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PROFILING THE ACTION OF ACETYLCHOLINE IN THE DROSOPHILA MELANOGASTER LARVAL MODEL: HEART, BEHAVIOR, AND THE DEVELOPMENT AND MAINTENANCE OF SENSORIMOTOR CIRCUITS

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

By
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Lexington, Kentucky

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ABSTRACT OF DISSERTATION

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Understanding the role of various chemical messengers in altering behaviors and physiological processes is a common goal for scientists across multiple disciplines. The main focus of this dissertation is on characterizing the action of an important neurotransmitter, acetylcholine (ACh), modulating larval *Drosophila melanogaster* neural circuits and heart. In this dissertation, I provide important insights into the mechanisms by which ACh influences the formation and performance of select neural circuits, while also revealing significant details regarding its role in additional physiological functions, including cardiac pace making. In Chapter 1, I provide a general overview of ACh action in mammals and flies with a particular focus on the physiological and behavioral effects of cholinergic signaling in the context of modulation of neural circuits and developmental impacts.

Chapters 2 and 3 are dedicated to the role of ACh in modulating larval *Drosophila* heart rate (HR). Previous analysis has been performed identifying neuromodulator influence on larval heart rate, and I add to the current understanding of chemical modulation of cardiac function utilizing a pharmacological approach to assess ACh regulation of HR. I provide evidence that ACh modulates larval HR primarily through muscarinic receptors. I follow this by employing an optogenetic approach to assess ACh and additional neuroendocrine modulation of HR in an intact system in Chapter 3, further illuminating ACh regulation of larval HR.

Chapter 4 is dedicated to describing the role of ACh in modulation of neural circuits underlying larval locomotion, feeding behavior, and sensorimotor circuit activity. I discuss the pharmacological approach taken to address this topic. Here, behavioral as well as electrophysiological approaches reveal a contribution from both ACh receptor subtypes in regulation of these behaviors. I leverage this information and describe the influence of a specific receptor subtype, the muscarinic acetylcholine receptor (mAChR) on the function of these circuits by using combined pharmacological and genetic approaches to strengthen the pharmacological assessment, discussed in Chapter 8.

An additional goal of this work is to refine the optogenetic technique in the larval *Drosophila* model. Chapter 5 discusses useful experimental paradigms that allow for investigation of repetitively activating light-sensitive opsins on neuronal physiology in the larval model. Chapter 6 discusses an intriguing, previously undefined identification of *Glutamic acid decarboxylase1* expression in larval body wall muscle, which was

identified using optogenetic approaches in concert with electrophysiology. Furthermore, I combine these approaches to discuss the development of an experimental paradigm to address the developmental impacts of altering sensory (cholinergic) input on the formation and maintenance of a specific mechanosensory circuit (Chapter 8). Chapter 7 discusses the implication of deep tissue injury on proprioceptive sensory function in two model proprioceptive organs in crab and crayfish.

KEYWORDS: Drosophila melanogaster, acetylcholine, cardiac physiology, neural circuits, behavior, optogenetics

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CHAPTER ONE

Acetylcholine signaling: From humans to flies

Neuromodulators and their role in neural circuit function

Our experiences are regulated by a vast network of billions of interconnected components that work in concert to guide our day-to-day activities. In order to fully grasp the neurobiology underlying behaviors, emotions, memories, and senses it is essential to break down the neuronal constituents that drive these processes. We must identify neuronal types that play a role in in these processes and ultimately determine how each of these components are wired into functional neural circuits. While our understanding of the role of neural circuits in coordinating behavior has progressed, this knowledge is still in its infancy. We have gained the ability to identify the molecular components that make up these circuits and understand how they regulate processes in individual cells, but how they ultimately regulate the function of entire neural networks is still a major challenge that neuroscientists around the world are keen to tackle. Because the mammalian brain is so complex, with billions of neurons forming synaptic connections that number in the trillions, each with unique properties, we are forced to scale down our efforts and isolate individual neurons, or focus on controlling activity in specific regions of the brain. In doing so, we may neglect how specific circuit alterations affect more broad neural networks. Recently, however, strides have been made to address these shortcomings with the advent of techniques that enable experimental manipulation of circuit activity *in vivo*. This has improved our ability to alter circuit activity and observe changes in behavior in an intact nervous system. Continued progression in refining these techniques will assuredly follow and a goal of this research is to improve these methods in a model organism amenable to advancing our endeavor. Moreover, it is essential to investigate how individual neuromodulators impact the function of neural ensembles, as their role as essential components in neural circuit formation and function is known.

Neuromodulators maintain the ability to alter the function of the nervous system without broadly reconfiguring neural circuits. An understanding of the mechanisms underlying the ability to alter the efficacy of neural circuits is integral in how the circuits function. It is known that the modulation of many behavioral outputs in response to changes in the environment is not determined solely by neural circuit rewiring, but by alteration in synaptic output or shape of an action potential (Dunlap and Fischbach, 1978), neuronal firing rate or bursting properties (Combes et al. 1997), and changes in membrane conductance (Harris-Warrick RM and Marder E. 1991; Meyrand et al. 1994). Understanding the role of individual neuromodulators and the receptors through which they act in regulating these processes is important in furthering insights into the properties of model neural circuits. Considerable work has focused on analyzing the functional significance of neuromodulators in a variety of model organisms, including *Drosophila*, yet their role in complex circuits *in vivo* necessitates further investigation.

Additionally, how these messengers play a role in directing development of neural circuits is necessary in fully understanding their impact on neural connectivity and function. Formation of neural circuits is a process that is guided by a number of intrinsic and extrinsic factors. While the fate of specific neuronal subtypes and the initial formation of circuits is driven primarily by the combinatorial action of various transcription factors and cell signaling molecules, the ultimate refining and establishment of connections is activity dependent, regulated by the coordinated signaling of a variety of chemical messengers. Alterations in synaptic size, synaptic bouton number, dendritic size, and axonal branching all represent plastic changes that manifest as a result of activity-dependent fine-tuning. Understanding the role of various chemical messengers in modulating neural circuits and guiding changes in response to that regulate distinct behaviors is a common goal among neuroscientists. Thus, the focus of this work is on utilizing an amenable model organism, *Drosophila melanogaster*, to scale down the daunting task of understanding the workings of a remarkably

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complex collection of components that guide our experiences. I further pare down this task by focusing on the role of a neuromodulator that is prominent in nervous systems across taxa: acetylcholine.

Acetylcholine is a prominent neurotransmitter and neuromodulator in the mammalian nervous system

Acetylcholine (ACh) has been identified as a prominent chemical messenger in mammals for over a century. Since the seminal, Nobel Prize winning work from Otto Loewi and Sir Henry Dale in the 1920s and 1930s, in which they identified ACh as the chemical messenger involved in neural regulation of heart rate, ACh has been a primary focus of study in the mammalian nervous system. This work was followed by the identification of ACh as the chemical transmitter released from motor neurons in the spinal cord, where it binds nicotinic acetylcholine receptors (nAChRs) on muscle at the neuromuscular junction (NMJ) (Dale et al. 1936). Their work pioneered investigations into chemical neurotransmission and neurotransmitter receptor function, and early explorations into the mechanisms of synaptic transmission focused largely on the cholinergic vertebrate NMJ   As a result of its action at the vertebrate NMJ, a great deal is known regarding ACh signaling and its role in neural communication.   Particularly, some of the earliest electrophysiological experiments were focused on nAChRs and the advent of patch clamp electrophysiology that enabled single channel recordings helped classify the properties of these ion channels (Neher and Sakmann 1976). Properties including channel gating (Katz and Miledi 1972; Neher and Stevens 1977), ion selectivity (Adams et al. 1980; Dwyer et al. 1980), and channel desensitization (Katz and Thesleff 1957) were all investigated and on vertebrate nAChRs. Furthermore, the first cloning experiments utilizing nAChRs from the electric organ of the ray, *Torpedo*, through expression in host cells and improved techniques for structural and high-throughput sequence analysis contributed to an enhanced understanding of receptor structure and function, including detailed

analysis of ligand-binding and channel conductance. Thus, much of our knowledge regarding ligand-gated ion channel structure and function, in general, stems from the pioneering work on acetylcholine and its receptors.

In addition to its role as the transmitter used at the NMJ in vertebrates, it also is a primary excitatory transmitter in the autonomic nervous system at conventional synapses. It is used by sympathetic preganglionic nerves coming from the spinal cord and is also released by pre and post-ganglionic parasympathetic nerves. Here it binds primarily ionotropic nAChRs, mediating fast synaptic transmission, but also acts through metabotropic muscarinic acetylcholine receptors (mAChRs) to regulate autonomic functions including regulation of cardiac pace making and atrio-ventricular conduction (reviewed in Dhein et al. 2001) in gastrointestinal function (reviewed in Abrams et al. 2006), in bladder function (reviewed in Abrams et al. 2006), and in salivary glands (reviewed in Abrams et al. 2006). While Ach acts primarily as a classical excitatory transmitter in the autonomic nervous system and at the NMJ, its actions in the brain are primarily thought to be modulatory. Acetylcholine is ubiquitous in the brain and, thus plays a regulatory role in a number of important processes. Cholinergic neurons project from the pontine tegmentum and forebrain nuclei to the thalamus, midbrain, hippocampus and cortex and form vast connections with targets within these regions. While individual varicosities of cholinergic neurons in the spinal cord synapse with dendrites of pre and post-ganglionic fibers, in these brain regions, nerve endings on cholinergic projections arborize widely and do not terminate at synapses (Descarries et al. 1997). Additionally, nAChRs, which are found in nearly every neural area (Woolf 1991; Changeux and Edelstein 2005; Dani and Bertrand 2007) and mAChRs, which are abundant in the neocortex, hippocampus, substantia nigra, pars compacta, ventral tegmental area and mammalian retina, are located both pre-and post-synaptically as well as throughout the brain, adding additional evidence that ACh actions in the brain are primarily through volume transmission, modulating release of neurotransmitters and modulators. How this broad regulation of neural

activity underlies important processes and contributes to nervous system disorders is a focus of intense research by neuroscientists and physiologists around the world.

Acetylcholine and its implication in nervous system disorders

Due to the abundance of ACh and its associative receptors in the mammalian CNS, it is not surprising that dysfunction in normal ACh signaling is involved in the progression of a host of nervous system pathologies. As mentioned, cholinergic fibers project from the forebrain to the hippocampus in mammalian brains and it is the degeneration of these fibers that is thought to be a primary cause of memory loss and intellectual disabilities associated with Alzheimer's disease (reviewed in Francis et al. 1999). The role of both nAChRs and mAChRs, which are abundant in the mammalian hippocampus, have been extensively studied and it has been shown cholinergic signaling is important in focus and attention (reviewed in Berry et al. 2014) and learning and memory (reviewed in Hasselmo 2006) through a variety of mechanisms including modulation of glutamate and GABA neuronal activity in the dentate gyrus (Radcliffe et al. 1999), presynaptic inhibition of excitatory feedback within cortical circuits via inhibitory mAChRs (Hasselmo and McGaughy 2004) and synaptic modifications enhancing long-term potentiation (Leung et al. 2003; Buccafusco et al. 2005).   Additionally, genetic disruption of key components in ACh signaling genes have been implicated in other disorders including attention deficit hyperactivity disorder (English et al. 2009), anxiety and depression-like behavior (Mineur et al. 2013) and, more recently, schizophrenia (reviewed in Raedler and Tandon 2006 and Terry 2008). However, perhaps the most widely investigated role of ACh signaling in the mammalian CNS is its involvement in reward processing.   Since tobacco use is the leading cause of preventable death in developed countries (Peto et al. 1996; Mathers and Loncar 2006; Benowitz 2008), it is of great interest to identify the mechanisms underlying nicotine dependence. The ubiquitous expression of nAChRs in multiple regions of the

brain associated with initiation of addiction points to a prominent role for multiple receptor subtypes in this process. It is well known that the dopaminergic pathway originating in the ventral tegmental area (VTA) and projecting to the prefrontal cortex and limbic and striatum structures, including the nucleus accumbens (NAc), is important in driving reinforcing behavior associated with drug addiction (Dani and Heinemann 1996; Corigall 1999; Di Chiara 2000; Mansvelder and McGehee 2002). nAChRs have been shown to potentiate dopamine release from these projections, as nicotine increases firing rate and phasic bursting rates of these midbrain neurons. The receptor subtypes associated with this enhancement of circuit efficacy and corresponding regulation of self-administration and/or conditioned place preference in rodent models are primarily the alpha4beta2, the alpha6beta2, and the alpha6beta3 receptors (Drenan et al. 2010; Mameli-Engvall et al. 2006; Picciotto et al. 1998; Pons et al. 2008). Additionally, alpha7 nAChRs are expressed in the VTA and are thought to modulate GABAergic inhibition and glutamatergic excitation of dopaminergic neuronal activity (Mansvelder and McGehee 2002; Jones and Wonnacott 2002). In addition to their involvement in initiation of addiction, nAChRs are also involved in modulating activity in the region where VTA projections terminate, including in the NAc. This structure is integral in regulating a number of reward-related behaviors including association with drug seeking and/or aversion behavior following exposure (Day et al. 2010). Here, evidence points to involvement of additional nAChR receptor subtypes in modulation of dopamine tone, including the alpha5beta2 receptor and alpha4beta2 receptor (Cachope et al. 2012).   Furthermore, while the role of specific nAChRs and mAChRs in the hippocampus in the context of addiction has not been widely investigated, the encoding of memories associated with drug-induced emotional states likely involves these receptors. A growing body of evidence suggests that the hippocampus is associated with context and cue-induced drug relapse (Azam et al. 2002). Additional work has implicated multiple nAChR subtypes expressed in the medial habenula-interpeduncular nucleus to play a role in nicotine

reinforcement and withdrawal (Grady et al. 2009). Therefore, multiple receptor subtypes play a role in addiction and we are likely only scratching the surface of our knowledge of ACh involvement in this process.

Furthermore, ACh signaling is integral in neural circuit development. Activation of nAChRs haven been shown to be essential for neuronal growth and differentiation, synapse formation, and in altering the signaling properties of other neuromodulators (Liu et al. 2007b). For instance, it is known that activation of nAChRs is important to regulation of GABA switch from an excitatory neurotransmitter to an inhibitory neurotransmitter in the mammalian hippocampus (Bruel-Jungerman et al. 2011). Endogenous cholinergic activity is thus essential in guiding the development of neural circuits. Based on this knowledge, much attention has been given to the impact of prenatal nicotine exposure. Even in an era where the detriments of smoking tobacco are well-known, embryonic exposure to tobacco smoke remains a problem. A number of developmental consequences that have been correlated with prenatal nicotine exposure in both humans and rodent models include increased mortality and low birth weight (Cornelius and Day 2000). Additionally, exposure during this developmental period increases one's susceptibility to nicotine addiction and alcohol abuse by adolescence. Moreover, this exposure is associated with attention deficit hyperactive disorder and behavioral affects in rodents show enhanced hyperactivity (Fergusson et al. 1998; Huizink and Mulder 2006; Cornelius and Day 2009).   This abundant research suggests a substantial role for nicotinic acetylcholine signaling in regulating neural circuit connectivity. While the exact molecular mechanisms that underlie this association with neurological and neuropsychiatric disorders aren't fully understood, it likely stems from an alteration of expression of nAChRs around the brain and correlative alteration in activity, as increased binding sites have been identified following prenatal nicotine exposure (Tizabi et al.1997; Slotkin et al. 2005; Nunes-Freitas et al. 2011). In assessing these mechanisms, the use of animal models where environmental factors can be controlled, is essential. For instance, controlling

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the concentration of nicotine that gets exposed to the nervous system, or exposing developing embryos to nicotine without the added toxins found in cigarette smoke, is feasible and may help to address the role of nicotine, more directly in these pathologies.

The complexity of acetylcholine signaling in the nervous system and beyond

As evidenced by the broad range of disorders associated with ACh signaling, one theme persists: acetylcholine transmission in the nervous system is extremely complex. This is demonstrated by the diversity of receptor subtypes expressed in the mammalian genome. In humans, seventeen known nicotinic receptor subunits are expressed (Zoli et al. 2015). These subunits can arrange in a variety of manners to form a functional receptor, each with unique properties. Although they share a common basic structure, the subunit stoichiometry influences their characteristics dramatically.   For instance, the alpha4beta2 receptor displays much higher affinity for nicotine than its counterparts, which may factor into their more prominent role in the initial stages of nicotine dependence (McGehee and Role 1995; Gotti et al. 2006; Albuquerque et al. 2009).   Furthermore, the various receptor subtypes are quite distinct in their ion permeability. While each receptor has been shown to maintain high Na^+ and K^+ conductance, there is a diverse difference in Ca^{2+} permeability. The alpha7 homomeric nAChR receptor, for instance, is much more permeable to Ca^{2+} than other known receptors (Shen and Yakel 2009). Recent analysis has shown that this receptor like plays a more prominent role in potentiating glutamatergic synapses through regulating AMPA receptor mobility in the rodent hippocampus (Halff et al. 2014). Thus, this receptor subtype may function more prominently in the mechanisms underlying classical calcium-dependent synaptic plasticity fundamental to learning and memory, although additional subtypes most likely play a role. Furthermore, the desensitizing characteristics of these two receptor subtypes are distinct, as the alpha7 receptor desensitizes much more rapidly

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than the alpha4beta2 (Miwa et al. 1999; Ibanez-Tallon et al. 2002). This wide variation in receptor properties may explain the vast expression difference in the brain, with each receptor subtype finely tuned to regulate specific processes.

Likewise, the mAChR family is also complex, with 5 individual receptors (M1-M5) expressed in the mammalian genome. These metabotropic GPCRs are further alternatively spliced, adding to this diversity (Maggio et al. 2016). Moreover, where the nAChRs are ion channels, mAChRs are G-protein coupled receptors (GPCRs) that regulate ion channel function, effector enzyme activity, and second messenger abundance and activity in a variety of ways. Therefore, while the differing components that make up nAChRs themselves contributes to their complexity, the ability to modulate a host intracellular processes contributes the to mAChR complexity. For instance, the M2 and M4 receptors are inhibitory receptors and they can exert their influence through multiple mechanisms. Acting through $G_i\beta\gamma$ they can directly modulate inwardly rectifying K^+ channel conductance and also reduce adenylyl cyclase activity, ultimately reducing cAMP concentration and intracellular Ca^{2+} (Logothetis et al. 1987) through $G_{i/o}$. The manipulation of cAMP concentration, in turn, has a broad impact on cell physiology and can alter additional enzymatic activity and ultimately change gene expression. In turn, the M1, M3, and M5 receptors are excitatory, acting primarily through $G_{q/11}$ (Wess 1996) to activate adenylyl cyclase, phospholipase C and additional cellular signaling pathways (Felder 1995; Brodde and Michel 1999). Like nAChRs, they are ubiquitously expressed in the mammalian brain and in visceral tissue, where they are known to modulate smooth muscle contraction, and cardiac function (Dhein et al. 2011). Thus, the diversity of action and expression of the mAChRs make this family of receptors complex as well.

Consequently, because of the anatomical and molecular complexity associated with ACh signaling in the mammalian nervous system and beyond, I turn my attention to a more amenable model. *Drosophila melanogaster* serves as an intermediate in investigating the intricacies that underlie neural circuit function. While not as simple as models that have been crucial in illuminating

basic principles in neural function, including *Caenorhabditis elegans,* the fly model provides opportunities to answer applicable questions that may not be possible in simpler organisms. We can feasibly investigate distinct neural circuits and their role in complex behavior, including associative learning and memory, for example. This has been evidenced, specifically pertaining to the role of cholinergic signaling in olfactory associative learning, in recent reports (Silva et al. 2015; Barnstedt et al. 2016). Coupled with unmatched ability to manipulate gene expression, this offers a remarkable combination to address the molecular, cellular, and network components that regulate nervous system function. Furthermore the fly model continues to serve as a useful model in addressing basic physiological questions underlying process including cardiac function. Thus, I've harnessed these capabilities to enhance our understanding of ACh modulation of neural circuit and cardiac function.

Drosophila as a tractable model in neurobiology

By sheer number of cellular components, the fly nervous system is exponentially less complex than rodent model counterparts. Consisting of approximately 10,000 neurons during larval stage (Ohyama et al. 2015) and 100,000 as adults (Peng et al. 2011) the fly brain provides a useful platform for investigation of the anatomy and function of neural circuits. Like in mammals, the nervous system is broadly divided into a peripheral and central nervous system, the latter consisting of a nerve cord (analogous to a spinal cord) and a central brain consisting of two lobes. The central brain is further subdivided into 2 main regions: the supraesophagal ganglia (the central brain) and the more posterior, ventral subesophagal ganglion, which serves as an intermediate, or gateway to the thoracic and abdominal ganglia of the nerve cord, much akin to the mammalian brain stem. The supraesophagal ganglion is further divided into three distinct regions arranged from anterior to posterior: the protocerebrum (PC), deuterocerebrum (DC) and tritocerebrum (TC). Each of these contains morphologically distinct neuropils that are dedicated to specific

functions. In the adult, these regions are the optic lobes (vision), the antennal lobes (olfaction), the mushroom bodies (associative learning) and the central complex, a set of four neuropils thought to serve as an integration center for sensory, motor, and learning processes (Wolff et al. 2015). The subesophagal ganglion in the posterior brain, which receives the vast majority of gustatory sensory input and aides in regulating feeding motor programming (Schoofs et al. 2014), gives way to the segmental thoracic ganglia and the more posterior abdominal ganglia of the ventral nerve cord, which extends down the ventral midline of the animal. The ventral nerve cord ganglia are involved in sensorimotor processing in the body segments and, in larvae, receive reiterative afferent input from mechanosensory sensory neurons that tile the cuticle, making up the peripheral nervous system. These sensory afferents send axon tracts via three nerves that are segmentally repeated: the segmental nerve, intrasegmental nerve and transverse nerve (Singhania and Grueber 2014). These tracts also contain motor neuron axons, which exit from soma located in cortex region (outer) of the ventral nerve cord and project axons away from the midline. Thus, the fly brain is made up of functionally and morphologically distinct regions that can serve as advantageous in studies for analogous regions in the mammalian brain.

While the neuroanatomical characteristics and relative reduced number of neurons allow for more feasible investigation of an intact nervous system, perhaps what makes the fruit fly model most useful is the ability to manipulate the genome. This has proven particularly useful in the field of neuroscience. At the forefront of novel genomic techniques that took root in this model are binary expression systems. Most noteworthy among these is the GAL4/UAS system (Brand and Perrimon 1993), which has now become invaluable to fruit fly researchers in multiple disciplines. This system has allowed for the targeting of gene/protein manipulation and imaging in select cells. In neuroscience, a primary endeavor in understanding neural circuit function is to identify the components that make up these connections. The use of activity-dependent

imaging techniques, including genomic calcium indicators, and labeling synaptic connections (GRASP) (Feinberg et al. 2008) in concert with the GAL4/UAS system allow for unprecedented visualization of circuit components. Moreover, the advent of techniques, such as thermogenetics and optogenetics, and the continued development of driver lines targeting interneuron popluations (Jenett et al. 2012) allow for manipulation of circuit activity with precise spatial and temporal control. Thus, the *Drosophila* model is unmatched in regard to the meticulousness with which one can alter circuit dynamics.

 Furthermore, as it relates to neural circuit properties in the context of plasticity and development, the fly has proven a remarkably amenable model. Studies in *Drosophila* have provided examples of synaptic homeostasis in neurons within the CNS, including that of Ping and Tsunoda (2012), who illustrate the combinatorial role of nicotinic acetylcholine receptors (nAChRs) and an additional ion channel in mediating a balance in neural activity. They showed that the Dalpha7 nAChR is upregulated after 24 hours of curare-mediated inhibition. In response to blocking nAChR-mediated excitatory input, they illuminated a novel mechanism guiding homeostatic plasicitity, identifying an activity-dependent increase in Shal K^+ expression in response to nAChR upregulation. This is a unique mechanism that may prove to be conserved across phyla as a mechanism involved in homeostatic plasticity (Ping and Tsunoda 2012). Likewise, recent analysis has shed light on activity-dependent formation of motor circuits with manipulation of sensory activity (Fushiki et al. 2013). Upon depriving embryos of sensory input during critical periods in neural development, this group shows that entire sensorimotor programs are altered at later developmental time periods. A similar study addressed the impact of manipulating interneuronal activity during embryogenesis on probability of seizure induction (Giachello and Baines 2015). They show enhanced seizure induction as a result of a sustained imbalance of excitation/inhibition in the CNS following manipulation of embryonic neural activity (Giachello and Baines 2015). Thus, the *Drosophila* model has served useful in addressing mechanistic

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questions relating to neural plasticity and activity-dependent development of neural circuits. Although our predictions that activity indeed sculpts neural circuitry are based on seminal work in the mammalian brain (Hubel and Wiesel 1962), the complexity of the mammalian nervous system makes investigating these hypotheses quite difficult, particularlty in intact systems.

While we have used *Drosophila* to address many questions underlying these processes we still lack is a full understanding of the role of specific neuromodulators in regulating neural circuits. Whether it's in guiding development, modulating mechanisms driving synaptic plasticity, or altering excitability of neurons within specific circuits, the role of modulators and the cellular mechanisms they influence are not well known. Furthermore, the receptor subtypes involved in regulating circuits and behavior in this model warrants further investigation. The aforementioned studies highlight the capabilities in utilizing the fly as a model to address mechanistic questions relating to neural plasticity and activity-dependent development of neural circuits. I want to dovetail this research and utilize the aforementioned techniques to address, more specifically the role of ACh in regulation of these processes, and I've helped develop a technique that will make this feasible in a specific sensory-CNS-motor (sensorimotor) circuit (discussed in Chapter 8). Moreover, I've provided a foundational work that identify receptors within defined circuits that may be targeted for modulation-a necessary step in addressing more comprehensive questions.

