K., Krall, R.M., Johnson, D., Mayo, S., Zwanzig, G., Anderson, H., Colgan III, W., Chung, W.-Y. Megighian, A. and Dupont-Versteegden, E.E. (2014). Citizen science with high school students and adults from around the world participating in analysis of synaptic transmission. Annual meeting of Society for Neuroscience. Washington, D.C, USA.
  52. Ritter, K., **Majeed, Z.R.**, Robinson, J., Blümich, S.L.E., Brailoiu, E. and Cooper, R.L. (2014). Acute actions of fluoxetine (Prozac) on neuronal and cardiac function. Annual meeting of the Kentucky Academy of Sciences. Nov. 14-16, 2014 at Lexington, KY.
  53. Dabbain, N., Schwarcz, E., Graff, J., **Majeed, Z.R.** and Cooper, R.L. (2014) How the inhibitory modulator GABA alters development, behavior and survival and heart function in *Drosophila*. Annual meeting of the Kentucky Academy of Sciences. Nov. 14-16, 2014 at Lexington, KY.
  54. Cooper, R.L., **Majeed, Z.R.**, Malloy, C., Blümich, S.L.E., Chung, W.-Y., and Putnam, R.W. (2015). Effects of intracellular pH on synaptic transmission: Differences in evoked and spontaneous release. Poster. Society for Integrative and Comparative Biology. Annual Meeting. January 3-7, 2015 West Palm Beach, FL.
  55. Cooper, R.L., DeCastro, C., Titlow, J., **Majeed, Z.R.**, Malloy, C., Vaughn, M., and King, K. (2015). Maintaining the *Drosophila* larval heart in situ: Modulators and stretch activated channels. Oral. Society for Integrative and Comparative Biology. Annual Meeting. January 3-7, 2015 West Palm Beach, FL.

56. Cooper, R.L., **Majeed, Z.R.**, Malloy, C., Zeidler-Watters, K., Krall, R.M., Johnson, D., Mayo, S., Colgan III, W., Chung, W.-Y., Megighian, A. and Dupont-Versteegden, E.E. (2015). Citizen science with high school students and adults from around the world participating in analysis of synaptic transmission. Oral. Society for Integrative and Comparative Biology. Annual Meeting. January 3-7, 2015 West Palm Beach, FL.
57. Blümich, S.L.E., Ritter, K., **Majeed, Z.R.**, Robinson, J., Brailoiu, E. and Cooper, R.L. (2015). Acute actions of fluoxetine (Prozac) on behavior and neuronal activity. 11th Göttingen Meeting of the German Neuroscience Society 2015. March 18 - 21, 2015 Göttingen, Germany.
58. deCastro, C., Titlow, J., **Majeed, Z.R.**, Vaughn, M., King, K. and Cooper, R.L. (2015) Maintaining the Drosophila larval heart in situ: Modulators and stretch activated channels. 3rd Annual meeting of the KY chapter of the American Physiological Society, Sullivan University College of Pharmacy, Louisville, KY. March 23, 2015.
59. deCastro, C., Titlow, J., **Majeed, Z.R.**, Vaughn, M., King, K. and Cooper, R.L. (2015). Maintaining the Drosophila larval heart in situ: Modulators and stretch activated channels. The 9th International Congress of Comparative Physiology and Biochemistry. August 23-28, Kraków, Poland.
60. Piedade, W.P., Koch, F. **Majeed, Z.**, Brailoiu, E., Blümich, S.L.E., Putman, R., and Cooper, R.L. (2015). Sensitivity of presynaptic pH on synaptic transmission: Differences in evoked and spontaneous release. Annual meeting of Society for Neuroscience. Chicago, IL., USA.
61. DMahmood, D., Dabbain, N., Graff, J., **Majeed, Z.R.** and Cooper, R.L. (2015). How the inhibitory modulator GABA alters development, behavior and neuronal circuit function in Drosophila. Annual meeting of Society for Neuroscience. Chicago, IL., USA
62. Sifers, J., D.Mahmood, D., Dabbain, N., Graff, J., **Majeed, Z.R.** and Cooper, R.L. (2015). GABA's action modulating development, behavior and survival as well as heart function in Drosophila. Annual meeting of the Kentucky Academy of Sciences. Nov. 13-14, 2015 at Northern Kentucky University.
63. deCastro, C., Titlow, J., **Majeed, Z.R.**, Malloy, C. Zhu, Y.-C., Vaughn, M., King, K. and Cooper, R.L. (2015). Maintaining the Drosophila larval heart in situ: Modulators and stretch activated channels. Annual meeting of the Kentucky Academy of Sciences. Nov. 13-14, 2015 at Northern Kentucky University.
64. Piedade, W.P., Koch, F. **Majeed, Z.**, Brailoiu, E., Blümich, S.L.E., Putman, R., and Cooper, R.L. (2015). Sensitivity of presynaptic pH on synaptic transmission: Differences in evoked and spontaneous release. Annual meeting of the Kentucky Academy of Sciences. Nov. 13-14, 2015 at Northern Kentucky University.
65. Koch, F., **Majeed, Z.R.**, Morgan, J., and Cooper, R.L. (2016) Manipulation of various neural circuits and the effect on locomotion behavior in Drosophila using optogenetics. Leipzig Veterinary Congress, Leipzig, Germany. January 14 to 16, 2016
66. Piedade, W.P., Koch, F. **Majeed, Z.**, Brailoiu, E., Blümich, S.L.E., Putman, R., and Cooper, R.L. (2016). Sensitivity of presynaptic pH on synaptic transmission: Differences in evoked and spontaneous release. Annual Meeting of the Kentucky Chapter of the American Physiological Society, March 24, 2016, Univ of KY., Lexington, KY.
67. deCastro, C., Titlow, J., **Majeed, Z.R.**, Malloy, C. Zhu, Y.-C., Vaughn, M., King, K. and Cooper, R.L. (2016). Maintaining the Drosophila larval heart in situ: Modulators and stretch activated channels. Annual Meeting of the Kentucky Chapter of the American Physiological Society, March 24, 2016, Univ of KY., Lexington, KY.

