

ABSTRACT OF DISSERTATION

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The Graduate School
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2007

INFLUENCE OF THE SEROTONERGIC SYSTEM ON PHYSIOLOGY,
DEVELOPMENT, AND BEHAVIOR OF *DROSOPHILA MELANOGASTER*

ABSTRACT OF DISSERTATION

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
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Lexington, Kentucky

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Lexington, Kentucky

2007

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The regulation and modulation of the serotonergic system is clinically significant in humans. Abnormally low levels of serotonin can result in depression and conditions like panic disorder, obsessive-compulsive disorder, social anxiety disorder, sudden infant death syndrome, and eating disorders. The mechanistic role of serotonin (5-HT) on the neural circuits related with these diseases is not definitively known.

Drosophila is a simple model system that provides an advantage over vertebrates to modify genetically and for electrophysiological studies on identifiable cells. In this organism the sensory-CNS-motor circuit is modulated by 5-HT, octopamine (OA), and dopamine (DA), which gives one insight that these neuromodulators are playing a role in central neuronal circuits. The role of 5-HT in the behavior and development of *Drosophila melanogaster* larvae is being studied. p-CPA (para-chlorophenylalanine) blocks the synthesis of 5-HT by inhibiting tryptophan hydroxylase. The development, behavior and physiology in 3rd instar larvae are affected after feeding this drug. MDMA (3,4-methylenedioxyamphetamine), an analog of methamphetamine is a drug of abuse that has been shown to cause depletion of 5-HT from nerve terminals. It causes the 5-HT transporter to work in reverse. Thus, a dumping of 5-HT results. In *Drosophila* 3rd instar larva development, physiology and behavior are effected when MDMA is fed throughout their development period. Also at the fly neuromuscular junction, (NMJ) MDMA is causing more evoked vesicular release of glutamate from the presynaptic nerve terminal. Also using anti-sense expression of the 5-HT_{2dro} receptor, role of 5-HT and one of its receptors is studied on development, physiology and behavior. Knock down of 5-HT_{2dro} resulted in developmental delay. Physiology and behavior were also abnormal in these animals.

KEYWORDS: Serotonin, *Drosophila*, sensory-CNS-circuit, MDMA, heart rate.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	III
TABLE OF CONTENTS.....	IV
LIST OF TABLES.....	VII
LIST OF FIGURES	VIII
LIST OF FILES	X
CHAPTER 1.....	1
INTRODUCTION	1
GENERAL BACKGROUND OF DEVELOPMENT	1
NEURAL ACTIVITY IN DEVELOPMENT.....	4
INFLUENCE OF HORMONES AND NEUROMODULATORS ON DEVELOPMENT.....	6
SEROTONIN (5, HYDROXY TRYPTAMINE, 5-HT).....	7
ROLE OF 5-HT IN DEVELOPMENT.....	9
EFFECTS ON DEVELOPMENT THROUGH RECEPTORS.....	12
MANIPULATIONS IN NEUROMODULATOR SYSTEMS ON FUNCTION	13
5-HT RECEPTORS AND EXPRESSION IN DROSOPHILA.....	17
USING THE DROSOPHILA HEART AS A BIOASSAY FOR 5-HT EFFECTS.....	19
CHAPTER 2.....	23
MODULATION OF SENSORY-CNS-MOTOR CIRCUITS BY SEROTONIN, OCTOPAMINE, AND DOPAMINE IN SEMI-INTACT DROSOPHILA LARVA.....	23
ABSTRACT.....	23
INTRODUCTION	24
METHODS.....	26
RESULTS	27
DISCUSSION.....	31
CHAPTER 3.....	42
INFLUENCE OF P-CPA AND MDMA ON THE SEROTONERGIC SYSTEM IN RELATION TO PHYSIOLOGY, DEVELOPMENT AND BEHAVIOR OF DROSOPHILA MELANOGASTER.....	42

ABSTRACT	42
INTRODUCTION	42
METHODS	44
RESULTS	48
DISCUSSION.....	54
CHAPTER 4.....	71
KNOCK DOWN OF 5-HT ₂ RECEPTORS ALTERS DEVELOPMENT, BEHAVIOR AND CNS ACTIVITY IN DROSOPHILA MELANOGASTER.....	71
ABSTRACT	71
INTRODUCTION	71
METHODS.....	74
RESULTS	77
DISCUSSION.....	83
CHAPTER 5.....	100
DIRECT INFLUENCE OF SEROTONIN ON THE LARVAL HEART OF DROSOPHILA MELANOGASTER.....	100
ABSTRACT	100
INTRODUCTION	101
METHODS.....	104
RESULTS	108
DISCUSSION.....	112
CHAPTER 6.....	123
DISCUSSION.....	123
REFERENCES	136
CHAPTER 1.....	136
CHAPTER 2.....	161
CHAPTER 3.....	169
CHAPTER 4.....	182
CHAPTER 5.....	191
CHAPTER 6.....	202

VITA..... 214

LIST OF TABLES

TABLE 5.1.....	123
----------------	-----

LIST OF FIGURES

Figure 2.1: Schematic diagram of the Drosophila larva preparation:	35
Figure 2.2: Representative traces of induced responses recorded in muscle 6 in various segments.....	36
Figure 2.3: Recruitment of motor units.....	38
Figure 2.4: The influence of neuromodulators in altering the sensory to motor neuron central circuit was examined.....	40
Figure 3.1: p-PCA growth curve.....	63
Figure 3.2: MDMA growth Curve.....	64
Figure 3.3: Body wall and mouth hook contractions.....	65
Figure 3.4: Spontaneous activity in 3rd instar CS larvae.	66
Figure 3.5: Spontaneous activity.....	67
Figure 3.6: Sensory-CNS-motor circuit.	68
Figure 3.7: Heart rate.....	69
Figure 3.8: HPLC analysis of 3rd instar larvae.....	70
Figure 4.1: Locomotory movements at room temperature.	90
Figure 4.2: Locomotory movements of larvae that are grown at high temperature. .	91
Figure 4.3: Locomotory movements at high temperature.....	92
Figure 4.4: Locomotory movements at 31-32C.....	93
Figure 4.5: Development curve for low temperature.	94
Figure 4.6: Development curve for room temperature.	95
Figure 4.7: Development curve for high temperature.....	96
Figure 4.8: Spontaneous activity.....	97
Figure 4.9: Sensory-CNS-motor circuit	98

Figure 4.10: Heart rate..... 99

Figure 5.1: Dorsal Vessel..... 120

Figure 5.2: Heart rate of 3rd instar semi-intact preparations..... 121

Figure 5.3: The effects of 5-HT on intact and on the isolated heart and aorta segments. 122

LIST OF FILES

Dasari.pdf 4.5mb

CHAPTER 1

INTRODUCTION

GENERAL BACKGROUND OF DEVELOPMENT

Developmental neurobiology is the study of how neurons grow and form connections. This includes understanding development and complex organization of the brain and its systems as well as factors affecting this process. This involves evaluating the sensory inputs and processing of those signals that are then responsible for defined motor patterns or behaviors.

The CNS during development is known to be dependent on the on-going electrical activity and synaptic transmission. Thus, the activity of sensory input to drive interneurons, which in return drives motor neurons and their targets are critical. Not only is activity important for developing a sensory-CNS-motor circuit but within the CNS the various internal circuits also require activity to establish themselves. The nervous system (NS) is very dynamic during development and depending on the animal model there can be a wide range in the rate of development of the NS. The CNS develops at different rates during the early life stages among animal species. In addition, particular regions of the NS develop at various rates. As one might expect vegetative functions such as regulation of the heart, blood pressure and breathing to develop earlier as compared to those for fine motor coordination, visual or olfactory senses.

Recently there is a vibrant interest in understanding more about neuronal replacement and treatments with stem cells that differentiate into neurons within the adult mammalian CNS (Encinas et al., 2006; Huang and Herbert, 2006). It is now

established that in the adult mammalian CNS there are cells that can migrate into neural tissue and start to grow processes, which connect into existing circuits (Lie et al., 2004; Hagg, 2005; Miller, 2006; Gage 1995; McDonald, 1999). It was shown in 1983 (Goldman and Nottebohm) that many new neurons are formed in adult song bird (canary) brain and that seasonal changes occur in singing birds resulting in new neurons that arise from stem cells, which became part of the neural circuitry involved with vocalization (Nottebohm et al., 1986). What is truly amazing is that development regresses over the winter and repeats itself the following year (Nottebohm et al., 1986). Along with research that is ongoing in birds, other groups (Monfils et al., 2006) discovered that when cells in the CNS are damaged in rodents parts of it filled back in with new neurons. It is now known that the sub-ventricular zones contain stem cells that could indeed transform into neurons and help repair damaged neural tissue (Lois and Alvarez-Buylla, 1993; Luskin, 1993). This brings one to the point of maintenance of the existing neural circuits by replacement after the initial establishment of the CNS. At the NMJ in adult rodents it has been known for sometime that synapses are not hard wired but are very dynamic in pairing back and re-growing at normal NMJs that are not undergoing regeneration or repair (see review Sanes and Lichtman, 1999; Purves and Lichtman, 1987).

Expanding on these earlier findings I wished to address if one altered synaptic communications by neuromodulators in the larval brain of *Drosophila* would there be consequences in further development or maintenance of functional neural circuits. As compared to other animal models *Drosophila* offers a rapidly developing CNS and this animal model serves as a spring board for genetic studies to address

similar questions that are being addressed in vertebrates by targeting specific genes and proteins.

In order to relay key aspects in neural development I will give a brief overview next on the mammalian systems since a wealth of information is available and some of the underlying principles are important for all animals. Mammalian neurogenesis begins with the formation of the neural plate that is a thickening of ectodermal cells on the dorsal aspect of the developing embryo. Ridges are formed at the lateral edges of the plate, which curl up to meet at the dorsal midline to form the neural tube. The internal cavity created by the tube is called the ventricle. As closure of the neural tube is occurring, specialized regions of the nervous system begin to emerge through differential cell division and migration. Major subdivisions of brain include the myelencephalon and metencephalon, the mesencephalon, and the prosencephalon, which matures into the diencephalon and telencephalon. Through this process, the subdivision of the developing brain lays the foundation for regional specialization in the mature brain. By the end of embryonic stages of an animal, neurons make connections with other neurons either locally or at distant central or peripheral target tissues. For example, retinal ganglion axons from the eyes enter the brain at the junction of optic nerve and diverge to the optic tectum and lateral geniculate nucleus. The numerous synapses and connections that are made go through the process of refinement, rearrangement and elimination are based on activity (Wiesel and Hubel , 1965; Levay et al., 1980). As for the vertebrate brain, the *Drosophila* larval brain also shows regions of similar function that can be quantified and examined for alterations in size (Iyengar, et al., 2006). Possible in the near

future synaptic connectivity within defined circuits will also be addressed.

NEURAL ACTIVITY IN DEVELOPMENT

Development and maintenance of neural circuits is dependent on the electrical activity. There are 2 general activities in the brain that can effect the development of the neural circuits – spontaneous activity that is devoid of any sensory or motor input and activity based on experience that is from input of sensory and motor units. Spontaneous activity is seen as bursts of activity for a few seconds or minutes in absence of neuronal stimulation. This activity was shown to have an effect on both synapse formation and elimination. For example, when newborn cats were deprived of any visual activity by closing both eyes; ocular dominance columns for both the eyes are still formed although the columns are obscured (Hubel and Wiesel 1965; Sherman and Spear, 1982). This was thought to be due to spontaneous activity. To prove this TTX (a blocker for sodium channels) was injected into both eyes of 2-6 weeks postnatal kittens. The experiment demonstrated that the lateral geniculate nucleus did not segregate into stripes (Stryker and Harris, 1986). This kind of activity was also seen in the developing auditory system of birds and the spinal cord of chicks (Lippe, 1994; O'Donovan et al., 1994; Kotak and Sanes, 1995).

Experienced based activity or use-dependent activity involvement in the development of neural circuits was shown by the pioneering work of Hubel and Wiesel (1963a,b) for which they received a Nobel Prize. Their work on visual deprivation in one eye of newborn cats showed that cortical neurons did not

responded to stimulation from the closed eye. In normal animals half the cortical neurons respond to one eye and the other half to the other eye. Also the closure of lids for 3 months led to blindness in the newborn cats and monkeys (Wiesel and Hubel, 1963b; Wiesel 1982). At the same time Hubel and Wiesel showed that the cortical region of the CNS, which would have been supplied by the deprived eye, shrunk whereas those of the other eye expanded. These results have shown cortical neurons in the visual system are developed and maintained based on activity. Since then many studies have shown that spontaneous electrical activity during the embryonic stages and experience based activity in early postnatal stages are important for the development and refining of the neural circuits (Penn and Shaatz 1999; Zhang and Poo, 2001).

However similar experiments carried out in adult animals had no effect on their ocular columns architecture or even the responses from cortical neurons and on blindness (Wiesel, 1982; LeVay et al., 1980). Hence they concluded that the age of animal when these experiments were conducted were important. The plasticity in the newborn animals is lost as the animal ages. The time at which the plasticity can occur is referred to as the "critical period". The critical period is defined as, period in early stages of development of an animal where it shows very high sensitivity to the external stimuli and experience. Critical periods are seen in many animals and in many sensory systems such as visual, auditory, sound localization, bird song, and olfaction. Critical periods are altered by various chemical compounds like hormones, neurotransmitters or neuromodulators and drugs of abuse like cocaine.

INFLUENCE OF HORMONES AND NEUROMODULATORS ON DEVELOPMENT

Hormones and neuromodulators are chemical compounds, which have many roles in an organism and are known to affect and regulate development of the whole animal as well as the nervous system. Hormones are known to regulate fate of some neurons in certain areas of the CNS, as well demonstrated in songbirds. The higher vocal center is more developed in males than females and thus the effects of testosterone were investigated for its role on growth of this key neural location. This center plays an important role in song acquisition and retention. It was shown that when female birds are injected with testosterone, the female could be induced to sing like a male (Nottebohm and Arnold, 1976; Nottebohm, 1980). In insects, it is well established that hormones such as ecdysone and juvenile hormone alter neural development and differentiation (Garen et al, 1977; Pak and Gilbert, 1987; Truman, 1996). The surge of ecdysone in the pupal stage of *Drosophila* likely plays a key role in inducing gross alterations in the neural circuitry (Kraft et al, 1998; Thummel, 1996; Truman and Reiss, 1988) and motor unit function (Li and Cooper, 2001; Li et al, 2001). It has also been demonstrated that the sequence of exposure of neuromodulators (serotonin and octopamine) and cocktails produce differential effects on synaptic modulation in other arthropods (i.e., the crustaceans) (Djokaj et al, 2001). Cocktails of various hormones and neuromodulators have not yet been investigated for their combined effects in developmental roles.

It is shown in various studies that neurotransmitter signaling is present before synaptogenesis (reviewed in Herlenius and Lagercrantz, 2004). But total knock-out of synaptic trafficking in mouse was shown to have no effect on the formation of

brain structure or synapses, however for survival of these synapses synaptic activity is needed (Verhage et al., 2000). Different neurotransmitters have different roles in the process of the brain development. For example, the noradrenergic system (norepinephrin) is essential for the brain development as it regulates the development of Cajal-Retzius cells, which are the first neurons to be formed. Cajal-Retzius cells are important for the migration of neuronal cells and laminar formation (Naqui et al., 1999,). Also a surge of norepinephrin at birth is important for formation of olfactory system and learning, that is important for recognition of one's mother (Insel and Young, 2001). Similarly 5-HT has been shown to affect neuronal differentiation, migration and synaptogenesis (Gaspar et al., 2003), acetylcholine (Ach) mediates synaptic connections and wiring of the circuits (Maggi et al., 2003), dopamine (DA) neurons appear in gestational period of development in rats (Olson and Seiger, 1972; Herlenius and Lagercrantz, 2004), humans (Sundstrom et al., 1993). Any disturbance in the development of dopaminergic system leads to various diseases like dyskinesia, obsessive compulsive disorder, etc (Zhou et al., 1995a,b). Other neuromodulators, which also serve as neurotransmitters, have also been of interest. Octopamine (OA), which is not found in vertebrates but is in invertebrates, has gain much attention because of its dramatic effect on behavior and development, particularly in insects (Barron et al., 2002; Schulz et al., 2002; Fox et al., 2006; reviewed in Roeder, 1999; Osborne, 1996; Monastirioti, 1999).

SEROTONIN (5, HYDROXY TRYPTAMINE, 5-HT)

One of the main neurotransmitters and neuromodulator that has been targeted over the years is 5-HT. It was identified as early as in 1930's with the name

enteramine and later name serotonin (Whitaker-Azmitia, 1999). The compound is commonly found in very simple to complex invertebrates and has been suggested to even be one of the 1st neurotransmitters in the evolution of animals (Whitaker-Azmitia, 1999). The role of 5-HT in invertebrates has been investigated for some time and is known to alter sensory, CNS and motor function (e.g., Marinesco and Carew, 2002) and it may even serve as an overall enhancer for animals similar to epinephrine via the sympathetic nervous system in mammals (review- Shuranova et al., 2006). Recently 5-HT is shown to have a role in mediating the structure of brain and also in neurogenesis (Yan et al 1997, Gould, 1999). Also for human and mammalian studies the role of 5-HT and its various receptor subtypes gained interest when it was established that medicinal herbs and synthesized compounds, like LSD, targeted 5-HT receptors which were responsible for altered behaviors (Nichols, 2004; Reissig et al., 2005; Gresch et al., 2005).

Rapport et al., (1948b) were the first investigators to show the structure of 5-HT and establish it as a vasoconstrictor (Rapport et al., 1948a). Twarog and Page (1953) showed for the first time that 5-HT is present in brain using dog, rat and rabbit brain extracts. Woolley and Shaw (1954) suggested 5-HT to have a role in brain development, as it is similar to the auxins, a plant growth hormone. Later Gaddum and Picarelli (1957) started reporting on various 5-HT receptors. To date seven classes of 5-HT receptors are known and classified pharmacologically into 14 distinct subtypes of mammalian receptors (Barnes and Sharp, 1999, Hoyer et al., 2002). 13 of these receptors belong to the category of G-protein coupled receptors (GPCR) and one receptor (5-HT₃) is a ligand-gated ion channel type of receptor.

In considering comparative studies based on pharmacology and genomic sequence data there are a considerable number of 5-HT receptor subtypes (Barnes and Sharp; 1999, Hoyer et al., 2002) and some share similar sequence homology between species (Barnes and Sharp, 1999; Saudou and Hen, 1994). Thus, using one model organism in examining regulation of particular receptor subtypes may shed light into the functions of other organisms, such as humans where such experimentation is problematic to investigate developmental topics.

ROLE OF 5-HT IN DEVELOPMENT

In general synaptic plasticity is the ability of synapses between two neurons or a neuron to a target cell to change in strength or number of connections in the network. There are various mechanisms by which synaptic plasticity is measured, such as how much neurotransmitter is released at the synapse or the responsiveness on the receiving cell. Even structural changes that occur in the circuit would be a form of synaptic plasticity. The underlying cause of behavioral plasticity is assumed to be due to synaptic plasticity within the neural circuitry of an animal. Gaspar et al (2003) has shown that 5-HT uptake is necessary for the normal development and refining of cortical sensory maps during the critical period of development in mouse. Role of 5-HT in synaptic plasticity has been shown in rodents (Mnie-Filali et al., 2006), chickens (Chen et al., 1997), *Aplysia* (Marinesco et al., 2004; Chang et al., 2003), and crustaceans (Harzsch et al., 1999; Cooper et al., 2003). Not only are there direct effects on electrical activity by 5-HT on neurons but indirect effects on the whole system. For example, it is known that 5-HT can alter the release of growth hormone in rats (Murakami et al., 1986) which then alters the

growth of the entire animal. In humans the effect of 5-HT on growth hormone is not well known. Studies have shown stimulatory (Mota et al., 1995), inhibitory (Casanueva et al., 1984) and no effect (Handdwerger et al., 1975) of 5-HT on secretion of GH. Such global effects are also known to occur even invertebrates such as crustaceans in which 5-HT can have an effect on the release of the hyperglycemic hormone (Lee et al., 2000, 2001; Escamilla-Chimal et al., 2002).

The levels of 5-HT during the development are very important. Either high or low levels of 5-HT during the critical period can lead to miswiring of connections (Gaspar et al., 2003). Miswiring of neurons can lead to various problems like drug addiction disorders, anxiety disorders and autism. The levels of 5-HT during the fetal stages and in young children are high and the levels come down as development progresses. But in autistic children the levels of 5-HT is maintained high (Chugani, 2002; Warren and Singh, 1996; Hanley et al., 1977). 5-HT is also associated with other disorders like anti-social behavior, depression, migraine etc. The drugs of abuse like cocaine, MDMA (3,4-methylenedioxymethamphetamine, ecstasy), LSD or even anti-depressants like SSRI when taken by pregnant women can effect the development and behavior of the offspring (Discussed in Chapter 1, section vii and chapter 3).

To illustrate the role of 5-HT, the serotonin-ergic system is commonly manipulated using pharmacological agents. A few pharmacological approaches used to deplete 5-HT in vivo are to block tryptophan from being used to make 5-HT (Drummond, 2006; Hood et al., 2006). Another method is to kill the seretonergic neurons using the neurotoxin 5,7-dihydroxytryptamine (5,7 DHT; Walker et al., 2006;

Shirahata et al., 2006; Jha et al., 2006). The use of p-chloroamphetamine (PCA; Eide et al., 1988) and p- chlorophenylalanine (PCPA), which are both inhibitors of rate limiting enzyme tryptophan hydroxylase in the 5-HT biosynthesis pathway (Jha et al., 2006; Cooper et al., 2001) are assumed to be a specific inhibitor of 5-HT biosynthesis. However, recent studies have shown PCPA to casue a significant effect on another neurotransmitter, norepinehrine (Jha et al., 2006; Dailly et al., 2006). Also the initial study that showed PCPA as depletor of 5-HT has reported small levels of dopamine and norepinephrine to be reduced in brain tissue (Koe and Weissman, 1966; Sanders-Bush and Massari 1977). The levels of 5-HT are increased by 5-hydroxytryptophan (5-HTP), immediate precursor of 5-HT in biosynthesis pathway (Pellegrino and Bayer, 2000; Fickbohm et al., 2005).

PCPA has been successfully used as a 5-HT depletor for many years and in many organisms like rats (Sinha, 2006; Jha et al., 2006; Koe and Wiessman, 1966), mouse (Khozhai and Otellin, 2006; Dailly et al., 2006; Koe and Wiessman, 1966), dog (Haga et al., 1996; Dourish et al., 1986; Koe and Wiessman 1966), *Drosophila* (Banerjee at al., 2004; Pendelton et al., 2002; Vaysse et al., 1988; Kamyshev et al., 1983), snail (Filla et al., 2004; Baker and Croll 1996; Baker et al., 1993), and crustaceans (Mattson and Spaziani, 1986; Cooper et al., 2001).

In *Drosophila*, PCPA was first used to study the role of 5-HT on locomotion of Canton-S (CS) adults by Kamyshev et al. (1983). They showed that locomotor acitivity increases upon administration of PCPA at 150 µg/ml of yeast-raisin media. Vaysse et al (1988) used 0.6g/ L of PCPA to study the learning behavior in

Drosophila. In Chapter 3 I report in detail the effects of PCPA on development, locomotor behavior and physiology of larval *Drosophila* system.

EFFECTS ON DEVELOPMENT THROUGH RECEPTORS

Generally hormones/neuromodulators bring about their biological responses by interacting with their receptors. Many different studies have shown that these receptors are either G protein linked or ligand gated ion channels, which have wide ranging effects on cellular function. As mentioned earlier 14 different types of 5-HT receptors are known to date in mammalian systems. There could be new additions to this list as 5-HT₄ and 5-HT₇ receptors are shown to have alternate splice variants (see review Hoyer et al., 2002). Also recently 5-HT₂ receptor is shown to have different RNA-edited isoforms (Burns et al., 1997, also see review Niswender et al., 1998). Now 5-HT receptors from many model organisms have been classified based on sequence or pharmacology (Monasoratti, 1999; Tierney et al., 2001). The *Drosophila* genome has been shown to have four 5-HT receptors named 5-HT_{1Adro} 5-HT_{1Bdro} 5-HT_{2dro} 5-HT_{7dro} (Saudou et al., 1992; Witz et al., 1990; Colas et al., 1999).

One particular receptor subgroup that has interested many researchers in vertebrate models is the 5-HT₂ receptor family containing 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}. These receptors are involved in many physiological functions like smooth muscle contraction, feeding behavior, sleep, mood, pain, learning and memory (Roth et al., 1998). Also 5-HT₂ receptors are of interest as these receptors are targets for many psychoactive drugs and drugs of abuse (Roth et al., 1998; Aghajanian and Marek, 1999).

Depending on the levels of the agonists as well as antagonists, receptors can undergo up- and down-regulation by alteration of their expression levels and/or by changing their densities on the cell surface (Azaryan et al, 1998). These receptors are regulated by altered cellular activity and developmental times. Also G protein receptor kinases are involved in receptor desensitization, which occurs in the presence of agonist like 5-HT and even antagonist (Hanley and Hensler, 2002).

MANIPULATIONS IN NEUROMODULATOR SYSTEMS ON FUNCTION

The role of the neuromodulators on the nervous system function, development and whole animal development is not fully known. It is likely that an alteration in the levels of these neuromodulators during the development are very critical not only for development but also maintenance of neural circuits. Exact amounts of these neuromodulators during the development are likely critical and either higher or lower levels might result in abnormalities in the development of an organism. Decreased levels of 5-HT in prenatal rats have shown abnormalities in formation of different layers of neocortex in differentiation and development of neurons (Khozhai and Otellin, 2006). In Down's syndrome (DS; trisomy 21), 5-HT levels were shown to be lower in postmortem brains (Mann et al., 1985; Whitaker-Azimitia; 2001). When compared to normal developing brains, DS brains have higher levels of 5-HT_{1A} receptor and by birth these levels drop below normal (Bar-Peled et al., 1991). Recently Gulesserian et al. (2002) showed in adult DS patients, serotonin transporter (SERT) levels are higher in frontal cortex. Many seretonergic

agents have been used in the treatment of DS, particularly to help self-injurious behavior and aggressive behaviors (Gedye, 1990; Gedye, 1991).

Usage of certain drugs before or during pregnancy in vertebrates can result in altering the physiological concentrations of neuromodulators, which may lead to abnormalities in the development of a fetus or the child. For example, cocaine, a major drug of abuse, which blocks the reuptake of dopamine and 5-HT at synapses (Woolverton and Johnson, 1992; Filip et al., 2005), causes an increase in cardiovascular toxicity in pregnancy. Maternal complications of cocaine ingestion are premature labor, placental abruption, uterine rupture, cerebral ischemia and death. Cocaine can rapidly diffuse across placenta to the fetus and cause severe vasoconstriction. Cocaine use in pregnancy causes subtle molecular and behavioral effects on fetal brain tissue. In postnatal life these effects are manifested in decreased IQ scores and learning deficiencies (Krzysztof 2003). Also recently Bae and Zang (2005) have shown that exposure of neonatal rats to cocaine causes apoptosis and hypertrophy of myocytes in postnatal heart. Fetus exposed to cocaine through a mother also effects the development of the brain. Due to the increased levels of 5-HT, serotonergic terminals are not formed properly (Whitaker-Azmitia, 1998). The serotonergic system is important during the development of the vertebrate brain.

Other prevalent drugs of abuse are amphetamines. These are a group of non-catecholamines that produce powerful stimulation and have a prolonged activity in the body (Krzysztof 2003). Methamphetamine is the most commonly abused type of amphetamine. Another drug of abuse is MDMA (3,4

methylenedioxyamphetamine), an analog of methamphetamine. MDMA is shown to have an effect on the developing fetus in various animal models. MDMA is known to decrease the embryonic motility in chicken embryos. (Lyles and Cadet, 2003). In 11-20 day old neonatal rats MDMA exposure (5-20 mg/Kg s.c, 2X/daily) causes dose-related impairments in sequential learning and memory (Lyles and Cadet, 2003). This time period of neonatal rats correspond to the late human trimester brain development (Lyles and Cadet, 2003).