Acetylcholine is a vital neurotransmitter in Drosophila melanogaster

Drosophila uses many of the same neurotransmitters as mammals. ACh is the primary neurotransmitter used in sensory neurons projecting into the CNS in invertebrates, including *Drosophila.* It is also a primary excitatory neurotransmitter and neuromodulator within the CNS (Lee and O'Dowd 1999; Su and O'Dowd 2003). Although it is not used at the NMJ in flies and other insects, it is widely ubiquitous, and thus, is thought to regulate a number of important

processes. While distinct differences in anatomical and functional properties of cholinergic neurons in the fly nervous system relative to mammals are clear, ACh and the components mediating cholinergic signaling exhibit comparable importance. Like in mammals, *Drosophila* ACh receptors (AChRs) consist of two major subtypes: the metabotropic muscarinic acetylcholine receptors (mAChRs), and the ionotropic nicotinic acetylcholine receptors (nAChRs), both of which are activated by ACh and the agonists, muscarine and nicotine, respectively. The *Drosophila* nicotinic receptors share a common architecture as part of the cys-loop family of ligand-gated ion channels that facilitates fast synaptic transmission (Livingston and Wonnacott 2009). Muscarinic receptors are metabotropic and act indirectly with ion channels through second messenger G proteins to generate a cellular response (Collin et al. 2013). Specifically, these receptors have been shown to couple to conserved 2nd messenger cascades through excitatory, $G\alpha_{q/11}$-PICβ-IP$_3$ and inhibitory $G\alpha_{i/o}$-AD-cAMP cascades (Ren et al. 2015). The *Drosophila* genome contains ten nAChR subunits and three mAChR types, A-type (encoded by gene CG4356), B-type (encoded by gene CG7918), and C-type (encoded by CG12796). As in mammals, the mAChRs couple to distinct 2nd messenger cascades that regulate a host of cellular processes (more detail in chapters 2 and 4). The A and C-type receptors have been identified as excitatory, while the B-type is inhibitory (Ren et al. 2015; Xia 2016). Their expression analysis shows that each subtype is expressed at each developmental stage throughout the nervous system; however, the pharmacological profiles of these receptor subtypes appear to be distinct (Collin et al. 2013). The A-type receptor can be activated by both low concentrations of ACh and muscarine, whereas the B-type receptor exhibits a 1000-fold reduction in affinity for muscarine (Collin et al. 2013). While it has been much more laborious to characterize the nAChRs, advancing the pharmacological profile of this receptor system in the nervous system is necessary for understanding how these receptors may modulate neural circuits underlying a variety of physiological processes. I have provided foundational research to progress this goal.

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The importance of cholinergic transmission in *Drosophila* is higilighted by previous work. The enzyme choline acetyltransferase (ChaT) and the degradative enzyme acetylcholinesterase (AChE) are highly expressed in afferent sensory neurons and neurons within the CNS (Buchner 1991). ACh synthesis is integral in *Drosophila* development as null mutations in these two enzymes involved in ACh metabolism result in embryonic lethality (Buchner 1991). In *Drosophila,* ACh and the components mediating cholinergic signaling are not surprisingly important in integrating sensory information given its role in sensory neurons. Recent work has enhanced our understanding of the role of ACh signaling in specific processes and behavior including olfactory information processing (Gu and O'Dowd 2006; Silva et al. 2015), motion detection (Takemura et al. 2011), nociception (Hwang et al. 2007; Titlow et al. 2014) and gustation (Schoofs et al. 2014; Huckesfeld et al. 2016; Schlegel et al. 2016). It is known that cholinergic neuronal activity is important in modulating neural circuits guiding larval locomotion (Song et al. 2007) in mediating escape response (Fayyazuddin et al. 2006) and in stimulating grooming, jumping, and hyperactive geotaxis ability (Bainton et al., 2000; Hou et al. 2003) in adult flies. While this work has illuminated an important role of for ACh signaling in the fly, the identification of receptor subtypes that mediate this signaling. Furthermore, their specific involvement in regulation of development and maintenance of defined neurocirctuiry as not been fully addressed. I have identified a role for receptor subtypes, nAChR and mAChRs, in modulating larval locotion, feeding, and sensorimotor ciruit activity. Additionally, I have developed a paradigm that will allow for investigation of specific receptor subtypes involved in regulating a development of a nociceptive circuit (discussed in Chapter 8).

Questions have arisen regarding the practicality of using the *Drosophila* in studies relating to addiction and learning and memory. Recent analysis has shed light on the developmental impact of nicotonic acetylcholine signaling in flies. As mentioned, in mammals, developmental consequences of embryonic exposure to nicotine are low birth weight, increased mortaility, and enhanced probability of

nicotinic dependence by asolescence (Cornelius and Day 2000). These studies have been recapitulated in the fly model and similar outcomes have been identified (Valzquez-Ulloa 2017). Particularly, flies display altered behavioral responses to nicotine and/or ethanol if exposed to nicotine in early development indicating changes in sensitivity. My work has also shown altered developmental rates in response to nicotine exposure and I've identified enhanced mortaility in the presence of particular concentrations of nicotine. Moreover, the Dalpha7 nAChR receptor plays a particularly prominent role in this sensitivity and it was shown this receptor was upregulated following chronic nicotine exposure (Velazquez-Ulloa 2017). This is consistent with other work aforementioned, which showed this receptor expression was altered following chronic antagonist exposure (Ping and Tsunoda 2012). Furthermore, an elegant study implicated a role for dopamine in mediating sensitivity changes to developmental nicotine exposure, suggesting that the molecular alterations observed here may impact circuit dynamics in a manner similar to mammals (Bainton et al. 2000). A recent paper also illuminates the role of ACh signaling, and specific nAChR involvement in olfacatory memory storage, with a role for dopamine in plasticity in the mushroom bodies in *Drosophila* (Barnstedt et al. 2016). Thus, this analysis suggests that this model is feasible in addressing basic questions regarding neural circuit plasticity in the context of ACh signaling. Further investigating circuits that may be regulated by these receptors and their activity-dependent modulation is warranted. It would be interesting to follow up these behavioral and molecular observations using electrophysiological approaches to examine changes in circuit efficacy. Although investigating the role of ACh as a modulator in the CNS has been performed, much of what we know is from work in vitro (Lee and O'Dowd 1999). Furthermore, a study using approaches similar to the techniques I discuss in Chapter 4 was performed, but the receptor subtypes involved in regulating ACh influence on excitatory pre-motor input was not analyzed (Rohrbough and Broadie 2002). Thus, I have provided more

comprehensive analysis of sensorimotor circuit function as a prelude to examination of receptor involvement in plasticity.

Drosophila as a model for analysis of cardiac physiology

      Acetylcholine has also been shown to modulate *Drosophila melanogaster* heart rate (Zornik et al. 1999; Malloy et al. 2016, 2017). I provided a pharmacological analysis of ACh modulation of larval heart rate. The larval *Drosophila* heart has become a popular model in which to study cardiac physiology and development.   Particularly, the larval heart has become a principle model for translational studies regarding the role of ion channels and modulators in regulating heart rate. *Drosophila* have an open circulatory system that consists of a simple dorsal vessel with a posterior heart and anterior aorta. The larval dorsal vessel is a myogenic tube that spans the rostral: caudal axis of the animal (Gu and Singh 1995). Hemolymph is drawn into the heart through ostia in the posterior pump and circulated through an aorta back into the visceral lumen (Molina and Cripps 2001). The pacemaker of the larval heart is located caudally and, like in the human heart is myogenic (Cooper et al. 2009; Desai-Shah et al., 2010; Dowse et. al 1995; Gu and Singh, 1995; Johnson et al. 1998; Rizki 1978). In the late 3rd instar there appears to be neurons innervating the rostral tissue of the aorta, but the function of this innervation have not been addressed (Johnstone and Cooper 2006).   Because of these characteristics and additional similarities in physiology and the ability to manipulate expression of genes, the *Drosophila* larval heart can be used as a model for ionotropic and chronotropic actions as well as investigations into the ionic basis for pacemaker activity. Our lab has previously described the role of cardioactive modulator influence on heart rate, displaying dopamine (Titlow et al. 2013), serotonin (5-HT) (Majeed et al. 2014) and octopamine (de Castro, unpublished) all modulate heart rate in a semi-intact preparation. I added to this, by identifying mAChRs as particularly influential in regulating ACh-meditate cardiac modulation. This served as an additional bioassay to address the pharmacological properties of

cholinergic receptors as well, which serves as a complement to the analysis in the nervous system.

Thus, *Drosophila melanogaster* is a significant model that is amenable for investigation of neural circuit function, nervous system development, and cardiac physiology. I harness these characteristics to enhance understanding of the role of ACh in modulating neural circuits and cardiac function and reveal the role of receptor subtypes in through which ACh imparts its influence. Chapter 2 discusses ACh modulation of heart rate in the larval model in greater detail, revealing the methodology and important findings from this investigation. I follow up this analysis with a different approach to address ACh and additional modulator influence on heart rate in an intact system. In this analysis I utilize an optogenetic approach to screen the influence of neural populations on regulation of heart rate. Since the larval *Drosophila* circulatory system is open, we can stimulate modulator release from the nervous system and observe how increasing circulating concentrations alter heart rate. I've done this by targeting cholinergic neurons and additional neural ensembles.

I then shift to describing cholinergic modulation of neural circuits using a pharmacological approach. This is the focus of Chapter 4. Specifically, I address how nicotinic and muscarinic acetylcholine receptors modulate neural circuit efficacy underlying larval locomotion, feeding, and sensorimotor activity. This analysis serves as a prelude to additional experimentation focusing on mAChRs and their influence on these behaviors. I add genetic approaches in concert with pharmacology to address muscarinic acetylcholine signaling involvement in these neural circuits. This is detailed in Chapter 8 and is currently in progress in the lab.

Additional goals of this research are to refine and improve experimental procedures for fly researchers around the world. As I mentioned previously, the use of thermogenetics and optogenetics has vaulted the fly model to the forefront in analysis of neural circuit function. However, as the popularity of these

methods increases, it is important to address shortcomings that may still exist in these relatively novel techniques. Thus, Chapters 5 and 6 discuss two projects that address improvements that can be made in utilizing optogenetics in the fly model. Chapter 5 highlights important insights I've uncovered as a result of repetively activating light sensitive opsins on neural excitability. Specifically, I illuminated behavioral and circuit accommodation in response to repetitive activation of a specific Channel-rhodopsin variant that is dependent on developmental exposure and feeding parameters. This serves as a prelude to a developmental assay that is currently in progress (discussed in Chapter 8). Chapter 6 discusses novel findings identifying *Glutamic acid decarboxylase1* expression in larval *Drosophila* skeletal muscle. In this analysis, I utilized intracellular recordings in larval body wall muscle 6 in association with optogenetics and observed synaptic responses upon optic stimulation of opsins in *dGad1-Gal4* expressing tissue. I used pharmacological and anatomical transection of the CNS to isolate responses directly in larval body wall muscle, removing any influence from CNS-motor neuron activation and sensory feedback and observed continued membrane depolarization while utilizing a specific, sensitive Channel-rhodopsin variant.   The *dGad1* driver also drove expression of a GFP fluorescent reporter in larval body wall muscle. This highlights the potential for off-target effects using optogenetics in concert with the GAL4/UAS system as this driver line is used to direct expression in the larval CNS.

Chapter 7 details a project that was one in a series of 3 papers in which I analyzed the characteristics of stretch-activated ion channels on proprioception in two proprioceptive organs: the PD organ in crab (*Callinectes sapidus*) and the muscle receptor organ (MRO) in crayfish (*Procambarus clarkii*). Specifically, I used these model organs to mimic the consequences of deep tissue injury on proprioceptive sensory function. Thus, this work deviates from the fly model, which has been the focus of most of my work. These projects were done in association with students in a Neurophysiology course and the students served as co-authors on the paper, which have all 3 been submitted for publication (2 in

Undergraduate Research Journals). Chapter 8 pulls together the compilation of work that I've completed to date and also details projects that are on-going that have been alluded to in this chapter. These projects relate to cholinergic signaling in the fly nervous system, with one in particular focusing more specifically on activity-dependent formation of a nociceptive circuit. I also discuss future directions and how I think these projects can be expanded upon.

CHAPTER TWO

Pharmacological identification of cholinergic receptor subtypes on the *Drosophila melanogaster* larval heart

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INTRODUCTION

The *Drosophila melanogaster* larval heart is a popular cardiac disease model for mammalian heart pathologies. Various studies have shown a number of genes in *Drosophila* regulating cardiac function, including muscle contractile proteins and ion channels, are similar to those in mammals (Bier, E. and Bodmer, R., 2004; Cammarato et al. 2011; Ocorr et al. 2007; Wolf et al. 2006;). In addition, because of the wealth of molecular tools available to alter expression of ion channels and membrane receptors, one can utilize this organism to better understand the physiological mechanisms which may underlie dysfunctions that are manifested in cardiac disease states. *Drosophila* use many of the same neurotransmitters and receptor subtypes as mammals and use similar mechanisms for transmitter release, recycling and general neuronal function (Martin and Krantz 2014). One of these neurotransmitters, acetylcholine (ACh), is prominent in the nervous system and has been confirmed to exhibit modulatory effects on various tissues within *Drosophila*. In vertebrates, ACh is a chemical transmitter of the autonomic, somatic, and central nervous system. In insects, it is the predominant excitatory neurotransmitter of the sensory neurons and interneurons within the central nervous system (CNS) (Martin and Krantz 2014). Acetylcholine receptors (AChRs) consist of two major subtypes: the metabotropic muscarinic acetylcholine receptors (mAChRs), and the ionotropic nicotinic acetylcholine receptors (nAChRs), both of which are activated by ACh and the

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agonists, muscarine and nicotine, respectively. The nicotinic receptor is part of the cys-loop family of ligand-gated ion channels that facilitates fast synaptic transmission (Wonnacott and Livingston 2010). Muscarinic receptors are metabotropic and act indirectly with ion channels through second messenger G proteins to generate a cellular response (Collin et al. 2013). The *Drosophila* genome contains ten nAChR subunits and mAChR types, A-type (encoded by gene CG4356) and B-type (encoded by gene CG7918), have been cloned in this organism (Collin et al. 2013). The expression of these subunits and pharmacological profile has not been   characterized in the larval heart.

     Drosophila have an open circulatory system that consists of a simple dorsal vessel with a posterior heart and anterior aorta. The larval dorsal vessel is a myogenic tube that spans the rostral: caudal axis of the animal (Gu and Singh 1995). Hemolymph is drawn into the heart through ostia in the posterior pump and circulated through an aorta back into the visceral lumen (Molina and Cripps 2001). The pacemaker of the larval heart is located caudally and, like in the human heart is myogenic (Dowse et. al 1995; Gu and Singh, 1995; Johnson et al. 1998; Rizki 1978) meaning action potentials in this tissue are initiated in the absence of neural innervation within the cardiac muscle itself (Cooper et al. 2009; Desai-Shah et al., 2010). In the late 3rd instar there appears to be neurons innervating the rostral tissue of the aorta, but the function of this innervation have not been addressed (Johnstone and Cooper 2006).   Because of these characteristics and additional similarities in physiology and use of manipulating developmental expression of genes, the *Drosophila* larval heart can be used as a model for ionotropic and chronotropic actions as well as investigations into the ionic basis for pacemaker activity.

     In mammals, the cholinergic system is implicated in a number of cardiac diseases, lending credence to the idea that ACh acts on cardiomyocytes. In fact, studies show that cardiac regulation by the parasympathetic nervous system is mediated primarily by ACh binding to the M_2 muscarinic ACh receptor (M_2-AChR)

in many vertebrates (Gavioli et al. 2014). In insects, neuromodulators travel in the hemolymph and affect non-neuronal tissues in addition to acting as the primary mediator of communication between cells of the nervous system (Majeed et al. 2014). A number of neuromodulators that are prominent in larvae, including dopamine (Neve et al., 2004; Titlow et al. 2013), serotonin (Dasari and Cooper 2006; Majeed et al. 2013) and octopamine (Johnson et al. 1997), have also shown to exhibit modulatory effects on the heart. It has previously been shown that ACh at concentrations between 1mM and 1M, decreases heart rate (HR) in *Drosophila* at the larval, pupal, and adult stages with no significant changes at concentrations lower than 1mM (Zornik et al. 1999); however, these studies were performed in the intact, whole animal with injections into the hemolymph. Many compounding actions may come into play with the stress of injections and the presence of other cardioactive substances other than those injected. Additionally, the pharmacological characterization of the cholinergic receptor subtypes involved in modulating HR has not been characterized in isolation of compounding variables with a well-defined physiological saline.   The pupal metamorphic stage is also an active period of transition in hormones and development not only for the skeletal muscle and the nervous system but also the heart (Consoulas et al. 2005; Zeitouni et al. 2007).

This stage in *Drosophila* development is commonly used for investigating cardiac function since the pupa is stationary for injection and observation, but the dynamic process in this transitional stage make it somewhat problematic. In addition, the adult heart is modulated by neuronal inputs, which complicates addressing the function of the intrinsic cardiac pacemaker and ionic regulation in an intact heart (Dulcis and Levine 2003, 2005). The larval heart is easily exposed, myogenic, and its activity can be maintained for hours with a newly developed physiological saline (de Castro et al. 2014). Whereas previous research has utilized intact pupa or larvae with drug administration via injection, we directly expose an open preparation with pharmacological agents at known concentrations. This technique isolates the heart from the nervous system and

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prevents the action of additional modulation from various endogenously released substances.

Because regulation of the *Drosophila* cardiac physiology by modulators remains poorly understood, it is important to determine how endogenous modulators separately act on, and influence cardiac pacemakers in altering HR. The aim of this research is to gain insight into the role of the cholinergic system and specific receptor subtypes in modulating the *D. melanogaster* larval heart. The findings of this study enhance our understanding into the role of modulators and ion channels in general which affect HR, adding to the ever-increasing knowledge regarding endogenous messengers on cardiac tissue. Homologous genes control early developmental events as well as functional components of the *Drosophila* and vertebrate hearts (Bier and Bodmer 2004); thus, the fly is a useful model in which to study cardiac function and the molecular mechanisms underlying heart disease in humans. Mutations affecting ion channels and second messenger systems are readily accessible in *Drosophila*, and it is important to understand the pharmacological profiles of specific receptors in order to utilize these mutants to study the mechanisms which regulate cardiac function.

MATERIALS AND METHODS

Fly rearing and stocks

Wild type *Canton S* (CS) flies were used for HR analyses via the semi-intact method. This strain has been isogenic in the lab for several years and was originally obtained from Bloomington Fly Stock. In order to obtain staged larvae, the flies were held for a few days at 25 C in a 12 hour light/dark incubator before being tested. All animals were maintained in vials partially filled with a cornmeal-agar-dextrose-yeast medium. The general maintenance is described in Campos-Ortega (1974).

Pharmacology

Acetylcholine (CAS # : 60-31-1), nicotine (CAS #: 65-31-6), clothianidin (CAS # 210880-92-5), muscarine (CAS #: 2936-25-6), atropine (CAS #: 51-55-8), and scopolamine (CAS #: 6533-68-2) were purchased from Sigma-Aldrich (St. Louis MO, USA) (Milwaukee WI, USA). Tubocurarine (curare) (Cat #:2820) and benzoquinonium dibromide (Cat #:0424), were purchased from Tocris Bioscience (Minneapolis, MN, USA) . Fly saline, modified Hemolymph-like 3 (HL3) (Stewart et al. 1994) containing: (in mmol/L) 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 1 $CaCl_2$, 5 trehalose, 115 sucrose, 25 N,N-Bis-(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) was used. The following modifications were made to the HL3 saline: pH was decreased from 7.2 to 7.1 and BES buffer was increased from 5.0 mmol/L to 25.0 mmol/L to maintain a stable pH (de Castro et al. 2014).

Heart rate assay

Semi-intact preparations were used throughout. After collection, early third instar larvae were pinned ventral side up on a glass plate and dissected in a droplet of saline (Cooper et al. 2009). The *Drosophila* heart is very sensitive to pH (Gu and Sing 1995); therefore, the saline is adjusted to pH 7.1 and maintained with the high concentration of buffer as described in de Castro et al. (2014). The larval dissection is described in detail by Gu and Singh (1995) and in video by Cooper et al. (2009). An illustration of the preparation used can be found in Desai-Shah et al. (2010). In short, third instar larvae were opened by an incision in the ventral midline and the internal organs were washed aside by saline in order to expose the intact heart to various solutions. The preparation was then left untouched for 2 minutes after dissection to allow the heart to recover from the larval dissection. The heart was then visualized through a dissecting microscope and the rate was measured by directly counting the contractions in the posterior "heart" region of the dorsal vessel. In order for ease of counting the HR, one can readily observe the trachea movements as a consequence of the heart pulling on the ligament attachments.   The baseline

counts were collected with saline and then the saline solution was carefully removed and exchanged with saline solutions containing various agents. The solutions, consisting of agonists and antagonists of both nAChRs and mAChRs at varying concentrations, were introduced onto the open preparation. After exchanging the saline with an agent of interest, the preparation was allowed to sit for 1 minute prior to counting the HR. Following a 1 minute waiting period, the heart contractions were examined for 1 minute, in order to calculate the HR in beats per minute (BPM). After the initial 1 minute count, the solution was left on the preparation for 10 minutes and a 2nd count was performed in order to measure the effects of the agents after a longer period. Hearts that did not continuously beat throughout the paradigm or did not reach 50 beats in 1 minute upon initial exposure to saline were not used in our analyses. As a control, fresh saline was used to replace the first saline solution. Once the HR was counted, the average BPMs and percent change in initial HRs as well as the percent change in the HRs after a 10 minute period were calculated and graphed. All the experiments were performed at room temperature (21–23°C) during the hours of 9-5 pm.

Statistical analysis

The data presented is expressed as mean +/- SEM. The program, SigmaPlot (version 12.0) was used for graphing and statistical analysis. One-way ANOVA test was used for multiple comparisons among the concentration treatments by each individual drug. Student's t-test was used in order to compare the heart rate treatments to the controls, with a confidence level of P ≤ 0.05 as considered statistically significant. Tukey's test was used as a post hoc test to compare the percentage changes of HRs.

RESULTS

Mechanical disturbance and time effect on HR

As previously reported, mechanical disturbance plays a role in altering HR in a semi-intact, open preparation (Majeed et al. 2013). In addition, the HR generally slows down over time. In order to obtain a baseline reading for the effects of mechanical disturbance and time, control experiments were conducted in which saline was washed out and exchanged for fresh saline of the same composition. The newly added saline was then left on the preparation for 10 minutes in order to analyze the role of time on HR. A simple saline exchange resulted in a small increase in HR initially and a decrease over a time period of 10 minutes (Figure 2.1).   In addition, the raw data for average BPM at five time points was recorded over a 10 minute period for each individual preparation (Figure 2.1b). The control experiment was used to account for changes in HR upon solution exchange when various compounds are added. Percent change in rates were compared to controls in order to obtain a true reading of the percentage change in HR due to the action of the added compounds. Results are provided as a percent change of basal rate since there were variations in baseline HRs among preparations, which were calculated based on initial saline counts for each separate trial. The initial change in HR increases 5.77 ±3.22 % (Figure 2.1a) after a saline to saline exchange and then drops 12.40± 7.03% after 10 minutes bathed in saline. Exchanging saline for a second time, after the preparation is untouched for 10 minutes, induces a positive percent change of 16.60±6.67 % before falling 25.40± 6.32% following an additional 10 minute period.

Acetylcholine dose-response relationship

After noting the change in HR induced by saline to saline exchange, the effect of ACh modulation on the heart was tested. Four different concentrations of ACh in saline were applied directly to the open preparation, and the percent change in HR after initial exchange and following a 10 minute period was

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determined. 100nM, 10µM, 100µM, and 1mM concentrations of ACh in saline were used. Each concentration of ACh induced an initial increase in HR when compared with the saline to saline control (Figure 2.2). At the intermediate concentration tested, the average HR increased significantly when a saline solution was exchanged for one containing 10µM ACh (Figure 2.2b). Applying 100nM concentration of ACh to the open heart induced an initial positive percent change of 26.3± 8.91 % from baseline, indicating an increase compared to control. The dose-response relationship reveals that increasing concentration of ACh did show a slight but insignificant increase in the mean percent change in HR (Figure 2.2b). This indicates the ACh receptors may be saturated and desensitized after exposure to ACh concentrations as low as 100nM. Data for each concentration of ACh was graphed and displays the variation in alteration in HRs over the 10 minute time course. The averages in the responses are shown in Figure 2a. The data indicates that there were variations among baseline rates among preparations; however, at each concentration, ACh displayed a positive effect on the HR. In addition, the preparations exposed to ACh did not show dramatic reductions in HR after a 10 minute period, suggesting that the addition of ACh to saline helped stabilize the hearts for a more extended period. This is in contrast to controls, which showed more dramatic reductions in HR over the full experimental time period (Figure 2.2a,d).

nAChR and mAChR agonists dose-response relationship

Following examination of the effect of ACh on the heart, the role of the three primary cholinergic agonists in modulating HR was examined. Nicotine and clothianidin concentrations of 100nM, 10µM, 100µM, and 1mM were exposed to open preparations. Muscarine concentrations of 100nM, 10µM, and 1mM were used in order to reveal a dose-response relationship. For each concentration tested, new larvae were used. The initial percent change after solution exchange as well as percent change after a 10 minute period was calculated and is shown in Figure 3a,. Average HR counts for hearts exposed to 10µM of each agonist

solution were also calculated and agonists that induced significant changes in HR were are presented (Figure 2.3b,c). In addition, the dose-response curve for each agonist was analyzed and displayed (Figure 3d). Exposure to nicotine at a concentration of 100nM increased HR, displaying a percent change of 25.54 ±9.82% from baseline (Figure 2.3a). Exposure to nicotine increased average HR significantly at a concentration of 10µM upon initial exchange (Figure 2.3b), displaying a percent change of 74.43 ±19.44 %. At higher concentrations, the percent change was not as dramatic. In addition, after bathing the preparations in nicotine, the HRs did not slow down as dramatically as preparations exposed to saline without added nicotine. The average decrease in HR after 10 minutes for each of the preparations exposed to nicotine was approximately -7.93 ± 6.04% BPM for all concentrations whereas the preparations bathed in saline alone showed a decrease of approximately -12.48± 7.03% BPM (Figure 2.3a). Nicotine induces a more dramatic change in increasing HR when exchanged compared to a simple saline to saline exchange and maintains a higher HR over the observed time period (Figure 2.3a). When the open preparation was exposed to an additional nAChR agonist, clothianidin, it was found that no significant change in HR resulted. There was an insignificant positive percent change of 18.20±5.09% when the preparation was exposed to 100nM clothianidin (Figure 2.3a). At increased concentrations, the HR did not show a positive change and even dropped in the presence of high concentration of clothianidin, signifying this agonist did not influence HR. This was in stark contrast to nicotine, which induced a significant positive percent change at a concentration of 10µM, suggesting nicotine may act via a separate mechanism to promote changes in HR.