68. Demers, B., Koch, F., **Majeed, Z.R.**, Morgan, J., Anderson, H., Cooper, R.L. (2016). Manipulation of various neural circuits and the effect on behavior in *Drosophila* using optogenetics: NGSS-Neurons, genetics, and selective stimulations. Annual Meeting of the Kentucky Chapter of the American Physiological Society, March 24, 2016, Univ of KY., Lexington, KY.
69. Greene, E., Demers, B., **Majeed, Z.R.**, Morgan, J., and Cooper, R.L. (2016) Manipulation of the serotonin neural circuit and the effect on locomotion behavior and physiology in *Drosophila* using optogenetics. Annual Meeting of the Kentucky Chapter of the American Physiological Society, March 24, 2016, Univ of KY., Lexington, KY.
70. Koch, F., **Majeed, Z.R.**, Morgan, J., and Cooper, R.L. (2016) Manipulation of various neural circuits and the effect on locomotion behavior in *Drosophila* using optogenetics. 8th European Conference on Comparative Neurobiology (ECCN). April 7-9, 2016. Munich, Germany.
71. Demers, B., Koch, F., **Majeed, Z.R.**, Morgan, J., Anderson, H., Cooper, R.L. (2016). Manipulation of various neural circuits and the effect on behavior in *Drosophila* using optogenetics: NGSS-Neurons, genetics, and selective stimulations. Bluegrass Chapter of Society for Neuroscience annual meeting. Lexington, KY. April, 21.
72. Greene, E., Demers, B., **Majeed, Z.R.**, Morgan, J., and Cooper, R.L. (2016) Manipulation of the serotonin neural circuit and the effect on locomotion behavior and physiology in *Drosophila* using optogenetics. Bluegrass Chapter of Society for Neuroscience annual meeting. Lexington, KY. April, 21.
73. Koch, F., **Majeed, Z.R.**, Morgan, J., and Cooper, R.L. (2016) Manipulation of various neural circuits and the effect on locomotion behavior in *Drosophila* using optogenetics. 8th European Conference on Comparative Neurobiology (ECCN). April 7-9, 2016. Munich, Germany.
74. Cooper, R.L., **Majeed, Z.R.**, Titlow, J.S., Malloy, C.A. and de Castro, C. (2016) What the *Drosophila* larval heart preparation has taught us about pacemaker modulation. 2016 International Congress of Entomology. September 25-30, 2016 in Orlando, Florida, USA.
75. Hall, K., D.Mahmood, D., Dabbain, N., Graff, J., **Majeed, Z.R.**, and Cooper, R.L. (2016) Optogenetic and pharmacological alteration in the GABAergic system within *Drosophila melanogaster* affects development, feeding behavior and locomotion. Annual Meeting of the Kentucky Chapter of the American Physiological Society, March 24, 2016, Univ of KY., Lexington, KY.
76. D.Mahmood, D., Hall, K., Dabbain, N., Graff, J., **Majeed, Z.R.**, and Cooper, R.L. (2016) Optogenetic and pharmacological alteration in the GABAergic system within *Drosophila melanogaster* affects development, feeding behavior and locomotion. Bluegrass Chapter of Society for Neuroscience annual meeting. Lexington, KY. April, 21.