MDMA was used in 1970's by psychiatrist in treating depressed patients. Patients when given this drug would be more open in discussing their problems. However MDMA was declared as an illegal drug by US government, so therapeutic usage was stopped. In 1990's MDMA became famous among teenagers as a party drug termed "Ecstasy". The long-term effects of MDMA assessed in rats, mice and humans, are depletion of 5-HT and DA from neurons. MDMA can induce neurotoxicity and cell death. In rats MDMA causes acute release of 5-HT from its stores, which would activate the 5-HT_{2A} and 5-HT_{2C} receptors on the GABA interneurons, decreasing GABAergic transmission and increasing the DA release and synthesis (Zhou et al, 2003). The excessively released DA can be transported into already depleted 5-HT terminals, at the same time excessive DA is metabolized by MAO within 5-HT terminals resulting in the excessive generation of free radicals and reactive oxygen species (Zhou et al, 2003). There is additional evidence, which supports that MDMA-induced neurotoxicity might occur because of the production of superoxides rather than hydroxyl radicals (Green et al., 2003).

There are various effects noted in humans by exposures to MDMA such as hallucinations, hypernatremia, hyperkalemia, psycho-stimulation, and long-term neuropsychiatric behaviors, such as depression and psychosis (Simantov, 2004). High doses (average of 1.04mg/L of blood) in humans results in death. In spite the commonality of this drug and all the data that is present in the literature the specific mechanism of action is not known. The popular model for MDMA's mechanism of action is through reversing the 5-HT transporter on the presynaptic nerve terminals increasing the amount of 5-HT within the synapse until the nerve terminal is depleted of 5-HT.

Because of all the effects of MDMA on different neurotransmitter systems especially on 5-HT and DA, it is possible that a fetus is developmentally affected. Some initial studies have shown that prenatal exposure of MDMA does not effect the development or behavior in rats (Colado et al., 1997). But a recent study in rats showed that perinatal exposure of MDMA has led to some developmental defects in learning and memory (Broening et al., 2001) and enhanced locomotor activity in later life (Koprach et al., 2003).

Since it has proved to be difficult in the intact vertebrate brain to fully understand the developmental consequences in neural circuits and responsiveness of 5-HT to neurons exposed to MDMA, I chose to use a more favorable system, the fruit fly. For several reasons the fruit fly can serve as a useful model. *Drosophila*, a genetically favorable system is widely used to study the role of neuromodulators and various studies have used flies as a model organism for the study of drugs of abuse (Rothenfluh and Heberlein, 2002; Willard et al., 2006). Using *Drosophila* one can

relate rapidly the role of neuromodulators in the development of neural circuits and effects on behavior. In addition the effects of MDMA on the development can be addressed in conjunction with potential perturbations in the neuromodulators, because it is an easy system to conduct pharmacological manipulations and introduce mutations. Chapter 3 gives details of effects of MDMA on *Drosophila* larval development, behavior and physiology. Also comparisons on effects of MDMA and p-CPA on *Drosophila* larvae are reported.

5-HT Receptors and Expression in *Drosophila*

Another means of examining the effects of the serotonin-ergic system on development and behaviors is not to target the biosynthesis of 5-HT but to alter the receiving end of the 5-HT, such as the receptors. Agonists and antagonists of various 5-HT receptors are commonly used to treat human disorders. For example, selective serotonin reuptake inhibitors (SSRI) like fluoxetine, clomipramine are used in the treatment of autism (Hollander et al., 2003; Namerow et al., 2003), 5-HT₃ antagonist alosetron is used in the treatment of irritable bowel syndrome (IBS) in females (Andresen and Camilleri, 2006), atypical anti-psychotic drugs in the treatment of schizophrenia (Meltzer et al., 2003; Stimmel et al., 2002). It is known that people with altered levels in expression of particular 5-HT receptors can show social and mental deficits (Whitaker-Azmitia, 2001; Sodhi and Sanders-Bush; 2004). Perhaps the lack of the appropriate 5-HT receptor expression throughout neural development is the cause for a number of ailments in humans that have yet to be correlated to molecular mechanisms. There are various polymorphisms in races of people for 5-HT receptors which are noted to be responsible for differential effects to

drug therapies (Bolonna et al., 2004; Reynolds et al, 2005). This is a growing interest of pharmaceutical companies as well as medicine in general in order to provide therapy based on one's genomic identity (Bolonna et al., 2004; Reynolds et al, 2005).

This emerging field of study in mammals is exciting for many reasons. One being that it will help to understand the interaction of receptor expression and more specific drug therapies to reduce side effects from broad spectrum agonists and antagonists but in time there will be more interest in the developmental consequences in slight to extreme modifications to particular neural systems, like the neural circuitry that is impacted by 5-HT modulation. This is one reason why I pursued the potential effects on development in *Drosophila* related to the alteration in the appropriate expression of 5-HT receptor subtypes.

Genomic analysis has shown that there are four receptor types for 5-HT in *Drosophila* (Witz et al., 1990; Saudou et al, 1992; Colas et al, 1994; Tierney, 2001; Peroukta; 1994). As mentioned earlier they are named as 5-HT_{7dro}, 5-HT_{1Adro}, 5-HT_{1Bdro} and 5-HT_{2dro} based on sequence and functional similarities with the mammalian 5-HT receptors 5-HT_{1A}, 5-HT₂ and 5-HT₇. Nichols et al (2002) showed that LSD in flies may be mediating its affects through 5-HT_{1Adro} and 5-HT_{2dro}. In general very little work has been conducted on *Drosophila* 5-HT receptors. The 5-HT_{2dro} is 40% homologous over the transmembrane domain of 5-HT₂ receptor of mammals. 5-HT_{2dro} is present on 3rd chromosome and right arm. Two transgenic lines have been made concerning this receptors that I have taken advantage of in my studies. One with an anti-sense strand of the gene under heat-shock promoter,

called Y32 and another with an anti-sense strand under heat shock Gal4-UAS system. In chapter 4, I report on 5-HT_{2dro} role in *Drosophila* development, behavior and physiology.

Using the *Drosophila* heart as a bioassay for 5-HT effects

Since I have focused on the role of 5-HT in CNS function and behavioral studies I wanted to use an additional physiological assays for examining the holistic effects of the serotonin-ergic system. During the progression of the dissertation studies I had become aware that the heart of insects and crustaceans is very susceptible to exogenous application of 5-HT. In fact, students in the laboratory were using the heart rate as a bioassay for social interactions in crayfish with the notion of testing if there was a correlation to aggressive and submissive roles (Listerman et al., 2000). The underlying assumption was that if aggressive individuals have a higher level of circulating 5-HT, as proposed in earlier studies (Livingston et al., 1980), then the aggressive animals should have a higher heart rate as compared to submissive ones. Investigating actions of 5-HT on *Drosophila* hearts I discovered that studies had been conducted in *Drosophila*. However, I also discovered some shortcoming in the past procedures used to examine the actions of 5-HT on the heart of larval *Drosophila*.

Thus in Chapter 5, I present a full study that has already been published on the effects of 5-HT to the exposed larval heart with and without an intact CNS. The regulation of the heart via hormonal and direct neural innervation had been conducted primarily in adult hearts (Dulcis and Levine 2003, 2005; Dulcis et al. 2005; Johnson et al. 2002; Miller, 1997; Papaefthimiou and Theophilidis, 2001).

However only recently using GFP expressing lines of flies was this investigated in larvae by a fellow student in the laboratory, Dr. Andrew Johnstone. Since my findings presented in chapter 5 indicated that there are differences in heart rate depending if the CNS is intact or not, a study was conducted to examine possible connections from the CNS to the heart. They found nerves from the CNS leading to the dorsal aorta and in electron micrographs nerve terminals containing synaptic vesicles, thus suggesting direct motor nerve regulation of the heart (Johnstone and Cooper, 2006).

It has been known for some time that the heart rate in larvae can be altered by neurotransmitters and neuromodulators, which are known to be present in the hemolymph (Johnson et al. 1997, 2000; Nichols et al. 1999; Zornik et al. 1999). This was primarily examined up by injections in 3rd instars and early pupa (P1 stage, transition between larva and pupa) of 5-HT, DA, Ach, octopamine (OA), and norepinephrine (NE) which all increase HR (Johnson et al. 1997). Injection of 5-HT (1 μ M/l) caused the HR to increase by 46% from base line (Johnson et al. 1997) and Zornik et al., (1999) showed, in the wandering 3rd instar larva, that 5-HT increases HR by 111% with a concentration of 10⁻⁵ M (10 μ M/l).

The thought that injection through the larval body wall or into a pupal case of biogenic amines can cause the activation or release of many other compounds struck my interest. This did not seem to draw attention by past investigators to control during the experiments. My thought was that even saline injection could induce stress and potential release of 5-HT. Thus, I wanted to try direct application on exposed hearts in a defined saline. The ability to investigate the sensitivity of the

heart to 5-HT also was of interest since I could use the preparation as a bioassay to the sensitivity to 5-HT in the studies in which the levels of 5-HT had been reduced by feeding larval p-CPA and MDMA. Additionally, this heart bioassay would serve of interest to the studies in which I was using the fly strains that had a suppressed expression of the 5-HT₂ receptors (Chapter 4). Considering there were many avenues in which the larval heart bioassay to 5-HT was going to be of use to my other studies I decided to do a complete investigation on the subject. The findings presented in Chapter 5 served as a baseline to compare results in the other studies related to 5-HT production (Chapter 3) and altering 5-HT receptor expression (Chapter 4). Also since MDMA has direct action on neuronal 5-HT receptors I continued studies with MDMA to examine potential direct action on the larval heart in order to parallel the 5-HT study on the CNS.

The specific aims of this dissertation research are:

- 1) Address the role 5-HT in the development of *Drosophila* and changes in central nervous system physiological response due to pharmacological manipulations (by p-CPA or MDMA) during the development.
- 2) Determine effects of MDMA on development and physiological response of central nervous system.
- 3) Address effects caused by the lack of the major receptor (5-HT_{2dro}) on development and responsiveness of the larval CNS to exogenous 5-HT, MDMA application.

Addressing these aims are very important in understanding processes in neuronal develop in relation to whole animal behavior and the impact of

neuromodulators. 5-HT as described earlier is an important molecule in the development of brain and whole animal. To dissect out the role 5-HT plays in mammals is difficult due to the complexity. Hence using a simpler organism, *Drosophila melanogaster*, is advantageous and the results obtained here can be extrapolated to higher organisms. I have shown that 5-HT and its receptor plays a vital role in the development of *Drosophila* and its physiology.

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CHAPTER 2

MODULATION OF SENSORY-CNS-MOTOR CIRCUITS BY SEROTONIN, OCTOPAMINE, AND DOPAMINE IN SEMI-INTACT DROSOPHILA LARVA

ABSTRACT

I have introduced an in-situ preparation to induce motor unit activity by stimulating a sensory-CNS circuit, using the 3rd instar larvae of *Drosophila melanogaster*. Discrete identifiable motor units that are well defined in anatomic and physiologic function can be recruited selectively and driven depending on the sensory stimulus intensity, duration, and frequency. Since the peripheral nervous system is bilaterally symmetric to coordinate bilateral symmetric segmental musculature patterns, fictive forms of locomotion is able to be induced. Monitoring the excitatory postsynaptic potentials on the prominent ventral longitudinal body wall muscles, such as m6 and m12, provides additional insight into how the selective motor units might be recruited within intact animals. We also introduce the actions of the neuromodulators (serotonin, octopamine and dopamine) on the inducible patterns of activity within the sensory-motor circuit. The powerful genetic manipulation in *Drosophila* opens many avenues for further investigations into the circuitry and cellular aspects of pattern generation and developmental issues of circuitry formation and maintenance in the model organism.

INTRODUCTION

Sensory input early in life sculpts central circuits, which can become relatively hard wired after defined critical periods. This was most elegantly shown in the 1960's experimentally for the visual system in cats and monkeys (Hubel and Wiesel, 1963a, b, 1968, 1970) and is clinically relevant to humans. Other parts of the brain also show similar dependences on sensory activity in development. The formation of cortical circuits is of interest since this controls thought processes and forms of learning (Pallas, 2001). Refined experimentation of sensory attributes defining CNS and motor units have been possible in relatively less complex organisms. A striking example is in the development of the asymmetric claws of lobsters (Lang et al., 1978) where Govind and colleagues demonstrated that juvenile lobsters depend on sensory stimulation for the asymmetry to occur (Govind and Pearce, 1986). When lobsters (*Homarus americanus*) are not allowed to manipulate objects in their claws they will develop two cutter claws, where as if one claw is exercised a crusher claw will develop over subsequent molts for the side that had prior enhanced sensory stimulation. Not only is the muscle phenotype, biochemistry, and cuticle differentiated but the number of sensory neurons and the central neuropile in the thoracic ganglion are modified during development of the asymmetry (Cooper and Govind, 1991; Govind and Pearce, 1985; Govind et al., 1988).

In the genetically favorable invertebrate *Drosophila*, Suster and Bate (2002) produced embryos with reduced sensory function, which results in abnormal peristalsis of embryonic movements, which suggests sensory activity is developmentally important in shaping central control of motor output within

invertebrates. However, the problem still challenging the field is in understanding the integration of sensory input that controls muscular movements in a coordinated fashion. Recent studies in pharmacological treatments of spinal cord injuries in cats and in humans have revealed that recovery of locomotion is enhanced by using selective agonists and antagonist of neurotransmitters involved in sensory-CNS-motor circuits (Chau et al., 2002; Rossignol, 2000; Rossignol et al., 2001, 2002). These recent studies are a breakthrough in manipulating selective sensory systems and higher order function in controlling motor output.

The ability to combine a genetically favorable system and pharmacological studies is opening new horizons in regulation of development in neural circuits. In addition, neuromodulators provide a rapid way in which animals can tune up or down activity within a neural circuit and may be responsible for rapid changes in behavior, as recently examined for aggressive behavior in *Drosophila* (Baier et al., 2002). We assessed three common neuromodulators of interest in arthropod neurobiology: serotonin (5-HT), octopamine (OA), and dopamine (DA). Voltage dependent potassium channels and heart rate are modulated by 5-HT in *Drosophila* (Johnson et al., 1997; Zornik, 1999). DA is known to alter sexual behavior, habituation (Neckameyer, 1998a, b) and increase activity in adult flies (Friggi-Grelin et al., 2003) but depress synaptic transmission at the NMJ in larval *Drosophila* (Cooper and Neckameyer, 1999). Behaviors in bees are also affected by DA (Taylor et al., 1992). OA expression is related to stress responses in *Drosophila* (Hirashima et al., 2000) and OA receptors are present in mushroom bodies in *Drosophila* CNS (Han et al., 1998). These past studies indicate that there is a precedence of 5-HT, DA, and OA

to have central effects in the *Drosophila* brain (Baier et al., 2002; Blenau and Baumann, 2001; Monastirioti, 1999). The purpose of these studies is present an in situ preparation of larval *Drosophila*, with intact sensory-CNS-motor circuits, to serve as a model system for investigating actions of neuromodulators on developing central circuits.

METHODS

Many of the procedures used here have been previously described in detail (Ball et al., 2003; Cooper and Neckameyer, 1999; Li and Cooper, 2001; Li et al., 2001, 2002). The staining of the nerve terminals with an antibody to HRP was described previously (Li et al., 2002). In brief, the following procedures and condition were used with the modifications emphasized.

Stock and Staging of Larvae

The common 'wild-type' laboratory strain of *Drosophila melanogaster*, Canton S, was used in these studies. The methods used to stage fly larvae have been described previously (Campos-Ortega and Hartenstein, 1985; Li et al., 2002). Larvae at the beginning of the "wandering" phase of the third instar were used in these experiments.

Dissection and physiological conditions

Dissections included removal of the heart and viscera which left a filleted larvae containing only a body wall, body wall muscles and the neural circuitry for the sensory, CNS and body wall (i.e., skeletal) motor units as described earlier (Cooper

et al., 1995). The HL3 saline was prepared in the lab from component reagents (Sigma) and contained: 1.0 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 70mM NaCl, 5mM KCl, 10mM NaHCO_3 , 5mM trehalose, 115mM sucrose, and 5mM BES (N,N-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid) (Stewart et al., 1994).

Electrophysiology

The recording arrangement was essentially the same as previously described (Neckameyer and Cooper, 1998; Stewart et al., 1994). Intracellular recordings in muscles were made with 30-60M Ω resistance, 3M KCl-filled microelectrodes. The amplitudes of the excitatory postsynaptic potentials (EPSP) elicited by Is and Ib motor nerve terminals in the various segments of muscles m6 and m12 were monitored. Intracellular responses were recorded with a 1 X LU head stage and an Axoclamp 2A amplifier. Stimulation of segmental nerve roots was provided by suction electrodes (Cooper and Neckameyer, 1999). The stimulator (S-88, Grass) output was passed through a stimulus isolation unit in order to alter polarity and gain (SIU5, Grass). Electrical signals were recorded on-line to a PowerMac 9500 and G4 Mac via a MacLab/4s interface. All events were measured and calibrated with the MacLab Scope software 3.5.4 version. All experiments were performed at room temperature (19-22°C).

RESULTS

In filleted 3rd instar larvae, each segmental nerve root and ventral body wall musculature is readily observed (Fig. 2.1A). Various identified muscles with a rather simplistic innervation profiles can be used to monitor motor neuron activity (Fig.

2.1B). In these studies, we utilized muscle 6 (m6) and muscle 12 (m12) because of the well characterized innervation and synaptic properties of the ls and lb motor nerve terminals (Fig. 2.1C) (Atwood et al., 1993; Kurdyak et al., 1994; Li et al., 2002). Each segmental nerve root can be stimulated to drive sensory input into the larval brain as well as stimulating motor neurons to the segmental muscles that particular root is associated. By transecting the root and only stimulating the distal aspect of the root, the motor neurons are devoid of CNS activity and defined patterns of stimulation can be given. Likewise, the proximal root can either be left intact or transected to drive sensory patterns to the CNS for a particular segment or segments when multiple roots are utilized. Here we used single intact segmental roots to drive central circuits and record motor unit activity in contra-lateral and ipsi-lateral segments to the segment being stimulated (Fig. 2.1B).

Since the innervation to m6 and m12 is well defined, one can assess which specific motor neurons are being recruited as a result of sensory stimulation by monitoring the EPSPs induced in these particular muscles. The responses that can be evoked in m6 in the various segments when stimulating the 3rd segmental nerve on the right side is shown in Figure 2.2A. When monitoring two muscles simultaneously, selective motor neurons that are recruited which innervate both m6 and m12 or motor units which exclude m12 are able to be observed (Fig. 2.2B). In addition, since the lb and ls motor nerve terminals that innervate m6 show different morphology and physiological responses they can be discerned individually or when they are recruited in unison. The terminals of the ls axon contain small varicosities along its length and give rise to large EPSPs in the muscle, where as the lb axon

has big varicosities on its terminals (Fig. 2.1C and 2.2C), but produces smaller EPSPs (Atwood et al., 1993; Kurdyak et al., 1994; Stewart et al., 1994). The induced depolarizations on these muscles are graded and are non-spiking.

To examine if recruitment of sensory axons, interneurons and motor neurons is dependent on stimulation, three stimulation conditions were used. First, we examined the response of the motor units to stimulus duration. An increase in the duration of a train of stimuli enhanced activity of motor units (Fig. 2.3A, 40Hz with 10 stimuli; B, 40Hz with 15 stimuli). In addition, increasing the frequency of stimulation recruited motor units rapidly as compared to lower stimulus frequencies (Fig. 2.3A, 40Hz with 10 stimuli; C, 60 Hz with 10 stimuli). The amount of motor activity is also dependent on the intensity of stimulation (Fig. 2.3D, 40Hz with 10 stimuli low stimulus voltage). In 5 out of 5 preparations, the higher the stimulation frequency (40 Hz to 60 Hz), the longer duration of the stimulation (10 pulses to 20 pulses at 40 Hz), and the higher the stimulation intensity (increased by 1 V to the stimulating electrode) all resulted in an increase in the average activity of the motor neuron. The percent change from 40 Hz with a 10 pulse train is used for comparison (Fig. 2.3E). For this analysis, five periods of 500 msec duration, every ten seconds, were obtained and an average number of EPSPs was determined. Increasing the stimulation duration had the greatest effect in enhancing motor unit activity. It should be noted if the stimulation intensity is too large a failure to evoke action potentials could occur. Thus, some sensory neurons may drop out of as stimulation increases to very large voltages.

To determine the effects of 5-HT, OA, and DA in altering the sensory to motor neuron central circuit a segmental root was stimulated while the evoked responses in the contra-lateral m6 were monitored prior and during exposure to neuromodulators. The neuromodulators were applied by rapidly exchanging the entire bathing media with a saline containing the desired concentration. A single preparation was used for a given manipulation. Since the degree of recruiting motor neurons varied in each preparation a percent difference in the firing frequency of the motor units was quantified (Fig. 2.4A). OA at 10 μ M resulted in massive waves of muscle contraction making it difficult to maintain an intracellular recording (n=6). Thus, a lower concentration of 1 μ M was used for OA as compared to 5-HT and DA. In all cases, OA enhanced the firing frequency of the motor units. 5-HT (10 μ M) showed biphasic effects in altering the frequency of evoked motor unit response. Initially an enhancement in the frequency was observed but within 1 to 2 minutes a decrease in the frequency of the evoked responses occurred. The frequency in the evoked responses was measured for the peak excitatory effect within the first minute and the frequency after 2 minutes. The results are shown for 5 preparations (Fig. 2.4A). Only a small excitatory effect was observed for DA (10 μ M), however like for 5-HT, a transitory effect was observed (See enlarged inset). Five preparations were used for each compound and in each case the direction of change was the same (p<0.05, n=5, non-parametric rank sum Wilcoxon test). To illustrate the biphasic response induced by 5-HT the first stimulus train and resulting EPSPs after exposure is shown (Fig. 2.4B₁) along with a stimulus train 1 minute and 26 seconds later (Fig. 2.4B₂).

In examining the direct effects of the neuromodulators at the NMJ, a transected segmental nerve was stimulated distally to evoke a combined response from the Ib and Is terminals on m6. A percent change in the amplitude of the composite EPSPs revealed that both OA and DA reduced the amplitude ($p < 0.05$, non-parametric rank sum Wilcoxon test) whereas 5-HT had no significant effect on the response (Fig. 2.4C).

DISCUSSION

The topic of rhythmic control of locomotion is an age old question since Sherrington's time (Sherrington, 1898). Significant breakthroughs have occurred over the years, however the regulation and neural integration of locomotion remains a significant hurdle for the field. In this report, I demonstrate that the model organism, *Drosophila melanogaster* offers a unique advantage to begin to address pattern generation involved in locomotion as well as the role various sensory inputs have that drive circuits. In addition, the role of neuromodulators which is now proving to be advantageous to alter locomotive patterns in spinal injury models in mammals can also be assessed in larval *Drosophila*. The powerful genetic manipulation of the organism opens many avenues for further investigations into the circuitry and cellular aspects of sensory integration. The goal of this technical report is to present one with a preparation to address physiological effects in development and maintenance of central circuitry that could possibly be correlated with behavior.

The projections of sensory neurons in the larva can influence later development of novel sensory neurons in the adult, thus pharmacological

manipulation or altered activity profiles in the larva can be examined in shaping the adult CNS of holometabolus insects. Targeting particularly gene mutations in *Drosophila* towards specific sensory neurons or even all sensory neuronal function by inducible tetanus toxin light chain expression (Suster and Bate, 2002) within neurons will allow refined and gross manipulations of the circuitry for assessment of function and adaptation. As with *C. elegans* (Francis et al., 2003), genetic alterations in the expression of proteins involved in synaptic transmission result in behavioral patterns that can be quantified in larval and adult *Drosophila* (Neckameyer and Cooper, 1998; Li et al., 2001).

In our initial investigations, we were interested in monitoring fictive locomotion from recordings of the segmental nerves in filleted and pinned larvae (Fig. 2.1). Rhythmic patterns do appear, but the patterns are not reliable between preparations. In addition, when bursts of activity are recorded, the frequency profiles run down rather quickly making it difficult for long term assessment of fictive locomotion patterns. Hence, we turned to an alternative approach of driving the motor units by sensory nerve stimulation and then assessing the role of neuromodulators on the circuit. A similar approach has been used in the semi-intact leech preparation where electrical stimulation of sensory roots produces a escape swim circuit (Weeks, 1981). The fictive swimming can also be induced by exposure of the ventral nerve cord to 5-HT (Willard, 1981). Like wise, locomotor activity in the isolated spinal cord of the lamprey can be induced by bath application of NMDA (Svensson et al., 2003). The stomatogastric ganglion (STG) of crustaceans also serves as a nice invertebrate model for investigating actions of neuromodulators on motor patterns. It

has been shown in the STG that neural circuits and the networks are modulated by biogenic amines and there is both convergence and divergence in their action (Marder and Thirumalai, 2002).

Our particular interests focus on the influences of hormones and neuromodulators in altering central circuitry, particularly the ones already known to have a role in altering synaptic growth and plasticity at the neuromuscular junction (Cooper and Neckameyer, 1999; Li and Cooper, 2001; Li et al., 2001; Neckameyer and Cooper, 1998; Ruffner et al., 1999). It is well established that hormones such as ecdysone and juvenile hormone alter neural development and differentiation in insects (Garen et al., 1977; Pak and Gilbert, 1987; Truman, 1996). The surge of ecdysone in the pupal stage of *Drosophila* likely plays a key role in inducing gross alterations in neural circuitry (Kraft et al., 1998; Thummel, 1996; Truman and Reiss, 1988) and motor unit function (Li and Cooper 2001; Li et al., 2001). Likewise, other hormones or cocktails of other hormones need to be investigated for their developmental roles, since it has been demonstrated that the sequence of neuromodulator exposure and cocktails produce differential effects on synaptic modulation in other arthropods (i.e., the crustaceans) (Djokaj et al., 2001).

Since in the intact organism, compensatory mechanisms may override experimentally induced genetic, hormonal or environmental alterations, one can now turn to whole CNS and body musculature culture of larval *Drosophila* to address specific questions (Ball et al., 2003). However many compounding variables need to be considered, such as the loss of normal movements and appropriate feedback responses in culture conditions. The physiological saline based on the composition

of larval hemolymph, HL3, preserves synaptic transmission as well as muscular function and integrity (Stewart et al., 1994). Slight modifications of the HL3 saline are used for culturing the preparation (Ball et al., 2003), but perhaps the recently developed HL6 saline (Macleod et al., 2002) should be examined. With the physiological method presented, genetic or pharmacological manipulation of neuromodulators over a long-term, in the whole animal or in culture, can be readily assessed. However, the challenge is now to determine where the neuromodulators are acting (i.e., sensory, interneurons, and/or motor neurons) and what receptor subtypes exists.