In addition to exposing preparations to various concentrations of nicotine and clothianidin, muscarine solutions were tested in order to observe the effects of this mAChR agonist on HR. Much like nicotine, exchanging saline with a 100nM muscarine solution induced a positive percent change in HR. In addition,

a 10μM muscarine solution induced a significant increase in average HR (Figure 2.3c), rising 53.53 ± 7.43% from baseline (Figure 2.3a). Exposure to the highest concentration of muscarine did not yield as dramatic an increase in HR, again suggesting these receptors may be desensitized at a lower concentration. Initial change in HRs was higher when compared to controls at each concentration, following the same trend observed with ACh and nicotinic solutions. The hearts of preparations exposed to saline containing low concentrations of muscarine displayed a smaller percent decrease on average after a 10 minute waiting period compared to controls (Figure 2.3a). Overall, two agonists, nicotine and muscarine, were capable of inducing positive initial change in HR when exchanged from saline and both maintained hearts at higher rates after a 10 minute period, indicating that adding these drugs to a saline solution enhanced the ability of the heart to maintain a more rapid beat over a prolonged period of time. Clothianidin, however, did not affect HR, which may suggest that nicotine could influence HR through alternative mechanisms due to characteristics unique to the drug.

nAChR and mAChR antagonist dose-response relationship

Various cholinergic receptor antagonists were examined to test their ability to block the action of the agonists. Antagonists for both receptor subtypes were used in this examination. A total of 4 antagonists were examined. Each antagonist in various concentrations was used to test effect on the HR. In addition, following analysis of the effect of each antagonist on HR, the solutions were exchanged a second time, and the third solution exchanged contained a 10μM concentration of either nicotine or muscarine in order to examine the ability of each antagonist to block the positive response induced by each agonist. The initial percent change in HR after each solution was exchanged was calculated and the change in HR after a 10 minute bathing was calculated as well (Figure 2.4a,b). In addition, the averages of HRs at exchange point was calculated as well for each intermediate concentration (Figure 2.4c,d.) As can be seen in

Figure 2.4a, nAChR antagonists, benzoquinonium dibromide (BD) and curare both displayed agonist-like characteristics, as they increased HR after initial exchange, inducing a positive chronotropic response. At a concentration of 100nM, BD induced a positive percent change in HR of 27.56±8.56%, indicating this compound is capable of acting as a potent agonist in this model. Changes in HR were not dramatic with increasing concentration. In addition, curare also increased HR after initial exchange from saline. When compared to saline to saline exchanges alone, curare induced a higher positive percent change in HR at low concentrations, but induced a negative percent change at a high dose (10µM) (Figure 2.4a). Both nAChR antagonists also were capable of maintaining higher HRs over a 10 minute period compared with controls. At 100nM, hearts exposed to curare displayed an increase in HR after 10 minute exposure and hearts exposed to BD displayed a small decrease of 4.19±5.36%. This compares to a decrease of 12.48±7.03% in hearts bathed in saline alone for a 10 minute period (Figure 2.4a).

In addition, mAChR antagonists atropine and scopolamine were examined for their effect in altering HR. Similar to nAChR antagonists tested, both mAChR antagonists induced a positive chronotropic response in HR upon initial exchange from saline. Specifically, at each concentration, both atropine and scopolamine increased HR from baseline. At 10µM, atropine increased HR 36.51±15.23% from baseline, a 31% difference in percent change when compared to a saline to saline exchange alone (Figure 2.4b). Scopolamine displayed agonist-like characteristics at a higher concentration, increasing HR 35.47±13.51% from baseline at 1µM (Figure 2.4b). Both displayed a greater ability to maintain the HR over the course of 10 minutes, which is longer than compared to saline alone (Figure 2.4b).

After examining the effect these antagonists alone had on HR, their ability to block the action of nAChR and mAChR agonists was tested. The same preparations were used, and a third solution exchange was performed after allowing the antagonist-containing solutions to sit on the preparations for a 10

minute period. The third solution contained a 10μM concentration of the agonist along with varying concentrations of the antagonists. Only one preparation is used for each antagonist- agonist combination trial.   As can be seen in figure 2.4a, curare displays a slight ability to block nicotine action initially, as the percent change in HR is lower after initial exchange with this solution compared to a saline to saline exchange; however, after a 10 minute period, the HRs do not decrease as substantially as they do when bathed in a solution containing saline alone. This is similar to what was found when nicotine was added to saline without the addition of curare, suggesting this drug does not block the action of nicotine over the observed time period. In addition, BD does not inhibit the ability of nicotine to induce a positive response at low doses, but does appear to attenuate the action of nicotine at higher concentrations (Figure 2.4a). Similarly, the mAChR antagonist scopolamine does not block the ability of muscarine to induce a positive percent change in HR. Muscarine induces a dramatic change in HR in solutions containing scopolamine, increasing HR as high as 87.17% from baseline (Figure 2.4b). In contrast, our analysis shows that the mAChR competitive antagonist, atropine attenuates the substantial increase in HR exhibited by a muscarine solution, suggesting this antagonists is capable of blocking muscarine action. In the presence of 10μM muscarine, a 10μM atropine solution induces a 5.02±3.99% reduction in HR after initial exchange (Figure 2.4b). However, the positive response in HR observed when atropine is in solution without muscarine is surprising. The averages for each intermediate concentration of antagonist tested was calculated and compared with saline averages. In addition, averages after exchange with a third solution containing antagonists plus each agonist were calculated and compared (Figure 2.4 c,d).

DISCUSSION

This analysis adds to the increasing understanding of *Drosophila* cardiac physiology, and aids in promoting the larval model as a useful tool in analyzing modulatory systems and diseases affecting the heart. The availability of a wealth

of molecular tools make this model attractive for genetic studies. In addition, *Drosophila* serve a valuable model in understanding physiology at the cellular level, particularly as it relates to regulation of cardiac function (Piazza and Wessells 2011). One can utilize this genetically tractable organism in order to screen for mutations in ion channels and receptors that may be crucial in regulating the *Drosophila* heart.

Mechanical disturbance activates stretch-activated ion channels

Control saline exchanges induced a positive percent change in initial HR. The small percent change examined is potentially indicative of a response resulting from activation of stretch-activated ion channels. It is well known that these ion channels are present in cardiomyocytes of vertebrates and are sensitive to mechanical stimuli (Baumgaertner et al. 2012). In addition, Piezo proteins are documented in *Drosophila* and are also sensitive to mechanotransduction (Coste et al. 2012). These ion channels are activated by mechanical disturbance and activation results in the intracellular accumulation of positively charged ions, such as Ca^{2+} and Na^+ (Baumgaertner et al. 2012). This leads to activation of downstream signaling cascades within the cell. The mechanical disturbance caused by exchanging solutions most likely activates these channels and induces cellular response.

Acetylcholine increases HR

Cholinergic receptors are known to play an integral role in cardio regulation throughout the animal kingdom (McCann 1970). A number of diseases of the heart are associated with dysfunctions of cholinergic receptors in mammals, and it is known that ACh receptors are ubiquitous in the CNS of *Drosophila*, but their expression in cardiac tissue had yet to be fully determined (Gundelfinger and Schloss 1989; Nurimen et al. 1991; Schuster et al. 1993; Wadsworth et al. 1988). Whether or not ACh acts through peripheral neurons to modulate *Drosophila* HR in adults is currently unknown. Activation of peripheral

neurons could lead to the release of ACh into the hemolymph where it would interact with cholinergic receptors in cardiac pacemaker cells even for larvae. Previous studies performed in intact larvae suggest that ACh and nicotine both decrease larval HR, but show contrasting modulation in the adult fly. In addition, no evidence had yet been provided suggesting the presence of muscarinic receptors in this tissue. Conflicting results in previous work suggest that receptors in larval cardiac tissue are not solely nicotinic (Zornik et al. 1999). In fact, our analysis may rule out the possibility of functional nicotinic receptor presence in the plasma membrane of cardiomyocytes altogether. The peculiar actions of nicotine may mask any findings resulting from studies of an intact animal, as it is know that this agonist is lipophilic and can have additional actions within the cell. This previous research does, however, provide evidence that cholinergic receptors are present in this tissue and their activation contributes to modulation of HR (Zornik et al. 1999). A more thorough investigation into the mRNA expression of the receptor subtypes present at the larval stage will help to delineate the role of the cholinergic system in modulating HR in this model.

Since we were able to maintain hearts in a physiological saline for long periods of time, we were now able to address the effects of modulators known to be in hemolymph on cardiac function directly. It was found that ACh increased HR at concentrations as low as 100nM. There was a substantial increase in HR upon exposure to 100nM ACh suggesting the presence of cholinergic receptors in larval heart tissue. Higher concentrations show little additional positive effect on HR, suggesting ACh desensitizes receptors at low concentrations, thus resulting in decreased sensitivity to additional ACh activation at concentrations above 10µM. In this analysis, semi-intact preparations were used allowing for the exposure of the larval heart directly to select compounds without the influence of compounding variables. We determined that ACh is capable of inducing an increase in HR suggesting this modulator is activating receptors present in cardiomyocytes, resulting in depolarization of the membrane and a positive chronotropic action on the heart in this model.

Muscarine and nicotine increase HR

Since ACh induces an increase in HR when exposed directly to the larval heart it is likely that cholinergic receptors are expressed in this tissue. Previous studies have shown that ACh decreases HR and the nAChR agonist, nicotine increases HR (Zornik et al. 1999); however, pupa were injected with the substance and compounds were not selectively examined directly on the heart in a well buffered saline.   There has been no evidence supporting the presence of muscarinic receptors in *Drosophila* larval cardiac tissue to date. In order to elucidate the cholinergic receptor subtypes which may play a role in altering HR, we first added various concentrations of nicotine, clothianidin or muscarine to the open preparations and then examined if selective antagonists blocked agonist actions. The findings indicate that functional mAChRs are likely present in cardiomyocytes at the larval stage. These receptors function to induce a significant enhancement in in pacemaker activity, resulting in an increase in HR. Although we cannot definitively rule out the expression of nAChRs in larval cardiac tissue, the finding that clothianidin does not affect HR and the inability of nAChR antagonists from blocking nicotinic action suggests the absence of functional nAChR in the plasma membrane of pacemaker cells. More thorough expression analysis is needed to confirm this finding.

The results demonstrate nicotine influences HR significantly when exposed to the heart directly. While our findings show there may be an absence of nAChRs in the plasma membrane, the influence of nicotine may very well act in an alternative manner to induce an increase in HR. It is known that nicotine not only activates plasma membrane receptors but is well known to have direct effects on intracellular function since the compound is lipophilic and crosses cell membranes rapidly, particularly in more alkaline environments (>6.5 pH) (Hukkanen and Benowitz 2005). Considering the saline solution used to bathe the open preparations is measured at a pH of 7.1, it is likely that nicotine exists in a more unionized state in this solution, and thus may cross the cell membrane

quickly. This is an important characteristic that likely enhances the action of nicotine within the cell. Once in the cell the role of nicotine in modulating HR remains poorly understood. However, recent imaging analysis of membrane proteins, including nAChRs, performed by Moonschi et al. (2015) shows evidence of nAChR receptor presence in Endoplasmic Reticulum (ER) derived microsomes. Not only does this group confirm the presence of nAChR subunits in microsomes, but they also, through the use of Ca^{2+} flux imaging, show that these receptors are functional. In addition, previous findings indicating rapid desensitization of membrane nAChRs, such as that of Colombo et al. (2013), could also support nAChR activity in the ER in other cell types, as these receptors could be desensitized prior to incorporation into the plasma membrane. Therefore, we speculate the presence of functional nAChRs in the ER that may act to dump Ca^{2+} in the presence of nicotine, inducing an increase in HR. Although difficult in larval cardiac pacemaker cells due to the trouble in fluorescent imaging of a contractile organ, one may test this hypothesis in additional cell types through a Ca^{2+} flux imaging experiment where nAChR release from the ER is blocked via Brefeldin A. One could then look for changes in calcium binding with a calcium sensitive fluorophore (fluo-4) upon exposure to ACh. The additional actions of nicotine, including the activation of other membrane receptors, such as the transient receptor potential A1 channel (Talavera et al. 2009), as well as the blocking of additional surface receptors including 5-hydroxytryptamine type 3 (5-HT$_3$) (Schreiner et al. 2014) could play a role of altering HR in vivo as well. These findings open the door to further investigation of the mechanistic actions of nicotine in modulating HR.

While the presence of functional nAChRs in the ER remain a possibility, our analysis suggests that the identity of cholinergic receptors on larval pacemaker plasma membranes are primarily muscarinic. In testing the role of muscarine, an agonist that activates metabotropic mAChRs, in regulating HR, it was found that muscarine increased HR at both low and high concentrations, suggesting the presence of mAChRs in larval cardiac tissue. As stated, two

subtypes of mAChRs are expressed in *Drosophila*, A-type and B-type. The activity of these two receptor subtypes are crucial in regulating the excitability of the cell. In mammals, 5 muscarinic receptor subtypes have been identified (M1-M5) and classified pharmacologically (Felder 1995). These subtypes have been grouped into two groups based on their mobilization of intracellular Ca^{2+} (M1, M3, and M5) or their ability to inhibit adenylate cyclase (M2 and M4) (Felder 1995). M2 receptor is known to be present on human hearts and acts to slow down HR by inhibition of adenylate cyclase and decrease of intracellular cAMP. The functional characterization of the two muscarinic receptor subtypes in *Drosophila* has been more problematic; however a comprehensive analysis of the function of A-type and B-type mAChRs in this model was performed by Collin et al. (2013). The group measured relative A-type and B-type mAChR expression at various stages of the life cycle by extracting mRNA from the head, thorax, and whole-body of individual animals. Their expression analysis shows that each subtype is expressed at each developmental stage throughout the body; however, the pharmacological profiles of these receptor subtypes appear to be distinct (Collin et al. 2013). The A-type receptor can be activated by both low concentrations of ACh and muscarine, whereas the B-type receptor is not responsive to muscarine binding (Collin et al. 2013). In addition, sequencing analysis shows the binding pocket for ACh in the A-type receptor is highly similar to the binding domain in mammalian M1-M5 receptors, but less so in the B-type receptor, suggesting the different pharmacological profile is most likely due to structural differences between the two receptor subtypes (Collin et al. 2013). In our analysis, the heart was responsive to low concentrations of both ACh and muscarine, suggesting the presence of A-type mAChRs in larval cardiac tissue. It is noted that the addition of muscarine significantly increases average HR when compared to controls, indicating a stimulatory effect and potential activation of a 2nd messenger cascade that mediates intracellular Ca^{2+} levels. As stated, M2 mAChR receptor subtype is present in mammalian cardiac tissue and was shown to attenuate adenylate cyclase activity, thereby reducing the intracellular levels of

cAMP through G_i (Felder, 1995). Our analysis suggest that the mAChRs present in cardiac tissue at the larval stage act through a stimulatory cascade that is not regulated by adenylate cyclase, as it has been shown that HR stimulation by 5-HT does not act through cAMP (Majeed et al. 2013). In a recent study by Ren et al. (2015), the group showed that A-type mAChRs couple to the $G_{q}/_{11}$ signaling pathway, whereas B-type mAChR couple to the $G_{i}/_{0}$ pathway. Their findings that A-type receptors do not act through the inhibitory $G_{i}/_{0}$ pathway supports our evidence that this receptor subtype is present in larval heart tissue, as the stimulatory effects on HR suggest. However, the tissue from which mAChR mRNA was extracted was not described in their analysis, so the 2nd messenger signaling pathway through which these receptors act in larval heart tissue must be examined.

Understanding how cardiomyocytes pace the *Drosophila* heart has been in question. A study by Desai-Shah et al. in 2010 provided a comprehensive analysis of the role of three important calcium pumps in modulating HR, the Na^+/Ca^{2+} exchanger (NCX), the plasma membrane Ca^{2+}-ATPase (PMCA), and the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). It was found that compromising these exchangers individually or together had a dramatic effect on the HR of a semi-intact preparation. The analysis lead to the conclusion that $[Ca^{2+}]_i$ and $[Na^+]_i$ are tightly regulated in *Drosophila* larval hearts. A proposed model indicates that when *Drosophila* hearts are in diastole, depolarization and a slow release of Ca^{2+} from the sarcoplasmic reticulum (SR) by ryanodine receptors (RyR) leads to a rise in $[Ca^{2+}]_i$. The SERCA pumps Ca^{2+} back into the SR and the NCX removes $[Ca^{2+}]_i$ in exchange for Na^+ ions across the plasma membrane of the cell. The influx of Na^+ ions leads to a depolarization of the plasma membrane, thus opening low voltage-gated T-type Ca^{2+} channels (VCa) (Huser et al. 2012) and potentially voltage-gated Na^+ channels. The influx of Ca^{2+} acts on the RyR to cause the ER (endoplasmic reticulum) to dump Ca^{2+} which results in a calcium induced inhibition of the RyR. Until the $[Ca^{2+}]_i$ is reduced by the SERCA and NCX, the RyR stays inhibited but will start leaking

Ca^{2+} as $[Ca^{2+}]_i$ returns to a low level to then repeat the cycle (Subramani and Subbanna 2006). In addition, it is understood that the pacing cells act as contracting myocytes and that they can also generate action potentials, suggesting the presence of voltage gated Na^+ channels (Gu and Sing 1995). Given the fact that ER nAChRs have been shown to permit Ca^{2+} influx (Moonschi et al. 2015) and A-type mAChRs act through a stimulatory signaling cascade, it can be determined that activation of these receptors could lead to an initial increase in Ca^{2+} concentration in the cell, as the Ca^{2+} conductance increases. This increased Ca^{2+} conductance in turn activates the NCX, which pumps Ca^{2+} out of the cell, and Na^+ into the cell, leading to membrane depolarization in cardiac pacemaker cells and an increase in HR.

nAChR and mAChR antagonists increase heart rate

In addition to testing the role the two cholinergic receptor agonists in regulating HR, classical competitive antagonists were tested in order to deduce their ability to block the action of nicotine and muscarine. It would be assumed that since it is evident that both agonists significantly increase HR, the addition of competitive antagonists in the presence of the agonists would block this response. Surprisingly, we found that each antagonist actually increases HR initially and only atropine displays the ability to block the action of the mAChR agonist (muscarine) at each concentration tested. Although this may seem rather peculiar, it is well established that the pharmacological profile of nicotine and nAChRs is quite complex. In numerous studies involving mice, including those by Buccafusco et al. (2009) and Paradiso et al. (2003), the description of nicotine as a simple nAChR agonist appears to be quite simplistic (Buccafusco et al. 2009). These studies, along with many others, have found that the actions of nicotine often mimic the actions of classic nAChR antagonists, including *d-*tubocurarine and α-bungarotoxin (Ropert and Krnjevic 1982). We found similar results testing BD and curare. As stated previously, this phenomenon may be explained by the ability of nicotine to activate and desensitize receptors quite

rapidly (Buccafusco et al. 2009) and compensatory upregulation of expression of nAChR subunits could result (Buccafusco et al. 2009). However, it is noted in this analysis, nAChR antagonists were bathed on the preparation prior to the addition of nicotine. Thus, the ability of nicotine to induce a positive response in the presence of these competitive antagonists may not be due to its ability to quickly desensitize receptors. Had the preparation been bathed in nicotine first, one could assume that a change in conformation of the receptors would alter the ability of competitive antagonists to block further agonist action. Instead, it can be assumed that the difference in nAChR pharmacology in this model may likely be explained by structural differences in the associated receptor proteins. Additionally, the actions of nicotine on ER nAChRs could also play a role in rapid desensitization.

In addition, the ability of mAChR antagonists to block the action of muscarine were tested. Based on the results observed of muscarine altering HR and comparison with previous studies, it is likely that A-type receptors are present in larval cardiac tissue. Pharmacological data provided by Collin et al. (2013) shows both scopolamine and atropine are capable blocking the action of muscarine in *Drosophila*. While we found that atropine did indeed reduce HR in the presence of muscarine, scopolamine surprisingly did not show an ability to block this agonist. Moreover, both antagonists displayed agonist-like characteristics of their own. Although the pharmacology provided here suggests the presence of A-type mAChRs in larval cardiac tissue, the question regarding 2nd messenger cascade activation by these receptor subtypes in this tissue remains. Further pharmacological inhibition of particular 2nd messengers may be required in future studies to elucidate the role of mAChRs in modulating HR.

CONCLUSION

Analysis of the effects of cholinergic compounds on HR have not been previously administered in a manner that isolates the actions of the desired compound. In contrast with current understanding, our pharmacological analysis

indicates cholinergic compounds modulate HR in larval *Drosophila*. Understanding the effects of neuromodulators on regulation of HR and cardiac development can aid in understanding how exposure to increased concentrations of cholinergic drugs, such as nicotine in early development may alter the normal development of this vital organ. Alterations in these modulatory systems have shown to dramatically affect HR, showing the potential detriment posed to human fetuses in embryonic development (Horta et al. 1997). In addition, this study aids in providing a pharmacological profile for this organism and helps lay a foundation for future analysis in characterizing cholinergic receptor subtypes in cardiac tissue. Future studies surrounding potential nAChR function in the ER membrane *in vivo* can be performed to enhance knowledge regarding nicotinic action not only in cardiac pacemaker cells, but in additional excitable cells as well. The genetic amenability of *Drosophila melanogaster* allow for thorough examination of functional expression of particular subunits of cholinergic receptors and the role of second messenger signaling cascades in regulation of cardiac physiology and development.

a

Heart Rate (% Change +/- SEM)

30
20
10
0
-10
-20
-30

P=.817
P=.172
P=.073
P=<.001

n=6

b

$1\text{----}>2_1\ 2_1\text{----}>2_{10}\ 2\text{----}>3_1\ 3_1\text{----}>3_{10}$

Heart Rate (BPM)