## UNDERGRADUATE AND HIGH SCHOOL STUDENTS MENTORED

1. Stacy, A. **Project title:** Characterization of 5-HT (serotonin) receptor subtypes in *Drosophila melanogaster* larval heart. (Fall 2012)
2. Swoveland, R. **Project title:** Role of serotonin (5-HT) in *Drosophila* development and behavior. (Spring 2013)
3. Bankemper, A. **Project title:** Characterization of 5-HT receptor subtype in sensory-CNS-motor circuit in *Drosophila* larvae. (Spring 2013)

4. Crosthwaite, T. **Project title:** Role of PLC-IP3-PKC pathway in 5-HT mediated heart rate modulation in *Drosophila* larvae.
5. King, K. **Project title:** Modulatory action of Octopamine in *Drosophila* larval heart. (Summer 2012)
6. Greene, E. **Project title:** Role of 5-HTergic neuron activity during *Drosophila* larval development on locomotor activity. (Fall 2105)
7. Koch, F. GERMAN exchange student. **Project title:** Manipulation of various neural circuits and the effect on behavior in *Drosophila* using optogenetics: NGSS-Neurons, genetics, and selective stimulations (Summer 2015)
8. Demers, B. **Project title:** Role of 5-HTergic neuron activity during *Drosophila* larval development on locomotor activity. (Spring 2016)

### Development of laboratory exercises

Laboratory exercises for Bio450 (Neurophysiology Lab) at the University of Kentucky, 2012. All of these labs will have a movie of “how to do the lab” in time. Currently, each one has a detailed protocol.

1. **Majeed, Z.R.**, Titlow, J., Hartman, H.B., Burns, E., and Cooper, R.L. (2012) Proprioception: Response properties of joint receptors.
2. **Majeed, Z.R.**, Titlow, J., Hartman, H.B., Burns, E., and Cooper, R.L. (2012) Tension receptors in crab limbs: Responses related to muscle force.
3. Titlow, J., **Majeed, Z.R.**, Nicholls, J.G. and Cooper, R.L. (2012) Identifiable neurons in the central nervous system of a leech.
4. **Majeed, Z.R.**, Titlow, J., Nicholls, J.G. and Cooper, R.L. (2012) Sensory field maps in the skin of a leech for touch, pressure and nociceptive neurons.
5. Titlow, J., **Majeed, Z.R.**, Nicholls, J.G. and Cooper, R.L. (2012) Electrophysiological examining synapse formation in culture between identifiable neurons: Central neurons of leech.
6. Titlow, J., **Majeed, Z.R.**, Hartman, H.B., Burns, E., and Cooper, R.L. (2012) Mechanosensory integration: Input and output of mechanosensory information in the cockroach wind escape reflex