FIGURES

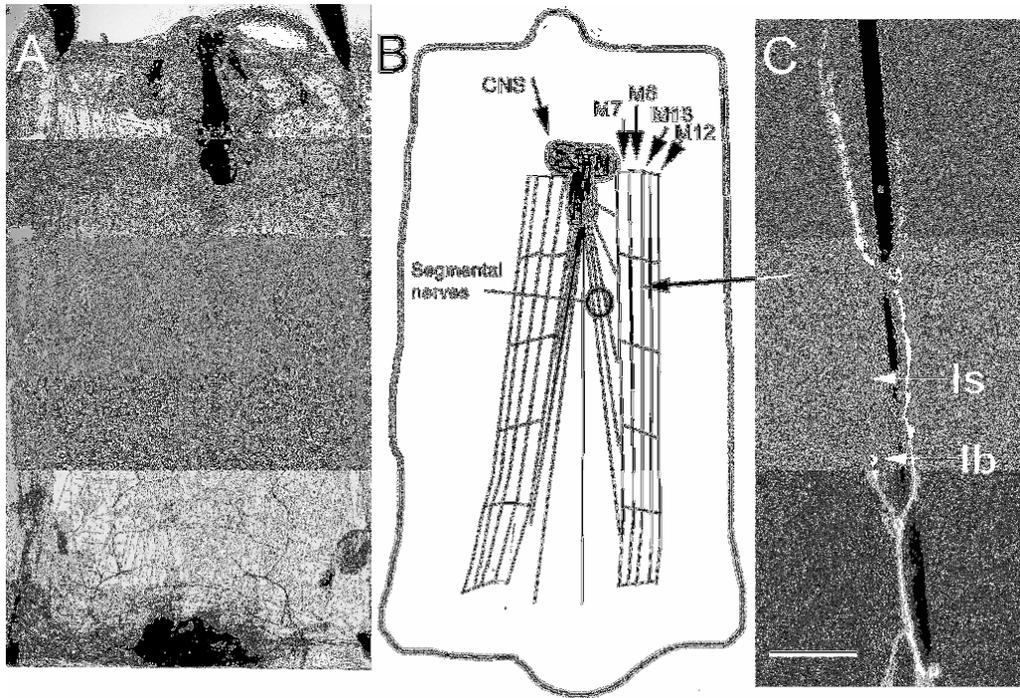


Figure 2.1: Schematic diagram of the *Drosophila* larva preparation:

(A) The preparation is pinned at the four corners to keep the preparation taut. The ventral abdominal muscles, m6 and m12, were used in this study. (B) The segmental nerves can be stimulated by placing the nerve into the lumen of a suction electrode and recruiting various subsets of sensory neurons. The segmental roots can be severed from the body wall to selectively stimulate sensory nerves orthodromically. (C) The terminals of Ib and Is on m6 and m7 are readily observed after treatment with fluorescently tagged anti-HRP antibody. Scale: 750 μm A & B, 90 μm C.

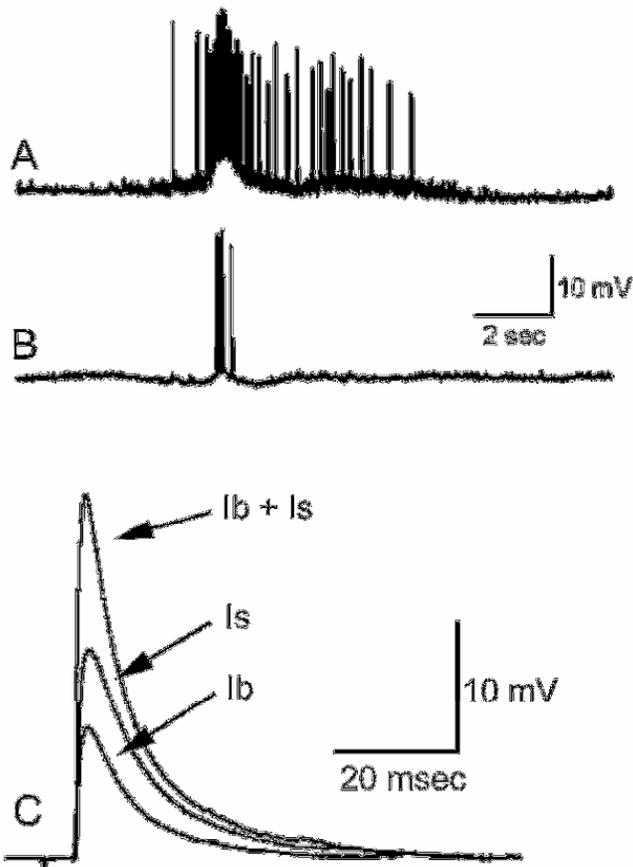


Figure 2.2: Representative traces of induced responses recorded in muscle 6 in various segments.

The 3rd segmental nerve on the right side of the larva was stimulated at a given voltage and frequency while responses were monitored in the m6 (A) and m12 (B) muscles on the contra-lateral side to the stimulated nerve root. In segment 3, contra-lateral to the segmental root being stimulated, EPSP responses in two different muscles m6 and m12 reveal that selective motor neurons can be recruited. The motor neuron RP3 innervates both m6 and m12 while the motor neuron 6/7b innervates m6 but not m12. Sometimes the lb is selectively recruited since only a response in m6 is observed. (C) Elicited responses in m6 is readily possible with a

intracellular recording as a consequence of stimulating the transected segmental root. Representative individual responses from the Ib and Is motor axons as well as the composite Ib and Is response are shown from late 3rd instars.

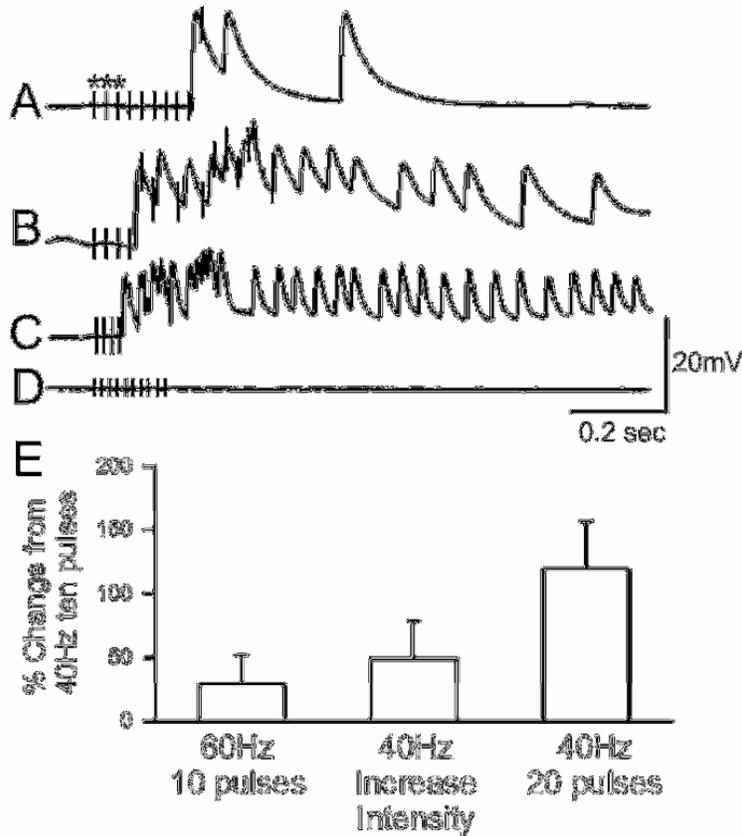


Figure 2.3: Recruitment of motor units. Recruitment of motor units is dependent on the duration of the stimulation.

(A, 40Hz, 10 pulses; B, 40Hz, 15 pulses), frequency of stimulation (A, 40Hz, 10 pulses; C, 60Hz, 10 pulses), and intensity (C, 60Hz, 10 pulses high stimulus voltage; D, 60Hz, 10 pulses low stimulus voltage). Represented are EPSPs recorded in m6 induced by stimulating the contra-lateral segmental root. At subthreshold (D) stimulation of sensory afferents no inducible responses are observed. However, recruitment occurs with an increased stimulation intensity (C). Stars in top trace indicate stimulus artifacts for the first three within the stimulus train. (E) An average percent change from 40 Hz with a 10 pulse train is used for comparison to a higher the stimulation frequency (60Hz), a higher the stimulation intensity (increased by 1 V

to the stimulating electrode), and a longer duration of the stimulation (20 pulses at 40 Hz) (at least $n=5$ for each condition).

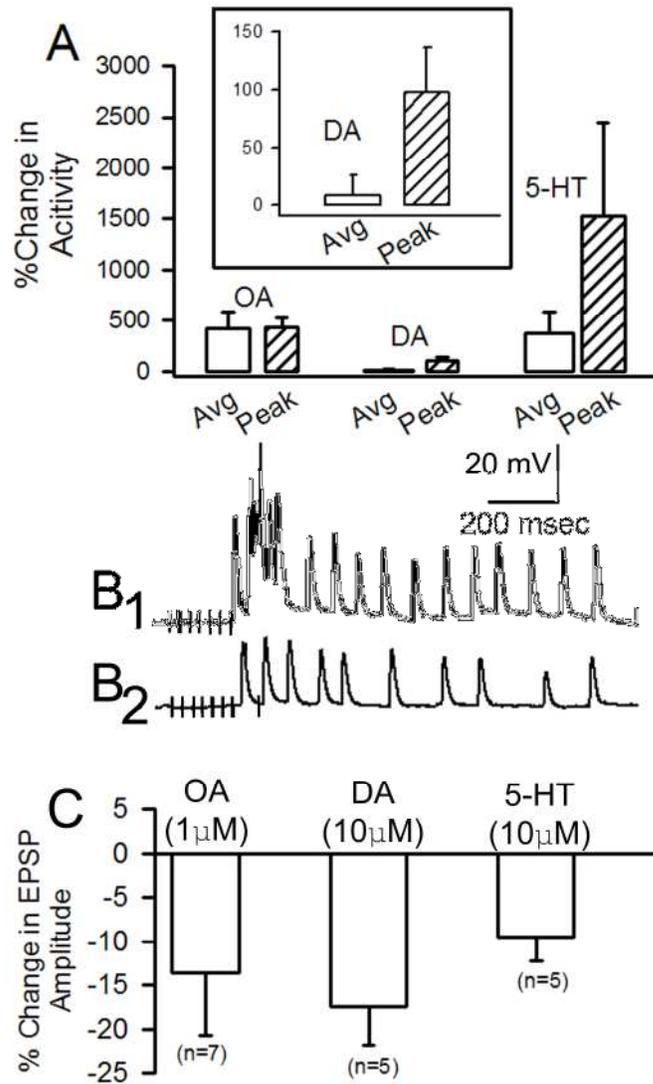


Figure 2.4: The influence of neuromodulators in altering the sensory to motor neuron central circuit was examined.

(A) A percent difference in the firing frequency of the motor units to m6 was determined before and during exposure to either serotonin (5-HT, 10 μ M), octopamine (OA, 1 μ M), or dopamine (DA, 10 μ M). Five independent preparations were examined for each neuromodulator. Since biphasic responses were observed for 5-HT, a peak enhancement in the firing frequency was measured within 1 minute.

The peak response and an average response for 2 minutes were used for analysis. The inset shows an enlarged view of the bar chart for the DA responses. A typical biphasic response induced by 5-HT is depicted by comparing B₁ (upon initial exposure) to B₂ (1 minute and 26 seconds later). (C) Direct assessment of OA (1μM), DA (10μM), and 5-HT (10μM) on the amplitude of evoked combined I_s and I_b EPSPs at the neuromuscular junction on m6 revealed that both OA and DA depressed synaptic transmission.

CHAPTER 3

INFLUENCE OF P-CPA AND MDMA ON THE SEROTONERGIC SYSTEM IN RELATION TO PHYSIOLOGY, DEVELOPMENT AND BEHAVIOR OF DROSOPHILA MELANOGASTER

ABSTRACT

Biogenic amines like serotonin (5-HT) are known to have a role in development and behavior. In this study the serotonergic system was altered using para-chlorophenylalanine (p-CPA) in order to study its role on development, behavior and physiology in larval *Drosophila*. Since MDMA is known to deplete 5-HT in neurons in mammals parallel studies to p-CPA were conducted. p-CPA and MDMA delayed time to pupation and eclosion. Locomotion and eating were reduced in animals exposed to these compounds. Sensitivity to exogenously applied 5-HT on a evoked sensory-CNS-motor circuit showed that the CNS is sensitive to 5-HT but that when depleted of 5-HT by p-CPA no enhanced sensitivity was observed. Larvae eating MDMA from 1st to 3rd instar did not show a reduction in 5-HT within the CNS; however, eating p-CPA reduced not only 5-HT but also dopamine content. Since the heart serves as a good bioindex to 5-HT exposure, it was used in larva fed p-CPA and MDMA, but no significant effects were noted to exogenously applied 5-HT in these pharmacologically treated larvae.

INTRODUCTION

Serotonin (5-HT), dopamine (DA) and octopamine (OA) are well known to act as neuromodulators in insects, particularly in *Drosophila melanogaster*, which when altered can produce behavioral and developmental defects as well as organizational

problems in the CNS circuits (Monastirioti, 1999; Osborne, 1996). 5-HT modulates voltage dependent potassium channels and heart rate in *Drosophila* (Johnson et al., 1997; Zornik et al., 1999). DA is known to alter sexual behavior, sensory habituation (Neckameyer, 1998a,b) and increase activity in adult flies (Friggi-Grelin et al., 2003) but depress synaptic transmission at the NMJ in larval *Drosophila* (Cooper and Neckameyer, 1999). OA expression is stress related in *Drosophila* (Hirashima et al., 2000) and OA receptors are present in mushroom bodies in *Drosophila* CNS which is a region important for learning in adults (Han et al., 1998). In fact, 5-HT, DA, and OA all have some central effects in the adult *Drosophila* brain related with learning or behavior (Blenau and Baumann, 2001; Monastirioti 1999). Recently, direct actions of these neuromodulators were shown to alter central neural activity (Dasari and Cooper, 2004).

These biogenic amines have broad differential effects on development and physiology in larvae as well as in adults. Here I focus on the serotonergic system and tissue sensitive to alterations in endogenous levels of 5-HT within larvae and pupa. The development and the distribution of 5-HT immunoreactivity neurons in the CNS are established (Valles and White, 1988). 5-HT has a role in many physiological process such as regulating locomotion and cardiac output (Dasari and Cooper, 2006; Kamyshev et al., 1983; Johnson et al., 1997; Nichols et al., 1999; Zornik et al., 1999; Johnson et al., 2000). Since 5-HT alters the activity of sensory-to-motor central circuits in larval *Drosophila* (Dasari and Cooper 2004) this opens the possibility that the serotonergic system could sculpt the formation of neural circuits by altering the neural activity in the developing CNS of *Drosophila*. Activity of

developing neural circuits is well established to play a major role in the patterning of the adult CNS in mammals prior to critical periods (Hubel and Wiesel, 1963a,b, 1968, 1970).

MDMA (ecstasy), a drug of abuse, modulates the homeostasis of the serotonergic system in humans and animal models (Green et al., 2003). Research is scant on the effects of MDMA in the developing CNS of mammals as well as in insects. Thus, I used the rapidly developing nervous systems of *Drosophila* larvae to provide an avenue to quickly screen the effects of MDMA on the larval CNS. The proposed mechanism of MDMA's action in mammals is an eventual depletion of 5-HT within neurons. I tested other means, through pharmacological manipulation, throughout early stages of larval development to deplete 5-HT for comparison with the effects induced by MDMA.

METHODS

Stock and Staging of Larvae

The common 'wild-type' laboratory strain of *Drosophila melanogaster*, Canton S, was used in these studies. The methods used to stage fly larvae have been described previously (Campos-Ortega and Hartenstein, 1985; Li and Cooper, 2002). All animals were maintained in vials partially filled with a cornmeal-agar-dextrose-yeast medium. All animals were kept on a 12:12 light-dark cycle. Introduction of pharmacological agents started with the 1st instar.

Behavioral assays

Early 3rd instar larvae were used for behavioral assays. Feeding and locomotory behavior was assessed as described in Neckameyer (1996) and Li et al. (2001). In brief, single animals were placed on a 2% agar surface and the number of body wall contractions was counted for 1 minute, after which an animal was placed in a 2% yeast solution overlaid on an agar plate (just covering the larvae with allowing the spiracles to reach out of the solution). In this condition, *Drosophila* larvae immediately feed, initiating a pattern of repetitive mouth hook movements. The number of full mouth hook contractions in 1 minute was counted (Sewell et al., 1975). The results of these behaviors are plotted as body wall contractions or mouth hook movements per minute.

Dissection and electrophysiological recordings

Wandering 3rd instar larvae were dissected as described earlier (Cooper et al., 1995). In brief larvae were dissected dorsally removing heart and viscera which left a filleted larvae containing only the body wall, body wall muscles and the neural circuitry for the sensory, CNS and body wall (i.e., skeletal) motor units. HL3 saline was prepared in the lab from component reagents (Sigma) and contained: 1.0 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 70 mM NaCl, 20 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5 mM KCl, 10 mM NaHCO_3 , 5 mM trehalose, 115 mM sucrose, and 5 mM BES (*N,N*-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid) (Stewart et al., 1994).

The recording arrangement was essentially the same as previously described (Neckameyer and Cooper, 1998 and Stewart et al., 1994). Intracellular recordings in muscles were made with (30–60 M Ω resistance), 3 M KCl-filled microelectrodes. The amplitudes of the excitatory postsynaptic potentials (EPSP) elicited by Is and Ib

motor nerve terminals of muscle m6 were monitored. Primarily body segments 3 and 4 were used throughout these studies. Intracellular responses were recorded with a 1xLU head stage and an Axoclamp 2A amplifier. To evoke a sensory-CNS-motor circuit the tail segmental nerves were cut and stimulated using the suction electrode while using an intracellular electrode in a m6 muscle fiber (Dasari and Cooper 2004). The stimulator (S-88, Grass) output was passed through a stimulus isolation unit in order to alter polarity and gain (SIU5, Grass). Electrical signals were recorded on-line via an A/D converter (powerlab 4s interface; ADInstruments). All events were measured and calibrated with Scope software 3.5.4 version (ADInstruments). All experiments were performed at room temperature (19–21 °C).

Developmental Assays

Eggs were collected after a 15-minute prepulse for 2 hrs and allowed to develop at 21°C. Eggs were transferred to vials (15/vial) containing food with p-CPA or MDMA so that 1st instars are in the food right after hatching. The food was made with 0.5 g of corn meal with 0.5 ml of water mixed with the appropriate concentration of drug. I used corn meal instead of yeast-water for the food since we found a high rate of death with the yeast paste. The deaths were probably due to CO₂ build up with the growth of the yeast. When wandering 3rds were seen, the vials were checked every 4 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 development.

Heart Rate

Heart rate in 3rd instar was examined and monitored in the same manner as detailed in Dasari and Cooper (2006).

Levels of Serotonin and Dopamine

The 1st instars were fed with pharmacological agents as described above. The wandering 3rd instars were used for selective measures on the CNS. Larvae were dissected in HL3 as described above. Brains were placed in dry ice immediately after dissecting. 25 brains were pooled for each sample set and stored in -80°C prior to HPLC analysis. HPLC analysis was performed at The Center for Sensor Technology (University of KY, USA).

The low level detections of 3-Hydroxytyramine (Dopamine; DA) and 5-Hydroxytryptamine (Serotonin; 5-HT) are performed as described earlier by Hall *et al* (1989). In brief an isocratic HPLC system (Beckman, Inc., Fullerton, CA), at a flow rate of 2 ml/min., which is coupled to a dual-channel electrochemical array detector (model 5100A, ESA, Inc., Chelmsford, MA), $E_1 = +0.35$ mV and $E_2 = -0.25$ mV, using an ESA model 5011 dual analytical cell. The compounds of interest are separated with reverse-phase chromatography, using a C18 column (4.6 mm x 75 mm, 3 μm particle size, Shiseido CapCell Pak UG120, Shiseido Co., LTD., Tokyo, Japan) with a pH 4.1 citrate-acetate mobile phase, containing 4.0% methanol and 0.34 mM 1-octane-sulfonic acid.

Statistical Analysis

When the basic assumption of parametric Student's-*t* test was valid it was used; otherwise, the non-parametric Wilcoxon rank sum test was used.

RESULTS

Developmental curves

The first set of general analysis was to examine the time it took 50% of the population to pupate. The time for 50% of the population to reach eclosion from pupation was also assessed. p-CPA or MDMA at various concentration was fed to larvae throughout 1st to 3rd instar stages. The time taken by each larva from egg (Time=0) to pupation was recorded. The highest concentration of p-CPA (50mM; 10mg/ml) showed a drastic delay in the development such that 50% of larvae took a mean time of ~225 hrs for pupation, whereas 50% of controls pupated at ~125 hrs (Fig. 3.1A, B). There was a high death rate in this group (64% \pm 5.8). There were a small number of deaths in controls (2% \pm 2) and at lower concentrations of p-CPA treated larvae. Larvae fed p-CPA at 50mM were generally smaller in size on average as compared to controls (this was based on casually observation and not statistically analyzed). p-CPA at 0.5 mM showed no delay from controls in the time for 50% of animals to pupate and 5 mM showed only a slight delay of 35 hrs from controls for pupation time (Fig. 3.1A, B). In calculating the amount of time taken for each pupa to eclose the time point at which pupa a formed was readjusted to a zero time point (Time=0). The time taken for p-CPA (50mM) fed larvae to eclose is longer than for controls (>35 hrs, Fig. 3.1C, D). They seem to catch up in eclosion time even though

there is delay in pupation time. A similar result was also seen with the other concentrations of p-CPA (Fig. 3.1 C, D).

Similar results to p-CPA were seen with MDMA fed larvae. MDMA at highest concentration (1mM) showed a delay in time to pupation by approximately 36 hrs for 50% of larvae to pupate (Fig. 3.2A, B). A 40% (\pm 7) death rate was seen in this group. The lower concentration 10 μ M MDMA fed larvae took less time (10 hrs) for 50% animals to pupate compared to controls and 100 μ M MDMA took ~10 hrs more than controls for 50% of the animals to pupate (Fig. 3.2A, B). Eclosion time for 1mM fed larvae is about 20 hrs more than the controls, whereas the other 2 concentrations did not show any significant difference from controls (Fig. 3.2C, D).

Behavior (mouth hook and body wall contractions)

Simple feeding (mouth hook movements) and a locomotor (body wall movements) behavior were tested for p-CPA (50mM) and MDMA (10uM) fed larvae. Larvae ate these compounds from 1st to 3rd instar stage and at the mid 3rd instar stage they were used for behavioral analysis. Each larva was first assayed for body wall contractions and then followed for quantifying mouth hook contractions. Compared to controls p-CPA and MDMA fed larvae showed significant lower body wall and mouth hook contractions (N=20, Fig. 3.3, ANOVA, $P < 0.0001$). Some of p-CPA larvae were seen to be crawling with difficulty and some just contracted their body once or twice.

Spontaneous activity (5-HT and MDMA)

The intrinsic activity of the CNS was assessed by monitoring motor commands to muscles m6 or m7. Both of these muscles receive the same innervation by two motor axons (Kurdyak et al., 1994). We noted substantial variation among preparations in the extent of the spontaneous activity and bursting frequency, but by pulling the edges of the cuticle taut in the experimental chamber the spontaneous activity can be insured to occur. Measures prior and during exposure to various compounds gives an approach to determine if the CNS circuit that initiates the motor commands is sensitive to the particular agent of interest. We utilized this experimental approach for examining the CNS sensitivity to 5-HT and MDMA at various doses. However, the approach has some compounding difficulties in that the preparation can show an enhanced response upon initial exposure but with rapid desensitizing after only a few minutes depending on the dose and compound. For example, application of MDMA resulted in a marked bursting behavior followed by short high frequency bursts (Fig. 3.4A for saline and 3.4B for MDMA). This was subsequently followed by decreased basal activity and inactivity to evoke a sensory-CNS-motor response by electrical stimulation of an afferent nerve root. The bursts over time became less frequent and shorter in duration (Fig. 3.4C).

In quantifying the bursting behavior I counted all the peaks within each burst and recorded the time of each burst. I then counted the frequency of burst per second and averaged all frequencies. A five second period was taken and a mean value per second was calculated. The mean frequency obtained before and after application of drug was used to calculate a percent difference (Fig. 3.5).

Spontaneous activity measured in saline controls showed a small increase of 20% in the mean (Fig. 3.5A) but this was not significant. In presence of 5-HT, control larvae showed a larger dose dependent increase in mean activity (Fig. 3.5A), whereas a lower concentration of MDMA, 100nM and 10 μ M, showed a smaller increase, however MDMA at 100 μ M showed a decrease in mean activity, but this was not significant (Fig. 3.5A). There was a substantial amount of variation from preparation to preparation.

Larvae fed 50mM p-CPA showed a slight increase in activity on application of 10nM and 100nM 5-HT whereas at 10 μ M a slight decrease in the mean response is seen but this was not significant (Fig. 3.5B). Probably at this high concentration of 5-HT the preparations are desensitized. MDMA (100 μ M) fed larvae showed an increase in neural activity at all concentrations of 5-HT, although it is a very small increase (Fig. 3.5C). Even in these preparations a lot of variation was observed. No statistical significance was detected among any of the groups examined with an ANOVA $P > 0.05$ as well also with a non-parametric rank-sum Wilcoxin test.

Sensory-CNS-motor circuit (5-HT and MDMA)

The sensitivity of central circuits to 5-HT and MDMA of various doses was examined by stimulating sensory nerves and monitoring motor units before and during exposure to the compounds. There is a clear dose-dependent effect to both 5-HT and MDMA, however in opposing actions, in altering the evoked CNS activity (Fig. 3.6A). Sham controls were performed since the disturbance of changing solutions could result in sensory activity and thus drive a motor response. In a few

preparations a slight increase occurred but the changes are non-significant for the group. The preparations were used once for each condition. The mean percent increase in response to 5-HT as well as the variability from 10 nM, 100 nM and 10 μ M (ANOVA $P < 0.0001$, $n = 7$). Tukey post hoc test showed only 10 μ M 5-HT exposure as significantly different from sham ($P < 0.05$). To our surprise, MDMA caused a decrease in evoked responsiveness which was dose-dependent except the variation among preparations was very consistent unlike that for 5-HT at higher doses (Fig. 3.6A). The MDMA effects are significantly different from shams. (ANOVA $P < 0.0001$).

p-CPA fed larvae when exposed to 5-HT showed a decreased response. The decrease in activity is dose dependent. 5-HT at 10 μ M showed an approximately 50% decrease compared to saline whereas 100nM showed a 30% decrease in activity (Fig. 3.6B). This was unexpected. Maybe preparations are desensitized very fast on application of 5-HT. On the other hand, MDMA fed larvae showed an increase in activity when exposed to 5-HT. Exposure to 10nM 5-HT showed a larger increase as compared to 100nM or 10 μ M 5-HT (Fig. 3.6B). But as compared to shams both p-CPA fed and MDMA fed larvae showed a significant difference (ANOVA $P < 0.0001$). Also MDMA fed larvae exposed to 10nM 5-HT showed a significant difference ($P < 0.05$; Tukey test).

Heart Rate

5-HT is known to have a role in cardiac function in *Drosophila* (Dasari and Cooper, 2006). So we examined if larvae altered their 5-HT sensitivity in cardiac

function to long-term depletion of 5-HT, the larvae were fed p-CPA from 1st to 3rd instar. In mid-3rd instar the larvae were dissected and heart rate monitored for alteration to exogenous application of 5-HT. Larval heart rate (HR) increases upon exposure to 5-HT (100nM); however, there is no significant increase above controls for larvae depleted of 5-HT (Fig. 3.7). Controls increased HR by 31% and p-CPA fed larvae by 28%. The significance to examine the HR is that it serves as another physiological measure, out side the CNS, for the sensitivity to 5-HT in these pharmacologically manipulated larva.

Levels of Serotonin and Dopamine

It is important to determine the amount of depletion of 5-HT in the CNS as well as whole body to correlate with the delayed development in p-CPA and MDMA treated animals. The brains from mid 3rd instar larvae were individually dissected out of the larvae for HPLC analysis. At least 5 samples with each sample containing 25 pooled brains were analyzed. The HPLC results showed that control brains (n=6) have about 12-14 pg/brain of 5-HT and DA (Fig. 3.8A). Treatment with p-CPA (50mM) caused a significant decrease in 5-HT levels in larval brains, approximately by 90% (n=5, ANOVA, P<0.05 Fig. 3.8A). Larvae that ate p-CPA also resulted in a significant decrease in DA levels (n=5, AVONA P<0.05, Fig. 3.8A). MDMA treatments did not produce a difference in the levels of DA or 5-HT (n=5, Fig. 3.8A). Treatment with 5,7-DHT, a compound that was perdicted to kill serotonergic neurons, showed a small decrease in the mean 5-HT levels but it was not significant (Fig. 3.8A).

Intact larvae were also collected from the same culture vials as above for each group and washed in water to remove food residues. 20 larvae were pooled together for each set and subjected to HPLC. Control larvae had approximately 500 pg/larva of DA and 300pg/larva of 5-HT. No significant differences in levels of 5-HT and DA occurred with either p-CPA or MDMA treated groups as compared to controls (Fig. 3.8B).