180
160
140
120
100
80
60
40
20

n=6

0 1 2 3 4 5
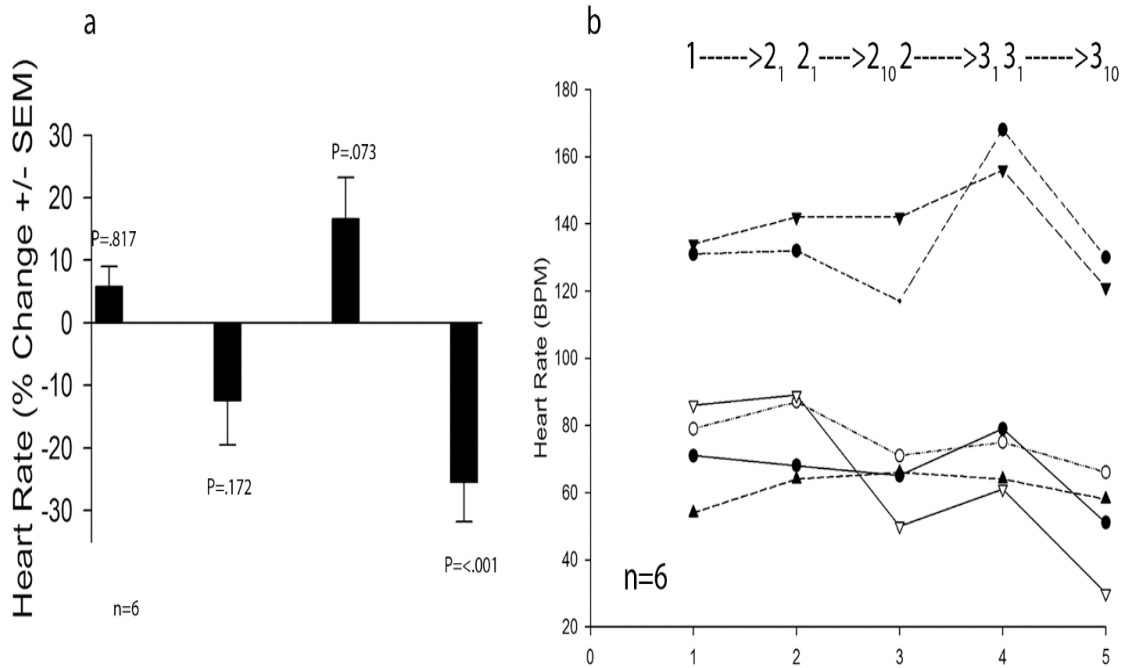
Figure 2.1. Change in HR as a result of mechanical disturbance upon changing solutions. a The percent change in HR after exchanging saline solutions. The preparations were left inside saline for 1 minute and then the rate was obtained for the following minute. Saline (1-Saline) was exchanged with saline (2-Saline). The preparations were left for 1 minute and subsequently rate was obtained over the next minute. The preparations were left for 10 min (subscript 1 to 10) and then the HR was counted for 1 minute. Saline (2-Saline) was exchanged with saline (3-Saline), the preparations were left for 1 min before counting the rate in the next minute. The preparations were left for 10 minutes and then the HR was obtained for 1 minute. Data are presented as mean ± SEM. b The raw change in HRs in response to saline to saline solution exchanges. The changes in solutions are noted, with the subscripts illustrating time points during which solutions were left on the preparations (1 minute to 10 minute period)
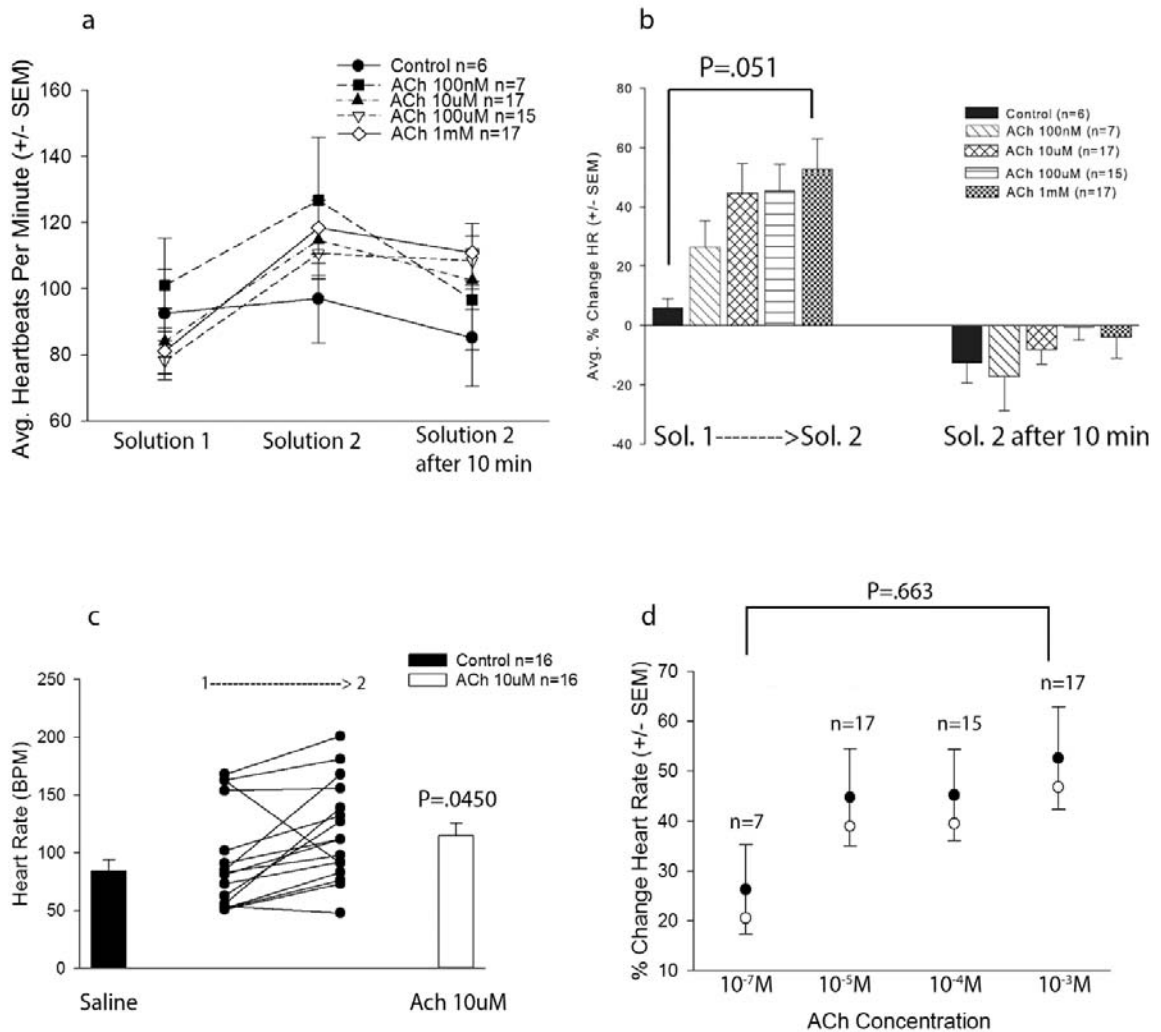
Figure 2.2**.** Change in HR in response to various concentrations of acetylcholine (ACh). a The average change in HR in response to saline (solution 1) to saline + ACh (solution 2) exchanges. At each concentration, ACh induced a more substantial change in beats per minute (BPM) when compared to controls as evidenced by the increased slope. In addition, preparations bathed in ACh solutions for 10 minutes displayed less dramatic reductions in HR after the time period. b The percent changes in HR after exchange from solution 1 to solution 2 c Change in average HR in exchange from saline to ACh 10µM with raw changes for each preparation. The addition of ACh induced a significant change in average HR. (Student's t-test was used for comparison) **d** Dose–response relation of Ach action on larval HR. Open circles represent the subtraction of control saline exchanges from various concentrations of ACh. (One-way ANOVA was used for comparison).

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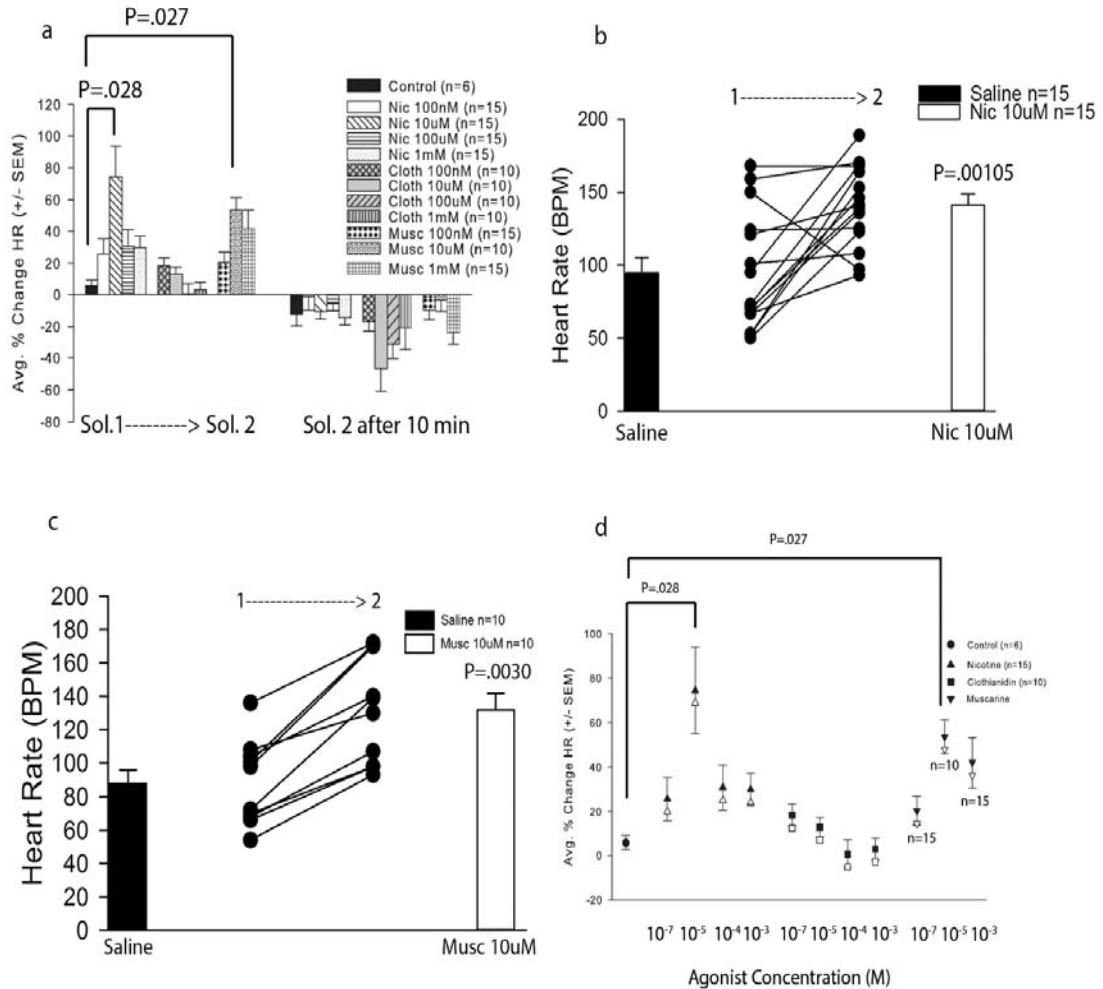
Figure 2.3 Change in HR in response to various concentrations of AChR agonists. a The percent changes in HR after exchange from solution 1 to solution 2. Solution 2 contained various concentrations of nicotine (Nic), clothianidin (Cloth) or muscarine (Musc), as indicated. The percent change in HR after 10 minutes is noted in the second group of columns. The addition of both agonists induced a positive percent change in HR. b Change in average HR in exchange from saline to Nic 10µM with raw changes for each preparation. The addition of Nic induced a significant change in average HR. c Change in average HR in exchange from saline to Musc 10µM with raw changes for each preparation. The addition of muscarine induced a significant increase in average HR. (Student's t-test was used for comparison.) d Dose–response relation of Nic and Musc action on larval HR. Open shapes represent the subtraction of control saline exchanges from various concentrations of agonists. (One-way ANOVA was used for comparison)

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a

b

P=.990   P=.967

Control n=6
Curare 100nM n=10
Curare 1uM n=10
Curare 10uM n=10
BD 100nM n=10
BD 1uM n=10
BD 10uM n=10

*Sol. 3 consists of antagonist + 10uM nicotine

Avg. % Change HR (+/- SEM)

Sol. 1---> Sol.2   Sol. 2 after 10 min   Sol.2---> Sol.3   Sol. 3 after 10 min

P=.156

P=1.0

Control n=6
Scopolamine 100nM n=10
Scopolamine 1uM n=10
Scopolamine 10uM n=10
Atropine 100nM n=10
Atropine 1uM n=10
Atropine 10uM n=10

*Sol. 3 consists of antagonist + 10uM muscarine

Avg. % Change HR (+/- SEM)

Sol. 1----> Sol. 2   Sol. 2 after 10 min   Sol. 2----> Sol. 3   Sol. 3 after 10 min

c

1-------->2   2-------->3

P=.197   P=1.0

Control n=10
BD 1uM n=10
BD 1uM + Nic 10uM n=10

Heart Rate (BPM)

Saline   BD 1uM   BD 1uM + Nic 10uM

d

1-------->2   2-------->3

P=.078   P=.550

Control n=10
Atropine 1uM n=10
Atropine 1uM + Musc 10uM n=10

Heart Rate (BPM)

Saline   Atropine 1uM   Atropine 1uM + Musc 10uM

45

Figure 2.4. Effects of AChR antagonists on HR. a The average percent change in HR when saline is exchanged for nAChR antagonists curare and benzoquinonium dibromide (BD). Solution 2 consists of saline + antagonist. The observed change after a 10 minute period is noted. In addition, the ability of each antagonist to block the action of Nic was tested. Solution 3 consists of saline+antagonist+ 10μM nicotine.   Curare displays an ability to block action of Nic at each concentration and shows agonist-like characteristic on its own. b The average percent change in HR when saline is exchanged for mAChR antagonists scopolamine and atropine. Solution 3 consists of saline+antagonist+ 10μM muscarine. Atropine blocks the positive action of muscarine at each concentration, but, like nAChR antagonists, displays agonist-like characteristics of its own. Scopolamine does not block muscarine action. c Change in average HR in exchange from saline to BD 1μM with raw changes for each preparation. The addition of muscarine induced an increase in average HR that was significantly significant. In addition, the change in average HR is recorded then solution 2 is exchanged with solution 3 containing 1μM BD + 10μM muscarine (Student's t-test was used for comparison.) d Change in average HR in exchange from saline to atropine 1μM with raw changes for each preparation. The addition of atropine induced an increase in average HR that was not statistically significant. In addition, the change in average HR is noted then solution 2 is exchanged with solution 3 containing 1μM atropine + 10μM muscarine (Student's t-test was used for comparison.

CHAPTER THREE

Using optogenetics to assess neuroendocrine modulation of heart rate in *Drosophila melanogaster* larvae

INTRODUCTION

The *Drosophila melanogaster* heart has rapidly become a principal model in which to study cardiac physiology and development. While the morphology of the heart in *Drosophila* and mammals differ, many of the molecular mechanisms that underlie heart development and function are similar (Bodmer et al. 1998). Additionally, the hearts are functionally assessed by comparable physiological measurements, such as, cardiac output, rate and time in systole or diastole (Choma et al. 2011). A number of studies have used the *Drosophila* heart to identify proteins that are crucial in regulating cardiac muscle contraction and ion transport (Bier and Bodmer, 2004; Wolf et al. 2006; Ocorr et al. 2007; Cammarato et al. 2011). These proteins are known to share similar functions in mammals. In addition, recent analyses have begun to shed light on endogenous modulators and hormones that directly influence heart rate (HR) and rhythmicity. It has been shown that abundant neuromodulators active in *Drosophila* and other insects, including acetylcholine (Zornik et al. 1999; Malloy et al. 2016), serotonin (Dasari and Cooper 2004; Majeed et al. 2014; Hillyer et al. 2015), dopamine (Collins and Miller 1977; Zornik et al. 1999; Titlow et al. 2013), glutamate (Ellison et al. 2015), octopamine (Johnson et al. 1997; Zornik et al. 1999) and melatonin (VanKirk et al. 2016) display modulatory effects on the cardiac pacemaker. Furthermore, many of the receptors mediating the chronotropic and ionotropic

action of these modulators have been identified in the aforementioned studies. All of these compounds are systemic in humans and many of these receptors that are targeted in these studies share human homologues. Thus, the release of modulators from the central nervous system (CNS) that may alter cardiac function in humans through activation of their receptors on the heart directly, or through modulation of release of cardio active substances from the nervous system into the blood, may display similar actions in the fruit fly model. These studies highlight important features that make the *Drosophila* heart a potentially significant model in providing insight into the molecular mechanisms fundamental to human heart function. Advancing our understanding of the role of endogenous compounds and their receptors in influencing cardiac function will help to foster investigation into potential pharmacological and genetic therapies for human cardiac pathologies. Although it is becoming well known that the *Drosophila* heart is quite sensitive to changes in circulating modulators/hormones as well as hemolymph pH (Badre et al. 2005; Desai-Shah et al. 2010; de Castro et al. 2014), it is important to continue to address the role of these hormones in regulating cardiac function.

The *Drosophila* circulatory system is an open system that consists of a simple dorsal vessel with a posterior heart and anterior aorta. The dorsal vessel is a tube that spans the rostral- caudal axis of the animal and is made up of multiple types of cardiomyocytes (Gu and Singh 1995; Lehmacher et al. 2012). Hemolymph is drawn into the heart through ostia in the posterior pump and circulated through an aorta back into the visceral lumen (Molina and Cripps 2001). The pacemaker of the larval heart is located caudally and, like in the human heart, is completely myogenic (Rizki 1978; Dowse et. al 1995; Gu and Singh, 1995; Johnson et al. 1998). During the majority of the larval stage, the heart is completely devoid of neural innervation; however, in the late 3rd instar there appears to be neurons innervating the rostral tissue of the aorta, but the

function of this innervation has not been addressed (Johnstone and Cooper 2006). Neural innervation persists into the adult stage.

While the pupal stage is commonly used for examining cardiac function due to the fact that it is immobile for injection and inspection, this stage in *Drosophila* development is highly dynamic. Vast neural circuit rewiring, muscle breakdown, and reassembly of internal and external structures occur during this stage, making analysis of specific neural circuit and/or hormonal influence on HR somewhat challenging. In addition, as mentioned previously, the adult heart is modulated by neuronal inputs, which complicates addressing the function of hormones directly on the intact heart (Dulcis and Levine 2003, 2005). Therefore, the larval stage in *Drosophila* development serves as an ideal model for observation of direct systemic modulation of cardiac function. In previous analyses performed by our lab utilizing larval *Drosophila*, a semi-intact method, in which the larvae were dissected and the heart exposed directly to solutions, was utilized. A distinct advantage exists in using such a technique as one can assess the direct actions of controlled concentrations of modulators on the HR without the influence of additional modulators or hormones that may circulate the hemolymph as a result of stress from injections or other alternative approaches. Analysis using this method has led to the discovery of direct modulation of HR of a number of modulators as well as the receptors through which they act in cardiac tissue in larval *Drosophila.* The pharmacological approach on the semi-intact preparation allows for the use of agonists and antagonists for identification of these important receptor subtypes without the need to rely on low level mRNA expression profiling. While this approach serves useful purposes and has provided necessary insights, it fails to simulate the role of neuroendocrine released modulators in regulating cardiac function. The use of an intact larval preparation allows one to investigate the role of specific neural populations, and the modulators/hormones they release, in pacing the heart. In addition, it has been noted that the HR is much higher in an intact larvae than in a dissected preparation bathed in a physiological saline. It is

likely that the saline often used in such analyses and the composition of the hemolymph in an intact, closed system is not equivalent, as saline lacks endogenous combinations of peptides and modulators that influence the heart. Thus, an intact approach more closely mimics changes in cardiac function in vivo as a result of variations in neural circuit activity in response to environmental stressors. To date, there are no studies, to our knowledge, that have been performed in larval *Drosophila*, that address the role of neural-derived modulators that may influence heart function while circulating the hemolymph in vivo.

Since the larval HRs are fairly high at room temperature (22°C) the more subtle effects of modulators within the hemolymph might not be as pronounced. We have noted in a previous study that intact as well as exposed hearts in dissected preparations are substantially slowed at 10°C (Zhu et al. 2016). The exposed larval hearts respond well to modulators (5-HT, OA, DA, Ach, tyramine) at this temperature. Thus, we examined the possibility of detecting the effects of exciting the specific neurons containing modulators as well as defined sensory neurons on intact larvae conditioned to 10°C to determine if the HR is altered. Even at 10°C the larval heart does beat well (50 to 100 beats per min) so any modulators released into the hemolymph can be readily circulated within the whole body cavity. Additionally, it has been shown in previous analyses that channel rhodopsin (ChR2) is functional at 10°C in acute conditions (Zhu et al. 2015).

The advent of optogenetics has revolutionized the ability to temporally control the activity of excitable cells. While the majority of its use has centered on driving activity changes in neural populations to deduce the neural basis of behavior, optogenetic drive of cardiac muscle has recently been introduced in model organisms (Alex et al. 2015; Zhu et al. 2016). However, the use of optogenetics in assessing indirect regulation of cardiac pace making has yet to be addressed. Here, we have chosen to utilize the light sensitive cation channel ChR2-XXL (Dawydow et al. 2014) to drive activation of specific neural populations. Specifically, we have targeted activation of cholinergic neurons

(Cha-Gal4), dopaminergic neurons (ple-Gal4), and serotonergic (5-HT) neurons (Trh-Gal4) through tissue specific expression of ChR2-XXL to assess how systemic release of the modulators synthesized by these neurons alters HR in vivo. In addition, we activated a subset of class IV dendritic arborization (da) sensory neurons (ppk-Gal4) known to be critical in mediating response to noxious stimuli (Hwang et al. 2007; Xiang et al. 2010; Johnson and Carder, 2012; Kim et al. 2013; Kim and Johnson 2014) to examine if they could lead indirectly to alterations in HR.

The channel-rhodopsin-2-XXL variant was recently created, placed under the control of a UAS activation sequence and cloned into *Drosophila* (Davydow et al. 2014). It has been shown to produce more robust and longer photocurrents due in large part to its increased expression, enhanced affinity for a cofactor, all-trans-retinal (ATR), and potential increased single channel conductance (Dawydow et al. 2014). Expression of this rhodopsin allows for low-light applications as to prevent off-target effects and could be of use in targeting deep neural and muscle tissue in other model organisms as well as in humans. We have chosen to use this variant to ensure robust neuromodulator release, so that influence on the heart can be evaluated.

MATERIALS AND METHODS

Fly rearing

All flies used for HR analyses were held for a few days at 22° C in a 12 hour light/dark incubator before being tested.   All animals were maintained in vials partially filled with a cornmeal-agar-dextrose-yeast medium. The general maintenance is described in Campos-Ortega (1974).

Drosophila lines

The filial 1 (F1) generations were obtained by crossing virgin females of the recently created ChR2 line (which is very sensitive to light) called y^1 w^{1118}; PBac{UAS-ChR2.XXL}VK00018 (BDSC stock # 58374) (Dawydow et al. 2014)

with males from each driver line targeting specific neural populations. The driver lines used in this study include: *Trh-Gal4* (BDSC stock # 38389), *Cha-Gal4; UAS-GFP* (BDSC stock #6793), *ple-Gal4* (BDSC stock # 8848), *ppk-Gal4* (BDSC stock # 32078). These lines were all purchased from Bloomington *Drosophila* Stock Center (BDSC) in Bloomington, Indiana, USA. In addition, we also used *UAS-ChR2-H134RII-mcherry; Trh-Gal4* (III) homozygous line, which was kindly provided by Dr. Schoofs et al. (2014). This line expresses a less sensitive Chr2 variant in 5-HT neurons. Adult flies from the driver (Gal4) lines and the UAS-Chr2-XXL line were crossed on standard fly food. Flies from a parental line, y^1 w^{1118}; PBac{UAS-ChR2.XXL}VK00018 (BDSC stock # 58374), were used as controls in assessing the influence of neural-based modulators on HR. Progeny from these adults were not crossed with a Gal4 line; therefore, expression of ChR2-XXL is absent in these larvae. The *Trh-Gal4* line (*UAS-ChR2-H134R-mcherry; Trh-Gal4* (III) homozygous) was crossed with the UAS-ChR2-XXL line and, therefore, carries two different UAS constructs. In the text from this point on, the tested F1 generation will be referred to simply as "*Trh-Gal4 X UAS-ChR2-XXL*" for simplicity.

Preparation of food supplemented with all-trans-retinal and flies prior to testing

All trans-retinal (ATR; Sigma-Aldrich, St. Louis, MO, USA) was diluted in standard fly food to a final concentration of 100μM or 1mM or and protected from light with aluminum foil. For control experiments, larvae were cultured in food that only contained the solvent (absolute ethanol in the same volume used for the ATR mixtures in the fly food). The ATR or ethanol food mixtures were left alone for 48 hours prior to adding larvae in order to allow some evaporation of the alcohol solvent from the mixture. It has been noted that larval development slows in the presence of ethanol, so this evaporative precaution was taken to limit its developmental influence. Adult flies from the driver (Gal4) lines and the UAS-Chr2-XXL line were crossed on standard fly food. Approximately 3 days following the

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cross, 2nd instar larvae were removed from standard food vials and placed in 1mM ATR-food mixtures and left for 48 hours prior to testing.

Monitoring heart rate in the intact larva

The movement of the trachea is commonly used to monitor *Drosophila* larval HR because of the clear contrast of the structures (White et al. 1992; Dasari and Cooper, 2006). Early 3rd instar larvae were stuck ventrally on a glass slip using double stick tape in such a way that mouth hooks are free to move. Care was taken not to stick the spiracles to the tape. The glass slip was placed on top of a dark surface in order to maximize contrast between the background and the translucent larval body wall. The HR was measured for 1 minute in white light, followed by 1 minute in blue light (470nm wavelength, LEDsupply, LXML-PB01-0040, 70 lm @ 700mA) from a high intensity LED that was focused on the specimen through a 10x ocular objective while the HR was counted (Titlow et al. 2014). The photon flux (number of photons per second per unit area) was measured with a LI-COR (model Li-1000 data Logger, LDL 3774), which produced around 550 uMol s-1 m-2 per uA on the surface of the larvae. Following initial 1-minute counts, HR was counted again every 10 minutes while larvae were exposed to blue light to detect changes over a longer period of time. The heartbeats were counted by an observer's eye with the use of a dissecting microscope.

Statistical analysis

All data are expressed as raw values or as a mean ($\pm$ SEM). The Mann-Whitney Rank Sum test was used to assess, within line at each time point measured, the difference in HR elicited in response to a + all-trans-retinal (ATR) diet versus a –ATR diet to evaluate the efficacy of the addition of ATR on altering HR as a result of activating select neural populations. In addition, a Mann-Whitney Rank Sum test was used to test differences in percent changes in HR for

experimental lines vs. a control line (y^1 w^{1118}; PBac{UAS-ChR2.XXL}VK00018parental line). The groups were separated based on their prior feeding (+ATR or –ATR) and the percent change in HR at each indicated time point was compared. Since larvae often displayed variation in baseline HR, raw data is presented and is provided as beats per minute (BPM) and also as percent changes in HR. Comparisons between the +ATR and –ATR fed larvae within lines as well as from the control line vs. experimental lines were made to assess the efficacy ATR supplementation, as well as the role of modulator release, on HR. This analysis was performed with Sigma Stat software. P of $\leq$ 0.05 is considered as statistically significant. The number of asterisks are considered as P $\leq$ 0.05 (*), P $\leq$ 0.02 (**), and P$\leq$ 0.001 (***).

RESULTS

Blue light influence on heart rate

It has long been known that larval *Drosophila* display negative phototaxis behavior. Upon exposure to light, larvae swing their anterior in avoidance (Jennings 1904; Mast 1911; Grossfield 1978; Sawin et al. 1994). Larvae display photoavoidance even in the absence of Bolwig's Organ (Xiang et al. 2010). Thus, it is assumed that blue light is significant in influencing neural circuit activity within the CNS. Because of this, we tested the potential influence of blue light in stimulating the release of cardioactive modulators. In an effort to control for this influence alone, avoiding any targeted neural populations, we utilized the *UAS-ChR2-XXL* parental line as a control. In addition, to avoid confounding variables, these larvae were separated into two groups based on the presence of ATR (Figure 3.1 a1,2) or absence of ATR (Figure 3.1 b1,2) in their food prior to testing. Due to the fact that there is a high degree of variability in baseline HR in larvae (generally between 160-200 beats per minute (BPM) at room temperature (22°C) and 80-100 BPM at 10°C), the data is presented as raw changes in BPM (Figure

3.1 a1, b1) at each time point indicated as well as percent change from one time point to another (Figure 3.1 a2, b2). The percent changes indicate percent change from the previous time point (i.e., continued negative percent changes indicate continued drop in HR from original baseline in white light counts and a percent change close to zero represents a HR that has stabilized over time). All succeeding analysis was performed in a similar fashion; however, for the sake of simplicity, and due to the fact that baseline rates change from preparation to preparation, only percent changes are indicated in subsequent figures. It is noted that, at 22°C the alteration in HR upon initial exposure to blue light, as well as after continuous (10, 20, and 30 minute) exposure, produced highly varied results. In the ATR-fed larvae, the initial exposure to blue light induced a negative percent change in 5 out of 6 preparations from baseline (white light), representing an average percent decrease for the 6 preparations of -1.48% (Figure 3.1 a2). After 10-minute continuous exposure, the HR further decreased by an average of 2.90%; however, 3 out of 6 preparations displayed a positive percent change following this 10-minute period. The average HR for this group continued to decrease on average to -0.53% after a 20 minute exposure before rebounding after 30 minutes (Figure 3.1 a1, a2). The final time point measured represented a slight positive percent change from the previous point as 2 out of the 6 preparations displayed a positive percent change in HR, which was not statistically significant. Therefore, in +ATR-fed larvae at 22°C, blue light does not induce a significant percent change in HR at any time point measured (Rank Sum Test p>0.05 at all time points). Likewise, larvae fed a diet without ATR supplementation exhibit similarly varied responses to initial exposure to blue light as well as longer (10-30 minute) exposure to blue light (Figure 3.1 b1, b2). Initial exposure to blue light induces an average negative percent change from baseline of -1.24%, with only 2 of the 6 preparations exhibiting a positive percent change (Figure 3.1 b2). The HR rebounds in 5 out of 6 preparations, representing a non-significant positive percent change of 1.24%, before reducing an average of 0.15% after 20 minutes of continued blue light exposure (Figure 3.1 b2). After 30 minutes, 4 out of 6

preparations exhibit a positive percent change; however, this does not represent a statistically significant increase. As in the +ATR group, blue light does not induce any significant percent change in HR at any time point measured in the –ATR group (Rank Sum Test p>0.05).

As previously mentioned, because it was noted that the HR in intact larvae at 22°C was quite high, we considered the idea that any further increases by light exposure would be difficult to deduce. To observe if slowing baseline rates allowed for easier observation of changes upon blue light exposure, we assayed control groups of larvae (+/-ATR) at 10°C. The same experimental paradigm was utilized at 10°C as described previously. It is noted that the variability that existed in the preparations at room temperature is shared at 10°C. The baseline rates dramatically decreased (between 80-100 BPM) (Figure 3.1 c1, d1) compared to 22°C; however, there was little difference in observed degree of change following blue light exposure. In both the +ATR and –ATR groups, a positive percent change was exhibited upon initial blue light exposure (Figure 3.1 c2, d2). In particular, 5 out of 6 preparations in the +ATR group displayed a slight increase in HR while 4 out of 6 preparations in the –ATR group showed an increase in HR, representing positive percent changes of 4.8% and 4.23 % respectively. After 10 minutes of continuous blue light exposure, the HR decreased 2 out of the 6 preparations in the +ATR group and 3 out of 6 preparations in the –ATR group (Figure 3.1 c2, d2). At the 20 and 30 minute time periods, the +ATR preparations displayed high variability in their changes in HR, with 4 out 6 preparations exhibiting a reduction in HR at the 20 minute time point and 2 out of 6 preparations exhibiting a negative percent change in HR from 20 minutes to 30 minutes (Figure 3.1 c2). The –ATR group also showed variability over time; however, at the 30 minute time point, 4 out of 6 preparations displayed a negative percent change, representing an average decrease of 10.1% (Figure 3.1 d2). Just as was observed in the room temperature environment, there was no significant change in percent measures of HR at any time point measured in both the +ATR and –ATR groups (Rank Sum

Test p>0.05).   Thus, the role of blue light, alone in modulating HR in both environments is minimal.

Efficacy of all-trans-retinal supplementation and neural- based influence on heart rate

We next assessed the efficacy of ATR supplementation in producing differences in responses. It has previously been reported that the photocurrent produced in cells expressing the *ChR2-XXL* variant is much greater compared to the less sensitive variants (Dawydow et al. 2014). In addition, it has been noted in larval behavioral analysis in experiments performed in our lab that the *ChR2-XXL*-mediated response to blue light is extremely robust. Even in the absence of ATR and when exposed to a white light stimulus, larvae expressing ChR2 in motor neurons exhibit strong contractions of their body wall muscles. This led us to test the efficacy of responses in HR in larvae exposed to 1mM ATR supplementation compared with no ATR supplementation within each line. The average percent changes in HR at each time point tested (1-5) were recorded for the groups and compared, and the differences between the +ATR and –ATR groups were recorded.   It is noted that the difference in HR within the lines for the +ATR group and –ATR group was minimal. At 22°C, out of 20 total time points tested among line (4 time points per line X 5 lines), a significant difference between the groups was only observed twice (Figure 3.2). The initial change from white light to blue light in the *Cha-Gal4 X UAS-ChR2-XXL* line with ATR supplementation exhibited an increase in HR that represented a significant difference compared to the –ATR group (p<.05; Rank Sum test) **(**Figure 3.2 a1). Additionally, the line expressing the less sensitive ChR2 variant (H134RII-mcherry) in 5-HT neurons displayed a significant change in HR after initial exposure to blue light with added ATR compared to the –ATR group (p<.05; Rank Sum Test) (Figure 3.2 d1).   Within these lines, no significant difference was observed between the + and – ATR

groups at any subsequent time points (Figure 3.2 a1, d1). The additional lines displayed no significant difference in HR at all time points tested (Figure 3. 2).

Likewise, the influence of added ATR on HR was minimal at cold temperature in each of the tested lines. Again, among all tested time points within each line, only two time points displayed a significant difference in the percent change in HR between groups. As at room temperature, the initial exchange from white light to blue light induced an increase in HR in the *Cha-Gal X UAS-ChR2-XXL* (+ATR) line that represented a significant difference in comparison with the –ATR group (Figure 3.2 a2). The subsequent time points following time point 1 displayed no significant difference between + and – ATR groups. Additionally, the percent change in HR at time point 1 in the *ple-Gal4 X UAS-ChR2-XXL* line displayed a significant difference between the groups (Figure 3.2 c2); however, in this case, the +ATR group displayed a less robust increase in HR compared to the –ATR group. Consistent with the room temperature data, no significant differences were observed in the *ppk-Gal4 X UAS-Chr2-XXL* or *Trh-Gal4 X UAS-ChR2-XXL* lines, and, unlike at room temperature, no significant difference arose within the *Trh-Gal4 X UAS-ChR2-H134RII-mcherry* line (Figure 3.2).

Chemical modulation of heart rate

Given that ATR supplementation was shown to produce a minimal difference in the responses when compared to the –ATR groups, we assessed the role of neural-based chemical modulation in flies fed a diet supplemented with ATR (1mM) to remove the additional dietary variables. As noted, we targeted several populations of neurons that release modulators and/or hormones that have previously been shown to influence HR in a semi-intact larval preparation. The average percent changes in HR upon exchange from white light to blue light, followed by a 1 minute waiting period (1-Figure 3.3), and at succeeding 10 minute time points following initial exchange (2-5-Figure 3.3) were calculated for each fly

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line and compared to a control line (Figure 3.3). This analysis was performed at 22°C (Figure 3.3a) and in a room with a constant temperature of 10°C (Figure 3.3b).

At 22°C, upon optic stimulation, release of acetylcholine and activation of target populations of cholinergic neuronal signaling resulted in an average positive percent change of 4.74%, which represented a significant difference compared to the control line, which displayed a negative percent change of -1.48% (Figure 3.3a; Rank Sum Test $p<0.05$) from baseline. Likewise, initial activation of *UAS-ChR2-XXL* in dopaminergic and serotonergic (5-HT) neurons induced significant increases in HR, with average percent changes from a white light to blue light stimulus of 3.87% and 7.95% respectively (Rank Sum Test $p<0.05$; $p<0.03$ respectively).   Because it has been shown that 5-HT exhibits a strong influence on HR in both room temperature and acute cold settings in situ (Majeed et al. 2013; Zhu et al. 2015), the expression of the less sensitive ChR-2 variant (*H134RII-mcherry*) was also driven in 5-HT neurons. At 22°C, activation of this variant induced a significant positive percent change in HR following a 1 minute waiting period as well (Figure 3.3a) (Rank Sum Test $p<0.05$). Only one line exhibited an inhibitory influence on HR upon activation. Blue light activation of class IV da neurons induced a slight negative percent change of -0.47% in HR, which does not represent a significant difference when compared with the change in the control line (Figure 3.3a). Therefore, after the initial stimulus and subsequent release of neuromodulators/hormones into the hemolymph, with the exception of the ppk line, activation of all targeted neuronal populations elicited a positive influence on HR.

While the influence of blue light stimulation induced a positive percent change in HR in 4 of the 5 lines tested at room temperature, the subsequent changes in HR at the succeeding time points were less predictable across each line. Ten minutes following the count after the preparations were exposed to blue light, 4 out of 5 lines displayed a negative percent change. These changes mirrored closely the change in the control line, which exhibited a -2.94%

reduction in HR from the previous time point (an additive drop of approximately 4.4% from baseline) (Figure 3.3a). The only line that displayed a continued increase in HR from the previous count was the 5-HT line expressing *ChR2-H134RII*, which showed a positive percent change of approximately 3.2% (Figure 3.3a). Therefore, it is apparent that the excitatory influence from the modulators or hormones released from the targeted neural populations in 4 out of the 5 experimental lines tested was diminished after 10 minutes. However, upon continued observation after an additional 10-minute period, an increase in HR was observed in 3 of the 5 experimental lines. Specifically, the line expressing *ChR2-XXL* in cholinergic neurons (*Cha-Gal4*) and the line expressing ChR2-XXL in dopaminergic neurons (*ple-Gal4*) displayed positive percent changes from the previous time point measured. The increase in HR continued following an additional 10-minute period in the *ple-Gal X UAS-ChR2-XXL* line, as the HR increased from the previous time point measured approximately 6.8% on average (Figure 3.3a). Additionally, the *Trh-Gal4 X UAS-ChR2-H134RII-mcherry* (serotonergic) line displayed a positive percent change of 3.5% from the previous time point (from 4 to 5) (Figure 3.3a). Unlike the positive percent changes in HR observed upon initial change from white light to blue light, the positive percent changes displayed by these lines at subsequent time points did not represent statistically significant increases relative to control (Rank Sum Test; $p > 0.05$). Likewise, negative percent changes observed at time points beyond initial change (time points 2 through 5) did not exhibit statistically significant reductions relative to the control line, with one exception: the *ppk-Gal4 X UAS-ChR2-XXL* line displayed negative percent change of -29.1%, representing a significant change (Rank Sum Test; $p > 0.05$) (Figure 3.3a). Therefore, it is apparent that the change in HR after a 10-minute exposure to blue light was minimal across all lines; however, the initial enhanced rate observed following initial exposure to blue light was sustained in 4 out of 6 lines tested, with the exceptions being the control line and the *ppk-Gal4 X UAS-ChR2-XXL (*class IV da sensory neurons) line. Only these two lines displayed HRs that dropped below the initial HR

observed under a white light stimulus, signifying that, although we did not observe a continued increase in HR upon constant blue light exposure, the release of the targeted modulators elevated rates throughout the entire experimental time period from the initial baseline counts.

As mentioned previously, the change in HR in response to activation of select neural populations was observed at 10°C. Upon initial change to blue light, a positive percent change in HR was observed in all 6 lines tested, including the control (Figure 3.3b). Specifically, 4 out 5 of the experimental lines displayed a higher percent change relative to the control, with the *Trh-Gal4 X UAS-ChR2-H134RII-mcherry* line displaying a significant positive percent change of 7.5% (Figure 3.3b) (Rank Sum Test; $p < 0.05$). While at 22°C the *ppk-Gal4 X UAS-ChR2-XXL* line exhibited a consistent negative percent change in HR throughout the experimental time course, there was a positive percent increase in HR in this line in the cold environment. Consistent with what was observed at room temperature, the initial increase in HR observed after exchange to blue light was abolished after a 10 minute period of constant blue light exposure in 4 out of 6 lines tested, with only the *Cha-Gal4 XUAS-ChR2-XXL* (2.3%) and *Trh-Gal4 X UAS-ChR2-XXL* (1.4%) lines displaying continued increases in HR (Figure 3.3b). Neither of these increases, however, were statistically significant relative to the control line, which exhibited an average negative percent change of   -8.4% (Figure 3.3b) (Rank Sum Test; $p < 0.05$). Additionally, following 20 minutes of constant exposure, from time point 3 to 4, the HR in all lines remained relatively constant, with 3 out of 6 lines displaying an average positive percent change in rates and two, *Cha-Gal4-X UAS-ChR2-XXL* and *Trh-Gal4 X UAS-ChR2-XXL*, displaying average negative percent changes of 4.2% and -2.1% respectively, which represents a stabilization from the previous time point. Likewise, the percent change from time point 4 to 5 also displayed minimal changes in HR in all lines tested, with no significant differences in rate changes relative to the control (Figure 3.3b).

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In both environments, the initial exchange from white light to blue light induced positive percent changes in HR in all experimental lines tested with one notable exception being the *ppk-Gal4 X UAS-ChR2-XXL* at 22°C. The control lines exhibited slight changes in HR upon exposure to blue light, with a negative percent change observed at room temperature and a small positive percent change observed at 10 °C. The significant changes observed upon initial exchange to blue light were diminished in each line, and a continued significant increase was not observed; however, at both temperatures the rates that increased in the experimental lines never fell back below baseline. Therefore, it was noted that the HRs stabilized after 10 minutes of constant blue light exposure (Figure 3.3b).

DISCUSSION

Here we present the first study on the role of targeted neural-based hormones/modulators on modulation of heart rate (HR) in *Drosophila melanogaster* larvae. We have illustrated that utilization of optogenetics is useful in assessing the neural-based influence of modulators on cardiac function. In adding to the ever-increasing literature regarding the chemical and mechanical modulation of HR, we have further enhanced understanding of cardiac function in *Drosophila* and progressed its use as a tractable model for translational studies. In doing so, we have also progressed understanding of the efficacy with which one can utilize optogenetics in studies related to physiological processes not directly pertaining to the neural-basis of behavior.

Influence of blue light stimulation alone is minimal in inducing changes in heart rate

We have shown that the influence of blue light by itself on the activation of endogenously released cardio-active modulators on cardiac function is minimal. In each setting utilized (i.e., cold and room temperature; +/- ATR), the role of blue light alone in stimulating release of modulators/hormones that may influence

heart function was not significant. While it may appear disingenuous, recent analysis has shown the influence of blue light on *Drosophila* larval behavior to be robust, even in the absence of the important visual organ (Xiang et al. 2010). Therefore, it is important to understand the potential impacts of utilizing optogenetics on cardiac function, particularly as it relates to long-term, developmental studies. In performing this analysis, we noted an interesting characteristic. As can be seen in the preceding figures, there is a high degree of variation in HR even within an individual preparation. We noted that when intact larvae are stuck to tape as was performed in this analysis, they still maintain their ability to initiate body wall contractions. The body wall contractions cause brief, periodic pauses in heart contraction, therefore modulating HR for a given time period. Others who have performed similar techniques have noted this occurrence. A study by Sénatore et al. (2010) identified a crucial mechanoreceptor, *Painless,* that is essential in mediating response to the body wall contraction-induced mechanical perturbation of cardiac tissue. Although we did not directly correlate contraction occurrence with altered HR, the variation within preparations could very likely be explained by this phenomenon.

Retinal supplementation effect is minimal in neural-based influence on heart rate

Additionally, we have shown that the supplementation of all-trans-retinal (ATR) at a concentration of 1mM is minimal in its influence in significantly changing the cardiac response to release of targeted modulators. Out of the total 20 time points tested in the two environments, a significant difference between the +ATR groups and –ATR groups was observed in the *Cha-Gal X UAS-ChR2-XXL* line (at both room and cold temp) and in *the Trh-Gal4 X UAS-ChR2-H134RII*-mcherry line (at room only) upon initial exchange from white light to blue light. In these cases, the ATR group displayed a significantly greater positive percent change relative to the –ATR group. Additionally, the +ATR group

displayed a less robust response in the *ple-Gal4 X UAS-ChR2-XXL* line at 10 °C relative to the –ATR group, which represented a statistically significant difference. While we have noticed in our behavioral analyses, using both larval and adult *Drosophila*, that ATR supplementation is significant in enhancing responses to a blue light stimulus, the results here suggest that the addition of this cofactor does not induce significantly varied responses between the majority of treatment groups expressing the ChR2-XXL variant in the targeted neurons. The difference in efficacy of ATR supplementation in regard to the functioning and expression of ChR2 variants, including ChR2-XXL, has been detailed previously (Dawydow et al. 2014). It has been shown that supplementation of ATR enhances ChR2 photocurrent amplitude in response to blue light when expressed in host cells and it is suggested that this is due in large part to the reduced degradation of the translated protein when associated with the ATR cofactor; however, retinal supplementation is not required for functioning of the ChR2-XXL variant (Dawydow et al. 2014). Therefore, the supplementation of ATR is assumed here, and has been shown previously, to increase the abundance of the channels in the cell membrane, likely the primary factor underlying the enhanced photocurrent in relation to a –ATR treated preparation (Dawydow et al. 2014). Thus, we predicted to see a significant difference between our +ATR and –ATR group as we suspected an enhanced release of targeted modulators in the lines expressing ChR2-XXL, even though it has been shown to function without retinal. Moreover, we predicted to see a significant difference between groups in the line expressing the less sensitive channel rhodopsin variant (ChR2-H134RII-mcherry) due to the idea that this variant has shown to be significantly less responsive without ATR supplementation (Dawydow et al. 2014). It is important to note that we did indeed notice a significant difference upon initial blue light stimulation in this line at room temperature, as there was a negative percent change in the –ATR group. This is likely due to the significantly reduced responsiveness and sensitivity of this variant in the absence of ATR. As for the ChR2-XXL expressing lines, it is of

interest that the differences across the lines were minimal between the groups.
We suspect the need for an abundant release of modulators/hormones into the
hemolymph to observe an effect on cardiac function.   While the difference in
quantity of neurotransmitter/modulator release at synapses in the nervous
system as a result of ATR presence may induce obvious changes in neural
circuit function, the action of enzymes in breaking down the released substances
or their re-uptake by neurons or glia may dampen their influence on tissue distant
from the source of release. High release of these neurotransmitters may even
desensitize target receptors within the CNS, which then affects activity properties
of a targeted neuroendocrine cell. It is not known if the neurons stimulated
directly raise the transmitter they release into the hemolymph as entire neural
circuits are also modulated by these compounds. Thus, the relative difference in
efficacy of responses in a non-neuronal tissue as a result of ATR-mediated
enhancement of neuromodulator release is likely less pronounced. We suspect
this to be the case in this situation, as the larval heart is not innervated directly by
nerves, and therefore, the modulator/hormonal action on the heart requires
transport through the hemolymph. It is assumed that the difference in modulator
release in the presence of ATR versus in the absence of ATR is insufficient in
producing a significant alteration in HR. Future experiments, including High
Performance Liquid Chromatography (HPLC), can be performed to test the
relative concentration of modulators released into the hemolymph following blue
light activation of various neural populations via different ChR2 variants in the
presence and absence of ATR to follow up on these questions as they pertain to
neuroendocrine influence on physiological functions.

Release of targeted modulators enhance heart rate upon initial stimulation

        The importance of investigating the neural basis on influence of vital
organs including the heart in *Drosophila* is highlighted by the fact that an
autonomic nervous system in invertebrates, including insect, is known to play a

crucial role in regulating the function of vital organs. Anatomical and behavioral studies of a potential autonomic system in invertebrates were started back in the 1920s and 40s by Ju. Orlov and A.A. Zavarzin (Nozdrachev, 1983; Shuranova et al. 2006). Just as for higher organisms, invertebrates require behaviors that allow for escape from predation or danger. *Drosophila* larvae show a nocifensive response with a characteristic "corkscrew-like roll" behavior when confronted with a parasitic wasp (Hwanget al. 2007; Sulkowski et al. 2011; Robertson et al. 2013) or strong aversive stimuli (Titlow et al. 2014). A rapid and robust movements of a larvae, which does not possess neural stimulation of the cardiac tube, may require humoral factors to increase HR for distribution of endocrine factors and nutrient supply to activate the skeletal muscles to maintain active escape responses. In addition, environmental factors such as cold may require the cardiac system to remain functional so that response to stimuli is maintained and appropriate nutrient dispersal for regulation through transitional stages, such as with cold hardening or conditioning for longer-term cold survival, are conserved. Cold conditioning in some insects involves osmolality changes, antifreeze proteins or compounds to be distributed throughout the organism (Ring, 1982; MacMillan et al. 2015). It is possible that neuroendocrine hormones help to maintain cardiac function during an environmental transition (Zhu et al. 2016b). Previous analysis in a semi-intact system has shown that the heart is stable at $10^{\circ}C$; however, the exposure to modulators had varying effects on the heart at this temperature, suggesting unique roles of modulation of the heart at low temperature. In addition, the average HR in the exposed heart is much lower at this temperature, compared to the HR in intact larvae. We have begun to address these questions by targeting subsets of sensory and interneuron populations that may be important in regulating larval heart rate in response to environmental changes. While previous analysis has implicated modulators important in regulating HR, whether these modulators affect cardiac function in a similar manner through release from the nervous system in response to changes in the state of the animal has not yet been addressed.

As stated above, it has been shown using semi-intact preparations that application of acetylcholine (Ach) (Malloy et al. 2016), dopamine (DA) (Titlow et al. 2013), and serotonin (5-HT) (Majeed et al. 2014) each induced increases in HR in a dose-dependent manner. Additional analysis has identified octopamine as an important modulator in regulating HR as well, as it has been shown to decrease HR in cold environment (Zhu et al. 2016). In these studies, semi-intact preparations were utilized for analysis, which enabled control of the concentration of the modulator that was exposed directly to the heart. While the concentrations of circulating modulators as a result of activation of our targeted neuronal populations is not clear here, we presume that the concentrations of the different modulators are greater than what has been identified in vivo. It has been shown that DA modulates peripheral organs through circulation at the micromolar range (Matsumoto et al. 2003). The circulating concentration of 5-HT and Ach are unknown under normal conditions, but given the abundance of cholinergic afferent sensory neurons and Ach and 5-HT interneurons we anticipate the release of Ach, DA, and 5-HT through targeting a host of cells augments circulating concentrations. Regardless, our findings here correspond remarkably similarly with the semi-intact analyses at room temperature, in that activation of neurons that release the modulators tested in these previous studies showed positive influences on HR. Specifically, initial increase in HR upon release of Ach, DA, and 5-HT at room temperature represented significant increases relative to the control line.   Of note, the only line that displayed a negative percent change after initial blue light stimulation and upon subsequent activation was the *ppk-Gal4 X UAS-ChR2-XXL* line. While it is assumed that the activation of all cholinergic neurons through use of the Cha-Gal4 driver likely causes a substantial increase in hemolymph Ach concentration, use of the ppk-Gal4 driver targets only a subset of dendritic arborization sensory neurons (class IV) and the corresponding increase in hemolymph Ach concentration is lower. Activation of this subset of neurons is known to be both required and sufficient in regulation of

response to nociceptive stimuli (Hwang et al. 2007; Xiang et al. 2010; Johnson and Carder, 2012; Kim et al. 2013; Kim and Johnson, 2014). The subsequent behavior initiated, including the strong 'corkscrew' like roll described previously, comes at an energetic cost. We therefore thought we may observe changes in HR as a result of activating circuits that may release cardioactive substances that could enhance HR to provide necessary endocrine factors assisting in skeletal muscle activation. It was surprising that we detected a decrease in HR that continued throughout the experimental time course.

Likewise, each experimental line tested at 10°C exhibited an average positive percent change with 4 out of the 5 lines displaying a change that was higher than the control line following initial blue light exposure. This result is rather interesting in that it has previously been shown that the application of exogenous DA and Ach after acute cold (10°C) exposure induces negative percent changes in HR; however, 5-HT induces a positive percent change in a semi-intact preparation (Zhu et al. 2016b). It is important to note that the baseline rates in the intact preparation and the semi-intact preparations vary greatly and this may be due to the lack of synergistic effect on HR that may be present in the whole animal, as the physiological saline used in the semi-intact approach may lack additional cardioactive substances. However, we show here that the enhanced responses in the cold temperature matched closely with the room temperature observations, suggesting the initial excitatory responses observed at room temperature were preserved at 10°C.