Larvae were fed different concentrations of p-CPA (0.5, 5, and 50 mM) and MDMA (10uM, 100uM and 1mM) and allowed to form pupa. Every 4 hrs vials were checked for newly eclosed adults. The adults were collected and frozen immediately. The heads were chopped off from these adult flies and 5 heads were pooled for each set. The analysis of HPLC data revealed no significant difference in DA or 5-HT levels in either of the groups as compared to controls except for the 1mM MDMA treated group (Fig. 3.8C, D). The larvae fed 1mM MDMA showed a significant increase above controls in levels of both 5-HT and DA (n=5, ANOVA, $P < 0.05$, Fig. 3.8C, D).

DISCUSSION

In this study I demonstrated that feeding larvae p-CPA or MDMA will retard development and decreases activity associated with crawling and eating behaviors. HPLC analysis of 5-HT and DA for the whole larvae did not show any effects of the drug treatments, where as selectively measuring the brains of larvae showed significant effects. The levels of p-CPA that slowed development also reduce the concentrations of 5-HT and DA in 3rd instar larval brains. This effect of p-CPA is dose-dependent. However, treatment

with MDMA did not show any alterations in the 5-HT and DA in the CNS of larvae but in newly eclosed adults, which ate MDMA as larvae, showed a dose-dependent increase in both 5-HT and DA. Initial exposure of the larval brain to MDMA caused an increase in spontaneous activity which was short lived (~3 minutes) and in fact decreased evoked sensory-CNS-motor activity afterwards. In contrast, 5-HT increased evoked activity significantly but not spontaneous activity in a dose-dependent nature. When the larvae were depleted of 5-HT throughout development they did not increase their sensitivity to the excitatory response of 5-HT but showed a more pronounced inhibitory action of 5-HT on the central circuit. Just the opposite effect occurred for larvae fed MDMA, in that treatment with MDMA throughout larval development resulted in the central circuit to be more responsive to 5-HT. The sensitivity of 5-HT on the larval heart was used as an additional measure of the effects of p-CPA treatment. There was no effect on sensitivity of heart to 5-HT in p-CPA fed larvae.

Since development in larvae fed p-CPA or MDMA showed a dose-dependent effect in slowing down the rate of development as well as increasing the mortality rate, it would appear the involvement of the CNS serotonergic system is vital to the health of larvae. Direct and indirect effects are both likely to contribute to the offset development. Since larvae fed p-CPA or MDMA showed reduced mouth hook movements one would logically ask, "Was a reduced diet a limiting factor?" We could assay the total amount of food within the digestive system of larvae with a dye tainted food technique but such approaches do not inform one if the nutrients are absorbed from the digestive system. Possibly animals absorbed even more nutrients

if retention within the digestive system was enhanced due to a decrease gut motility from treatments with p-CPA or MDMA. Whole animal HPLC assays did not show a significant decrease in 5-HT with p-CPA treatments and given that 5-HT can modulated gut motility in many invertebrates (Ayali and Harris-Warrick, 1999; Katz and Harris-Warrick, 1989) possible there was no effect on digestive processes. But just as likely there may well be neural regulation of gut motility in larval *Drosophila* which is consequentially altered by the decrease in 5-HT within the CNS. Autonomic neural function of digestive properties in insects (Copenhaver and Taghert, 1989, 1991; Penzlin, 1985; Zavarzin, 1941) and crustaceans (Shuranova et al., 2006) is established but how the autonomic digestive function maybe regulated by serotonergic circuits within the CNS has not been addressed.

The development of some circuits within the CNS of arthropods are known to be dependent on 5-HT. The olfactory neurons in *Manduca sexta* (moth) are 5-HT sensitive (Kloppenburg and Hildebrand, 1995) and the correct development of neuronal processes within the olfactory center is dependent on 5-HT (Hill et al., 2003). The central development of the olfaction circuits in crustaceans (Sandeman et al. 1995) and *C. elegans* (Nuttley et al., 2002) are even dependent on 5-HT. In the eyes of flies (Chen et al., 1999), as well as crustaceans (Aréchiga and Huberman, 1980), 5-HT alters the visual sensitivity. This in turn could readily have a substantial effect in development of the neural circuit as it is well established from invertebrates (Payne, 1911; Roach and Wiersma 1974; Scott et al., 2003; Cooper et al., 2001) to mammals (Hubel and Wiesel, 1970) that activity in visual system sculpts the peripheral and central circuits. Since neuroendocrine axis in invertebrates are

sensitive to 5-HT (Lee et al., 2000) this could alter development of the whole animal as well as specific neuronal processes and neurohormones with very broad actions (Nässel, 2002). Likewise, if the circadian patterns were altered due to decreased neuronal 5-HT or direct action of MDMA on 5-HT receptors then one would expect endocrine related developmental abnormalities.

Genes that regulate tryptophan hydroxylase for the biochemical synthesis of 5-HT are known in *Drosophila* and there are two different genes encoding for two different forms of the enzyme. One that is expressed in the periphery and one in the CNS (Coleman and Neckameyer, 2005). When there are defects in phenylalanine hydroxylase in mammals a disease state of phenylketonuria can occur (Lenke and Levy, 1980). Phenylalanine is precursor for tyrosine. In this pathological state it is known that tyrosine hydroxylase and TPH are inhibited which leads to reduced 5-HT and associated neuronal damage (Curtius et al., 1981; Roux et al., 1995) which impinged on developmental rates, thus a similar situation may occur in *Drosophila*. The associated higher death rate with larvae fed MDMA and p-CPA in high doses points to some significance in the serotonergic system for overall health since it is likely the primary site of drug action.

The slowed developmental time from egg to pupation and pupation to eclosion with p-CPA and MDMA treatments is surprising since MDMA did not result in a reduced 5-HT in the larval brain. Thus, the retardation in development cannot be solely due to reduced 5-HT, but may likely be due to altered central neural activity since exposure to p-CPA or MDMA changed spontaneous as well as evoked patterns of central circuits. An easy approach to index rate of development was a

time measure for 50% of the larva to pupate or pupa to eclose but this does not provide a full spectrum of the developmental dynamics, thus we took the laborious task of checking fly cultures each 4 hrs throughout the day and night for weeks to obtain the developmental curves. Likewise analysis is best approached to span the entire curve instead of a single time stamp.

The behavioral similarities, as with overall development, associated with both p-CPA and MDMA indicates that the overall health of the animal might be comprised by treatments in a dose-dependent manner. Despite 5-HT levels not declining with MDMA treatments possible just the dis-synchronization of appropriate neural activity did not allow the animal to perform coordinated motor commands. Where as the depletion of 5-HT in the CNS by p-CPA must have produced altered neural activity by a different means from that of MDMA. 5-HT is associated with modulation of eating/digestion in crustaceans (Shuranova et al., 2006) and humans (Aubert et al., 2000) as well as in motor unit coordination (LeBeau et al., 2005; Dasari and Cooper, 2004; Strawn et al., 2000; Weiger, 1997) and behavior (Bicker 1999; Toth et al., 2005; Barnes and Sharp, 1999) in a wide variety of animals. Likewise MDMA in humans promotes mastication and heightened activity for motor function. The few minutes of increased central motor commands, in the larvae to exposure of MDMA, prior to the decrease evoked responses is contrary to what was expected since we predicted that a further increase in synaptic 5-HT release would mimic the exogenous application of 5-HT. However the proposed synaptic model of MDMA action in mammals (Green et al., 2003; Simantov, 2004; Sprague and Nichols, 2005) may not hold for *Drosophila*. In addition, the surprising results of p-CPA reducing not

only 5-HT but also DA beacons careful assessment of extrapolating mechanisms of drug action noted in mammals to invertebrates. Such oversights have also been noted to occur for pharmacological serotonergic agents used in crustacean behavioral research (Sparks et al., 2003) which is due to various cellular cascades and receptor subtypes (Clark et al., 2004; Tierney, 2001).

5-HT and MDMA showed an increase in spontaneous activity. p-CPA and MDMA fed larvae also showed an increase in presence of 5-HT. An opposite effect was seen with spontaneous activity. Sensory-CNS-motor circuit is modulated by 5-HT (10 μ M) in a biphasic manner (Dasari and Cooper, 2004). Here I have shown that 5-HT alters evoked activity in a dose dependent manner. In *Tritonia*, increased levels of 5-HT enhances the ability of dorsal swim neurons to initiate rhythmic activity in swim motor neurons. (Fickbohm et al., 2000). To my surprise the firing frequency for motor neurons in p-CPA fed larvae reduced in presence exogenous 5-HT. Since 5-HT levels are reduced throughout development, the receptors are not expressed properly. But opposite effects are seen with MDMA. Application of MDMA decreased the firing frequency but MDMA fed larvae showed an increase in activity with 5-HT application. As mentioned earlier, MDMA may not be completely working through the 5-HT system. In humans one of the mechanisms by which MDMA works is by reversing 5-HT transporter. *Drosophila* 5-HT transporter (dSERT) is homologous to human and rat SERT (51%). Demchyshyn et al. (1994) showed that dSERT is not similar to hSERT pharmacologically. The affinity of dSERT is different for antidepressants like imipramine. MDMA may not be acting on dSERT in the same manner as for hSERT.

I was fortunate to have carried out the HPLC analysis, as I would not have realized the broad action of p-CPA in reducing dopamine in parallel with serotonin. It appears that p-CPA likely works on tryptophan hydroxylase (TPH) as well as the biochemical pathway for synthesis of DA in *Drosophila*. The likely enzyme targeted for by p-CPA in DA production is tryptophan-phenylalanine hydroxylase. This enzyme is also used in part for 5-HT production for *Drosophila* (Coleman and Neckameyer, 2005). The long-term treatment of larva that were followed throughout pupa and eclosion offered even more fodder for speculations since the actions were dose-dependent in increasing 5-HT as well as DA in adults when treated with MDMA. The p-CPA decreased both 5-HT and DA in new adults much the same as was observed for larva. The speculation at present is that MDMA promotes production of 5-HT and DA by either turning off inhibitory feedback balance or directly stimulating biosynthesis in the CNS. We also noted that levels of 5-HT or DA in the CNS do not parallel whole larval body analysis and p-CPA treatments had no discernable effect on the whole body 5-HT levels. Tissue specific analysis is required in this model organism for 5-HT and DA. It is possible that p-CPA may only work on the TPH enzyme produced centrally since it is coded by a different gene than for the one expressed peripherally (Coleman and Neckameyer, 2005). As in vertebrates there are differential expressed TPH genes centrally and peripherally which produce different enzyme isoforms (Walther et al., 2003). It is possible even if the gene sequence is the same they may undergo alternative splicing differentially making one form more sensitive to drug treatments. Enzyme splicing variants which have various drug affinities as well as various 5-HT receptor variants and affinities

are now commonly observed in multiple species (Kishore and Stamm, 2006; Krobert and Levy, 2002). With the dual effects of p-CPA on 5-HT and DA concentrations in the CNS, one now has to determine if the observed behavioral changes are related to alterations in 5-HT or DA as well as the delayed development. As in vertebrates, DA in *Drosophila* has an effect in behavior and locomotion (Kume et al., 2005; Cooper and Neckameyer, 1999; Neckameyer, 1996, 1998).

The larval heart did not show any altered responsiveness to exogenously applied 5-HT (10uM) in the animals fed p-CPA most likely since peripheral 5-HT levels were not altered. Since peripheral 5-HT levels were not altered as determined by HPLC we did not expect a change. The heart does serve as a independent assay for alteration in the CNS responses to 5-HT since the heart is also responsive to 5-HT (Dasari and Cooper, 2006); however, the aorta of the larval heart does appear to be innervated (Johnstone and Cooper, 2006) and neuromodulatory action on this innervation is not known. This is one reason in my studies that the CNS was removed when conducting the heart assay.

These studies have produced some unexpected findings with MDMA's actions, particularly that 5-HT was not depleted in the brains of larvae and raised in pupa/adults for larvae fed MDMA. Possible enzymatic assays would resolve if MDMA could stimulate synthesis. The pronounced inhibitory effects of 5-HT after p-CPA treatment also was surprising since acute application produced excitation of the central circuit. In addition, the reduction of dopamine by p-CPA treatment in larvae was serendipitously found which could be accounted for by p-CPA not only blocking TPH but also tryptophan phenylalanine hydroxylase and tryosine hydroxylase that is

used to produce dopamine. To resolve this issue enzymatic analysis is needed. The excitatory and depressing effects of 5-HT could be accounted for by alternative regulation of the four known 5-HT receptor subtypes or even alternative splicing of the D5-HT₂ subtype as is known to happen in mammals (Kishore and Stamm, 2006; Pauwels, 2000). Since out of the four (5-HT_{7Dro}, 5-HT_{1ADro}, 5-HT_{1BDro}, and 5-HT_{2Dro}) receptor subtypes, that are analogous to mammalian systems in classification, a possible up regulation of the 5-HT₁ subtypes could cause a depression of cellular excitability (Barnes and Sharp, 1999; Tierney, 2001; Nichols et al., 2002). Where in a neuronal circuit the locations of the particular receptors subtypes could result in excitation on one set that ultimately inhibits motor neurons through a GABA-ergic path. Obviously more work is needed to know receptor localizations, expression regulation and cellular responses to make sense of the full sensory-CNS-motor circuits described in this study as well as in studies on vertebrates neuronal circuits (McMahon et al., 2001; Sodhi and Sanders-Bush, 2004; Vitalis and Parnavelas, 2003). With more experimentation, one may understand the mechanistic cellular actions of various pharmacological agents and the potential role of endogenous neuromodulators on the serotonin-ergic circuits and developmental influences on the larval brain as well as the brain during the pupa to adult transformation.

FIGURES:

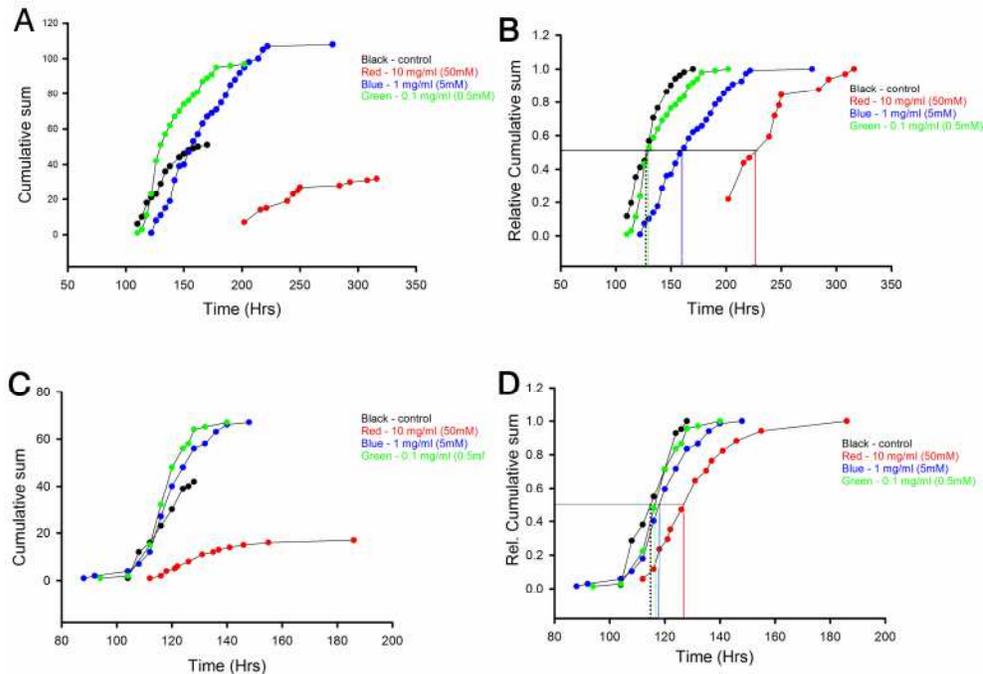


Figure 3.1: p-PCA growth curve:

p-CPA was fed from 1st instar to 3rd instar stage. The time to pupation from eggs and time to eclosion from pupation is calculated for each larva. A) Cumulative sum for time to pupation from eggs at different concentrations of p-CPA. B) Relative cumulative sum for A. Larva fed highest concentration of p-CPA (50mM) took the longest time to pupation. 50% of larva took 100 hrs longer to pupate as compared to control and lower concentration of p-CPA. C) Cumulative sum for time to eclosion from pupa formation for different concentrations of p-CPA. D) Relative cumulative sum for C. Time for each pupa is adjusted to '0' and calculated time taken from pupation to eclosion. The larva fed 50mM pupa took longest time to eclose but they seem to catch up with the others in the eclosion.

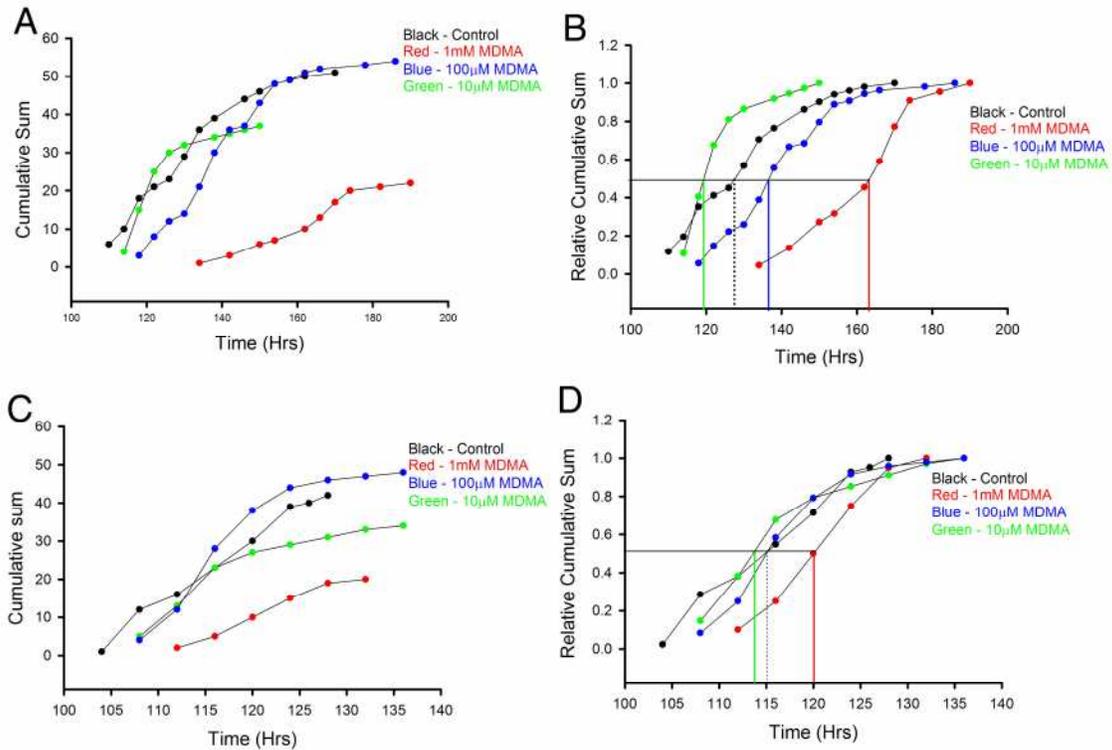


Figure 3.2: MDMA growth Curve.

MDMA was fed from 1st instar to 3rd instar stage. The time to pupation from eggs and time to eclosion from pupation is calculated for each larva.

A) Cumulative sum for time to pupation from eggs at different concentrations of MDMA. B) Relative cumulative sum for A. 50% of larvae took ~ 165 hrs for pupation at the highest concentration of MDMA, 1 mM.

C) Cumulative sum for time to eclosion from pupa formation for different concentrations of MDMA. D) Relative cumulative sum for C. As described earlier, time for each pupa is adjusted to '0' and calculated time taken from pupation to eclosion. Pupa of 1mM MDMA fed larvae took a little longer (~ 5 hrs more than controls) to eclose as compared to lower concentrations.

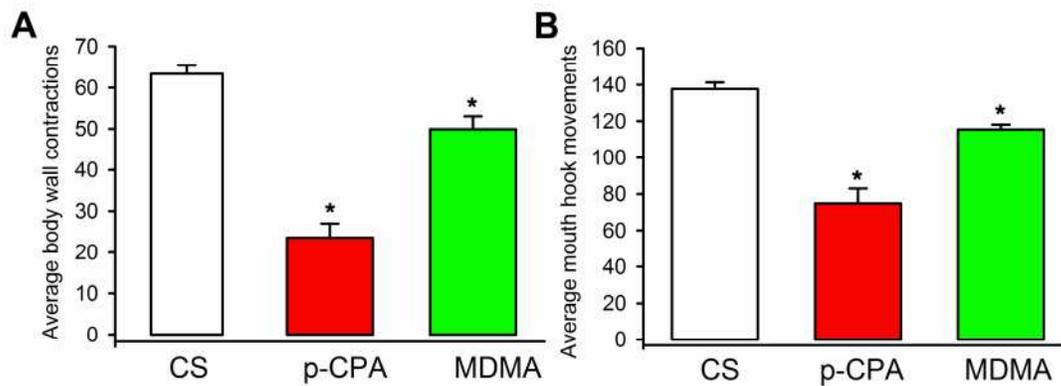


Figure 3.3: Body wall and mouth hook contractions.

A) Body wall contractions were counted for one minute and averaged per minute. p-CPA or MDMA fed larvae showed a decrease in body wall movements as compared to controls (N =20, ANOVA<math>< 0.05</math>). B) Mouth hook contractions were counted in yeast solution for one minute and averaged per minute. Both p-CPA and MDMA fed larvae showed a decrease in mouth hook movements as compared to controls (N =20, ANOVA<math>< 0.05</math>)

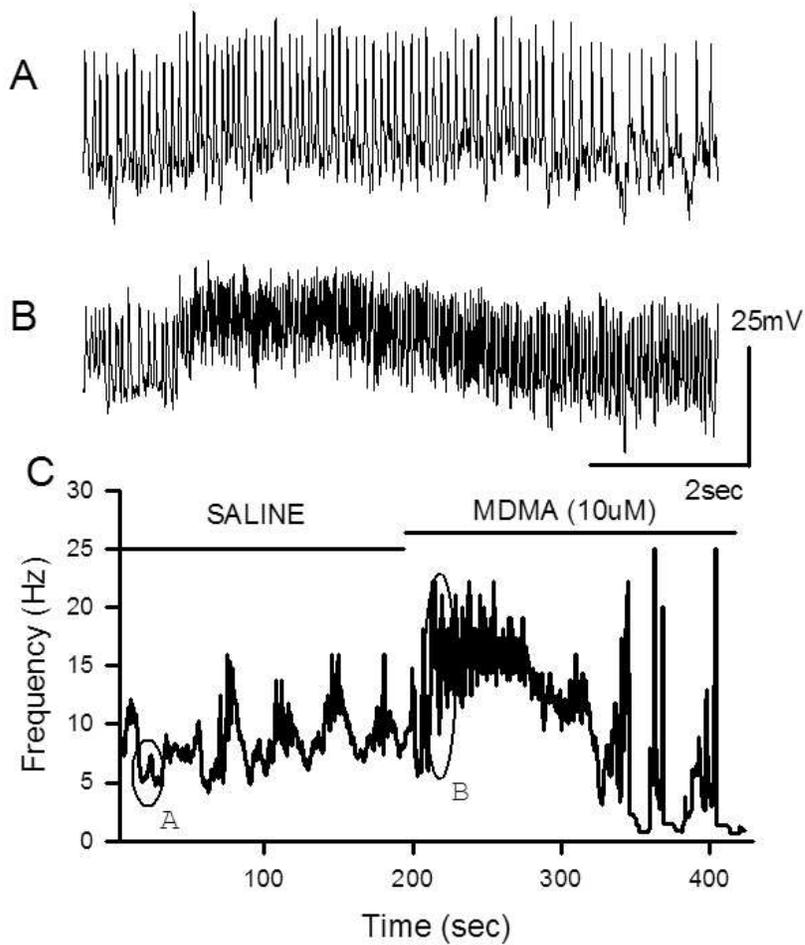


Figure 3.4: Spontaneous activity in 3rd instar CS larvae.

A) Spontaneous activity in saline. B) Spontaneous activity in presence of 10 μ M MDMA. C) Total recording of spontaneous activity in presence of saline and MDMA 10 μ M. Activity in presence of MDMA initially increased for first few 100s of seconds and decreased thereafter.

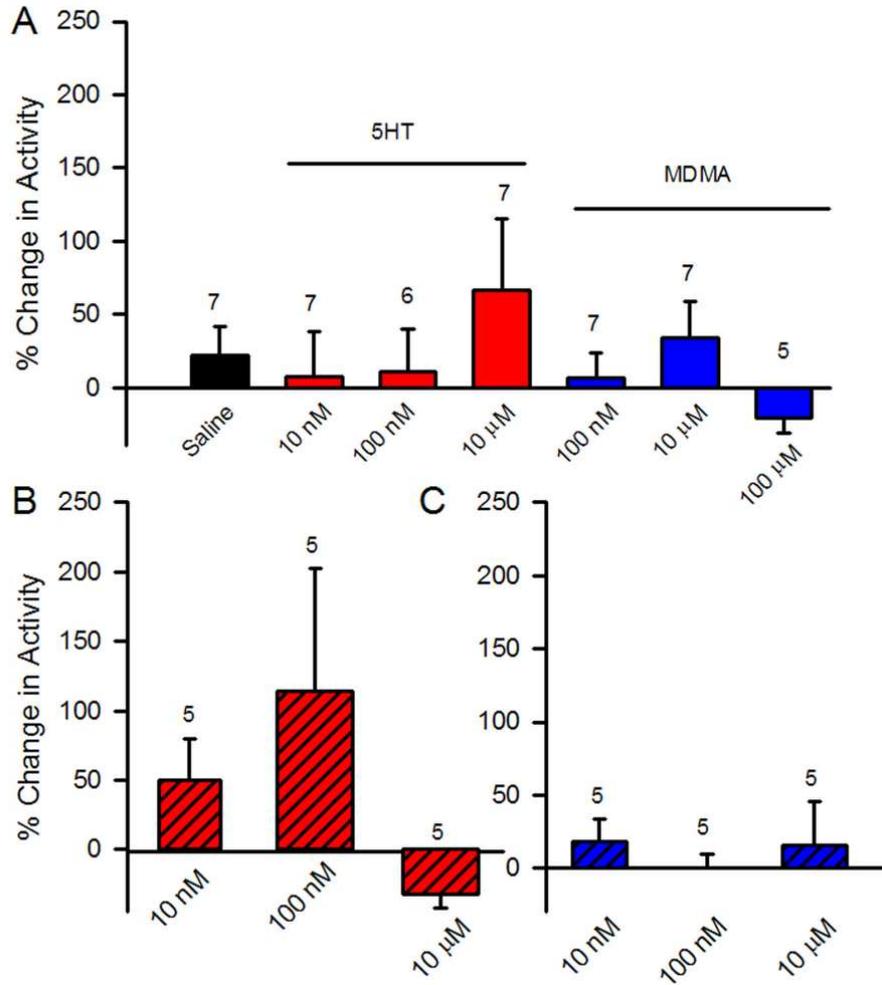


Figure 3.5: Spontaneous activity.

The % change in average frequency per second (activity) from saline in CS larvae (A), p-CPA fed larvae (B) and MDMA fed larvae (C). 3 different concentrations of 5-HT and MDMA are used on CS larvae and for the p-CPA and MDMA fed larvae 3 different concentrations of 5-HT are used in comparison with saline. The number above the bar shows the sample size.