In addition, it was noted that the elevated responses observed following blue light activation were suppressed after 10 minute, 20 minute, and 30 minute continued exposure. The relative stability of HR in the experimental lines following these time periods show that the action of the modulators in driving an increase in HR was diminished; however, as noted, the HRs in each case were elevated above baseline throughout the experimental time course. It is therefore

apparent that the excitatory responses exhibited in response to the circulation of the modulators in the hemolymph were sustained. Interestingly, the change in the rates over time in the intact animal and in the semi-intact preparations followed an amazingly similar trend. In each case, the initial response to the application of a controlled concentration of modulator was noted and the change in HR following a 10-minute continued exposure was calculated for Ach, 5-HT, and DA (Titlow et al. 2013; Majeed et al. 2014; Malloy et al. 2016). In response to each modulator, the first minute following the exchange of a solution with an added modulator, the HRs displayed positive percent changes; however, after a 10-minute exposure, the positive percent change was dampened but remained above baseline (Titlow et al. 2013; Majeed et al. 2014; Malloy et al. 2016).

We initially considered the possibility that the stabilization in HR over time observed here could be due to reduced probability of release of our targeted modulators from the nervous system. We considered that the continuous exposure to blue light might desensitize the rhodopsin channels, thereby reducing cation current and subsequently reducing vesicle fusion and release. Alternatively, we considered the potential that enhanced Ca^{2+} and/or Na^+ influx over time may induce depletion of readily releasable vesicles or may cause neuronal refractory though Na^+ channel inactivation or Ca^{2+}-dependent K^+ channel activation. While there may be some contribution due to synaptic depression, additional use of optogenetics in our behavioral analyses, whereby excitatory responses at the neuromuscular junction are observed well beyond the time course observed here, suggest that this is not likely the cause in diminished enhancement of HR over time. Although it has been shown that there is spike frequency adaption in neurons expressing different ChR2 variants, including Chr2-H134RII (Pulver et al. 2009), in response to constant blue light, the release of modulators persists and the robust behavioral effects observed in our analysis suggests the channels remain functional. We suspect the diminished increase in HR corresponds to reduced responsiveness of the heart to continued modulator

interaction. The similarities observed in the semi-intact studies help to corroborate this notion. We reason that the action of these modulators increases HR enough to meet the energetic demands of the animal through initial activation of receptors expressed in cardiac tissue. The energetic cost of continued enhancement in HR in response to prolonged modulator action is likely unnecessary and inactivation of intracellular cascades and/or receptor desensitization in cardiac tissue may result. Follow-up examinations manipulating receptor expression in the heart in the presence of continued blue-light activation may help to address this question and also may shed light on the receptor subtypes that may be important in regulating the changes in HR in response to physiological and environmental changes. This may help to strengthen the work that has been done in previous analyses identifying receptors that mediate the positive chronotropic responses and increased rates in *Drosophila* larvae.

CONCLUSION AND FUTURE STUDIES

We have identified, through the use of an optogenetic technique, neural populations that display modulatory effects on HR in an intact larvae and have shed light on the probability that humoral factors are likely at play in modulating HR under a variety of conditions.   The responses in HR correlate with what has been observed in semi-intact preparations in prior studies. We have shown that Ach, DA, and 5-HT may play important roles in regulating HR in response to environmental changes. While the neurons targeted in this study are known to release these modulators, we cannot rule out the synergistic effects that may arise from release of other cardioactive substances that were not directly targeted in this study. Given that activation of these neural populations likely causes release of additional modulators/hormones, it would be important to address the circulating concentrations of other endocrine factors that may modulate HR. Future analysis of the hemolymph may be performed using High

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Performance Liquid Chromotography (HPLC) or mass spectrometry following optogenetic stimulation of various neuronal ensembles to address this, and subsequent analysis using multiple techniques can be used to identify additional compounds that may be crucial in regulating *Drosophila* cardiac function. Further investigation into the receptors that mediate these neuroendocrine influences should be performed as well. Cardiac tissue-specific RNAi knockdown of various receptor subtypes and neuronal activation and analysis of cardiac response may help to assess the mechanisms underlying neuroendocrine regulation of HR in addition to what has already been reported. Additionally, the use of optogenetics in long-term developmental assays is coming to the forefront. As a result, it is important to identify potential detriments that may arise from chronic stimulation of subsets of excitable cells. Due to the fact that a number of targeted neuronal populations used in this study have been shown to release cardio active modulators, it stands to reason that use in long-term studies targeting similar cells may affect heart function and development. Not only is it important to identify the effects of systemic neuromodulator and hormones on acute heart function, but also allows for the investigation of the potential detrimental effects of long-term optogenetic studies involving indirect influence on vital organs.
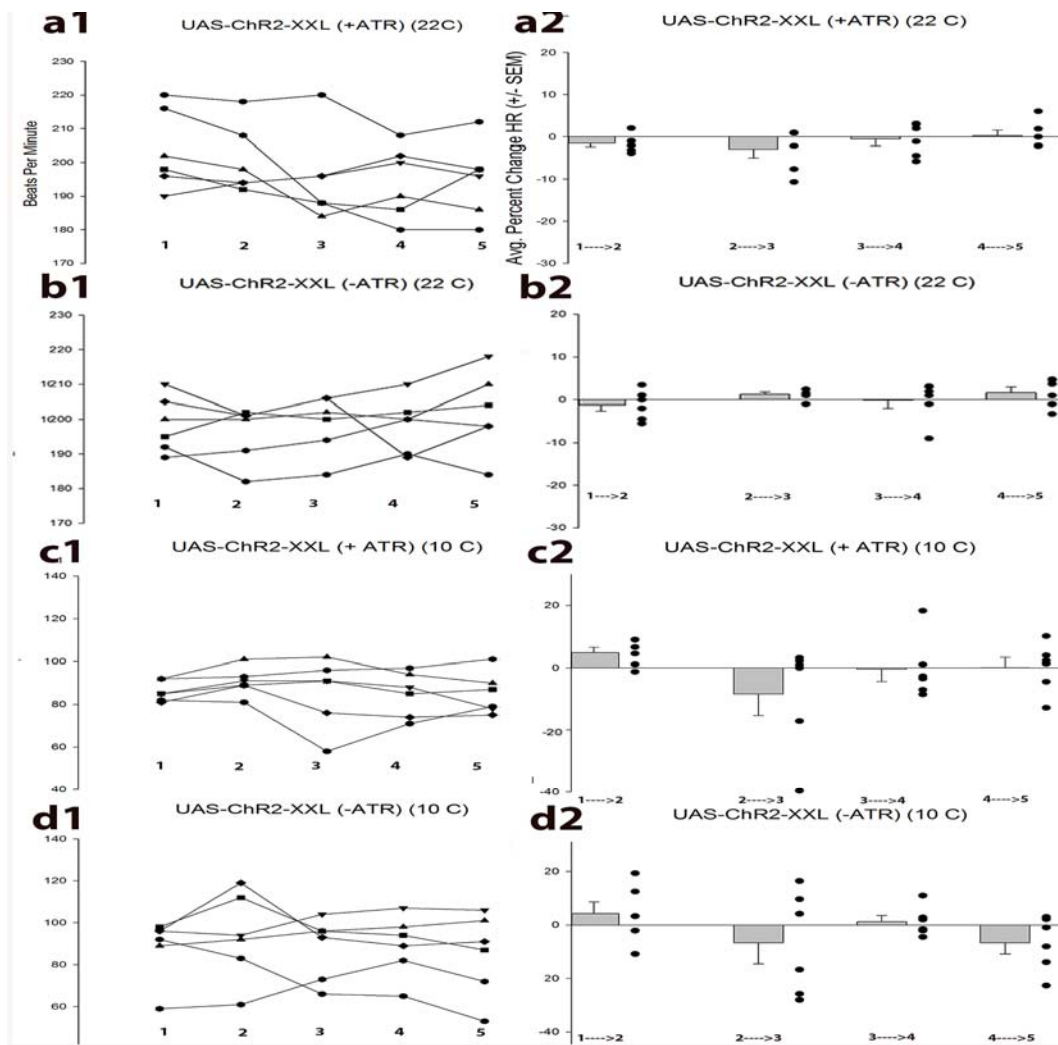
Figure 3.1. Blue light influence on heart rate of parental control lines. (Column 1): Raw average heart rates were calculated at 5 time points: (1) under white light (2) 1 minute following exposure to blue light (3-5) subsequent 10 minute intervals following exchange to blue light in room (22 C) and cold temperatures (10 C)(a-b and c-d respectively). (Column 2) The average percent changes in HR and individual percent changes for each preparation were calculated at 4 time points: (1) 1 minute following blue light exposure and subsequent 10 minute intervals following the initial change in order to examine the change in HR with continued blue light exposure (2-→5) for room temperature (22 C) (a-b) and cold temperature (10 C) (c-d). The influence of blue light alone on a control line was minimal.
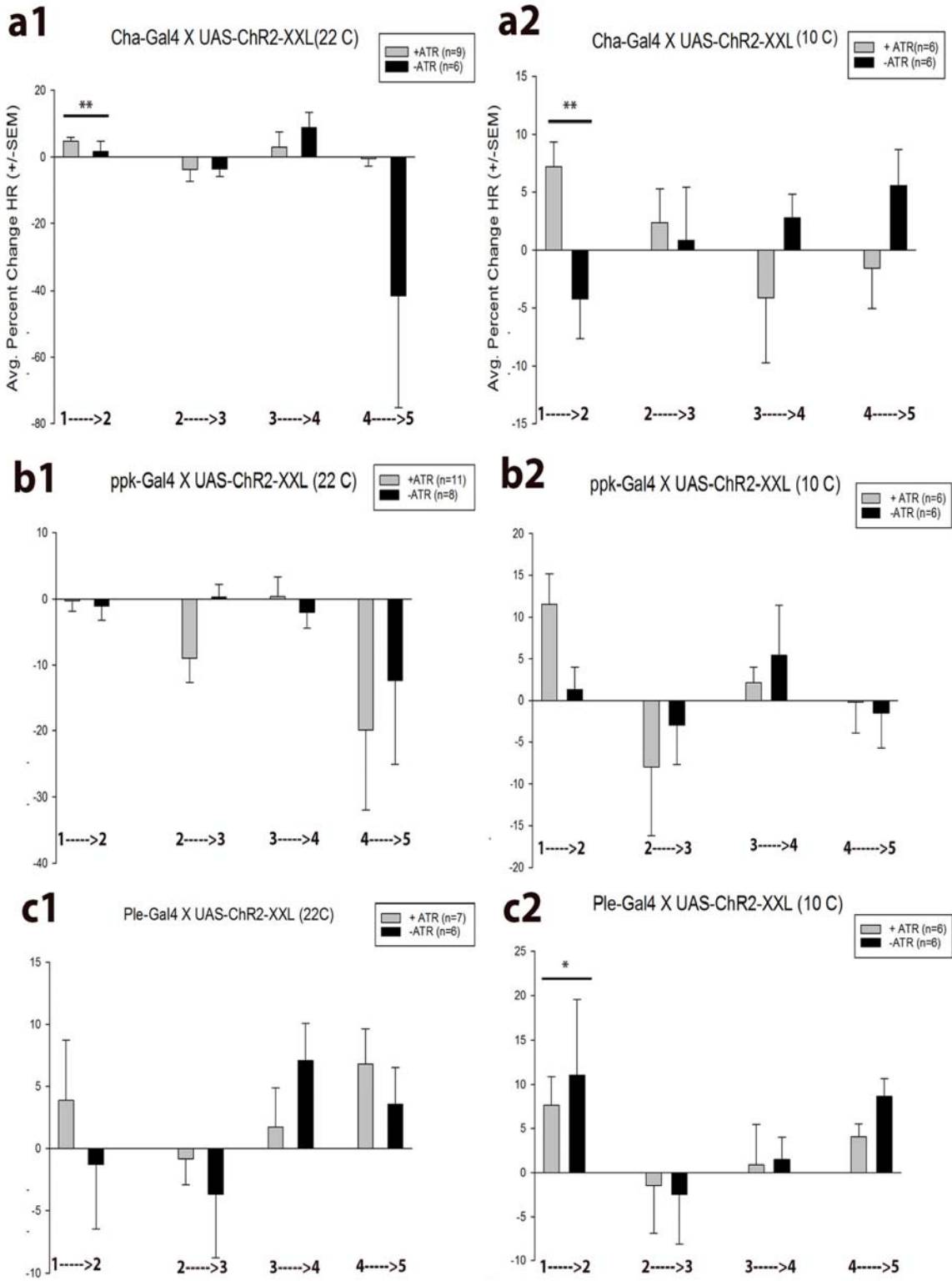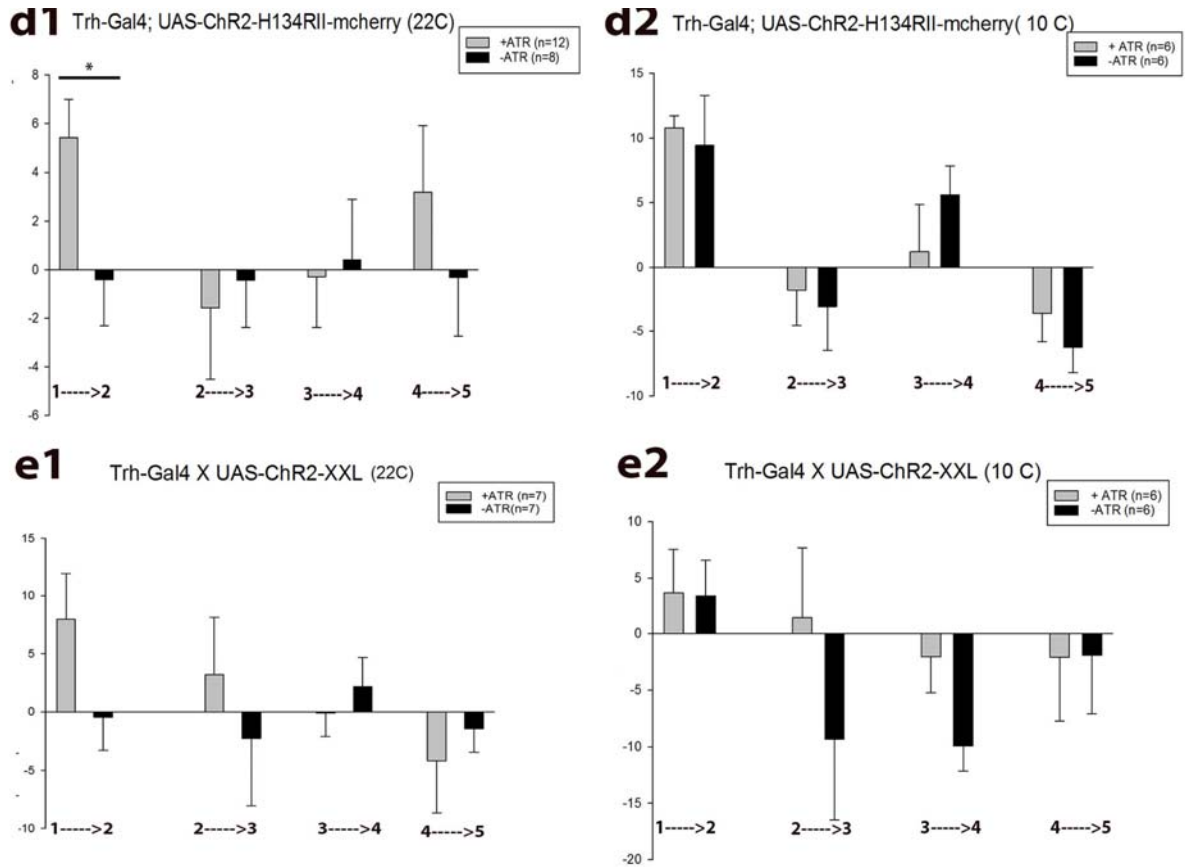
Figure 3.2 Efficacy of all-trans-retinal supplementation. The average percent changes in HR were calculated at 4 time points: (1) 1 minute following blue light exposure and subseuquent 10 minute intervals following the intial change in order to examine the change in HR with continued blue light exposure (2-→5) in room temperature (22 C) (column 1) and in cold temperature (10 C) (column 2). The average percent changes at each time point Mann-Whitney Rank Sum Test was used for analysis. (***: p<.001; **: p<.01; *: p<.05). The influence of ATR was shown to be minimal in inducing significant differences between the groups.

a

All Drosophila Lines + ATR (22 C)

Legend:
- ■ UAS-ChR2-XXL (control)(n=6)
- ▨ Cha-Gal4 X UAS-ChR2-XXL (n=9)
- ☐ ppk-Gal4 X UAS-ChR2-XXL (n=11)
- ▨ ple-Gal4 X UAS-ChR2-XXL (n=7)
- ▨ Trh-Gal4 X UAS-ChR2-H134RII-mcherry (n=12)
- ▨ Trh-Gal4 X UAS-ChR2-XXL (n=7)

Y-axis: Avg. Percent Change HR (+/- SEM)

X-axis: 1---->2, 2---->3, 3---->4, 4---->5

b

All Drosophila Lines +ATR (10 C)

Legend:
- ■ UAS-ChR2-XXL(control)(n=6)
- ▨ Cha-Gal4 X UAS-ChR2-XXL (n=6)
- ☐ ppk-Gal4 X UAS-ChR2-XXL (n=6)
- ▨ ple-Gal4X UAS-ChR2-XXL (n=7)
- ▨ Trh-Gal4 X UAS-ChR2-H134RII-mcherry (n=6)
- ▨ Trh-Gal4 X UAS-ChR2-XXL (n=6)

Y-axis: Avg. Percent Change HR (+/- SEM)

X-axis: 1---->2, 2---->3, 3---->4, 4---->5

Figure 3.3. Chemical modulation of heart rate. The average percent changes in HR were calculated at 4 time points: (1) 1 minute following blue light exposure and subsequent 10 minute intervals following the intial change in order to examine the change in HR with continued blue light exposure (2-→5) for room temperature (a) and cold temperature (b). The average percent changes at each time point Mann-Whitney Rank Sum Test was used for analysis. (***: $p<.001$; **: $p<.01$; *: $p<.05$). The average percent changes were compared to a control line and the significant indicators display differences compared to control. Initial exchange to blue light induces an increase in HR in 4 out 5 lines at room temp (22 C) and 5 out of 5 lines in cold temperature (10 C).

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CHAPTER FOUR

Pharmacological identification of cholinergic receptor subtypes: modulation of behavior and neural circuits in *Drosophila* larvae

INTRODUCTION

For nearly a century, acetylcholine (ACh) has been documented as a neurotransmitter both in the central nervous system (CNS) and in the peripheral nervous system (PNS) and plays a crucial role in a variety of CNS and PNS functions in mammals. In the mammalian CNS, it is known to function primarily as a neuromodulator, and has recognized roles in modulating synaptic strength in various brain regions, including the striatum (Cachope et al. 2012; Threlfell et al. 2012) and hippocampus (see Hasselmo 2006). Specifically, through multiple receptor subtypes expressed throughout the mammalian brain, ACh is known to modulate neural circuits underlying important cognitive processes including focus and attention (Berry et al. 2014), reward processing (Cachope et al. 2012), and learning and memory (see review Hasselmo 2006). Genetic disruption of key components in ACh signaling genes have long been implicated in a number of disorders including attention deficit hyperactivity disorder (English et al. 2009), Alzheimer's (see review Francis et al. 1999) and anxiety and depression-like behavior (Mineur et al. 2013) and, more recently, schizophrenia (see review Terry 2008 and Raedler 2006). Although the modulatory action of ACh in the nervous system of the organism *Drosophila melanogaster* is not as well characterized, its role as a vital chemical transmitter in this model and in other invertebrates is well documented. ACh is the primary neurotransmitter used in

sensory neurons projecting into the CNS and is also a primary excitatory neurotransmitter and neuromodulator within the CNS (Lee and O'Dowd 1999; Yasuyama and Salvaterra 1999; Su and O'Dowd 2003). The enzyme choline acetyltransferase (ChaT) and the degradative enzyme acetylcholinesterase (AChE) are highly expressed in afferent sensory neurons and neurons within the CNS (Buchner 1991). ACh synthesis is integral in *Drosophila* development as null mutations in these two enzymes involved in ACh metabolism result in embryonic lethality (Buchner 1991). In *Drosophila,* ACh and the components mediating cholinergic signaling are not surprisingly important in integrating sensory information given its role in sensory neurons. Recent work has enhanced our understanding of the role of ACh signaling and the specific receptor subtypes that regulate the processing of a number of sensory modalities including olfactory information processing (Gu and O'Dowd 2006; Silva et al. 2015), motion detection (Takemura et al. 2011), nociception (Hwang et al. 2007; Titlow et al. 2014) and gustation (Huckesfeld et al. 2016; Schlegel et al. 2016; Schoofs et al. 2014). It is known that cholinergic neuronal activity is important in modulating neural circuits guiding larval locomotion (Song et al. 2007) in mediating giant fiber escape response (Fayyazuddin et al. 2006) and in stimulating grooming, jumping, and hyperactive geotaxis ability (Bainton et al., 2000; Hou et al. 2003) in adult flies. Additionally, whole-cell current and voltage-clamp recordings in larval motor neurons have illuminated an excitatory role for ACh within the larval CNS (Rohrbough and Broadie 2002); however, a comprehensive analysis of the receptor subtypes regulating this excitatory pre-motor input have not been fully investigated. Specifically, the role of particular receptor subtypes and a pharmacological screening of in an intact nervous system has not been addressed in larval *Drosophila* in earnest. Nonetheless, these studies implicate ACh as an integral neuromodulator in the CNS of this model organism and suggest an extensive role of multiple cholinergic receptor subtypes in mediating sensory-CNS-motor circuits.

Given the relative complexity of the cholinergic system in relation to other neuromodulatory systems, the functional classification of cholinergic receptor subtypes within the *Drosophila* CNS has proven somewhat problematic. Acetylcholine receptors (AChRs) consist of two major subtypes: the metabotropic muscarinic acetylcholine receptors (mAChRs), and the ionotropic nicotinic acetylcholine receptors (nAChRs), both of which are activated by ACh and the agonists, muscarine and nicotine, respectively. The nicotinic receptor is part of the cys-loop family of ligand-gated ion channels that facilitates fast synaptic transmission. Muscarinic receptors are metabotropic and act indirectly with ion channels through second messenger G proteins to generate a cellular response (Collin et al. 2013). The *Drosophila* genome contains ten nAChR (Dα1-Dα7 and Dβ1-Dβ3) subunits and mAChR types, A-type (encoded by gene CG4356), B-type (encoded by gene CG7918), and C-type (CG12796) have been cloned in this organism (Collin et al. 2013; Xia et al. 2016). The activity of these two receptor subtypes is crucial in regulating the excitability of the cell and, while important insights have recently shed light on the pharmacological properties of mAChRs that can serve as useful in translating to whole neural circuits, the characterization of nAChRs has not been as fruitful. Even with the recent identification of important pharmacological properties of mAChRs, their role in behavior in larval *Drosophila* has not been widely investigated. This work provides further insights into the important pharmacological properties while also providing important understandings into their roles in altering neuronal excitability, which may help to illuminate their roles in neural circuit function.

The *Drosophila* nervous systems lends itself to easy experimentation and provides direct correlation of structure and function at identified, single cells, as well as at individual synapses. This model nervous system not only provides for an assessment of the effects of neuromodulators on well-characterized behaviors, but also allows correlation between identified synapses and certain behavioral components, thus permitting the identification of specific, cellular mechanisms underlying synaptic differentiation. However, recording synaptic

responses within the CNS has proven a challenge due in large part to the relative inaccessibility for individual cell recordings. Much of the work that has been done investigating synaptic transmission within the CNS in this model has been in cultured preparations as a result. Therefore, we've utilized an approach that allows for the observation of activity changes within the CNS in the presence of applied AChR agonists and antagonists *in vivo.* Through dissection and exposure of the nervous system, one can identify changes in activity of a given sensory-CNS-motor (sensorimotor) circuit in the presence of various agonists and antagonists of controlled concentrations. This illuminates the receptor subtypes present within the CNS and helps to define a pharmacological profile in the organism. Additionally, we've aimed at providing useful drug delivery paradigms that may prove efficacious in studies on pharmacological analysis in a model that provides unique challenges. While injection procedures have been utilized extensively, the stress of injections at the larval stage may confound assessment of the rapid effects of the injected drug. Here, for intact analysis, we utilize a feeding paradigm to assist in providing information regarding the time course of drug action through consistent food consumption. We couple this with the aforementioned electrophysiological approach, which provides a powerful combination enabling insight into the pharmacological properties of AChRs in an intact nervous system.

Simple and well-defined behaviors are readily assessable in larval *Drosophila*. We have chosen to examine two essential behaviors that offer unique opportunities to investigate how AChRs modulate defined neural networks regulating these behaviors. As stated, ACh has been implicated as important in modulating the circuits underlying both feeding and locomotion; however, the classification of these receptors through which ACh action is mediated has not been thoroughly addressed. Recent studies of larval *Drosophila* feeding behavior have begun exploring the neural circuitry driving motor output in the pharyngeal nerves innervating the muscles guiding larval

mouth hook movements (Schoofs et al. 2014; Huckesfeld et al. 2015). Additional analysis has helped unravel circuits underlying nutrient sensing and integration in processing of satiation (Melcher and Pankratz 2005) and we have sought to investigate the role of AChRs in modulating these circuits. Lastly, because larval locomotion is a useful in studying the mechanisms underlying regulation of rhythmic motor patterning, many studies have examined the neural circuitry fundamental to this behavior; however, the AChRs that may be important in modulating these neural networks have not been fully addressed.   Furthermore, our electrophysiological analysis focused on activity at larval abdominal muscle 6, which is integral in locomotion; thus, our *in vivo* electrophysiological recordings help shed light on AChR modulation of a motor program underlying this behavior. Therefore, these multiple neural circuits offer distinct platforms for which to study the molecular underpinnings of modulation of circuit activity. Thus, the goal of this work is to provide important information regarding the effect of multiple pharmacological agents in the *Drosophila* CNS and to provide insights into the functional role of these receptor subtypes in regulating larval behavior.