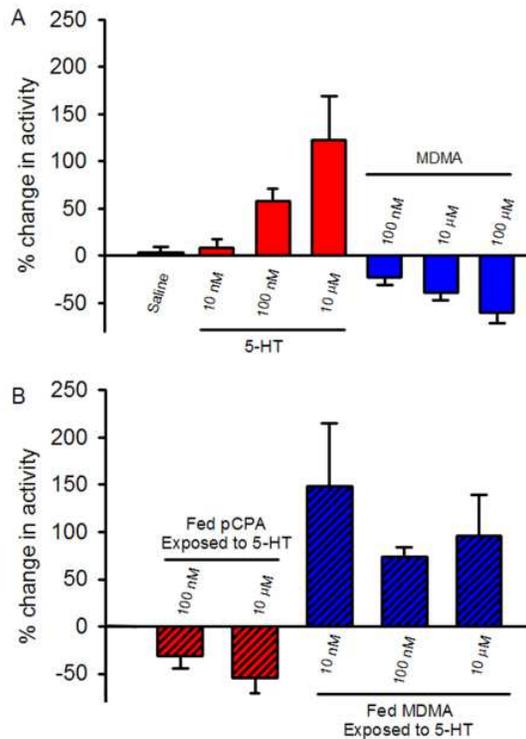


Figure 3.6: Sensory-CNS-motor circuit.

A) A percent difference in the activity of motor units to muscle 6 before and after application of 5-HT or MDMA. Saline shams showed a small increase. 5-HT showed a dose depended significant increase, whereas MDMA showed a dose depended significant decrease in activity from shams (ANOVA, $P < 0.0001$).

B) 50mM p-CPA fed larvae and 100μM MDMA fed larvae were exposed to 5-HT of different concentrations. The percent difference in activity is recorded before and after 5-HT exposure. p-CPA fed larvae showed a significant decrease in sensitivity to 5-HT whereas MDMA fed larvae showed a significant increase in activity in presence of 5-HT. (ANOVA, $p < 0.0001$).

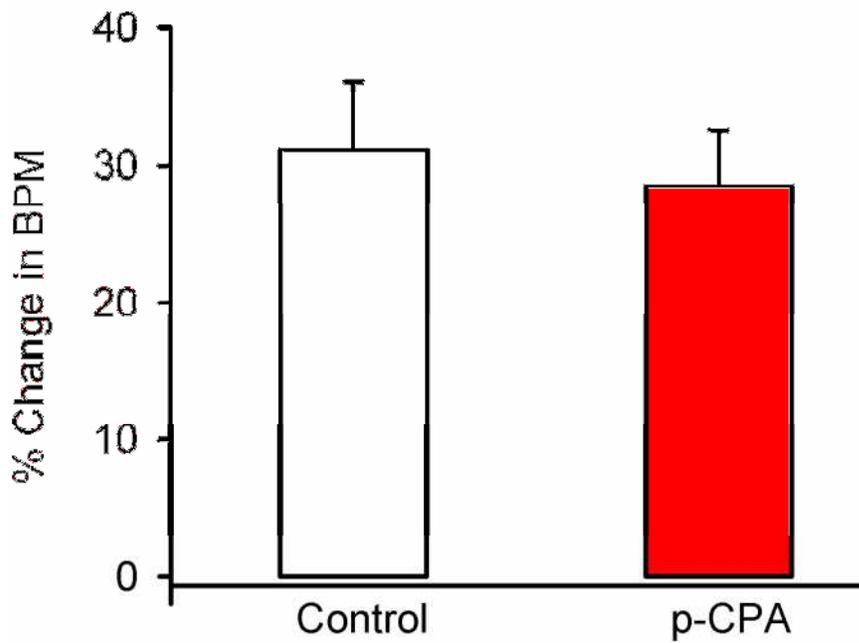


Figure 3.7: Heart rate.

The percent change in heart rate upon exposure to 5-HT (100 nM). Both control and larvae fed p-CPA showed a similar and significant effect ($n=7$; $P<0.05$, Student's t -test) increase in heart rate without a significant differences between the groups.

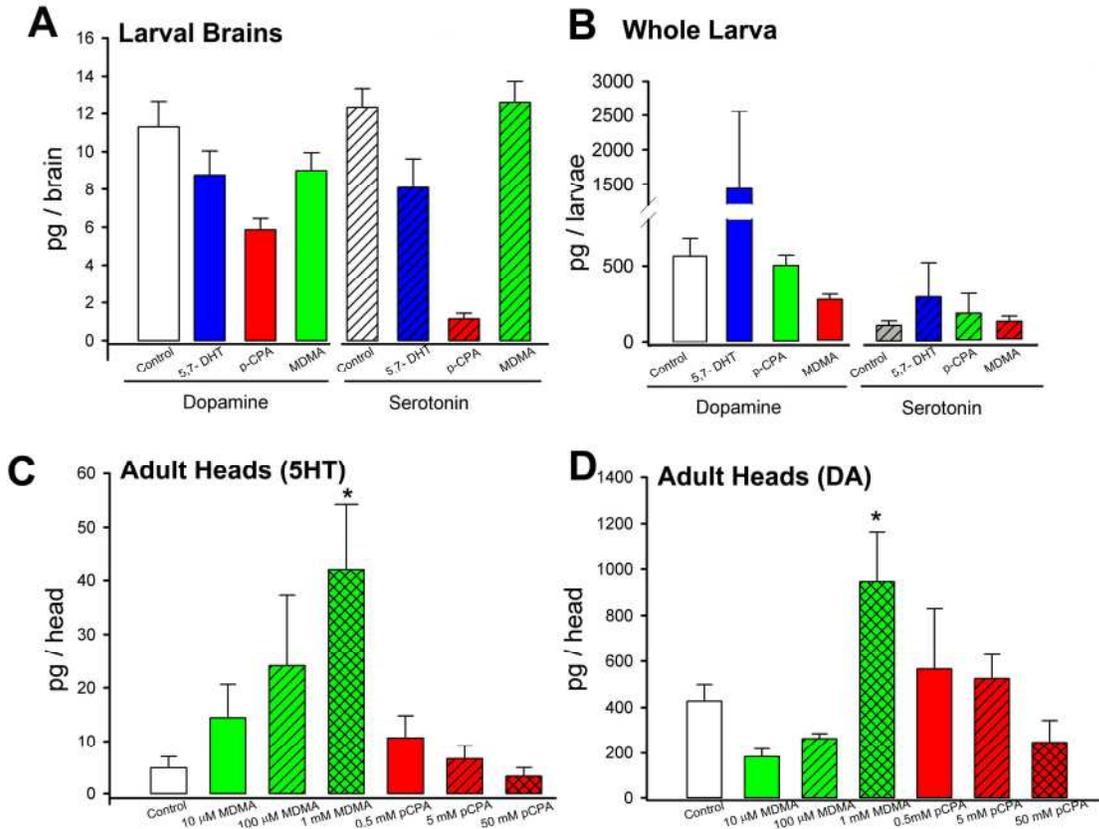


Figure 3.8: HPLC analysis of 3rd instar larvae.

(A) HPLC analysis on 3rd instar larval brains. P-CPA treated larval brains (n=5) showed a decrease in DA levels (open bar, ANOVA <0.05) and 5-HT levels (hashed bars, ANOVA, P<0.05). MDMA and 5,7-DHT didn't show a difference (n=5 and 3 respectively). (B) DA and 5-HT levels in whole larvae. None of treated groups showed any significant difference from the control group. (n=5). (C) DA levels in adult heads, collected after they have been treated with drugs from 1st to 3rd instar stage. 1mM MDMA showed a significant increase in DA levels (n=5, ANOVA, P<0.05). (D) 5-HT levels in adult heads. Except for 1mM MDMA, the other groups did not show any difference in levels compared to controls. (n=5).

CHAPTER 4

KNOCK DOWN OF 5-HT₂ RECEPTORS ALTERS DEVELOPMENT, BEHAVIOR AND CNS ACTIVITY IN DROSOPHILA MELANOGASTER

ABSTRACT

The expression of anti-sense 5-HT_{2dro} receptor retards larval development and produces slower body movements and gustation. When expressed from 1st instar, induced by heat shock, a high degree of death occurred and few reached 3rd instar. The CNS of larva increases its excitability when exposed to 5-HT, however when the 5-HT_{2dro} receptor is reduced in expression the sensitivity to exogenously applied 5-HT was decreased. Evoked sensory-CNS-motor circuits as well as spontaneous motor neuronal activity is reduced in larvae in which the 5-HT_{2dro} receptor is knocked down. Like CNS activity, heart rate (HR) in larva is sensitive to 5-HT. The knock down of 5-HT_{2dro} receptors from 1st instar to early 3rd instar resulted in no effect to sensitivity to HR although the initial HR was lower. Thus, the 5-HT_{2dro} receptor required for normal body development and CNS responsiveness to 5-HT.

INTRODUCTION

Serotonin (5-HT) is a major neurotransmitter and neuromodulator in both vertebrates and invertebrates. It has been shown to have a role in development and behaviors of various vertebrates and insects (Whitaker-Azmitia, 2001; Lucki, 1998; Monastirioti, 1999; Osborne, 1996; Chapter 3 for this dissertation). Recently, it was shown that 5-HT increases activity in a sensory-CNS-motor circuit in larvae (Dasari and Cooper, 2004). When reducing the production of 5-HT, by pharmacological

means through feeding p-CPA to larva, there is a delay in the rate of development and slowed body wall as well as eating behavior (Chapter 3 for this dissertation; Dasari et al., 2006). Likewise, exposure to MDMA (ecstasy), a drug of abuse, which impacts on the serotonergic system, also results in slowed larval development and reduced bodily movements (Dasari et al., 2006). Thus, altering the production or the level of 5-HT has broad ranged effects in larval *Drosophila*, however to understand the mechanisms by which these alterations occur one needs to address the effectors of 5-HT which relay the signals to cells.

5-HT acts through multiple receptors to mediate its many functions in various tissues. There are 14 known 5-HT receptors in vertebrates (Barnes and Sharp, 1999) whereas four are known to occur in the *Drosophila* genome (Tierney, 2001). Hence *Drosophila melanogaster* forms an attractive model organism to study the role of 5-HT mediated by these receptors. The four known receptors are named as 5-HT_{7dro}, 5-HT_{1Adro}, 5-HT_{1Bdro}, and 5-HT_{2dro} (Witz, 1990; Saudou et al, 1992; Colas et al, 1994; Tierney, 2001).

The 5-HT_{2dro} receptor subtype is of particular interest since in humans 5-HT₂ receptor is known to be associated with many diseases such as schizophrenia, depression, and anxiety (Leonard 1994; Fuller 1991). In addition, some drugs of abuse work on the 5-HT₂ receptor subtypes to relay the effects sort after by addicts (i.e., lysergic acid dimeythlamide) (Roth et al., 1998). *Drosophila* offer some advantages in the ability to address the developmental and mechanistic understanding of neuromodulators, particular 5-HT since this model organisms is

being increasing used to address physiological related inquiry as well as the genetic understanding of diseases that inflict humans (Kendler and Greenspan, 2006).

Colas et al. (1999a,b) showed that 5-HT_{2dro} is essential during embryogenesis and that it is the major receptor subtype in 3rd instar larvae (Colas et al., 1994). One approach to determine the role of 5-HT_{2dro} is to selectively knock down its expression. In my investigation I use two temperature sensitive transgenic lines, HsZ2 and Y32 which were designed to specifically reduce the functional expression of the 5-HT_{2dro} receptor. HsZ2 strain is a 5-HT_{2dro} anti-sense under heat shock GAL4-UAS construct. The Y32 strain expresses a 5-HT_{2dro} anti-sense under a heat shock promoter. Since these two strains had not been fully characterized for temperature induction of the anti-sense and the role on development, I conducted studies at 18, 21 and 33 °C. To determine the effects of reduced 5-HT_{2dro} expression on behavior and on CNS receptivity to 5-HT studies at low and heat shock induced temperatures were compared for sham controls and the two strains.

Johnson et al., (1997) have shown heart rate to be modulated by 5-HT in *Drosophila*. Also recently we have shown direct effects of various doses of 5-HT on *Drosophila* heart (Dasari and Cooper, 2006). 5-HT_{2B} receptor in mammalian model organisms have been shown to regulate cardiac embryonic development and cardiac adult functions. Hence these receptors might be involved in cardiac pathophysiology in adults. We measured heart rate in transgenic lines to investigate if 5-HT_{2dro} receptor regulates it.

METHODS

Stock and Staging of Larve

The common 'wild-type' laboratory strain of *Drosophila melanogaster*, Canton S, was used in these studies as controls. HsZ2 and Y32 are used as experimental lines. HsZ2 is a 5-HT_{2dro} antisense under GAL4-UAS system and Y32 is a 5-HT_{2dro} antisense under heat shock promoter (gifts from Dr. L. Maroteaux, IGBMC-CNRS-INSERM, Universite de Strasbourg, France). The methods used to stage fly larvae have been described previously (Campos-Ortega and Hartenstein, 1985; Li and Cooper, 2001). All animals were maintained in vials partially filled with a cornmeal-agar-dextrose-yeast medium. All animals were kept on a 12:12 light-dark cycle.

Behavioral Assays

Early 3rd instar larvae were used for behavioral assays. Feeding and locomotor behaviors were assessed as described in Neckameyer (1996) and Li et al., (2001). In brief, single animals were placed on a 2% agar surface and the number of body wall contractions was counted for 1 minute, after which an animal was placed in a 2% yeast solution overlaid on an agar plate (just covering the larvae with allowing the spiracles to reach out of the solution). In this condition, *Drosophila* larvae immediately feed, initiating a pattern of repetitive mouth hook movements. The number of full mouth hook contractions in 1 minute was counted (Sewell et al. 1975).

Dissection and electrophysiological recordings

Wandering 3rd instar larvae were dissected as described earlier (Cooper et al., 1995). In brief larvae were dissected ventrally removing heart and viscera which left a filleted larvae containing only a body wall, body wall muscles and the neural circuitry for the sensory, CNS and body wall (i.e., skeletal) motor units. The HL3 saline was prepared in the lab from component reagents (Sigma) and contained: 1.0 mM CaCl₂·2H₂O, 20 MgCl₂, 70 mM NaCl, 5 mM KCl, 10 mM NaHCO₃, 5 mM trehalose, 115 mM sucrose, and 5 mM BES (*N,N*-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid) (Stewart et al., 1994).

The recording arrangement was essentially the same as previously described (Neckameyer and Cooper, 1998; Stewart et al., 1994). Intracellular recordings in muscles were made with 3 M KCl-filled microelectrodes (30–60 MΩ). The amplitudes of the excitatory postsynaptic potentials (EPSP) elicited by Is and Ib motor nerve terminals in the various segments of muscles m6 were monitored. Intracellular responses were recorded with a 1×LU head stage and an Axoclamp 2A amplifier.

Sensory-CNS-Motor circuit

To induce a sensory-CNS-motor circuit the tail segmental nerves were cut and stimulated using the suction electrode (Dasari and Cooper 2004). The stimulator (S-88, Grass) output was passed through a stimulus isolation unit in order to alter polarity and gain (SIU5, Grass). Stimuli were given in short bursts at a frequency of 40Hz (10 stimuli at 40Hz). The amount of voltage given was adjusted so as to recruit sensory axons to produce at least a few responses in the muscles so that if any further increase or decrease occurred due to the effects of a neuromodulator the

change could be obtained. This sensory stimulation leads to interneurons and motor neurons to be activated, thus inducing a response in muscle 6.

In cases where the intrinsic spontaneous activity was measured all the segmental nerves were left intact. The intrinsic CNS activity probably is induced by the fact the animal has been cut down the dorsal midline and stretched out on a dish by pinning four corners of the animal down. The burst frequency and frequency within a burst were measured for experimental comparisons. All experiments were performed at room temperature (20–22 °C).

Heart Rate (HR) measures

The same microscopic method as for behavioral movements was used to record HR but with the exception of a 2x base objective to obtain a higher resolution of the heart and trachea. Early 3rd instar larvae were dissected ventrally and pinned on 4 corners. The heart and trachea are exposed in these semi-intact preparations. The movements of the trachea or heart were used for direct counts (Dasari and Cooper, 2006). Effects of 5-HT were observed in CS, HsZ2 and Y32 larvae allowed to grow at different temperatures.

Developmental assays

Eggs were collected after a 15-minute prepulse for 2 hrs and allowed to develop at 21°C until hatching. At which time 3 different developmental temperatures were investigated for the effects on development and I tested if room temperature might be a temperature at which a low expression level of the heat shock promotor

might be active which could result in anti-sense production in the two experimental strains.

1st instars (about 15) were transferred to vials containing food and placed at given temperature. The food was made with standard corn meal diet (Neckameyer, 1996; Li and Cooper, 2001). When wandering 3rds were seen, the vials were checked every 4 hrs and each individual pupa was marked on the walls of the tube to record the time to pupation. This was done throughout the day and night. The time to pupation and the time spent as a pupa, until eclosion, were indices for development.

RESULTS

Behavior – Mouth hook and body wall movements

Locomotion (body wall) and feeding (mouth hook) movements were measured for CS, HSZ2 and Y32 strains at permissive (room temperature) and restrictive temperatures to induce the heat shock antisense production of the 5-HT_{2dro} receptor. There was no significant difference seen among the three lines in either body wall or the mouth hook movements when larvae were raised in room temperature (ANOVA, $N \geq 10$, Fig. 4.1). To activate 5-HT_{2dro} antisense production, early 3rd instars were given a 4 hr heat pulse at 33°C and then the locomotor and feeding behaviors were measured at room temperature within 5 minutes of taking them out of 33°C. Each larva was taken from the incubator and placed on a test plate. This allowed the larvae to become familiar with the test plate for a minute. Then body wall and mouth hook movements were measured. HsZ2 line showed

lower average body wall movement than CS (Fig. 4.2 ANOVA, $P < 0.01$, $N = 25$). Y32 also showed lower body wall movements than CS (Fig. 4.2 ANOVA, $P < 0.05$, $N = 25$). But no significant difference was seen in mouth hook movements among the 3 lines that were heat shocked (Fig. 4.2, $N = 25$).

To control for the temperature variations I measured these feeding and locomotor behaviors at 33°C after a 4hr heat pulse at the same temperature. Here again no significant difference in the mouth hook movements occurred (Fig. 4.3B, $N = 10$) but this time there was no significant difference for body wall movements (Fig. 4.3A, $N = 10$).

To see the effects of a reduced expression of 5-HT_{2dro} throughout the development stages, I grew larvae from 1st instar to 3rd instar at 31-32°C. Early 3rd instars were used for measuring body wall and mouth hook movements at room temperature. To my surprise there was no significant difference between HSZ2 and Y32 compared to CS (Fig. 4.4A, B, $N \geq 10$).

Larval Development

Developmental assay were carried out at 18°C, room temperature (21-22°C) and 31-32°C for CS, HsZ2 and Y32. Time was assessed for 50% of population to reach pupation or eclosion. The time eggs were collected is taken as a zero point. At low temperature, where the anti-sense for 5-HT_{2dro} is not being expressed, we did not anticipate to see any major difference in the development patterns. But a small variation was observed between the 3 groups for time to pupation (Fig. 4.5A, B). 50% of the population in the CS group took approximately

244 hrs for pupation, whereas for Y32 50% took 280 hrs and 50% of HsZ2 took ~ 8 hrs more than the CS group. This small variation continued for the time from pupation to eclosion. For calculating the amount of time taken for eclosion for each pupa, the time point at which pupa formed was set to '0' (zero) and number of hrs are calculated to eclosion. 50% of pupa for CS and HsZ2 took ~ 190 hrs and ~ 180 hrs respectively, whereas Y32 took ~ 220 hrs (Fig. 4.5C, D). This is a small difference for CS, HsZ2 and Y32 in both time to pupation and eclosion for 50% of the population. To statistically test for a difference in the distributions a Kolmogorov-Smirnov (K-S) test was performed. D and P values for pupation among all groups were not significant (For CS and HsZ2: D is 0.1507 and a P of 0.985, CS and Y32: D, is 0.30 and a P of 0.417, HsZ2 and Y32: D, is 0.2549 and a P of 0.610). Similarly eclosion at room temperature is not significant (CS and HsZ2: D, is 0.0981 and a P of 1.0, CS and Y32: D, is 0.1593 and a P of 0.908, HsZ2 and Y32: D, is 0.1 and a P of 1.0)

Similarly, I observed the developmental pattern for room temperature in all 3 groups. Here again I found a small variation in the time taken for 50% of population to pupate and eclose. 50% of CS and HsZ2 took almost the same amount of time to pupate (~ 132 and 133 hrs respectively), whereas Y32 took ~ 140hrs (Fig. 4.6A, B). For eclosion time 50% of HsZ2 took ~100hrs where as Y32 and CS took ~2 and 4 hrs less than HsZ2. A K-S test was used to assess for statistical significant differences in the distributions. No significant difference was seen for pupation in any groups (CS and Y32: D, is 0.1442 and a P of 0.996, CS and HsZ2: D, is 0.1462 and a P of 0.999, HsZ2 and Y32: D, is 0.1625 and a P of 0.993). No significant difference

is seen in eclosion time for any groups (CS and HsZ2: D, is 0.2273 and a P of 0.914, CS and Y32: D, is 0.1697 and a P of 0.986, HsZ2 and Y32: D, is 0.1697 and a P of 0.986).

Developmental effects at the high temperature in which the anti-sense for 5-HT_{2dro} is actively expressed from 1st instar stage had pronounced effects. 50% of CS population took 120 hrs to pupate whereas Y32 and HsZ2 took 140 hrs (Fig. 4.7A, B). The major difference observed was when the majority of the CS larvae reached pupation. Only 3 of 100 HsZ2 and 30 of 100 Y32 had pupated. K-S test showed a statistical difference in pupation time between CS and HsZ2 (D is 0.5294 with a corresponding P of 0.010) and HsZ2 and Y32 (D is 0.4794 with a corresponding P of 0.019) but no difference between CS and Y32 (D is 0.3706 with a corresponding P of 0.123) is present.

Examining the effects on pupation proved to be futile since all of Y32 pupa and HsZ2 died and 20% of CS pupae eclosed. 50% of CS pupae that did eclose took 76 hrs (Fig. 4.7A, B).

Spontaneous activity

The intrinsic activity was measured from m6 or m7 muscles as both these muscles receive the same innervation by two motor neurons Ia and Ib. The spontaneous activity is seen when the larva is stretched and pinned, which likely keeps sensory neurons active. A lot of variation was observed from preparation to preparation as noted in my earlier studies (Chapters 2-3 of this dissertation). Two sets of experimental paradigms were used here: (1) larvae were grown at room

temperature and neural activity assessed at room temperature; (2) larvae were grown at 31-32°C and intrinsic activity was measured at room temperature saline. The sensitivity to 100nM 5-HT was examined for all the groups in these two conditions.

Intrinsic activity was recorded for 2 min in saline and then another 6-10 min in the presence of 5-HT. For quantifying, I calculated the average frequency per second by counting all the peaks within each burst. Also the time for each burst was recorded. The average frequency per second in saline and in presence of 5-HT was used to calculate the percent difference in activity from saline.

At room temperature 5-HT (100nM) sensitivity of the CS increased by approximately 15%, whereas HsZ2 and Y32 showed a decreased responsiveness (Fig. 4.8A). There is no statistical significance seen among CS, HsZ2 and Y32 (N = 5, ANOVA, Fig. 4.8A). However, the CS and HsZ2 larvae grown at 31-32°C and exposed to 5-HT (100nM) showed the opposite effects (Fig. 4.8B). CS and HsZ2 showed a decrease response whereas Y32 showed a significant increase in activity from saline (N = 5, ANOVA, $P < 0.013$ and Tukey post hoc test, $P < 0.05$, Fig. 4.8B). Y32 did not have much activity in saline when compared to that of CS and HsZ2. Introducing 5-HT increased the spontaneous activity in Y32.

Sensory-CNS-motor circuits

The sensitivity of central circuits to 5-HT in transgenic lines was examined by stimulating sensory nerves and monitoring motor units before and during exposure to the compounds. CS showed an increased in motor activity in presence of 5-HT

(100nM) at room temperature. Similarly HsZ2 and Y32 showed an increase in activity at room temperature in response to 5-HT (100nM). No statistical difference was seen among 3 groups (N = 5, ANOVA, $P < 0.15$, Fig. 4.9A). When antisense 5-HT_{2dro} was activated, sensitivity of CNS-circuit to 5-HT (100nM) is changed. CS and HsZ2 that were grown at high temperature (31-32°C) showed a very small increase in motor activity. Here again there is lot of variation in preparations. On the other hand Y32 grown at high temperature showed a decrease in activity. Y32 larvae grown at 31-32C have a lower activity in saline and there was a fast run down in the Y32 preparations. The frequency of stimulation had to be increased in some preparations to 60Hz from the 40Hz, as used for all the other preparations, in order to evoke motor nerve activity. Again no statistical significance is seen among the 3 groups (N =5, ANOVA, $P < 0.23$, Fig. 4.9B).

Heart rate

5-HT is known to modulate heart rate in *Drosophila* larvae (Dasari and Cooper, 2006) and also recently the larval heart was noted to be innervated (Johnstone et al., 2006). Hence, I wished to measure the sensitivity of the heart to 5-HT in transgenic larvae with a reduced expression in the 5-HT_{2dro} receptor to determine if this receptor subtype had any role. Mid 3rd instars were dissected and heart rate was monitored. To quantify the responsiveness a percent difference is calculated for each preparation from saline to 5-HT and an average value is compared (Dasari and Cooper, 2006). CS, HsZ2 and Y32 larvae grown at room temperature did not have any significant difference in sensitivity to 5-HT. All the 3

groups have shown an increase in the heart rate in presence of 5-HT, though HsZ2 showed a little higher increase, but not statistically significant (N = 10, ANOVA, P < 0.1), than both CS and Y32 (Fig. 4.10A). Larvae that were grown at 31-32°C were taken out one at a time right before the experiment and heart rate counted at room temperature for one min in saline and another 3 min in presence of 5-HT. The total time taken for each preparation is about 5 min. The initial heart rate in saline was low for all three strains CS, HsZ2 and Y32 as compared to the ones that were raised at room temperature. CS larvae showed a small variation in its initial heart rate as compared to that of room temperature. Y32 had a very low HR with some preparations not beating at all, but when the 5-HT containing saline was introduced HR increased. When initial heart rates are compared among the three groups that were raised at high temperature there is a statistically significant difference (Fig. 4.10C) Because of the initial low heart rate, a percent difference was calculated for comparisons, but in some cases a large difference is then obtained. No statistical difference is present among the 3 groups (N =10, ANOVA, P< 0.41 Fig. 4.10B).

DISCUSSION

In this study two lines of 5-HT_{2dro} transgenics (Y32 an anti-sense 5-HT_{2dro} under a heat shock promoter and HsZ2 an anti-sense 5-HT_{2dro} under heat shock Gal4-UAS promoter) were used to asses effects that would result from the lack of a 5-HT_{2dro} receptor. These transgenics had developmental, behavior and physiological defects. The development of Y32 and HsZ2 at room temperature and low temperature are not different from controls. However at high temperature (31-32°C)

most of CS formed pupa but just a few Y32 (30%) and HsZ2 (3%) larvae pupated. Only 20% of CS pupa eclosed and none from the transgenic lines. But no significant difference is present in simple locomotor movements (body wall and mouth hook movements) in larvae that are grown at high temperature. However, the physiological responses of the CNS to 5-HT application in transgenic larvae grown at high temperature had a decreased responsiveness compared to controls. Though initial HR was lower in transgenic larvae as compared to that of at room temperature, no effect on sensitivity to 5-HT was seen.

Studies altering synaptic transmission of adult vertebrate motor nerve terminals has provided evidence that established synapses and nerve terminals maintain a high degree of plasticity. A classic example is the increased branching and growth of motor nerve terminals when the terminal is exposed to botulinum toxins that block synaptic transmission (Shupliakov et al., 2002). Many of the same regulating factors in synaptic maintenance in adult animals are likely utilized during developmental stages, so understanding how synapses develop may have ramifications in mature synapses. Sokolowski (1980) showed that the behavioral repertoire of *Drosophila* larvae requires flexible synaptic inputs. It is likely that studies conducted at the relatively simplistic NMJ of *Drosophila* in synaptic regulation are paralleled in the larval CNS.

The development in the larval *Drosophila* CNS is a topic that has not been intensively studied but has been gaining a substantial interest in recent years (Bossing and Brand, 2002, 2006). Since the larval CNS contains substantially fewer neurons (~2000, Nassif et al., 2003) as compared to the 100,000 (Iyengar et al.,

2006) or more in the adult, the regulatory factors might be easier to assess and quantify in the larval brain. In addition, since associative learning assays are now established for larva correlative studies of CNS development or manipulations in development can be approached in this simpler CNS. It has also been recently established that the larval CNS is modular in neuropiles (Nassif et al., 2003; Younossi-Hartenstein et al., 2003) that can be an asset to quantifying alterations in subdivisions as compared to other regions (Iyengar et al., 2006). Since some of the regions in the larval CNS are known to be associated with particular sensory neurons, i.e. olfaction or gustatory senses, then those regions in the CNS can be examined for behavioral transgenics in olfactory learning screens.

Most of the interest in larval development of synapses has been directed at understanding factors that regulate NMJ formation and maintenance. The basic principles that are being uncovered at the NMJ are similar in some regards to the development within the CNS but with different cues than from muscle. The synaptic connections at the NMJ formed during embryogenesis are not "hardwired". In target-removed experiments, Cash et al. (1992) showed that motor neurons could innervate other muscle fibers when their own targets are missing. These alternate synapses are physiological functional. However, little or no investigation has been approached as to the potential effects of altered CNS function from neuromodulators on the development and maintenance of the NMJs.

The 5-HT_{2dro} of *Drosophila* is orthologous to mammalian 5-HT₂ receptor subfamily. Colas et al., (1999a,b) reported that 5-HT_{2dro} mRNA expresses during embryogenesis and gastrulation. With the use of these 5-HT_{2dro} transgenics it has

been shown that the receptor must have some functional role in embryogenesis since ectoderm extension, during embryogenesis, is delayed and hence embryonic lethality is observed (Colas et al., 1999a,b). In my studies, I activated the heat shock promoter at 1st instar stage, which retarded the development of larvae and reduced the number that reached the pupal stage. The ones, which did pupate, however did not eclose. Like in other animals 5-HT is thought to have a major role during development. 5-HT is detected in sea-urchins (Buznikov et al., 2005), chicken and frogs during zygotic divisions in which gastrulation and neurulation takes place. Nebigil et al., (2000) had shown that mice deficient in 5-HT_{2B} receptor even have a defective heart.

Since 5-HT modulates locomotive and behaviors in insects and that 5-HT containing neurons are known to innervate guts, pharyngeal muscles and ring gland in *Drosophila* (Valles and White 1988), I assumed that the knock down of the 5-HT₂ receptor would produce very pronounced effects on eating behaviors. It was surprising that no significant effect on locomotor or feeding behavior were obvious. The rationale for explaining the lack of an effect is probably that these behaviors are regulated by other 5-HT receptors and not the 5-HT_{2dro}. Recently the 5-HT₁ receptor was shown to have a role in sleep behavior in *Drosophila* (Yuan et al., 2006). Unlike *Drosophila*, the 5-HT_{2A} in mammals is involved in feeding behavior and 5-HT_{2C} in regulating ones size of meals (Lucki, 1998).

Other neuromodulators that are known to be in the hemolymph and released into the neuropile of the larval CNS, such as octopamine and dopamine, are likely to have key roles in shaping the development of the neural circuits (Monastirioti, 1999).

Other neuromodulators or peptides may even have a more dramatic role than 5-HT. Recent work (Pauly et al., 2006) in which larva were feed AMVT (10mg/ml of food), a drug that blocks tyrosine hydroxylase and thus blocks the synthesis of dopamine, causes sever developmental problems. If 1st or 2nd instars are fed this compound they die, however if 3rd instar are fed a majority die as pupa. The ones that do eclose have white eyes and the cuticle is pale. I have examined dopamine and octopamine on the CNS circuit of 3rd instar in an earlier study (chapter 2) and showed that dopamine (10 μ M) does increase the activity of the circuit, however octopamine at the same concentration resulted in the preparations to undergo massive waves of contraction.

Since there is such a pronounced effect of octopamine on the neural activity one would expect a deficit in the production of the receptors to results in substantial changes in CNS development. As far as I am aware, no such studies have yet to be conducted. The effects of octopamine and a related compound, tyramine, have been examined in relation to honey bee behavior and flight (Fussnecker et al., 2006). In order to block the action of these compounds antagonists were injected into the animals. So similar pharmacological approaches could be used in larval *Drosophila* however feeding the compounds might be less problematic. One does have to worry that the compounds mixed in with food and fed to larva or adults might be broken down during digestion.

It is likely that similar developmental cues such as activity and local retrograde signaling factors hold for synapse formation and maintenance in the larval CNS as established in other model preparations, however detailed studies on

defined tracts are necessary to substantiate the various possibilities. Possible by using a fly construct that expressed GFP in a subset of sensory neurons in the Y32 or HSZ fly strains and then imaged within a 3rd instar larval CNS that had the 5-HT_{2dro} receptor knocked down, the effect of the 5-HT_{2dro} on the development of the sensory circuits could be addressed. The established techniques of GFP expression (Brand, 1999), Gal4/UAS (Brand and Perrimon, 1993), and the MARCM (Lee and Luo, 1999) offer one a powerful approach to examining identified neurons during development and their reactions to manipulations in signaling cues and activity in relation to synaptic communication. Combining physiological measures of larval CNS circuits with the anatomical profiles along with behavior assays will undoubtedly be an area of significant interest in the next few years.

5-HT and its receptors have many physiological functions in animals including cardiovascular physiology (Nebigil et al., 2001; Hoyer et al., 2002). 5-HT was initially isolated from blood and found to be a vasoconstrictor (Rapport et al., 1948), making it an attractive neurotransmitter for regulating cardiovascular functions. Loss of *Tph1* gene (peripherally expressed TPH) in mice results in abnormal cardiac function (Cote et al., 2003) and knock out of the 5-HT_{2B} receptor in mice results in lethality due to defects in heart development (Nebigil et al., 2000). 5-HT_{2A} mediates arterial vasoconstriction and 5-HT_{1B} receptor mediates contraction of pulmonary arteries (Hoyer et al., 1994; MacLean et al., 1996). 5-HT receptors are shown as potential targets to treat hypertension and other peripheral vascular diseases (Frishman and Grewall; 2000). 5-HT_{2B} receptor is the major receptor 5-HT₂ subfamily present in heart. (Choi et al., 1997; Choi and Maroteaux, 1996; Lauder et al., 2000). In my

studies 5-HT_{2dro} transgenic lines have a lower initial heart rate when compared to that of room temperature or controls that were grown at high temperature, but sensitivity to 5-HT is not changed. Probably in *Drosophila* cardiac function is not modulated by 5-HT_{2dro} completely. Probably other 5-HT receptors have a role in cardiac function. Detailed pharmacological studies with agonists and antagonists of all 4 5-HT receptors of *Drosophila* would give more insight to this issue.

Clinically in humans, in addition to the role of these receptors during embryogenesis, they may be important as determinants in migraine, hypertension, heart failure, neurodegenerative diseases and brain maturation disorders such as schizophrenia or autistic behaviors. This study is significant since it will provide pertinent information, which addresses the degree in synaptic performance at the release sites over developmental stages in a model system of *Drosophila* when a defined receptor subtype is knocked down in expression. The findings can be correlated to the underlying factors of the serotonergic system during development which provides a frame work for future studies in knocking down the other three 5-HT receptor subtypes or even sensitivity to other neuromodulators such as dopamine or octopamine. Thus, there is premise that understanding the fundamental basics of synaptic transmission in this model system will be directly relevant to all neural systems, including humans.

FIGURES:

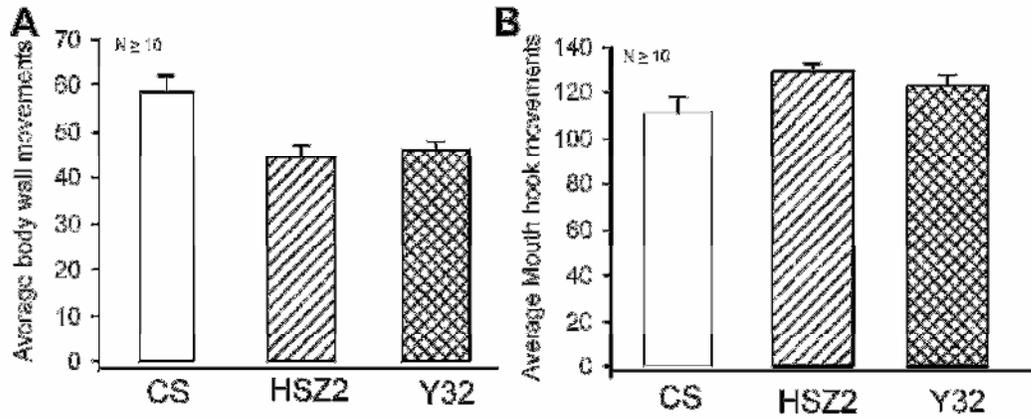


Figure 4.1: Locomotory movements at room temperature.

A) Body wall and B) mouth hook movements at room temperature. Larvae for CS, HsZ2 and Y32 were grown at room temperature and early third instars were used for behavior measurements. No significant difference was seen among 3 groups ($N \geq 10$, ANOVA).

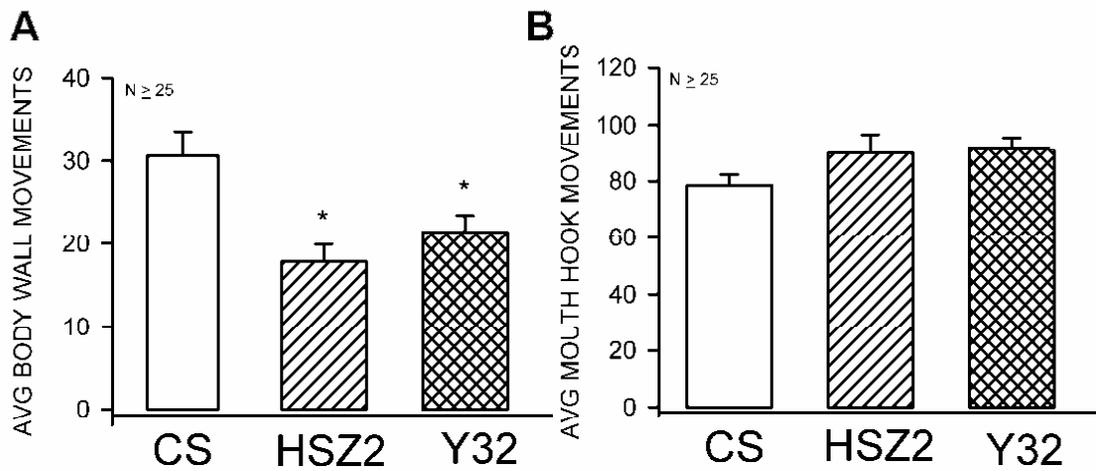


Figure 4.2: Locomotory movements of larvae that are grown at high temperature.

A) Body wall and B) mouth hook movements at room temperature. Early 3rd instar larvae of CS, HsZ2 and Y32 were given a heat pulse of 33°C and measurements were carried out at room temperature. Body wall movements were significantly reduced in transgenic lines when compared to CS ($N \geq 25$, ANOVA $P < 0.05$). No significant difference was seen among 3 groups for mouth hook movements ($N \geq 25$, ANOVA).

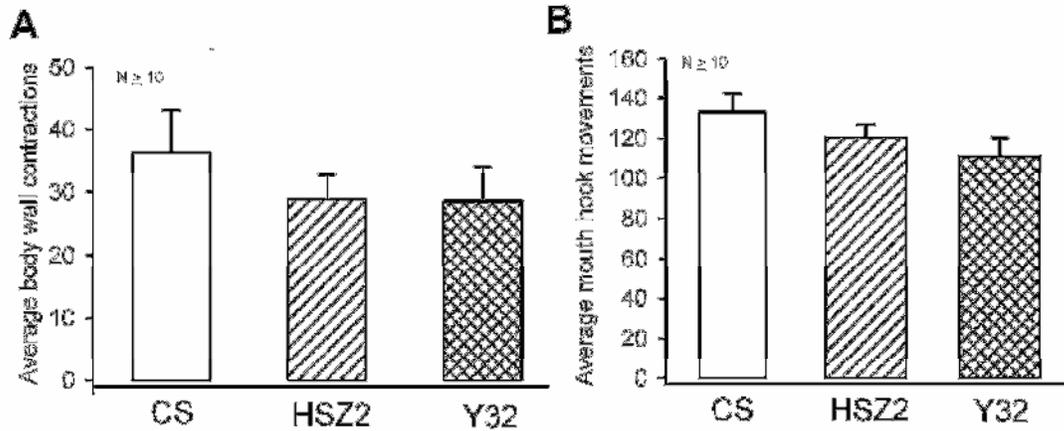


Figure 4.3: Locomotory movements at high temperature.

A) Body wall and B) mouth hook movements at 33°C. Early 3rd instar larvae for CS, HsZ2 and Y32 were given a 4Hr heat pulse and behavior measurements were carried out 33°C. No significant difference was seen among 3 groups ($N \geq 10$, ANOVA) for body wall or mouth hook movements.

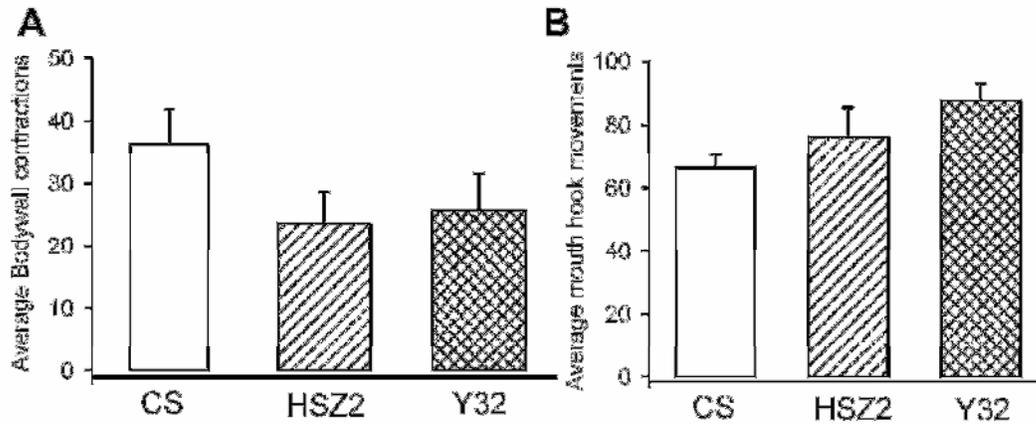


Figure 4.4: Locomotory movements at 31-32°C.

A) Body wall and B) mouth hook movements were measured for larvae that were grown 31-32°C. Early 3rd instar larvae for CS, HsZ2 and Y32 were used for behavior measurements. No significant difference was seen among 3 groups for body wall or mouth hook movements ($N \geq 10$, ANOVA).

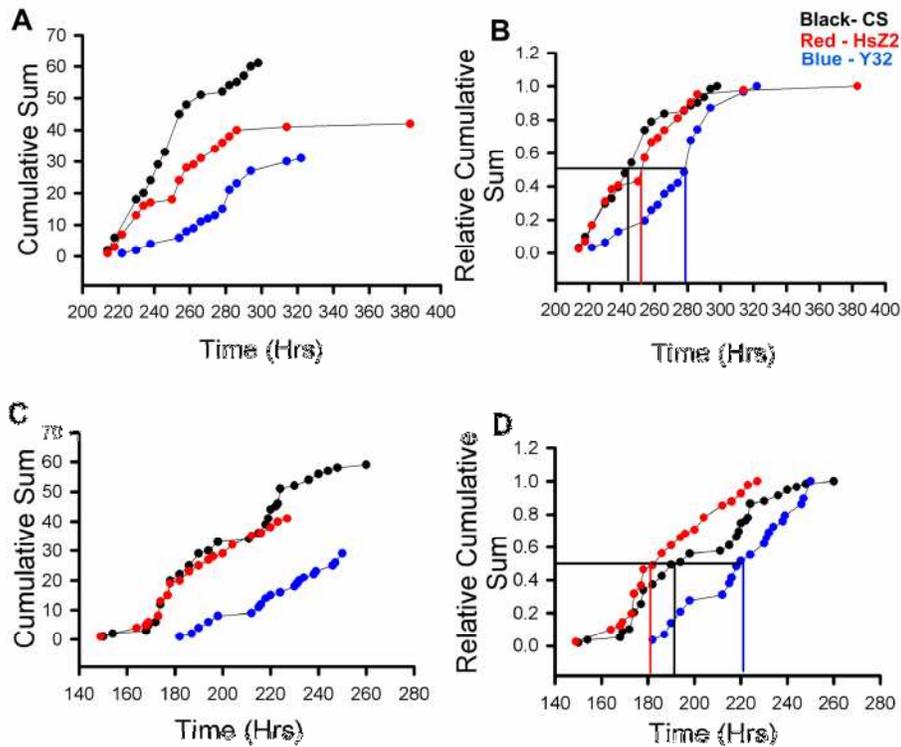


Figure 4.5: Development curve for low temperature.

CS, HsZ2 and Y32 were grown from 1st to 3rd instar at 18°C. A) Cumulative sum for time to pupation from eggs for CS, HsZ2 and Y32. B) Relative cumulative sum for A. No significant difference was seen among the 3 groups (K-S test). C) Cumulative sum for time to eclosion from pupa formation for CS, HsZ2 and Y32. D) Relative cumulative sum for C. Time for each pupa is adjusted to '0' and calculated time taken from pupation to eclosion. No significant difference for time to eclosion was observed for 3 groups (K-S test).

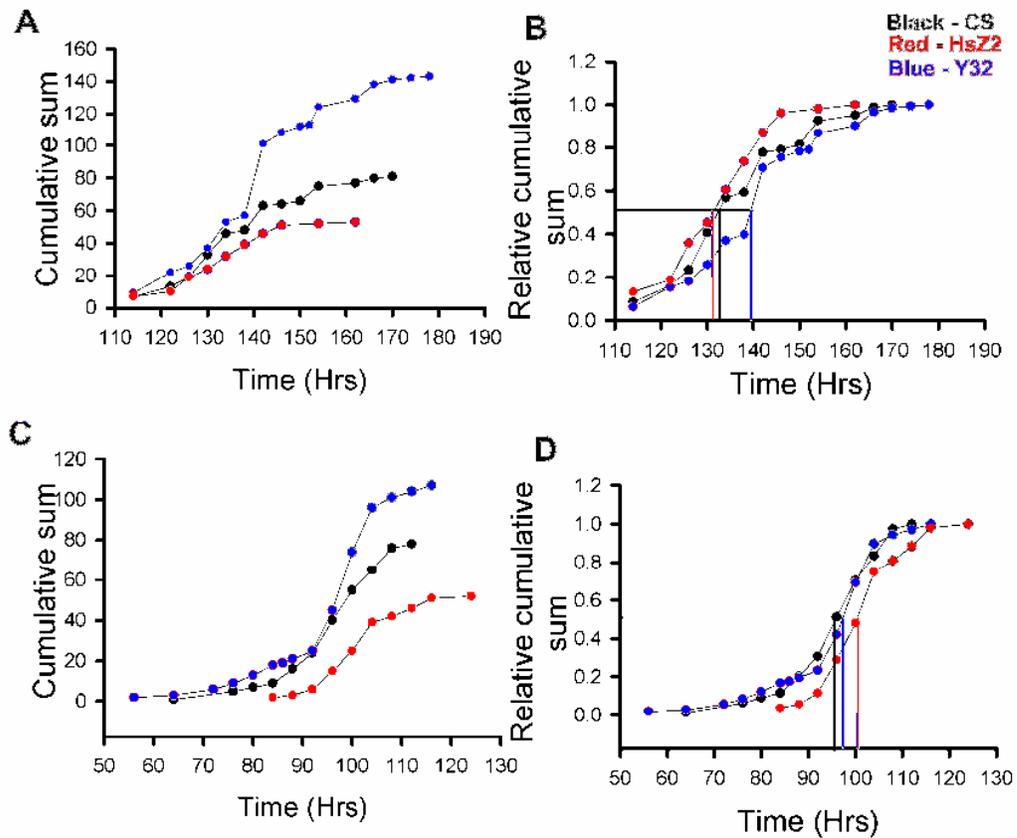


Figure 4.6: Development curve for room temperature.

CS, HsZ2 and Y32 were grown from 1st to 3rd instar at room temperature (21-22°C).

A) Cumulative sum for time to pupation from eggs for CS, HsZ2 and Y32. B)

Relative cumulative sum for A. No significant difference was seen among the 3

groups (K-S test). C) Cumulative sum for time to eclosion from pupa formation for

CS, HsZ2 and Y32. D) Relative cumulative sum for C. Time for each pupa is

adjusted to '0' and calculated time taken from pupation to eclosion. No significant

difference for time to eclosion was observed for 3 groups (K-S test).

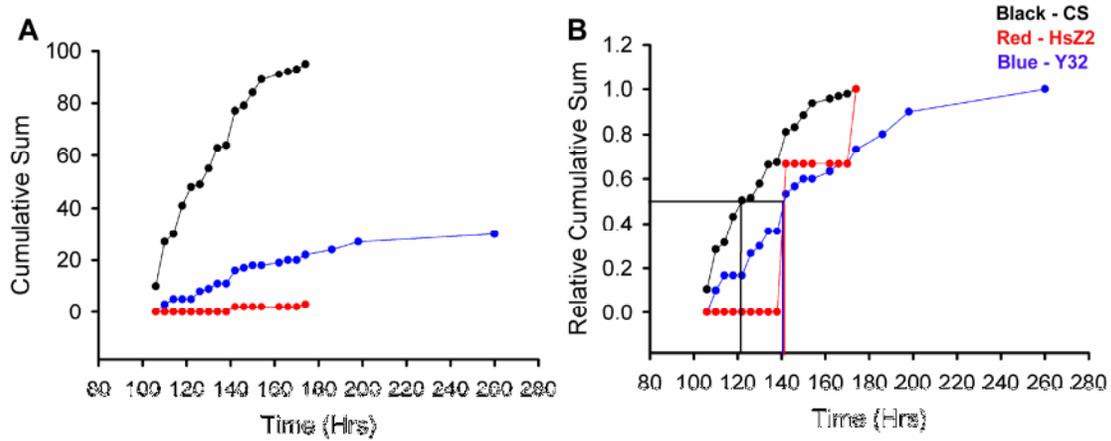


Figure 4.7: Development curve for high temperature.

CS, HsZ2 and Y32 were grown from 1st to 3rd instar 31-32°C. A) Cumulative sum for time to pupation from eggs for CS, HsZ2 and Y32. B) Relative cumulative sum for A. 50% of CS larvae took 120 hrs for pupation whereas HsZ2 and Y32 took about 140 hrs. But very few of HsZ2 and Y32 larvae reached pupation at this temperature. HsZ2 showed a significant difference from CS and Y32 (K-S test, $P < 0.01$).

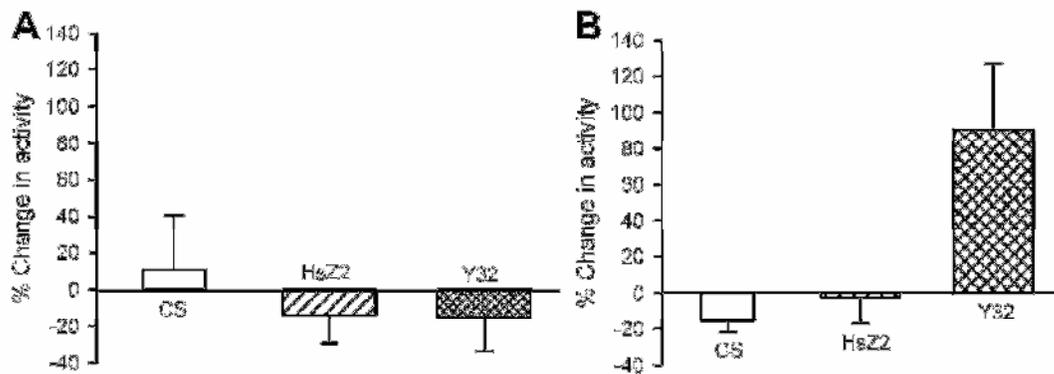


Figure 4.8: Spontaneous activity.

The percent change in frequency per second (activity) from saline to 5-HT (100nM) is measured in CS, HsZ2 and Y32 larvae grown at permissive temperature (A) and non-permissive temperature (B). A) At room temperature no significant difference in sensitivity for 5-HT 100nM was seen among the 3 groups (N=5, ANOVA). B) CS and HsZ2 larvae that were grown at high temperature showed a decrease in activity in presence of 5-HT (100nM) but not significant (N=5, ANOVA). Whereas Y32 showed a significant increase in activity (N = 5, ANOVA, $P < 0.01$)

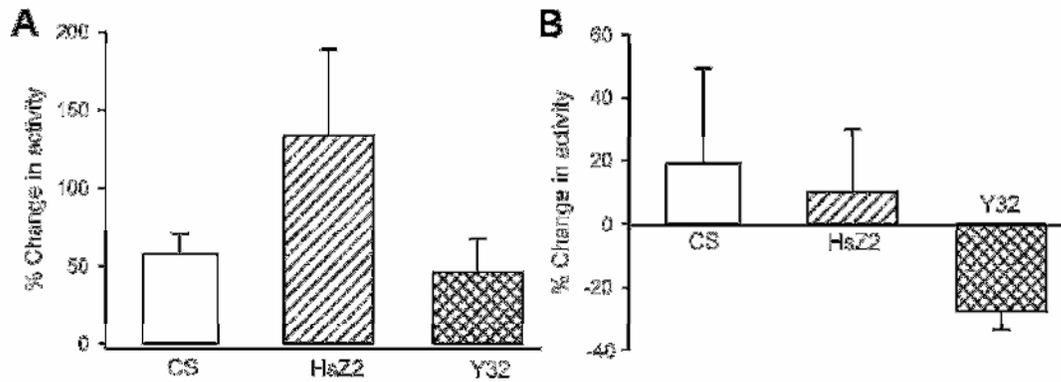


Figure 4.9: Sensory-CNS-motor circuit.

A) A percent difference in the activity of motor units to muscle 6 before and after application of 5-HT (100nM) in larvae grown at permissive temperature. All the 3 groups showed an increase in activity but no statistical difference seen (N =5, ANOVA). B) A percent difference in activity exposed to 5-HT (100nM) for larvae that were grown at non-permissive temperature. CS and Y32 showed an increase in activity but Y32 showed a decrease in activity. No statistical difference seen among the 3 groups (N=5, ANOVA).

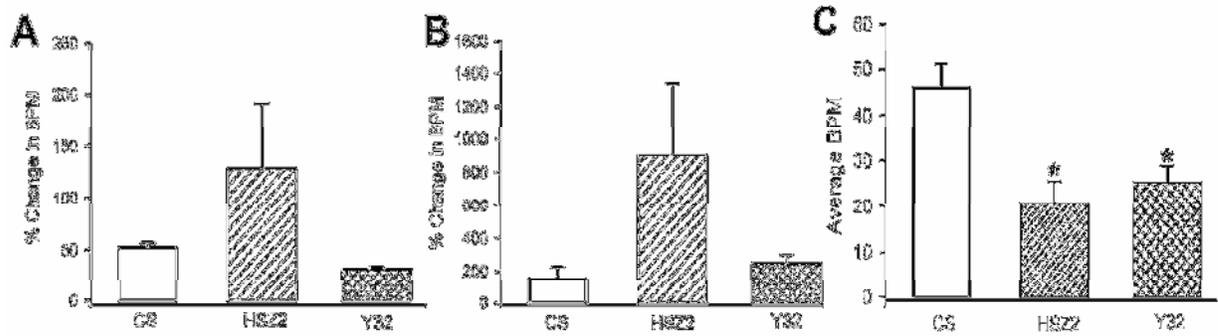


Figure 4.10: Heart rate.