MATERIALS AND METHODS

Fly maintenance and stocks

   Canton S (CS) flies were used in all behavioral assays. This strain has been isogenic in the lab for several years and was originally obtained from Bloomington Drosophila Stock Center. CS flies were used in all electrophysiological experiments. All flies were raised on standard cornmeal-agar-dextrose-yeast medium in vials kept at room temperature (22-23°C) under a 12 hour light/dark cycle. The general maintenance is described in Campos-Ortega (1974).

Pharmacology

Acetylcholine (CAS # : 60-31-1), nicotine (CAS #: 65-31-6), clothianidin (CAS#:) muscarine (CAS #: 2936-25-6), atropine (CAS #: 51-55-8),scopolamine (CAS #: 6533-68-2), piperonyl butoxide (pestanal) (CAS#:51-03-6) and methyllycaconitine citrate salt (MLA)(CAS#: 112825-05-5) were purchased from Sigma-Aldrich (St. Louis MO, USA) (Milwaukee WI, USA). Tubocurarine (curare) (Cat #:2820) and benzoquinonium dibromide (BD) (Cat #:0424), were purchased from Tocris Bioscience (Minneapolis, MN, USA). Fly saline, modified Hemolymph-like 3 (HL3) (Stewart et al. 1994) containing: (in mmol/L) 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 1 $CaCl_2$, 5 trehalose, 115 sucrose, 25 N,N-Bis-(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) was used. The following modifications were made to the HL3 saline: pH was decreased from 7.2 to 7.1 and BES buffer was increased from 5.0 mmol/L to 25.0 mmol/L to maintain a stable pH (de Castro et al. 2014).

Larval development and maintenance

To control for variation in age of flies tested, 6-hour egg collections were employed and embryos were selected and moved to vials housed at room temperature (22-23° C). Larvae were raised until early 3rd instar stage on standard cornmeal-agar-dextrose-yeast medium (Campos-Ortega 1974). They were then selected and moved to food containing various concentrations of food mixed with the compound being assayed at early 3rd instar (pre-wandering larval) stage.

Drug delivery and behavioral analysis in 3rd instar larvae

Ensuring larvae are exposed to a desired concentration of drug for intact feeding is difficult. However, a study by van Swinderen and Brembs (2010) in which flies were fed 0.5mg/mL methylphenidate showed that this concentration was effective in initiating physiological responses similar in time and efficacy to

human administration. In addition, food coloring may be added to the drug-food mixture and observed passing through the digestive tract in the abdomen to ensure the larvae are sustaining feeding (see Schoofs et al. 2014). Thus, controlled concentrations of each drug were added to a food mixture and larvae were placed in this mixture for two time periods to assess the time effect of drug administration. Specifically, the drugs were dissolved in one milliliter (mL) of distilled water and mixed with 2 grams of standard fly food to avoid soaking larvae. Multiple concentrations were used to generate a dose-response effect and are indicated in molar (M) in the figure legends. The concentrations used for each drug were kept consistent. A control (water only), 0.001M, 0.01M, and 0.1M concentration of each drug were utilized in order to maintain a relatively high concentration under the assumption that a reduced concentration would be exposed to the nervous system. In each behavioral test, for each time period, the populations of 3rd instar larvae were collected and fed each concentration plus a control to limit intra-population variability. The concentrations used for each drug were kept consistent. Larvae that did not survive the feeding paradigms were discarded from analysis unless otherwise noted.

For behavioral tests, AChR agonists, nicotine, muscarine, and acetylcholine were assayed.   AChR antagonists scopolamine, curare and benzoquinonium dibromide (BD) were also tested. For electrophysiological recordings on the larval CNS, additional drugs were screened, including acetylcholinesterase inhibitor pentanal, nAChR agonist clothianidin, mAChR antagonist, atropine, and nAChR Dα7 –specific antagonist, methyllycaconitine (MLA) in addition to the aforementioned compounds.

Locomotive behavioral analysis (body wall contractions)

Early 3rd instar locomotive behavior was evaluated as described in Neckameyer (1996) and Li et al. (2001). In brief, single animals were moved to an apple-juice agar (1% agar) surface following exposure to a controlled

concentration of ACh agonist, or antagonist in a food vial. The number of body wall contractions, quantified by recording posterior to anterior peristaltic contractions, was counted for 1 minute under dim lighting in room temperature (22°C-23°C). All behavioral analyses took place between 2-5 pm. Larvae were age-matched as previously described.

Feeding behavior analysis (mouth hook movements)

For feeding behavior assessment, an animal was placed in a water and dry yeast paste solution following feeding of the agonist or antagonist. In this condition, when presented the dry yeast paste, *Drosophila* larvae immediately feed, initiating a pattern of repetitive mouth hooks movements that allows for food intake. This method also stabilizes the larvae making it easier to observe mouth hook extensions and minimizes the contribution of mouth hook extension as a superfluous artifact of larval crawling. The number of full mouth hook contractions in 1 minute was counted. Larval development, selection, and feeding prior to examination were as previously described. The rate of mouth hook extensions can be correlated with the amount of food ingested, and thus can be used to show alterations in food intake (Joshi and Mueller 1988).

Electrophysiology in 3rd instar larvae

The technique utilized is described in Dasari and Cooper (2004). In short, a longitudinal dorsal midline cut was made in 3rd instar CS larvae to expose the CNS. Two of the last segmental nerves were cut and sucked into a suction electrode, which is filled with HL-3 saline and connected to an AxoClamp 2B amplifier. Sharp microelectrodes (3 M KCl) were used for monitoring larval muscle fiber 6. The segmental nerves were stimulated with trains of pulses, with the paradigm maintained at 10 pulses per train at 40-60Hz (S88 Stimulator, Astro-Med, Inc., GRASS Co., USA). There was a 10 sec delay from first stimulation to the next stimulation train. The voltage was dependent on the initial observation of evoked responses, and generally varied between 4-10 volts

because the suction electrodes, which were used to stimulate the segmental nerves in each preparation, were slightly different. Depending on how tight the seal is with the suction electrode and the nerve, the voltage must be adjusted to evoked action potentials in the sensory nerves. Thus, segmental nerves were stimulated with a controlled frequency and voltage until a response was observed from an intracellular microelectrode that was stabbed into muscle fiber 6 (m6) contralateral (across the midline) to the stimulus.   This allows for the examination of activity within the CNS associated with a controlled stimulus and the associated motor output.   The excitatory postsynaptic potentials (EPSPs) were observed and analyzed with LabChart 7.0 (ADInstruments, USA). The traces were measured by averaging the EPSP frequency in 5 stimulation trains made with normal saline and 5 stimulation trains after exchanging saline with various compounds, unless otherwise stated in figure legends (see Figure 4). Once the saline was exchanged, the solution was left on the preparation for 2 minutes before analyzing EPSPs, unless responses were observed more rapidly. In some cases, solution was left on the preparation to observe potential changes over a longer time course. This is noted in the Results section when pertinent. To ensure preparation viability following the application of each drug, the compounds were washed out and replaced with normal saline. The average frequency of EPSPs the 5 evoked responses was recorded from each animal and the means from each treatment group were compared. Data is recorded as percent change from a saline (HL-3) solution to a saline solution containing the added drug of varying concentration in order to generate a dose-response relationship.

Statistical Analysis

The data presented is expressed as mean +/- SEM. The program, SigmaPlot (version 12.0) was used for graphing and statistical analysis. For behavioral analysis a One-way ANOVA, or One-Way ANOVA on Ranks was used for multiple comparisons among the concentration treatments by each individual

drug with a confidence level of P ≤0.05 as considered statistically significant for each drug administration time course. Additionally, individual points are presented, which represent each individual animal tested in order to display the variation in responses within each population. Tukey's test or Dunn's test (for One-Way on ranks) was used as post-hoc tests for to compare the mean responses. The electrophysiological analysis is presented as percent change from control (saline only) as there is considerable variation among baseline EPSP frequency from preparation to preparation. The average percent changes for the given samples were calculated and compared via Mann-Whitney Rank Sum analysis for comparison of each percentage change at each concentration relative to a control (saline to saline exchange). P of ≤ 0.05 is considered as statistically significant. The number of asterisks are considered as $P \leq 0.05$ (*), $P \leq 0.02$ (**), and $P \leq 0.001$ (***) for all analyses.

RESULTS

Impact of Oral Supplementation of Acetylcholine on Larval Locomotion and Feeding

As stated, a number of techniques to increase circulating concentrations of endogenous modulators and/or exogenous drugs that may mimic or block modulator action have been attempted. We have utilized a feeding technique that enables larvae to be consistently exposed to the added drug via normal feeding by mixing 1mL of solution with added drug with 2 grams of food. The control group was exposed to food that had been supplemented with the water (solvent) only. Larvae were collected at 3rd instar stage and subjected to food mixed with varying concentrations of ACh (.001M-.1M) in order to develop a dose-response relationship. Additionally separate populations of larvae were subjected to two different feeding durations: an acute 20-minute duration and a 24-hour duration, which has been shown to induce molecular alterations that may

be illuminated in circuit performance (Ping and Tsunoda 2012), in order to assess time course of action.   Locomotion was analyzed after removal of the larvae from the food and body wall contractions were counted on an apple juice agar plate. For feeding analysis, larvae were placed in a dish containing a water/dry yeast paste mixture, which stimulates feeding behavior to analyze alterations in food intake following persistent agonist/antagonist intake.

We found that acute feeding of ACh (20 minutes) induced a biphasic response, with low-dose (0.001M) and high dose (.1M) inducing reduced locomotion (Figure 4.1 A; $p<0.001$; $p<0.01$ respectively; One-Way ANOVA). Conversely, after 24 hour feeding, high dose ACh significantly increased locomotion (Figure 4.1 A). While acute feeding did not significantly change larval mouth hook movements, 24 hour feeding of high dose ACh significantly suppressed feeding behavior (Figure 4.1 B; One-Way ANOVA $p<.05$) in stark contrast to the impact on locomotion. Thus, it appears there is a disparity in the manner with which Ach is modulating these separate circuits.   Nonetheless, a significant alteration in both behaviors is observed after a 24-hour consistent exposure and suggests ACh modulates both of these circuits, which is consistent with previous analysis and our findings utilizing alternative approaches.

Acetylcholine receptor agonist modulation of larval locomotion and feeding

While the findings indicating ACh modulation of locomotion and feeding are interesting our goal was to illuminate the receptor subtypes that are integral in regulating Ach-mediated alteration in circuit efficacy. As mentioned, nicotine has been shown to significantly alter behavior in adult flies (Bainton et al., 2000; Hou et al. 2003; Fayaduzzin et al. 2006) yet its role in modulating larval behaviors has not been fully investigated. Likewise, although mAChRs have been shown to be important in modulating circuits underlying olfactory aversive learning (Silva et al. 2015), their function in larval *Drosophila* behavior is virtually

unknown. Thus, we used the same feeding approach to assess the behavioral changes arising in response to AChR agonist feeding. To evaluate the impact of nicotinic cholinergic signaling on locomotion and feeding behavior, varying concentrations of nicotine were supplemented in the food (0.001M-0.1M) and to analyze muscarinic cholinergic modulation, the same concentrations were utilized. Additionally, the feeding duration times were controlled as previously described. We found that nicotine exposure significantly reduced larval locomotion after both acute and 24-hour feeding at high doses (Figure 4.2 A1; One-Way ANOVA $p<.05$), while muscarinic exposure enhanced body wall contractions after 20-minute feeding, but exhibited a similar, yet less efficacious influence in reducing locomotion after a 24-hour feeding period (Figure 4.2 A2; One-Way ANOVA $p<.05$). Likewise, nicotine significantly reduced mouth hook movements after just 20 minutes and also following a 24-hour feeding period at high doses (Figure 4.2B1; One-Way ANOVA $p<.05$). It is noted that the 24-hour exposure to 0.1M nicotine represents a lethal dosage, with 53 out of a total 55 (96%) larvae tested in each behavioral paradigm dying after 24-hour exposure (Figure 4.2 A1,B1). In a similar manner, high dose muscarine exposure significantly reduced mouth hook movements following 24-hour exposure; however, muscarine stimulated a general increase in feeding after acute exposure, which is similar to what was observed in the locomotion assay (Figure 4.2 B2). While both agonists appear to reduce both feeding and locomotion after a 24-hour exposure, nicotine displays a higher efficacy, reducing levels of body wall more robustly than muscarine (Figure 4.2). Additionally, nicotine is more potent in altering feeding behavior, as the .01M concentrations induced a significant decrease in mouth hook movements after just 20 minutes. Nonetheless, both agonists modulate both locomotion and feeding behavior, with some distinctions in their action.   The difference in agonist influence on locomotion after 24-hour feeding in comparison with ACh is intriguing and may highlight important details regarding their influence on their targets.

Acetylcholine receptor antagonist modulation of larval locomotion and feeding

To follow up on the analysis of AChR agonist influence on larval locomotion and feeding behavior, we tested the ability of classical nAChR and mAChR antagonists to modulate the behaviors of interest in order to further shed light on the pharmacological properties of AChR receptors that influence the efficacy of the circuits. We tested the role of two non-selective, competitive nAChR antagonists, tubocurarine (curare) and benzoquinonium dibromide (BD), which have previously been tested on the larval heart (Malloy et al. 2016) and in an additional functional assay on cultured embryonic neurons in *Drosophila* in order to block synaptic responses in the embryonic CNS (Lee and O'Dowd 1999; Ping and Tsunoda 2012). Additionally, we tested the competitive mAChR antagonist scopolamine in our behavioral analysis. Scopolamine has shown to block ACh and muscarine action on *Drosophila* mAChRs in heterologous expression systems (Collin et al. 2013; Xia et al. 2016), and in olfactory associative learning (Silva et al. 2015). While we predicted to see responses that opposed our agonist-induced behavioral outcomes, we instead observed a number of interesting results. Acute feeding (20 minutes) of both curare and BD produces an increase in locomotion, with significant increases at high doses (Figure 4.3 A1,A2; One-Way ANOVA $p<.05$). However, after 24-hour exposure to high doses of both drugs, body wall contractions are significantly reduced, similar to what was observed with nicotine feeding (Figure 4.3 A1,A2; One-Way ANOVA $p<.05$). Additionally, both 20-minute and 24-hour feeding of both curare and BD induced a general decrease in feeding behavior, with significant reductions observed at high doses after each time course with significant reductions at the highest doses tested (Figure 4.3 B1,B2; One-Way ANOVA $p<.05$). Therefore, the responses to the two nAChR antagonists tested were quite remarkably similar, but the similarity in regard to nicotine action in

regulating the circuit performance was surprising and points to the potential for nicotine-induced nAChR desensitization at high doses. Likewise, our mAChR antagonist analysis yielded some unexpected findings given our results uncovered with the muscarine treatment.   Both acute and 24-hour feeding of scopolamine produced a dose-dependent significant reduction in locomotion and feeding behavior (Figure 4.3 A3,B3; One-Way ANOVA p<.05) The action of scopolamine appeared was particularly potent, with the lowest dosage tested (0.001M) decreasing both behavioral responses after 20 minutes of feeding (Figure 4.3 A3,B3). This illuminates the potential for mAChRs and nAChRs to play an important role in circuit efficacy guiding these behaviors. We show here that both nAChRs and mAChRs are integral in modulating circuit activity underlying both feeding and locomotion in larval *Drosophila*.   Additionally, this implies the need for longer-term feeding for and could highlight the difficulty in penetrance of these drugs to the nervous system.

Acetylcholine modulation of sensorimotor circuit activity

To follow up our behavioral analysis we utilized an electrophysiological approach to examine the influence of nicotinic and muscarinic cholinergic signaling on sensorimotor circuit activity at the cellular level.   It is noted that using the feeding paradigms, while effective in analyzing effects over extended periods, is difficult to determine the concentration of the drugs that gets directly exposed to the CNS. Thus, to circumvent this confound, we utilized an approach in which 3rd instar larvae were dissected open and the CNS was exposed directly to an added saline containing a known concentration of our compound of interest. We can, therefore, directly examine the influence on a defined, evoked sensory-CNS-motor circuit by controlling the activity of afferent sensory neurons and analyze the impact of these compounds on sensory integration and associated motor output. Excitatory post-synaptic potentials (EPSPs) were recorded from body wall muscle 6, which is integral in guiding larval locomotion, so we can correlate activity changes in our electrophysiological analysis with

changes in behavior. The stimulus paradigm utilized was held constant from preparation to preparation, as described previously, with a notable exception being the input voltage and stimulus frequency within a train, which was adjusted to compensate for slight variations in the suction electrodes utilized in order to recruit motor neurons. The stimulus voltage was determined based on the identification of observed responses, and once responses (EPSPs) were observed in the muscle, the voltage and stimulation frequency were held constant for the entirety of the experiment. A two-minute waiting period was used following solution exchange to avoid alterations in activity as a result of mechanical disturbance. In some cases activity was altered within 2 minutes and persisted throughout experimentation. These instances are noted.

Again, we first tested the influence of ACh modulation of a sensorimotor circuit through application of 100nM-1mM concentration of ACh. We found that low dose ACh (100nM) application induced an increase in EPSP frequency relative to a control, representing a positive percent change of 189.0 +/- 116.7 (Figure 4.4 A,D). Specifically, 5 out of 6 preparations tested displayed positive percent changes following 100nM ACh application (Figure 4.4D). As we increased the concentration of Ach to 10uM and 100uM, we noticed a reduction in the positive percent change observed at low dose. A positive average percent change of 8.4± 15.8% and 5.8 ±17.7% for 10uM and 100uM concentrations were observed (Figure 4.4D). The individual preparations displayed quite varied results, as 3 out of 6 preparations displayed increased EPSPs at 10uM and 4 out of 5 at 100uM displayed a positive percent change. At the highest dosage tested, 1mM, 4 out of 5 preparations displayed a positive percent change, which represented an average of 116.8% (Figure 4.4 D).

To compare the responses observed by augmenting ACh concentration through exogenous application, we tested the ability of a specific acetylcholinesterase (AChE) inhibitor to alter the activity within the larval CNS. This served to enhance endogenous ACh activity through inhibition of synaptic

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degradation. We tested a specific compound, pestanal, which serves as a prominent commercial insecticide. Because previous work investigating AChE inhibitor influence on nervous system development in *Drosophila* larvae suggests the use of lower concentrations, we used concentrations ranging from 1ppm to 1000ppm (Kim et al. 2011). We noted that, within 30 seconds following application of 1ppm pestanal, a burst of activity was observed and EPSP frequency drastically increased. The activity persisted throughout the experimental time course, and was present even in the absence of sensory stimulation. Because of this, we could not assess EPSP frequency changes in association with the sensory stimulation due to the persistent spontaneous activity. Higher doses of pestanal also enhanced activity in a similar manner (see sample Figure 4.5 C). Thus, the exposure to pestanal stimulated a relatively more robust increase in CNS activity in response to acute exposure relative to exogenous ACh application.

Acetylcholine receptor agonist modulation of sensorimotor activity

Nicotine has been implicated as an excitatory agonist in the CNS of larval *Drosophila in vitro* (Lee and O'Dowd 1999)*;* however few studies have investigated the impact on circuit efficacy *in vivo*. Furthermore, the pharmacological properties of *Drosophila* mAChRs have been identified in heterologous expression systems (Collin et al. 2014; Ren et al. 2015); however, their role in behavior and circuit physiology has not been fully addressed. Thus, we sought to these to address their roles in modulation of an intact sensorimotor circuit.

We used our electrophysiological approach to more elegantly control for the concentration being exposed to the CNS. We observed reduction of both locomotion and feeding that was enhanced as the concentration was increased when larvae were exposed to nicotine, which was surprising given nAChRs excitatory role in the nervous system. Upon application we noticed that low

doses of nicotine (100nM-10uM) induced a significant enhancement in EPSP frequency, causing a high burst of activity upon stimulating sensory afferents (Figure 4.5 A1,B1). The positive percent changes of 20.6 ± 24.1% and 36.1 ±13.9% at 100nM and 10µM respectively represented increases relative to control (Figure 4.5 B1). However, we noticed rather drastic change as we increased the concentration 10-fold to 100µM. Upon application of 100µM nicotine, we observed an initial burst of activity and then a rapid shutdown of activity, which lasted throughout the experimental period (Figure 4.5 A2). Subsequent stimulations did not elicit EPSPs in muscle 6 and we observed a negative average percent change of -98.1 ±0.7% (Figure 4.5 B1). Likewise, we noticed a similar response at 1mM, where a negative average percent change of -97.52 ±0.7% was observed (Figure 4.5 B1). In each case, the reduction of activity was observed with 20 seconds of application. We considered the possibility that high dose nicotine was rapidly desensitizing nAChRs within this sensory-motor circuit.   In the mammalian nervous system, nicotine often exhibits antagonistic-like properties due to its ability to rapidly desensitize receptors. Additionally, it is known to be highly lipophilic and may act to alter cell physiology by means other than via activation of nAChRs (Hukkanen et al. 2005).   Thus, to observe if our rapid shutdown of EPSP activity was unique to nicotine, we tested an additional non-selective nAChR agonist, clothianidin, on the exposed CNS. Surprisingly, we found clothianidin to more potent in abolishing activity in response to sensory stimulation (Figure 4.5 B2). Like nicotine, low dose clothianidin (100nM) enhanced circuit activity, inducing an average positive percent change of 11.0 ± 28.5% (Figure 4.5 B2) however, as the concentration was increased 10-fold, a robust shutdown of activity was observed within 30 seconds of drug application. Specifically, 10µM application induced an average negative percent change of -92.1 ±2.1% and 1mM application induced an average negative percent change of -94.5 ±.8% (Figure 4.5 B2). Since we observed an abolishment of activity as low as 10µM that persisted in the presence of 1mM, we omitted the 100µM concentration. This suggests that

clothianidin, like nicotine excited sensorimotor activity at low doses, but rapidly desensitizes receptors as the concentration increases. It was surprising to us that it was more potent in reducing activity at a concentration as low as 10μM. Taken together, our intact and semi-intact analysis suggests high dose nicotine may be rapidly desensitizing nAChRs, resulting in reduced motor output that is recapitulated by an additional nAChR agonist, clothianidin.

Our behavioral analysis suggested an acute enhancement of activity of circuits underlying locomotion and feeding in the presence of muscarine, so we tested the its role in modulating sensorimotor activity upon direct exposure to the CNS. We identified a dose-dependent increase in sensorimotor activity, with 1mM muscarine application inducing an increase in EPSP frequency relative to control (Figure 4.5 A3,B3). While 100nM and 10μM concentrations produced variable responses, increasing the concentration to 100μM and 1mM enhanced circuit activity, with the highest dosage producing a positive percent change of 200.6 ±2.1% (Figure 4.5 B3).

Acetylcholine receptor antagonist modulation of sensorimotor activity

After observing that both agonists displayed significant role in altering sensorimotor circuit activity, we tested the ability of classical nAChR and mAChR antagonists on circuit efficacy. Because we observed initial excitatory responses in the presence of low dose nicotine, and what we interpret to be rapid desensitization as the concentration was increased, we predicted that the two assayed non-selective nAChR antagonists would reduce activity with high potency. However, similar to what was observed in our behavioral tests, both curare and BD were not potent in reducing circuit activity. Only 10μM and 1mM application of BD induced a reduction of EPSP frequency at muscle 6, represented as a negative average percent change of -4.7 ± 26.0% and -97.6 ±9.7% respectively following a two-minute delay post-application (Figure 4.6 B2).

Curare induced positive average percent changes at each concentration tested; although differential responses were observed from preparation to preparation (Figure 4.6 A1,B1). Since we observed similar enhancement of activity in our behavioral analysis following acute exposure, which ultimately reduced after 24 hours, we decided to test the efficacy of both antagonists after a longer bathing period. This finding intrigued us and, in light of our behavioral analysis, which showed reduced responses after 24 hours of feeding, we observed alteration in activity following a 15-minute period. After this time, we noticed a small reduction in activity relative to the previous time points, but activity essentially returned to baseline (saline).   Previous analysis has noted, similarly, the significant length of time required for curare to reduce activity *in vivo* (Rohrbough and Broadie 2002); however we maintained a consistent paradigm as a means of comparing the potency of AChR agonists and antagonists. This shows that, although longer exposure may reduce activity, as indicated in our behavioral assessment, these two non-selective antagonists are not potent in blocking nAChR-mediated modulation of circuit activity, and in the time course of experimentation, only 1mM BD significantly reduced EPSP frequency.