The percent change in heart rate upon exposure to 5-HT (100 nM). A) CS, HsZ2 and Y32 larvae that were grown at room temperature showed an increase in heart rate with no statistical difference (N=10, ANOVA). B) CS, HsZ2 and Y32 larvae that were grown at high temperature showed an increase in heart rate with no statistical difference (N=10, ANOVA). But the initial heart rate in HsZ2 and Y32 are lower giving an abnormal % change in BPM. C) Initial heart rate for CS, HsZ2 and Y32 grown at high temperature. The initial heart rate for HsZ2 and Y32 is statistically lower as compared to that of CS (ANOVA, $P < 0.0012$). Also this group has a lower initial heart rate as compared to that of room temperature raised CS, HsZ2 and Y32. (BPM – beats per minute).

CHAPTER 5

DIRECT INFLUENCE OF SEROTONIN ON THE LARVAL HEART OF DROSOPHILA MELANOGASTER

ABSTRACT

The heart rate (HR) of larval *Drosophila* is established to be modulated by various neuromodulators. Serotonin (5-HT) showed dose dependent responses in direct application within semi-intact preparations. At 1nM HR decreased by 20% and increased at 10nM (10%) and 100nM (30%). The effects plateaued at 100nM. The action of 5-HT on the heart were examined with an intact CNS and an ablated CNS. The heart and aorta of dorsal vessel pulsate at different rates at rest and during exposure to 5-HT. Splitting the heart and aorta resulted in a dramatic reduction in pulse rate of both segments and the addition of 5-HT did not produce regional differences. The split aorta and heart showed a high degree of sensitivity to sham changes of saline and no significant effect to 5-HT. Larvae fed 5-HT (1mM) did not show a significant change in HR. Since MDMA is known to act as a weak agonist on 5-HT receptors in vertebrates, I tested exogenous application however no significant effect from 1nM to 100uM was observed in larvae with and without an intact CNS. In summary, direct application of 5-HT to the larval heart had significant effects in a dose-dependent manner and MDMA had no effect.

INTRODUCTION

The various functional aspects of heart regulation in development to normal maintenance throughout the larval life and metamorphosis of *Drosophila melanogaster* are being extensively addressed (Ashton et al., 2001; He and Adler, 2001; Molina and Cripps, 2001; Ponzielli et al., 2002; Sláma and Farkaš, 2005; Wessells and Bodmer, 2004). In addition, regulation of the heart via hormonal and direct neural innervation continues to be an active research area using this model organism (Dulcis and Levine 2005; Dulcis et al. 2005; Johnson et al. 2002; Miller, 1997; Papaefthimiou and Theophilidis, 2001). Relatively recently 5-HT receptors (5-HT_{1B/1CR} and 5-HT_{2A/2BR} subtypes) were shown to be present in the vertebrate aortic and mitral valve cells of the heart (Fitzgerald et al. 2000; Roy et al. 2000) and linked to some forms of cardiac valve disease with altered regulation (Jian et al. 2002). Since the larvae develop quickly and the age can be precisely timed, the development of the heart from embryo throughout growth of larval stages, as well as through metamorphosis from larvae to adult, can be readily examined within a matter of 1 to 2 weeks. High throughput screening is possible to assess multitudes of pharmacological agents or mutational screens (Gu and Singh 1995).

The *Drosophila* heart, better known as the dorsal vessel, is a continuous tube extending from the last abdominal segment to the dorso-anterior region of the cerebral hemisphere. The heart is divided into anterior aorta and posterior heart (Rizki, 1978). The heart does not pulsate constantly as noted when larvae are preparing to crawl (Rizki 1978). When a larva is engaged in feeding the pulsation of

the heart and the motion of the mouth hooks are seen to be functionally related. This is partly due to the fact that the terminal ligaments of the aorta are attached to the pharyngeal region of the alimentary canal.

In the larvae the origin of heartbeat is known to be myogenic (Dowse et al. 1995; Johnson et al. 1997) however, the larval heart has not been shown to have innervation to date. Where as in the adult, Dulcis and Levine (2003) have shown that the heart is innervated. Thus, the current dogma was that the larval heart is myogenic without innervation. Johnstone and Cooper (2006) did show that the aorta is innervated but they did not observe the true region of heart to be innervated. (It should be noted that this observation was noted after this chapter had been published. Therefore some interpretations of this chapter should be considered in regards to the published current findings, Johnstone and Cooper, 2006) The HR in larvae can be altered by neurotransmitters and neuromodulators present in the hemolymph (Johnson et al. 1997, 2000; Nichols et al. 1999; Zornik et al. 1999). Injections in 3rd instars and early pupa (P1 stage, transition between larva and pupa) of serotonin (5-HT), dopamine (DA), acetylcholine (Ach), octopamine (OA), and norepinephrine (NE) raise the HR (Johnson et al. 1997). Effects on HR of Drosophila peptides have also been examined (Johnson et al. 2000; Nichols et al., 1999; Zornik et al., 1999).

In pupa, injection of 5-HT (1 μ M/l) caused the HR to increase by 46% from the base line (Johnson et al. 1997). A similar study by Zornik et al. (1999) also showed, in the wandering 3rd instar larva, that 5-HT increases HR by 111% with a concentration of 10^{-5} M (10 μ M/l) injected into the animal. In the Zornik et al. (1999)

study it was determined that the adult heart is more responsive to 5-HT than the wandering 3rd instar larvae or the pupal heart.

Injection through the larval body wall or into pupal case of biogenic amines can cause the activation or release of many other compounds, related to stress or injury, into the hemolymph from endogenous sources that cannot be controlled for during the experiment. Thus, we opened the larva in a bath of physiological saline that washes away the hemolymph to test the direct effects of compounds on the heart within a defined saline. Since we are not aware of any attempt to clearly determine if the larval heart is or is not innervated we assayed the effects of compounds with and without the CNS intact. Also since exogenous application of a neuromodulator could cause the local release of neuroendocrine compounds we wanted to eliminate the possibility. In addition, we have shown earlier that OA, DA, and 5-HT have direct actions on the larval CNS neural activity (Dasari and Cooper, 2004). Also in adult flies its known that the rate of stress with age is correlated with heart failure. In addition, HR decreases with age (Wessels et al, 2004). So to avoid the stress induced variables on HR that could occur with injections, we examined if feeding larvae 5-HT would have an effect on heart rate in intact preparations.

Many drugs of abuse and mind altering therapeutic medicines have actions through the serotonin-ergic system, with some altering the presynaptic reuptake mechanisms or acting directly as an agonist/antagonist at the 5-HT receptors. One compound of particular interest to understand is MDMA (3,4-methylenedioxymethamphetamine, ecstasy) and its direct action on 5-HT receptors. Invertebrates have proven to be useful on this front in studying drugs of abuse since

they possess physiological systems with few compounding variables and relatively fast developmental times (Hirsh, 2001; Rothenfluh and Heberlein, 2002; Sparks et al., 2004; Wolf and Heberlein, 2003). MDMA is a ring-substituted amphetamine and is a widely abused drug among young people worldwide. MDMA is a potent releaser and also reuptake inhibitor of 5-HT, DA and NE (Green et al., 2003). In humans the acute adverse effects that are seen after MDMA ingestion are elevated blood pressure, increased heart rate, nausea, chills, sweating tremor, bruxism probably through actions on the autonomic nervous system (de la Torre and Farre 2004; Green et al., 2003; McCann et al., 1996; Peroutka et al., 1988). Previously it was demonstrated that MDMA has direct action on neuronal 5-HT receptors in an arthropod; thus, we also examined in parallel to 5-HT's effects the action of MDMA on the *Drosophila* heart with an intact and ablated CNS.

The purpose of this study is to observe the acute effects of 5-HT and MDMA on heart rate in intact and semi-intact 3rd instar larval *Drosophila* preparations. In addition, we wanted to know if an intact CNS altered the responsiveness of the heart to these compounds possible through direct innervation of the heart or aorta.

Preliminary results of this study were presented in abstract form (Dasari et al., 2004).

METHODS

Stock and Staging of Larvae

The common 'wild-type' laboratory strain of *Drosophila melanogaster*, Canton S, was used in these studies. The methods used to stage fly larvae have been described previously (Campos-Ortega and Hartenstein, 1985; Li and Cooper, 2001).

All animals were maintained in vials partially filled with a cornmeal-agar-dextrose-yeast medium. Larvae of the early third instar phase were used in these experiments. The general dissection technique and HL3 saline content has been previously reported (Cooper and Neckameyer, 1999; Stewart et al., 1994). The HL3 saline was derived from direct measures with ion sensitive electrodes of larval hemolymph and maintains normal function of larval neuromuscular junctions and the CNS (Ball et al., 2003; Dasari and Cooper, 2004). In brief, the HL3 saline was prepared in the lab from component reagents (Sigma) and contained: 1.0 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 20 mM MgCl_2 , 70mM NaCl, 5mM KCl, 10mM NaHCO_3 , 5mM trehalose, 115mM sucrose, and 5mM BES (N,N-bis [2-Hydroxy-ethyl] -2-aminoethane-sulfonic acid). The HL3 was freshly controlled for pH and temperature prior to experimentation, as the pH will drift during storage.

Heart Rate (HR) measures

With a microscopic method, the movements of the trachea or heart were used for direct counts of HR. A microscope (adjustable zoom 0.67 to 4.5; World Precision Instrument, FL, USA) fitted with a 10X eye objective was used for visual observations. With visual inspection one can readily observe the heart beating or the trachea movements as a consequence of the heart pulling on the ligament attachments (Fig. 5.1A). The movements of the trachea are commonly used to monitor *Drosophila* larval heart rate because of the clear contrast of the structures (Dasari and Cooper, 2005; Johnson et al., 1997; Miller, 1985; White et al., 1992).

Semi-intact preparation

The HR was also used to examine the direct effects of MDMA and 5-HT, at different concentrations, in exposed hearts of larvae with an intact and ablated CNS. These semi-intact animals were of early 3rd instar that had been dissected ventrally and pinned on four corners (Fig. 5.1B). Guts and all the visceral organs (including the brain for deinnervation) were removed in such a way that the heart is intact and still attached to rostral and caudal ends of the larvae. This dissection technique has been used to directly assess pharmacological agents on the heart of *Drosophila* larvae (Gu and Singh, 1995). The dissection time was 3-6 min. The preparation was allowed to relax while bathed in HL3 saline for 3-5 min after dissection. The heartbeats were counted for a minute at every 5 min for 20 mins, initially in saline and then with 5-HT containing saline. Time '0' is taken at the 1st min of counting in saline. 5-HT is added to the preparation at the 7th min and a fresh dose is added again at 12th min. The average heart beat at the 1st and 5th min for saline and at 15th and 20th min for 5-HT or MDMA was used for analysis.

The HL3 saline was carefully controlled to be at pH 7.2 since HR slows at high pH and speeds up at lower pH (Badre et al., 2005). Gu and Singh (1995) used pH 7.0 for the pharmacological analysis of the heart and also showed maintained viability. During these trials the saline remained aerated by agitation of solution through repetitively injecting saline, through a 21-gauge needle, into a beaker.

To quantify HR either direct observations were used or the images were recorded on to VHS tapes and analyzed by a photodiode. In the cases in which the photodiode was used, the detector (model 276-142, Radio Shack, USA) was placed

in the back of a black plastic 35mm film canister and the open end was held over the region on the monitor screen in which the heart and caudal end of the larvae was magnified. The output of the photodiode was amplified by use of an impedance amplifier. The impedance detectors (UFI, model 2991) allowed HR to be monitored as a measure of dynamic change in the light path across the photodiode during each heart contraction. These signals were recorded on-line to a PowerMac 9500 via a MacLab/4s interface (ADInstruments, Australia). Events were measured and calibrated with the MacLab Chart software version 3.5.6 (ADInstruments) with an acquisition rate set at 4kHz. The HR was determined by direct measures with a window discriminator, which measured a running average of instantaneous events. The values were then converted to beats per minute (BPM). Similar procedures in the use of an impedance amplifier were used as described in earlier studies for obtaining heart rates and ventilatory rates in crayfish (Dasari and Cooper 2005; Li et al., 2000; Listerman et al., 2000; Schapker et al., 2002).

Aorta and Heart separation

Wandering 3rd instars are opened as described above. HR was measured for 2 min and then heart and aorta are separated by severing them at the junction with fine scissors. HR was measured for another 2 min before applying saline containing 5-HT.

Intact preparation

Early 3rd instar larvae were glued ventrally on a glass slip using super-glue in such a way that mouth hooks are free to move. Care was taken not to glue the spiracles so that only ventral aspect within the mid-length was adhered to the glass. Yeast mixed in either 5-HT or water was placed over the head of the animal. The yeast stimulated the animal to eat, thus consuming the yeast and the compounds. The larvae were glued down and HR measured for 2 minutes with water and yeast followed by another 15 minutes while feeding on 5-HT containing solution. Heart beats were counted for 30 sec intervals starting initially at the time periods between 1-2 mins, 6-8 mins.

Statistics

All the groups were first tested with one-way ANOVA and followed with Bonferroni's posthoc analysis. Some of groups were also analyzed using Student's t-test.

RESULTS

Semi-intact preparation

The direct exposure of the heart tube allows individual compounds to be directly assayed on the heart without compounding variables introduced from endogenous hormones and substance contained in the hemolymph. After pinning the dissected larvae open, the saline bathing solution was changed to one containing 5-HT or MDMA. Six different concentrations of 5-HT and three different concentrations of MDMA were examined. The 1nM 5-HT exposure resulted in a

significant decrease in HR (Fig. 5.2A, ANOVA $p < 0.05$, $n = 10$) as compared to the saline sham exposures. Saline exchange by itself results in a slight increase in HR in some preparations but not a significant effect for all animals. However, we felt that experimentally it was important to provide sham controls, as this is a valid issue when examining the effects of 5-HT and MDMA in isolated heart segments (see below). The increased concentrations of 5-HT at 10nM produced a substantially increased HR (Fig. 5.2A, ANOVA $p < 0.05$, $n = 10$). However at a 100nM the increase in HR from sham control reached a maximum effect (ANOVA $p < 0.05$, $n = 10$) compared to the 1 μ M, 10 μ M, and 100 μ M. There was no further significant increase at these higher concentrations compared to 100nM but all were significantly greater in increased HR from the saline control (ANOVA $p < 0.05$, $n = 10$). Thus, the 5-HT receptors were likely saturated at 100nM with only modest increases in the mean HR for the higher concentrations. It should be noted that each preparation was treated individually and not exposed to a series of experiments. At concentrations of 10 μ M 5-HT and above, the heart is in tetany and cannot relax well between beats. This is likely the prime reason for the saturating effect in further increases of HR with exposure to higher concentrations.

Exposure to MDMA at various concentrations did not have a significant effect on the HR (Fig. 5.2A,B). The animals were followed for at least 20 minutes without any notable consequences related to HR.

Since there is no known innervation from the brain to the dorsal vessel we examined larva with the CNS left intact (Fig. 5.2A and 5.2B) and in ones where it was carefully removed (Fig. 5.2C and 5.2D). To test whether a potentially de-

innervated heart showed any differences in HR compared to larvae with an intact CNS, the dissected larvae with and without the CNS were exposed to 5-HT and the change in HR noted. There was significant decrease in HR to 5-HT at 1nM (Student's t-test $p < 0.05$, $n = 10$) but no significant decrease with 5-HT at 10nM and MDMA at 1 μ M ($n = 10$) as compared to saline exposure. As with the intact CNS preparations 100nM 5-HT showed a significant increase in HR (ANOVA $p < 0.05$, $n = 10$, Fig. 5.2C). Comparing the differences for intact and ablated CNS the effects of MDMA caused a decreased HR at 1 μ M in larvae without a CNS (Student's t-test $p < 0.05$, $n = 10$). In addition, a reversal in the effects of 10nM 5-HT in ablated CNS preparations was observed. Without the CNS the 10nM 5-HT had a significant effect in reducing the HR (Student's t-test $p < 0.001$, $n = 10$; compare Fig. 5.2A and 5.2C for 10nM). The mean, median, 95% confidence levels and the range of the distributions in the data sets are shown in the whisker box plots for the intact CNS (Fig. 5.2B) and for the preparations with an ablated CNS (Fig. 5.2D).

Heart and Aorta separated

During the initial experiments when exposing the hearts with a ventral dissection it became apparent that the dorsal vessel had a different rhythm along its length. These regional differences in pulse rates were not observed in the intact preparations. In addition, exposure of the entire dorsal vessel in the ventral dissected preparations to 5-HT suggested regional differences in the actions of 5-HT. To address this issue the heart and aorta were transected at their junction. The rate of beating of aorta and heart was observed in saline and during exposure to 5-

HT (100nM). We chose 100nM concentration as this level produced a significant increase in HR from saline without inducing a tetanic state of the heart in whole dorsal vessel. MDMA was not used in these preparations, as it didn't show any effect in direct exposure to the heart.

The number of beats within the aorta and heart were measured prior to transection and then again after a saline wash or a saline wash containing 5-HT. Comparisons were made by determining a percentage difference for the separated aorta and heart to the intact state of the aorta and heart. The effect of direct exposure of 5-HT (100nM) produced a substantial increase in HR (Fig. 5.3A, ANOVA $p < 0.05$, $n = 10$) in the heart when the dorsal vessel is left intact. However after transection the preparation became very sensitive to changes in the bathing media. When sham controls for mechanical disturbances on the heart rate were compared to saline changes containing 5-HT, there is no significant effect of 5-HT. Saline exchange alone increased the rate of the aorta significantly (Fig. 5.3B, ANOVA $p < 0.05$, $n = 14$). A small effect was also produced on the true heart with saline exchange (Fig. 5.3B, ANOVA $p < 0.05$, $n = 14$). The exposure to 5-HT also had a substantial effect on the rate of both the heart and aorta but not significantly over the sham control effects ($n = 7$). Therefore, we concluded that 5-HT did not show any differential effects on the rate of the two segments when the dorsal vessel segments were isolated from each other.

Intact Preparation

In examining the possibility of 5-HT gaining access to the hemolymph and altering HR, we fed early 3rd instars 5-HT (1mM) mixed with yeast. Control experiments of water and yeast were also conducted for the same period of time. The controls maintained a steady HR for 8-10 minutes, however the larvae eating a 5-HT tainted diet after 6-8 minutes dropped in HR for some larvae but not others. The change was not significant (Student's t-test) for 5-HT fed larvae compared to controls. The same larvae feeding on 5-HT were followed further for 12-15 minutes, and in some preparations HR was not noticeable even though the larvae were alive as indicated by mouth hook movements. The cardiac arrest was not due to tetany of the heart. In other larvae no change in HR occurred before or after 15 minutes of eating the 5-HT containing food. Thus, there was a large variation observed in the 5-HT fed larvae as compared to controls.

DISCUSSION

In this study we showed that the larval *Drosophila* heart can be examined in a semi-intact state in which the hemolymph can be exchanged with saline in order to reduced compounding variables of endogenous neuromodulators or induced release due to systemic injections of pharmacologic compounds. The minimal HL3 saline maintains the HR, thus pharmacological assays can be run with an intact or ablated CNS for high output screening as to direct actions on cardiac function. The larval heart in a semi-intact state (heart and aorta connected) decreased its rate at 1 nM 5-HT but increased in rate with exposure higher than 10nM with a plateau in the enhancement at 100nM. Ablating the CNS resulted in a decline in HR for 5-HT

exposure at 10nM however increased for higher concentrations. Separating the dorsal heart tube into the true heart and aorta while exposure the segments to 5-HT or saline both resulted in an increase HR with no discernable effects to 5-HT alone over the sham effects of changing the saline bath. It is of interest to note for future pharmacological investigations that the heart is very sensitive to exchange of the bathing media. This was also noted in earlier studies in examining the effects of pH on HR (Badre et al., 2005). Lastly, 5-HT introduced in the diet at a high concentration of 1mM did not alter HR significantly but did result in an increased variability in HR, thus suggesting feeding larvae this particular biogenic amine to address effects on HR might not be feasible.

Since direct exposure of the heart tube to 5-HT resulted in biphasic changes in HR with low concentration decreasing HR while higher concentrations increased HR beacons the possibility of different 5-HT receptor subtypes with varying degrees of binding affinity and/or activated second messenger cascades on the heart tissue (Johnson et al., 2002). It is known that the *Drosophila* genome contains 4 different 5-HT receptor-coding sequences (Colas et al., 1995; Monastirioti, 1999; Saudou et al., 1992; Witz et al., 1990). However, posttranslational modifications or regulated RNA editing of the 5-HT receptors in *Drosophila* might occur as known in vertebrates for some 5-HT receptors subtypes (Paupard et al., 2000; Slominski et al., 2003; Visiers et al., 2001). Differences in RNA editing is also a possibility over the development of the larvae as shown for different types of receptors in mammals (Lee et al., 2001), thus one should consider pharmacological and molecular analysis at various life stages for a complete assessment in subtypes of 5-HT receptors. In this study we

only examined early 3rd instars, which predominantly express the 5-HT_{2dro} receptor subtype (Colas et al., 1995), however previous studies did not specifically address expression in heart tissue developmentally. A complete pharmacological profile of agonists and antagonists to 5-HT receptors would be advantageous in helping to address the receptor subtypes in cardiac modulation within *Drosophila*. Although one should also be careful in assuming the vertebrate pharmacological profiles correctly characterizes the invertebrate systems (Sparks et al., 2003). Such differences might explain why the heart is not sensitive to MDMA but that the larval CNS is very sensitive to exogenous application (Dasari et al., 2004).

A number of studies have addressed the effects of biogenic amines and peptides on cardiac function in larval and pupal stages (Johnson et al. 1997, Zornik et al., 1999). The pupal stage offers the advantage in that it does not require to be restrained. Even the larval heart can be viewed in freely wandering larvae but it is considerable harder to maintain a good assessment of heart function continuously (Dasari and Cooper, 2005). Thus, gluing or taping larvae have been preferred methods to restrain the animal for viewing of the tracheal movements or the heart directly. The introduction of pharmacological agents has also been preferred by injection through the cuticle into the hemolymph. Although such methods have certain advantages one is not confident to the effects of the stress in relation to the animal dumping various endogenous biogenic amines and peptides into the hemolymph. One also needs to consider heat stress is known to alter biogenic amine levels in *Drosophila* (Hirashima et al., 2000) In addition circadian patterns of 5-HT are known to occur and could alter the responsiveness to exogenous

applications (Fowler et al., 1972). This was one reason that we chose to dissect the larvae and expose the heart directly to 5-HT in a defined physiological saline at a given temperature and period of day (12:00-18:00). This semi-intact preparation also offers the advantage to introduce various compounds over time and wash out previously introduced substances (Gu and Singh, 1995). An additional advantage of the dissected preparation that has not previously been addressed is the fact that the CNS can be left intact or removed. Leaving it intact and using a small volume of bathing media one can address if compounds from the CNS can be induced for release for examining an effect on cardiac performance. In addition if any neuronal connections do exist on the larval heart they can be removed or left intact for assaying various exogenous agents. Earlier studies in which TTX was bathed on the exposed heart did not produce any significant alterations in HR (Gu and Singh, 1995) and thus it was assumed that the larval hearts are myogenic but this does not prove that the hearts are not modulated by neuronal innervation as for mammals. Since we did note that differences do occur in sensitivity to 5-HT in preparations with an ablated CNS, this suggests that there may indeed be a neural input that is modulated by 5-HT possibly at presynaptic nerve terminals on the heart as for skeletal muscles in crustaceans (Southard et al., 2000). The activity of the CNS circuitry in larvae is known to be influenced substantially by 5-HT as well as by octopamine and dopamine (Dasari and Cooper, 2004). This issue of innervation of the heart tube remains to be addressed anatomically in larval *Drosophila*.

Earlier reports have shown that injection of 5-HT into 3rd instars and P1 pupal stage of *Drosophila melanogaster* Oregon R and Canton S strains respectively,

causes an increase in HR (Table 5.1). In this study, we have shown that direct exposure of heart to 5-HT in 3rd instars of Canton S strain also causes it to increase at high concentrations but at low concentrations of 1nM it decreases. In the previous concentration ranges examined 5-HT produced a larger increase in HR than what we report for semi-intact preparations. The possibility of synergistic actions of endogenous compounds with the injected 5-HT is one possibility for enhanced effects of 5-HT. As shown in crustaceans and leech, 5-HT has different effects in the presence of octopamine as compared to 5-HT exposure alone (Djokaj et al., 2001; Mesce, 2002). Given that there are many active substances contained in the larval hemolymph, the biogenic/peptide cocktails remains an interesting avenue for further research in isolated preparations. On the other hand the dissection of the larvae and exposure to HL3 physiological saline might not provide the correct ionic environment for cardiac function. The HL3 saline was originally developed and assayed for skeletal neuromuscular transmission, however the ions were determined from larval hemolymph samples (Stewart et al., 1994).

The ability to feed larval and adult *Drosophila* compounds to alter endogenous neuromodulators has been used with success (Neckameyer et al., 2001) but this approach is likely not feasible for readily degraded compounds by an acidic environment or that are photo liable such as 5-HT (Lisi et al., 2003; Strawn et al., 2001). Saliva of adult *Drosophila* contains enzymes known to digest chitin as well as amylose and cellulose (Gregg et al., 1990), thus possibly larvae also contain such abilities. Thus, we are not convinced that feeding the larvae food tainted with 5-HT (1mM) resulted in any significant change in hemolymph levels since injection into

larvae or direct application on to the heart at lower concentrations resulted in a change of HR.

Much to our dismay the transected heart tube into the true heart and aorta became very sensitive to any alterations in changing of the bathing media which made it difficult to assay differential effects of the aorta and the true heart to exposure of 5-HT. It is established that the heart tube contains pacemaker activity but the extent and control of a major pacemaker in the heart and aorta segments has not been addressed in relation to biogenic modulation. Transecting the heart tube into the true heart and the aorta resulted in the two segments beating at their own intrinsic rates. The aorta significantly slowed down and the true heart increased in rate. Thus, uncoupling the caudal-anterior communication within the heart tube had some effect on possible unmasking some feedback or inhibition of rate in the intact true heart where the lack of the master pattern activity of the heart on the aorta resulted in the slower aorta pacemaker activity, which is analogous to mammalian hearts among the pacemaker regions. Possibly after the heart tube is separated there is a reduction in filling the aorta or less peripheral resistance for the heart between pulses which could then effect myogenic stretch related activity. It is also conceivable that the micro-environments in the cut vesicles related to perfusion of the saline to luminal surface of the vessel is altered which might explain the heightened sensitivity to saline exchange and exposures to 5-HT.

Developing a means to introduce agents without causing mechanical disturbance of the bathing media will help in examining localized effects of pharmacological agents on the true heart and the aorta separately. We are currently

examining explant cell culture of *Drosophila* heart tissue to determine its feasibility. Electrophysiological recordings would give an insight into the ionic properties of the pacemakers and the mechanistic effects of the various known modulators on the *Drosophila* heart.

Table 5.1

Effects of 5-HT on HR as percent difference from base line as reported in various studies for comparison.

	1mM	100 μ M	10 μ M	1 μ M	0.1 μ M	0.001 μ M	Referenc e
Injection 3 rd instar	↑ 120 ± 6	↑ 123 ± 5	↑ 111 ± 4	NS	-	-	Zornik et al., 1999
Injection P1 stage	↑ 75	↑ 55	↑ 28	↑ 46	↑ 15	-	Johnson et al., 1997
Direct application 3 rd instar	-	↑ 38 ± 5	↑ 29 ± 4	↑ 27 ± 3	↑ 31 ± 5	↓ 17 ± 3	This study

The effect of 5-HT on heart rate. The percent difference in heart rate from the base line after injection of 5-HT as reported in earlier work is shown. Zornik et al. (1999) injected different concentrations of 5-HT in 3rd instar larva. Johnson et al. (1997) injected 5-HT into P1 stage, the transition stage between 3rd instar and pupa.

FIGURES:

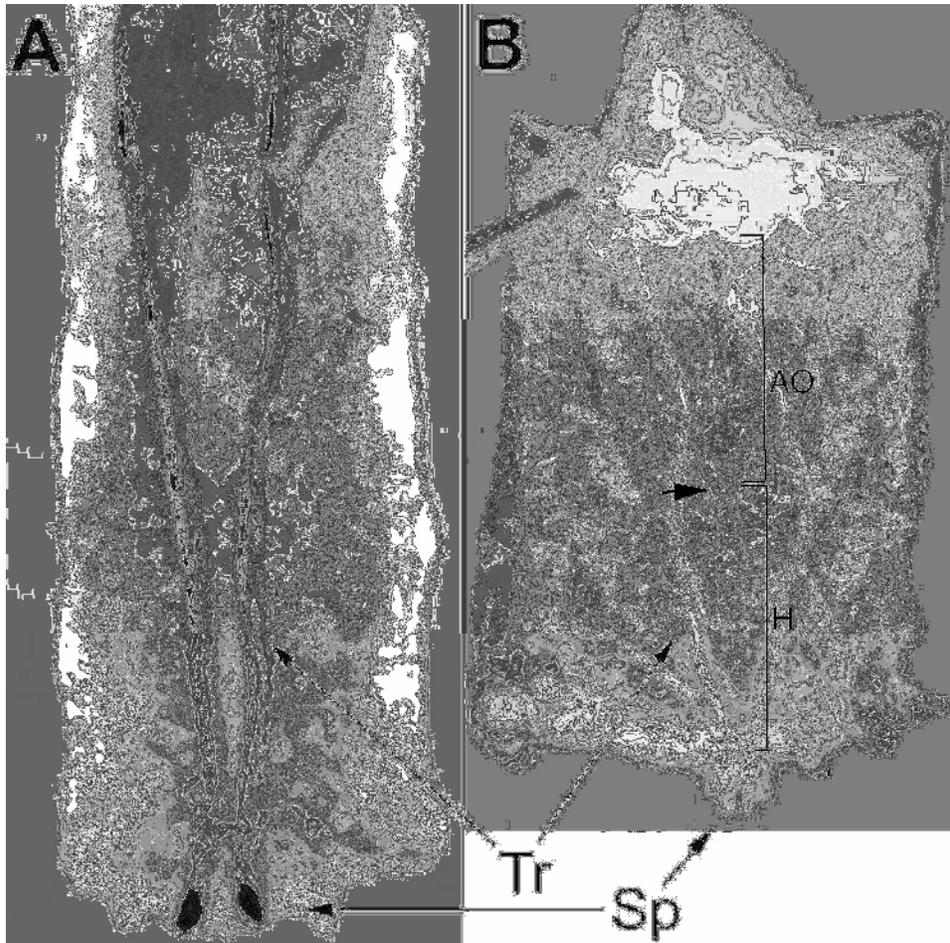


Figure 5.1: Dorsal Vessel.

A) Dorsal view of an intact 3rd instar larva. The movement of trachea due to pulling of the attachments from heart is used to observe the heart rate. B) Ventral dissection of a 3rd instar to view the heart directly. Pinning of the animal on its back after dissection is used to directly apply the compounds on the heart with or without the CNS intact. Small arrow indicates where the heart and aorta are separated for transected dorsal vessel studies (see below). Tr, Trachea; Sp, Spiracles; H, Heart; AO, Aorta.

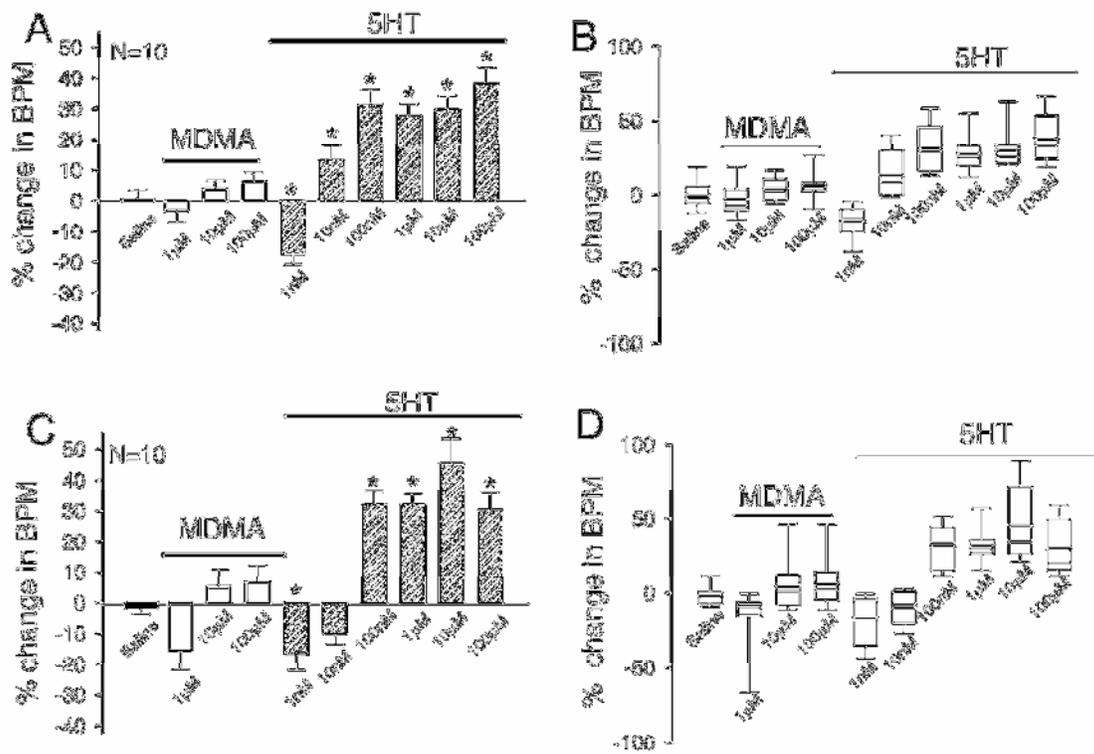


Figure 5.2: Heart rate of 3rd instar semi-intact preparations.

A) % change in heart rate from saline per minute in innervated preparations (brains attached). B) 95% confidence intervals for the same data set as in A. C) % change in the heart rate from saline in deinnervated preparations (CNS ablated). D) 95% confidence intervals for the same data set as in C. BPM, beats per minute. A star denotes ANOVA, $P < 0.0001$, followed by Bonferroni test, $P < 0.05$.

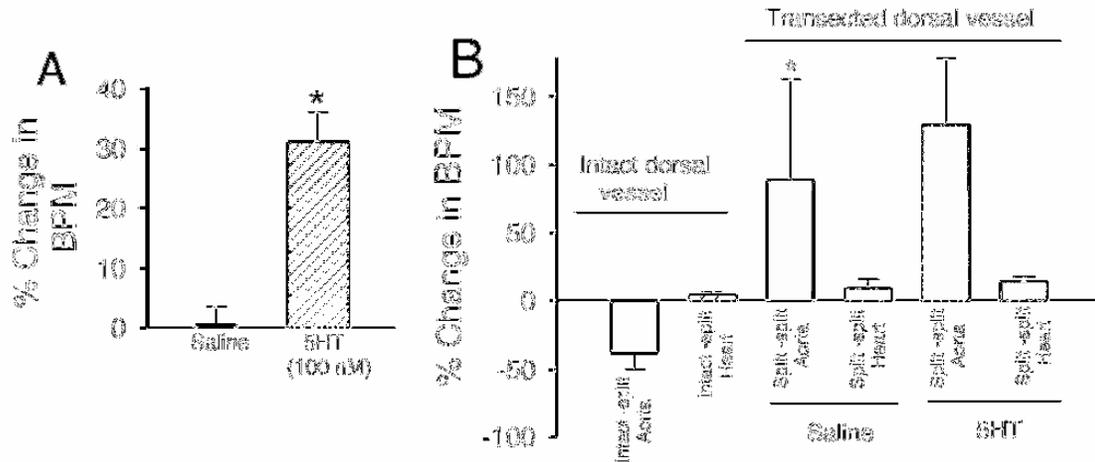


Figure 5.3: The effects of 5-HT on intact and on the isolated heart and aorta segments.

The number of beats were measured before and after severing the dorsal vessel at the aorta and heart junction. A) Percent change in the heart rate from saline to exposure to exogenous 5-HT (100nM) in an intact dorsal vessel. B) The beat rate was measured in aorta and heart separately before and after dividing the vessel. A percent change is calculated for intact to split aorta and heart in saline (n =14). The percent change in HR before and after saline wash in the split aorta and heart preparations revealed a significant increase in rate (n=14; ANOVA, $P < 0.0002$, Bonferroni test, $P < 0.05$). Exposure to 5-HT (100nM) produced a larger mean effect but there was also considerable variation among the preparations, thus no trend to 5-HT exposure was observed as compared to the sham saline effects (n=7).

CHAPTER 6

DISCUSSION

In these studies I have shown that the neuromodulators 5-HT, OA and DA have a role in modulating the CNS in larval *Drosophila melanogaster*. In order to examine the effect of these neuromodulators on the larval CNS I developed an in situ preparation consisting of an intact sensory-CNS-motor central circuit in which exogenous applications could be applied. All three neuromodulators resulted in an increase in the firing frequency in the circuit; however, exogenous application of OA and DA at NMJ caused a decrease in EPSP amplitudes where as 5-HT showed no effect. Also, I have shown that 5-HT modulates heart rate (HR) in *Drosophila* larva. Cardiac activity is an important physiological function that I used in parallel in addressing issues related to manipulating the levels of 5-HT in the whole animal as well as 5-HT receptor subtypes. The heart 5-HT assay serves as an additional tissue to examine along with CNS function. The modulatory effect on the sensory-CNS-motor circuit and the potential relationship on behavior, synaptic growth and plasticity interested me in pursuing their role on developmental processes in larval *Drosophila*. Many drugs of abuse such as cocaine, amphetamine and MDMA (ecstasy) are known to alter the serotonergic system in mammals. These drugs also have the potential of effecting synaptic transmission, behavior and development.

In vertebrates, 5-HT is known to play a role in development, physiological process, behaviors, and to be associated to many pathological diseases. In this study I have shown, when 5-HT is depleted in *Drosophila* larvae by feeding p-CPA, the developmental rate is retarded. Also, the percent of death from 1st to pupal stage

is (p-CPA) dependent of 5-HT. Hence, 5-HT plays a very important role in larval *Drosophila* development. Locomotor behavior (body wall movements) and mouth hook movements are reduced in larvae fed p-CPA. These behavioral responses may in part be accounted for by the decreased responsiveness of the sensory-CNS-motor circuit to exogenous application of 5-HT in p-CPA fed animals. Since there is a reduced responsiveness to exogenous applications it is likely that synaptically released 5-HT would also produce a reduced response. However the detailed action of 5-HT on the neural circuits those that are responsible for eating and crawling behaviors are not known.

Unlike the p-CPA fed larvae, exogenous application of 5-HT on sensory-CNS-motor circuit in larvae fed MDMA showed a significant increase in sensitivity to 5-HT. Also MDMA fed larvae showed a significant decrease in locomotor movements but no alteration in feeding behavior. Such a differential effect helps one in dissecting the pharmacological differences among these behavioral circuits. If one knew the specific action of MDMA such as binding affinity for the particular 5-HT receptors and transporters that MDMA targets then one could propose neuronal wiring diagrams for these differences in behaviors. In humans MDMA causes a euphoric sensation, elation and a state of stimulation. MDMA causes reversal of 5-HT transporter and release of 5-HT. Another drug available in market, fluoxetine (Prozac), a selective 5-HT uptake inhibitor, is used as an antidepressant. Fluoxetine blocks the 5-HT transporter from re-uptaking 5-HT from synapses. Though both these drugs increase concentration of 5-HT at synapses they show opposite effects. This could be because of various effects of MDMA just not only on 5-HT system but

also other neurotransmitter like dopamine, norepinephrine, acetylcholine. Also fluoxetine inhibits MDMA or its metabolites entry into presynaptic terminal, thus giving its neuroprotective effects against MDMA (Sanchez et al., 2001). Using behavior studies along with physiological studies such distinctions can be made. I had assumed that the *Drosophila* model could offer some insight into these matters, but it appears that MDMA does not deplete 5-HT from nerve terminals.

Altering 5-HT function by changing the target instead the production end was another approach that I undertook in these studies. I was fortunate that fly strains were made in which the 5-HT receptor (5-HT_{2dro}) can be reduced in expression by an antisense approach (Chapter 3). In these strains 5-HT receptor knock downs affected both growth and locomotor movements. I also discovered that even at room temperature the heat shock induced antisense production might be activated since development was slowed as compared to when the strains were raised at 18°C. With a significant knock down in the receptor expression from 1st to 3rd instar stage, growth and the sensitivity to 5-HT in the central circuits were also affected. Hence I showed that 5-HT as well as 5-HT_{2dro} receptor plays an important role in the development of *Drosophila melanogaster*.

The results I obtained, in regards to p-CPA treatments, are similar to those for other animals. Koe and Wiessman (1966) showed that administration of p-CPA to rats decrease their brain 5-HT levels and when administered to pregnant rats, slowed down the neuronal differentiation in brain regions of pups containing 5-HT terminals or known to have high 5-HT content in adults (Lauder and Kerbs, 1978). Even pregnant rats exposed to p-CPA on a critical period for CNS development

resulted in a decrease in 5-HT in embryonic neurons, showing that the maternal p-CPA treatment effects the development of the embryo (Butkevich et al., 2003). The mRNA for many of the 5-HT receptors mRNA are present throughout the development process of the brain in many animals (Whitaker-Azmitia et al., 1996; Hellendall et al., 1993). This is probably why a deficiency in 5-HT effects brain development in animals and in some cases the consequences are seen in the postnatal life of the organism. This is also very important with antipsychotics related to the 5-HT axis since no standards are being followed for breast-feeding mothers and potential effects on neonatal development.

It is possible that a reduction in neural activity either by alterations in dopamine or 5-HT could reduce the growth related hormone production and/or release. A reduction of ecdysone production can prolong larval stages for a week (Li and Cooper, 2001). The neural regulation for feedback/release of ecdysone and juvenile hormone is well established (Gu et al., 1996; Song and Gilbert 1996). Neural factors like PTTH stimulate prothoracic gland to stimulate and release ecdysone which is later converted into a usable form, 20-hydroxyecdysone, in other tissues. The conversion to 20-hydroxyecdysone is regulated by inositol 1,4,5-triphosphate (InsP₃) and cAMP signaling pathway (Venkatesh et al., 2001). In one of the 5-HT signaling pathways both InsP₃ and cAMP act as 2nd messengers. Hence altered levels of 5-HT might effect the release/ secretion of ecdysone.

My study is the first to quantitatively show, by HPLC, how much 5-HT is reduced by feeding p-CPA to larval *Drosophila*. It is interesting to note that the reduction was observed for the CNS but not for whole larva. It is not surprising since

the CNS may only contain a small amount in respect to the whole body. Studies in mice have shown 2 distinct isoforms of TPH (Walther et al., 2003), TPH 1 and TPH 2. TPH 1 is expressed in the peripheral regions (non-neuronal) and is scarce in the CNS. On the other hand, TPH 2 is expressed only in brain. Zhang et al., (2004) showed that TPH 2 is the main source for 5-HT synthesis in brain. Recently in *Drosophila*, it is shown that there are two different forms of TRH enzyme, one that is specific to the brain, called as DTRH, and another (DTPH, *Drosophila* tryptophan-phenylalanine hydroxylase) is in the periphery (Coleman and Neckameyer, 2005). So perhaps p-CPA selectively targets a DTRH in *Drosophila*. Also another significant finding from the studies utilizing HPLC was that 5,7-DHT did not result in a drop of 5-HT production in the CNS and therefore the 5-HT producing neurons were probably not killed out. Such a finding stresses the importance of not extracting pharmacological phenomena that is well established in vertebrates and assumes that the same approach will work in an invertebrate species.

5-HT is involved in motor unit co-ordination in other animals, including invertebrates, so it is not so surprising that some effects in bodily movements are observed in *Drosophila* related to 5-HT (Sewell et al., 1975; Kamyshev et al., 1983). For example, leech swimming behavior is modulated by 5-HT (Kristan and Nusbaum, 1982). Central pattern generators involved in chewing and respiration are also affected (Jacobs and Fornal, 1993). Likewise, P-CPA administered intraperitoneally in rat pups showed a marked decrease in their growth, posture and locomotor activity (Myoga et al., 1995). Of course to compare studies one has to be cognizant of dosage and scaling between animals for body size but also for

metabolic rates (White and Seymour, 2005; West et al., 2002). p-CPA (10mg/ml) fed to adult *Drosophila* flies showed a decrease in their ability to fly (Banerjee et al., 2004) but their locomotor activity increases when treated with 150 ug/ml (Kamyshev et al., 1983). This difference observed in adults and larvae in the action of p-CPA might be because of the developmental changes. The circuits in the larval fly system are also different from adult flies. Given that the larval CNS is reorganized as compared to the adult CNS it would be expected that locomotion between the different stages would differ in response to 5-HT as well as eating behaviors. Colas et al., (1999) also showed that the 5-HT receptor subtypes even vary over the larval stages, so I would expect that locomotion as well as eating responses might vary differentially at the different stages to such pharmacological manipulations as feeding p-CPA. Studies of this nature have not been undertaken yet, but are worthy of investigation.

Also p-CPA is not a specific inhibitor of TPH but likely also inhibits phenylalanine hydroxylase (Koe and Weissman, 1966; Miller et al., 1975, 1976), the rate-limiting enzyme in tyrosine synthesis (Neckamayer and White, 1993). In my HPLC study, I found a small decrease in the levels of DA in brains of p-CPA fed larvae. Hence the behavioral responses we measured may not be completely due to 5-HT depletion, but depletion of DA or even a combinational effect of the two being reduced. Even in mammals, effects of p-CPA on locomotor activity are shown to be divergent in reducing activity (Fibiger et al., 1971; Pirch, 1969) to enhancing movements (Borbely et al., 1973; Marsden et al., 1976). These differences seem to be based on the dosage of p-CPA and the environment in which the experiments are

carried out (Dringenberg et al., 1995). Also p-CPA can cause a reduction in food intake (Borbely et al., 1973; Marsden et al., 1976). Besides 5-HT is present in intestinal tissue and helps in gastrointestinal function so a reduction could alter dietary/digestion factors. The alteration in 5-HT level affects the mobility of the gut and is associated with irritable bowel syndrome (Crowell, 2001). The larvae treated with p-CPA were smaller in size as compared to controls and this might be a part of the developmental retardation seen because reduced food intake and absorption.

MDMA, in mammals is known to work through the serotonergic system (Green et al., 2003). Also it affects dopamine (DA) and acetylcholine (ACh) function, which can then result in very broad actions in the CNS. Feeding MDMA to *Drosophila* larvae caused a delay in development, in a dose dependent manner. The higher the dose the greater the delay in development. However in rats that were administered MDMA (Day 11-20, this is similar to the human late third trimester stage in humans; Broening et al., 2001) no effect on survival was observed but spatial and sequential learning and memory was reduced (Broening et al., 2001). Since MDMA did not deplete 5-HT in the larval CNS, as noted by the HPLC studies, one would assume that MDMA is not functioning in the same manner in *Drosophila* as in mammals. *Drosophila* 5-HT transporter (dSERT) is homologous to human and rat SERT by only 51%. Demchyshyn et al. (1994) reported that dSERT is not similar to the hSERT pharmacologically. The affinity of dSERT is different for antidepressants like imipramine as compared to hSERT. Thus, MDMA may not be acting on dSERT the same way as on hSERT. It would be interesting to compare, possibly with radiolabel binding assays, if indeed the 5-HT transporter in fly neurons

have a different binding affinity for MDMA than that of vertebrates. Possibly the molecular sequence would reveal substantial differences in the binding domains to account for the differential effect among species.

The acute effects of administration of MDMA in rats immediately results in an increased locomotion (Callaway et al., 1990). This hyperactivity is based on the release of 5-HT by the transporter working in reverse (Callaway et al., 1990). The dumping of 5-HT in neurons is reduced when rats are treated with p-CPA (Callaway et al., 1990). 5-HT_{1B} (Callaway et al., 1990; Rempel et al., 1993) and the 5-HT_{2A} receptors (Kehne et al., 1996) were shown to be activated by MDMA and hyperactivity in rats is dependent on the activation of these receptors. But in *Drosophila* larvae fed MDMA from 1st to 3rd instar stage no increase was observed but instead a decrease in locomotor movements occurred. An aspect that I did not investigate was the potential variation due to gender. Recently Palenicsek et al., (2005) showed that female rats are more susceptible to acute locomotor stimulating effects of MDMA than male rats. All the other effects of MDMA were seen to be similar in male and female rats (Walker et al., 2006). Such sexual differences in the effects of MDMA in *Drosophila* larvae as well as in adults could be followed up in future studies.

Just as 5-HT modulates heart rate in humans by either increasing (Harris et al., 1960) or decreasing (Hollander et al., 1957) the rate depending on the dosage, 5-HT has a pronounced effect on the heart rate in larval *Drosophila*; however, the effect is directly on the heart tissue. MDMA also causes an increase in the heart rate and blood pressure in humans (Freedman et al., 2005; Mas et al., 1999);

however, in rats a decrease in heart rate was observed at a dose of 1 mg/Kg (O'Cain et al., 2000) and at a dose of 20mg/kg., Aa dose considered to be neurotoxic produced intense bradycardia (O'Cain et al., 2000). 5-HT is involved in cardiovascular function in humans as well as insects (Chapter 3) and their close cousins the crustaceans (Listerman et al., 2000). 5-HT_{2B} receptors are present on valves, and defects in these receptors result in valvular cardiopathies in mammals (Setola et al., 2005; Kaumann and Levy, 2006). It is known that a reduction in 5-HT synthesis by a loss of TPH1 in mice can produce abnormal heart rate and lead to heart failure (Cote et al., 2003). In my studies, exogenous application of 5-HT caused an increase in heart rate in a dose dependent manner with the slightest effect being noted at 10nM. Interestingly MDMA did not have any significant effect on the *Drosophila* larval heart rate at doses ranging from 1µM to 100µM. P-CPA fed *Drosophila* larvae also did not show any difference in the heart rate as compared to that of controls. This might be because 5-HT levels were not reduced in the whole larvae feed p-CPA, so the heart might have been exposed to normal levels of 5-HT throughout the feeding regimen of p-CPA. In addition, it is not known if MDMA binds to *Drosophila* 5-HT receptors. Recently it was shown by my colleague, Dr. Andrew Johnstone, that the aorta of larval heart is innervated (Johnstone and Cooper, 2006); however, the effects of 5-HT on this innervation has not been investigated. Dulcis and Levine (2003) showed, in adult *Drosophila*, that the heart is innervated and that the nerve terminals are glutamatergic, but they did not investigate neuromodulation of this innervation. The mechanism of 5-HT's action on the *Drosophila* larval heart is not known since the receptor subtype has not been investigated yet.

Pharmacological studies are underway in the Cooper laboratory. Once the receptor subtypes are known then logical predictions of why MDMA does not produce a direct effect on the larval heart as compared to the CNS can be made.

Neural circuits are conserved across phylogenetic orders. For example, central pattern generators of locomotion in spinal cords are conserved from lamprey to rats (Katz and Harris-Warrick, 1999). Formations of various neural circuits are known to be dependent on signaling cues from neurotransmitters and neuromodulators. Neurotransmitters and neuromodulators are expressed in high amounts during certain stages of developmental periods as compared to others, which is key to their role. Neuromodulators can regulate critical periods of a development with altered stimulation when required for correct development within given developmental windows.

Hubel and Wiesel (1970) showed that there is a critical period for effects of visual deprivation. Critical period of monocular deprivation in kittens is at 3 and 4 week of age in layer IV and slowly shifts to other extragranular layers at 6 weeks and end at 1 year of age (Hubel and Wiesel, 1970; Daw et al., 1992). The sensitivity of the visual system in binocular competition is regulated by extracellular inputs like serotonergic and cholinergic system (Kojic et al., 2001). 5-HT has been proposed to be a factor that determines critical period for synaptic plasticity in the rat visual cortex of rats (Edagawa et al., 2001). 5-HT is shown to increase with development and suppress induction of long-term potentiation (LTP) in rat visual cortex (Edagawa et al., 2001). Similarly 5-HT has been shown to be involved in critical periods in the development of other neural circuits. Treatment of 9-day pregnant rats with p-CPA,

critical day for normal serotonergic system development in fetus, caused alteration 5-HT levels and also decreased the sensitivity of pain in offspring (Butkevich et al., 2003). In insects like the bee (*Bombus terrestris*), the 2nd larval stage is the critical period for caste determination (worker and queen bee). During this critical period, juvenile hormone (JH) biosynthesis is eight to ten times higher in queen larvae than worker larvae (Cnaani et al., 2000). In *Drosophila*, abolishing activity of acetylcholine esterase enzyme (AChE) during embryogenesis is lethal showing that there is a critical period in CNS during embryogenesis where AChE function is required (Greenspan et al., 1980). I have also shown that 5-HT is important during the larval development of *Drosophila*. In addition, Colas et al (1999) did show that 5-HT_{2dro} receptor function is important during embryonic development. As a future step, using pharmacological manipulations and genetic tools during critical periods, where 5-HT signaling is important during development, will help to advance the understanding of neural development.

The sensory input controls CNS and motor unit development and function. Invertebrate models like *Tritonia* (mollusk), somatogastric ganglion (STG) of crustaceans, have proven to be very useful in learning about rhythmic activity. 5-HT modulates CPGs in both vertebrates and invertebrates. STG of crustaceans is shown to be modulated by biogenic amines (Marder and Thirumalai, 2002). In *Tritonia* dorsal swim interneurons (DSIs) are a set of serotonergic neurons that are intrinsic to swim CPG (Katz et al., 1994). Fickbohm et al. (2000) showed that increasing 5-HT in *Tritonia* enhances the ability of DSIs to elicit rhythmic activity in swim interneurons but also DSIs were silent or less active in between bouts of

activity. In isolated lamprey spinal cord, a vertebrate model, an increase in 5-HT reduces fictive swim burst frequency (Harris-Warrick and Cohen, 1985). In *Drosophila* the pharyngeal muscles are also involved in feeding and it is known that exogenous application of cholinergic agonists in a 3rd instar semi-intact preparation induced bursting activity in muscles (Gorczyca et al., 1991). 5-HT is also present in pharyngeal muscle fibers (Valles and White, 1986) and exogenous application of 5-HT also initiates activity in these muscles (Budnik and White, 1987b). Suster and Bate (2002) showed that though the circuit for peristalsis and crawling is formed in embryos and larvae of *Drosophila* in complete absence of sensory input, the actual patterns of locomotion and its integration into the circuit is lost. In 3rd instar semi-intact larval preparation I have shown that 5-HT (10 μ M) increases motoneuron firing frequency in a biphasic manner. Decreasing 5-HT levels during development decreased this firing frequency. Octopamine (OA) at 10 μ M also caused a rhythmic movements in semi-intact preparation and caused an increase in firing frequency so it is not only the presence of the compounds but at what concentration. The varied concentrations at particular periods in develop could be crucial but as far as I am aware no one has investigated this topic. Recently, OA was shown to modulate CPGs for larval locomotion (Fox et al., 2006) so it would be of interest to see the long term consequences of excess or depletion of OA on the CPGs.

There are many continuation projects that have stemmed from these studies, many of which I have already highlighted above. Some of the tangible projects that would be immediately beneficial to the field in modulation of neural development are outlined next. The effects of the 5-HT deficiency during larval development can be

extended to examine the consequences on neural development in the adult CNS. 5-HT is known to be abundant in adult eyes (Rodriguez Moncalvo and Campos, 2005) and involved in learning/memory (Tempel et al., 1984; Alshuaib et al., 2003). Deficiency of 5-HT during larval and pupal development might cause defect in the formation of 5-HT nerve terminals within the adult CNS. Testing vision, learning and memory in adult flies, as well as in larvae, after various bouts of p-CPA feeding would help us in further understanding the role 5-HT in development and modulation of these behaviors. The role of the 5-HT receptors in these same behaviors of course could parallel such studies in the production of 5-HT. Many studies have shown *Drosophila* as model organism for discovering the mechanisms of action of drugs of abuse like, cocaine. Such additions to the reasons why MDMA resulted in behavioral differences while not depleting the 5-HT in the CNS is of interest.

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CHAPTER 1

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PUBLICATIONS:

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