Additionally, we began to address specific nAChR subtypes that may be prominent and functional within a sensorimotor circuit. We tested an α7-specific antagonist, (MLA), that has been shown to be a potent agonist selective for this subtype in mammalian preparations (see Halff et al 2014).   In flies, the alpha 7 subunit plays a significant role in adult motor response to a sensory, 'lights-off' stimulus (Fayaduzzin et al. 2006) and is highly expressed in the *Drosophila* CNS (Chantapalli et al. 2007; Celniker et al. 2009; Gramates et al. 2017). Likewise, it has also been shown that the Dα7 nAChR forms a functional homomeric receptor (Landsdell et al. 2012), and the Dα7 sequence displays high similarity (~42-43%) with its mammalian counterpart (Grauso et al. 2002).   Additionally, we suspect the high dose nicotine treatments are shutting down activity through rapid desensitization, and it is known that the Dα7 receptor undergoes

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desensitization much more rapidly than additional subtypes, including the $\alpha4\beta2$ (Gott et al. 2009; Albuquerque et al. 2009), which, along with the $\alpha7$, are the two most abundant nAChRs in the mammalian CNS. Therefore, we tested the effect of MLA on the CNS of a semi-intact preparation to deduce the influence of the D$\alpha7$ subtype on sensorimotor activity. Upon bathing the preparation in high dose (1mM) MLA we noticed rapid and robust shutdown of activity, similar to what was observed with high concentration nicotine (Figure 4.6 A3). We noted that both evoked activity pertaining to the sensory stimulation and any spontaneous EPSPs were completely abolished within 10 seconds post-application, suggesting rapid reduction of endogenous, tonic activity in addition to evoked sensorimotor activity. We also noted mini-EPSPs (mEPSPs) were still present during the recording suggesting that this drug was not blocking glutamate receptors (GluRs) at the NMJ. This was observed in 7 out of 7 preparations tested and lasted the entirety of the experimental timecourse (continued abolishment of activity after 2-minutes post-application). The overall average negative percent change of (-77.0 $\pm$ 16.8%) (Figure 4.6 B3). As the concentration was reduced, the complete abolishment of EPSPs was not observed, but in 3 out of the 4 concentrations tested, a reduction in EPSP frequency was observed (Figure 4.6 A3,B3). Therefore, of the nAChR antagonists tested, MLA was the most potent in reducing activity, and this points to a prominent role for the D$\alpha7$ receptor in modulating sensorimotor circuit efficacy in the larval CNS.

Furthermore, we tested the role of two classical, competitive mAChR antagonists in modulating sensorimotor circuit activity. We revealed that scopolamine reduced locomotive behavior after 20-minute and 24-hour exposures, and we predicted to observe a reduction in sensorimotor activity in response to scopolamine application. We also tested an additional mAChR competitive antagonist, atropine. Upon exposure to high dose (1mM) scopolamine, sensorimotor circuit activity rapidly shut down, reducing EPSP

frequency to 0 within 20 seconds (Figure 4.6 A4,B4). Specifically, the presence of 1mM scopolamine produced an average negative percent change of -97.1 ±0.4%, representing a reduction relative to control (Figure 4.6 A4, B4). We again noted that mEPSPs were present throughout the recording, suggesting the reduction of acitivity observed at muscle 6 was not a result of GluR inhibition. As the concentration was reduced, the robust shutdown was not observed, but a reduction in EPSP frequency was detected at 100µM, 10uM, and 1mM (Figure 4.6 B4). While high dose scopolamine reduced activity reliably, atropine exposure did not induce consistent responses. Exposing the exposed nervous system to 100nM-100µM atropine resulted in increased EPSP frequency in half the preparations tested and decreased in half, displaying a wide variation in action (Figure 4.6 B5). 1mM atropine did reduce activity in 5 out of 6 preparations tested, inducing a negative percent change of -48.3 ±18.8% (Figure 4.6 B5)

DISCUSSION

While strides have been made in identifying the pharmacological properties and contribution of cholinergic receptor subtypes to neural circuit activity in the fruit fly model, considerable work remains. Insights into acetylcholine receptor (AChR) properties have expanded through the use heterologous expression systems; however, how these properties are translated to the level of neural circuits have not yet been fully addressed. Neural circuits are dynamic and the function of these receptor subtypes in response to fluctuations in ACh 'tone' or agonist/antagonist and their role in modulating circuit efficacy is important to address. In this study, we utilized a pharmacological approach to investigate the role of nicotinic cholinergic and muscarinic cholinergic signaling in fundamental behaviors in larval *Drosophila*. We have provided a comprehensive pharmacological assessment of the function of both ACh receptor subtypes in larval locomotion, feeding, and in modulating activity of an evoked sensorimotor circuit. The role of important neuromodulators in

modifying neural circuit properties in the *Drosophila* CNS warrants further investigation, as much of our current knowledge stems from analysis performed *in vitro*. This work can serve as a foundation for more comprehensive analysis on, for instance, simple associative learning and addiction and distinct cell intrinsic mechanisms underlying plasticity in the CNS. Furthermore, the *Drosophila* model is becoming a more amenable model for the investigation of the development effects of nicotine exposure (Bainton et al. 2000; Velazquez-Ulloa 2017). One can use the insights presented here to address the role of ACh and its targeted receptors in regulating molecular mechanisms underlying this conserved developmental impact.

Oral supplementation of acetylcholine displays differential modulatory influence on assayed neural circuits

We were not surprised to identify a significant influence on these behaviors in response to ACh exposure. We have noted in additional experimentation using thermogenetic and optogenetic approaches that activating or silencing cholinergic neurons inhibits locomotion and feeding and renders larvae unresponsive to tactile touch. The phenotypes observed utilizing these approaches suggest ACh excites motor neurons guiding both locomotion and mouth hook movements, as tetanus-like contraction of the muscles mediating these behaviors is observed, and, conversely, a relaxation phenotype upon neuronal silencing is detected. This supports the findings of previous studies, which showed focal application of ACh excites motor neurons (Rohrbough and Broadie 2002) and *TrpA1*-meditated activation of cholinergic interneurons excites motor nerves innervating the larval feeding apparatus (Schoofs et al. 2014). Thus, we predicted to observe enhanced feeding behavior and locomotion as a result of increased excitability of the neural ensembles guiding both locomotion and feeding in response to increase ACh tone. What was interesting was the differential modulation observed between the time courses of treatment and the

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circuits observed. It is possible that the concentration of ACh that gets exposed to a synapse is much lower than what is found when manipulating synaptic transmission using alternative approaches, where concentrations as high as 1mM upon evoked vesicle fusion in the synaptic cleft are observed (Edmonds et al. 1995). It was our goal to attempt to simulate high concentrations of agonists/antagonists, mimicking evoked modulator release, but we are not able to directly measure the concentration exposed to neural tissue. The general excitatory responses observed in our electrophysiological analysis support previous *in vivo* examination (Baines and Bate 1999; Rohrbough and Broadie 2002) and suggests that enhanced locomotion observed after 24 hour ACh feeding is likely the result of enhanced motor output with sustained increase in circulating hemolymph ACh. What was surprising, however, was that we noticed varied responses as the dosage was increased. For instance, in 6 preparations tested, 3 exhibited a reduction in EPSPs when exposed to 10μM ACh. It is possible that imAChR (mAChR-B), which shows significantly lower affinity for ACh (Collin et al. 2013) may be activated in this circuit with higher concentrations, or nAChRs may even be blocked as ACh dosage is increased as channel block may occur at high doses (Barik and Wonnacott 2009). The increase in activity in response to exogenous ACh application was recapitulated following AChE exposure; however, a drastic difference in activity was observed. While exposing the semi-intact preparation to exogenous ACh did not induce a substantial increase in spontaneous activity, pestanal application significantly enhanced EPSP frequency even in the absence of sensory stimulation. The robust increase in spontaneous activity in the presence of low dose pestanal suggests that this drug penetrates into the CNS quite readily. This also illuminates the potential that the endogenous concentration at synapses within the CNS may be higher than the applied doses when CNS circuits are spontaneously active, and the 1mM dosage may represent, more closely, the endogenous concentration at active synapses.

The varying effects on these behaviors illuminate the potential that AChRs modulate the circuits differentially. We noted that the potency of each of our agonists and antagonists was generally greater on the mouth hook movements, which may suggest that more subtle changes in signaling in a high-fidelity, repetitively active circuit may be more easily identified. Additionally it also points to the potential for differential AChR receptor expression and regulation of these two distinct neural circuits. In addition to the more specific pharmacological assessment utilized to address specific receptor subtype modulation of locomotion and feeding, molecular genetic techniques in association with pharmacological approaches can be utilized to corroborate our findings. It is of particular interest to continue investigation regarding the modulatory influence of ACh on larval feeding. Our pharmacological findings oppose what we've observed previously, and what others have shown. An interesting series of papers have identified a group interneruons that a substantial role in modulating larval feeding (Melcher et al. 2006; Bader et al. 2007b; Schoofs et al. 2014). This population of interneurons releases a neuropeptide, *hugin*, which is involved in regulation of feeding across phyla (Schlegel et al. 2016). When these neurons are activated, feeding behavior is reduced. This group also found that ACh is released from these neurons, and that the synthesis and release of ACh is necessary in regulating the effect on feeding (Schlegel et al 2016). Thus, manipulating activity of a reduced number of cholinergic neurons has shown the ability to reduce mouth hook extensions. Therefore, ACh modulation of feeding behavior may be dose-dependent, and we show here that supplementing the larval diet with ACh reduces feeding behavior over time. An alternative possibility is that sustained feeding for a 24-hour period induces receptor desensitization; however, the enhancement of activity in the locomotive circuit matched our predicted outcomes and was supported by our electrophysiological analysis showing ACh excitation of a motor program underlying larval crawling. While the circuit components underlying these two behaviors are distinct, and the desensitizing properties are likely receptor-dependent, the correlative excitation

of locomotion points to the inability of ACh at these concentration to drastically reduce behavioral responses. Although we cannot definitively rule out desensitization in the feeding circuit, it would be interesting to tease apart the impact of ACh on satiation or nutrient sensing, and examine this separately from direct modulation of motor output to the mouth hook motor apparatus, which was showin elegantly in Schoofs et al. (2014). A longer period feeding may drastically alter neuroendocrine regulation of feeding behavior, and could illuminate a separate role for Ach and specific receptor subtypes in this process.

Nicotine modulates larval feeding, locomotion and sensorimotor activity and displays potential desensitizing properties in the larval CNS

The importance of nAChRs in the *Drosophila* CNS is quite evident, as they are the targets of important insecticides, including neonicotinoids (Matsuda et al. 2001). In spite of this, the functional characterization of these receptors in an intact nervous system has been problematic. To date, only three of the ten nAChR subunits expressed in the Drosophila genome have been implicated in regulation of behavior: $\alpha 3$, $\alpha 4$ and $\alpha 7$. A primary cause for this is that successful reconstitution of these receptors in heterologous expression systems has been difficult (Landsdell et al. 2012). While insights have shed light on the ability of nAChRs to form homomeric α-subunit receptors, the inability to reconstitute receptors containing the β subunit has hindered functional characterization of endogenous channels in *Drosophila* (Landsdell et al. 2012). As a result, non-native β subunits from other species are utilized, which limits full understanding of native receptor function (Landsdell et al. 2012). Furthermore, immunohistochemical and precipitation/purification experiments, while informative for expression analysis, do not provide resolution regarding the functionality of the receptors *in vivo*. Thus, we have utilized this pharmacological approach to shed light on nAChR properties in an intact nervous system and

their roles in modulating behavior and sensorimotor circuit physiology. We uncovered a number of intriguing results, with a primary conclusion that the nAChRs in these circuits desensitize in the presence of nicotine in a dose-dependent manner.

In our electrophysiological analysis we noted low concentration application of nicotine induced high frequency bursts of activity, which was significantly enhanced relative to control. However, as the concentration was increased, the change in EPSP frequency was robust. Application of a high concentration, 100µM and 1mM, nicotine directly to the CNS exhibited consistent initial bursts of activity in muscle 6 followed by rapid abolishment of EPSPs. Within approximately 5-10 seconds following the bursts of activity, subsequent evoked responses were abolished. This suggests that the nAChRs in the CNS rapidly desensitize in the presence of these high concentrations of nicotine. Surprisingly, the dampened responses are remarkably vigorous, as the number of EPSPs dropped to zero in every preparation. We expected to observe some desensitization due to the properties described in some mammalian subtypes, including $\alpha4\beta2$ and $\alpha7$ (Miwa et al. 1999; Ibanez-Tallon et al. 2002); however, the fact we observed a complete shut down of sensory-CNS evoked potentials lends credence to the idea that there is an abundant expression and a vast functional role in of nAChRs within the sensory-CNS-motor circuit evoked in our analysis. Likewise, this suggest the presence of receptor subtypes that display similar kinetics and properties with mammalian $\alpha4\beta2$ and $\alpha7$ receptors, which are known to desensitize in the presence of high concentrations of nicotine. We were able to show that a saline washout, which required multiple washes especially with the highest (1mM) dose, 'rescued' the activity within the CNS. Interestingly, after 100µM and 1mM exposure, activity recovered and often led to enhanced EPSP frequency when compared to activity prior to nicotine exposure. This suggest, perhaps that residual, low concentration nicotine was likely present and enhanced excitation similar to what we observed in the controlled paradigm

with low doses of the drug.   We also noted that the additional agonist, clothianidin, which is synthesized and utilized as a commercial insecticide, abolished activity within 20 seconds of application. The ability of nicotine to cross cell membranes in an alkaline environment is well documented (Hukkanen et al. 2005) and can, thus, have an influence on cell function not directly related to action on nAChRs. We were surprised to note that application of clothianidin displayed a similar ability to reduce EPSP frequency. Clothianin displayed a higher potentcy, abolishing activity at a lower concentration (10µM) than nicotine with similar efficacy.

Surprisingly, curare and BD were not efficacious in reducing locomotion and feeding as expected given our findings with the nicotine treatment. Acute feedings of both antagonists induced an increase in locomotion at high concentrations. The similarity among responses between the two drugs strengthens the notion that acute feeding induces an enhancement of circuit activity that drives locomotion. Therefore, the results of this increase in locomotion may be an initial excitation of motor output. It has been shown previously that curare blocks central cholinergic transmission both in vitro (Lee and O'Dowd 1999) and *in vivo* (Rohrbach and Broadie 2002) and has been utilized as an agent to reduce cholinergic transmission (Ping and Tsunoda 2012). While longer feedings did reduce both locomotion and feeding, we show here that acute exposure to each non-selective antagonist induced initial enhancement in activity. Furthermore, we did not observe a robust reduction in activity in association with the evoked stimulation in the presence of these antagonists after application to the exposed CNS in our electrophysiological analysis. Only low dose (100nM) and high dose (1mM) BD induced a negative percent change in EPSP frequency in muscle 6 after a two minute application.   It was noted that bathing the preparation in curare for 15 minutes brought activity back down to baseline, or even reduced activity; however, activity within the evoked circuit was never abolished suggesting that the potency of curare is

reduced relative to BD, which shut down motor output at a concentration of 1mM. We also noted that enhanced locomotion occurred in response to 20-minute feeding of both of these antagonists, suggesting that the excitatory responses observed in semi-intact preparations were corroborated in our behavioral analysis. It has been noted previously and others have shown GABA and glutamate inhibit spontaneous activity in motor neurons (Rohrbach and Broadie 2002). Additionally, it has been shown *in vitro* that curare can bind with high affinity to both 5-HT$_3$ and GABA$_A$ receptors (Barik and Wonnacott 2009). Given the importance of GABA and 5-HT transmission in modulating locomotion (Silva et al. 2014; Majeed et al. 2016) and sensorimotor circuit activity (Majeed et al. 2016), it is a plausible assertion that the high concentrations in our behavioral and electrophysiological analysis may target GABA$_A$ and 5HT-3 receptors, inducing 'off-target' alterations in circuit efficacy. Therefore, in utilization of these antagonists in an intact nervous system, it is important to be mindful that longer exposures may be required to reduce cholinergic transmission within the larval CNS. Furthermore, much of the analysis utilizing these drugs were in cultured neurons, and the impact on multiple inputs may be different in the intact preparation, illuminating differences in circuit influence.

Likewise, the role of nicotine in regulating feeding is well documented in mammalian studies. There is an expansive expression profile of nAChRs outside the nervous sytem. Expression in adipose tissue, for instance is implicated as a player in altering weight in smokers (Voorhees et al. 2002). Following the intake of nicotine, immediate effects are observed on food intake, appetite, hunger and fullness (Grebenstein et al. 2013), likely due to the activation of melanocortin system (Mineur et al. 2011; Picciotto et al. 2012). However, the short time with which activity was reduced suggests that nAChRs are likely more prominent in modulating motor output to the mouth hook apparatus than regulating satiation and we suspect this mechanism to be more prominent in driving behavioral changes observed in this analysis.

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Nicotinic Dα7 receptor is prominent in modulating sensorimotor activity

      The rapid, and robust shutdown of activity in response to high doses of nicotine coupled with the action of the non-selective nAChR antagonists suggested to us that nicotine was inducing desensitization of these receptors in the CNS. Because it is known that, in rodent models, the α7 receptor desensitizes rapidly and is known to play a role in a sensorimotor behavior in the adult fly (Fayaduzzin et al. 2006), we reasoned that this receptor was abundant in the larval CNS and likely plays a functional role in modulating sensorimotor activity in larvae.   Thus we tested the action of a well-known α7-specific antagonist, methyllycaconitine (MLA), in blocking activity in the larval CNS. Application of high dose (1mM) MLA induced rapid and robust abolishment of EPSPs at the NMJ in response to afferent stimulation. Not only did we notice that CNS activity in response to sensory stimulation was abolished, but any spontaneous activity unrelated to the sensory stimulation was abolished as well. Only mEPSPs were observed when this antagonist was applied, which shows the abolishment of activity is not due to any blocking of glutamate receptors on the muscle, and rather, is due to inhibition of excitatory input onto motor neurons. This suggests that this antagonist is potent in blocking α7 nAChRs in the CNS and points to a prominent role for this receptor subtype in modulating sensorimotor activity.   We noted that the responses when exposed to this particular drug were abolished much more rapidly (within 20 seconds) than the additional antagonists tested, suggesting also that these receptors display a high affinity for this antagonist.   Not surprisingly, the lower concentrations, while reducing activity, were less efficacious in abolishing EPSPs in muscle 6, but reduced activity was noted in 3 out of 4 concentrations tested. The robust nature of abolishment was intriguing and it supports the notion that the Dα7 nAChR is prominent in the larval CNS.

      The α7 receptor is known to play a crucial role in potentiating neural circuits in the mammalian hippocampus and promoting glutamatergic synapse

formation and maintenance (Lozado et al. 2012; Halff et al. 2014). Specifically, glutamatergic synapses in the rodent hippocampus through regulating AMPA receptor mobility and function, thus playing a prominent role in plasticity underlying learning and memory. It has also been highlighted as a target for activity-dependent modulation in the fly CNS (Ping and Tsunoda 2012) and its expression has been identified in the mushroom bodies (Kremer et al. 2010; Christiansen et al. 2011; Nakayama et al. 2016) suggesting it may play a conserved role in synaptic plasticity underlying associative learning in this model. Furthermore, a recent report identified this receptor subtype in playing a role in nicotine-induced effects on survival, developmental rate, and nicotine and ethanol sensitivity in flies (Velazquez-Ulloa 2017). This study showed the receptor is upregulated following developmental exposure to nicotine (Velazquez-Ulloa 2017). While Ping and Tsunoda (2012) identified upregulation in response to antagonist exposure, this study shows nicotine can induce similar changes in receptor expression, and it may be through desensitization. Nevertheless, the identification of a significant role in modulation of a defined neural circuit in larval *Drosophila* can be informative in identifying this subtype as a target for genetic manipulation and more comprehensive analysis in its role in various forms of plasticity in this model. It would be an interesting follow-up to address the modulation of sensorimotor circuit activity following chronic nicotine exposure to illuminate alterations in efficacy, to follow up on previous behavioral analysis (Velazquez-Ulloa 2017). A worthy complementation to this study can be to assess the role of this receptor in plasticity and can be expanded to address its role in habituation to touch or other associative learning assays, along with additional components of cholinergic signaling mentioned previously. Here, we've illuminated an important role of a specific receptor subtype in modulation of sensorimotor activity and advance understanding of the receptor properties adding to previous investigations of its role in behavior (Fayaduzzin et al. 2006) and development (Velazquez-Ulloa 2017).

Muscarine exposure enhances sensorimotor circuit activity but reduces locomotion and feeding after chronic exposure

While characterizing the pharmacological and physiological properties of nAChRs in the fly model has been a particularly arduous task, recent analysis has shed light on the pharmacological properties of the three mAChR receptor subtypes that are found in the *Drosophila* genome. Furthermore, a previous study identified the 2nd messenger cascades involved in mAChR regulation of cellular function (Ren et al. 2015). It has been shown that the A and C-type receptors to couple to excitatory 2nd messenger cascades that excite neurons. Both of these receptors are sensitive to acetylcholine, muscarine, and the antagonists scopolamine and atropine (Collin et al. 2013; Xia et al. 2016). Conversely, the B-type mAChR couples to $G_{i/o}$, inhibiting adenylyl cyclase (*rutabaga*) and reducing cell excitability (Ren et al. 2015). Moreover, this receptor has been shown to be significantly less sensitive to ACh and muscarine (1,000-fold) and is not blocked by atropine or scopolamine (Collin et al. 2013). All of these receptors have been shown, either through GAL4/UAS-driven fluorescent reporter expression in our analysis (A and B-type), or in quantitative expression analysis, to be expressed in larvae or adults (Collin et al. 2013; Xia et al. 2016). The A-type receptor is activated by both low concentrations of ACh and muscarine, whereas the B-type receptor is not responsive to muscarine binding (Collin et al. 2013).   Thus, this recent analysis has shed light on how mAChRs may differentially alter neuronal excitability, which helps to provide important insights into their influence potential influence on intact circuits.

Due to previous pharmacological assessment, we suspect that the majority of responses are regulated by the A-type and C-type receptors. While high concentrations of muscarine may influence mAChR-B receptor-mediated signaling, our findings displaying scopolamine and atropine as potent in reducing activity in locomotion, feeding and sensorimotor activity suggest that these circuits are regulated prominently by the A-type and/or C-type receptor. Our

analysis shows that initial exposure to muscarine enhances circuit activity underlying larval locomotion and feeding, as 20-minute feeding of muscarine induced a dose-dependent increase in both behaviors, with the feeding increasing significantly upon acute exposure. However, 24-hour exposure significantly reduced both locomotion and feeding. As noted upon supplemeting ACh for these time periods, a similar response in feeding behavior was observed. While the significant reduction in mouth hook extensions after an acute exposure to scopolamine suggests mAChRs play a role in regulating motor output to the mouth hook apparatus, the possibility that the reduction in feeding over a longer time course may be through neuroendocrine regulation. The endocrine influence on nutrient sensing and cell metabolism may serve as a mechanism in reducing feeding behavior. Our analysis showing 24-hr exposure to ACh reduces feeding suggests also that its action may be through excitatory mAChRs (A-type and C-type) in driving reduced gustatory response upon presentation of yeast. It is possible that ACh, and muscarine as evidenced here, may potentiate inhibitory circuits underlying higher-order nutrient or satiation processing. In both cases, exposure to ACh and muscarine induces initial enhancement in feeding behavior, suggesting they may act through mAChR to enhance motor output; however sustained feeding and increased concentration may induce more subtle changes to the feeding circuit through endocrine control. The *hugin/ACh* neurons aforementioned have been shown to project to the pars intercerabalis in the protocerebrum, a crucial site of neuroendocrine regulation in the larval CNS (Schlegel et al. 2016). This region contains insulin-like peptide producing cells (IPCs) that project to the ring gland, the primary endocrine gland in larval *Drosophila* (Schlegel et al. 2016). It has been previously shown that mAChRs are highly expressed in this region (Cao et al. 2014). Thus, it is possible that, while the excitatory influence on motor output to the feeding apparatus may subside over a 24-hr time course, the neuroendocrine influence may play a crucial role in regulating feeding behavior. It is possible that ACh, and muscarine as evidenced here, may potentiate inhibitory circuits underlying higher-order

nutrient or satiation processing in the subesophagal zone (SEZ) or pars intercerabalis, where gustatory sensory input and second-order gustatory processing occurs, respectively (Schlegel et al. 206).   The opposing influence of scopolamine, however, warrants further investigation, but the potential for off-target effects certainly exists, as scopolamine is to influence 5-HT receptors (Lochner and Thompson 2016).   Futhermore, the reduced locomotion observed in our analysis also suggests that prolonged exposure to muscarine may desensitize receptors, reducing activity in an excitatory circuits driving locomotion. We noted enhancement of sensorimotor activity upon exposure to musacrine, suggesting it drives excitation to motor neurons innverating muscle 6, thus we suspect the long-term reduction in locomotion may shed light on potential desensitization, as scopolamine also reduced locomotion after chronic exposure.

Future analysis should center on a combined pharmacological and genetic approach, whereby combination of genetic manipulation of specific receptor subtype expression coupled with pharmacology is utilized. This would corroborate our findings and help to address, more specifically, the receptor involved in modulating these circuits. The potential for off-target effects is important to consider, and the use of a combined approach would help address these potential confounds. The change in behaviors following longer feeding is intriguing and it would be of interest to follow up the role of mAChRs specifically in modulating larval feeding. Specific receptor knockdown can be performed using a host of interneurons drivers, including those that target the pars intercerabalis, directly. It would be important to tease apart the influence of mAChRs on modulating motor output through neuroendocrine regulation, and approaches including mass spectrometry and/or HPLC to detect changes in circulating concentrations of hormones, including insulin-like peptides, can be used in mAChR mutants to assess regulation of hormones known to influence gustation and cell metabolism. Furthermore, recording intracellular responses in

mouth hook muscles or via en passant recordings of mouth hook motor nerves following agonist/antagonist feeding or in receptor mutants upon activation of interneurons can be performed to directly assess modulation of motor output, as described by Schoofs et al. (2014).

CONCLUSION AND FUTURE DIRECTIONS

Questions still remain regarding the exact neurons that may be impacted by altering cholinergic activity within the larval CNS. Moreover, we do not know exactly how many synaptic connections are altered as a result of the sensory stimulations. The larval sensory system is made up of reiterative subsets of cell that send projections to the VNC, targeting a host of interneuron populations (Singhania et al.2014). Given that the afferent nerves stimulated in this approach are made up of different sensory subsets, our stimulation likely targets a host of interneurons. Identification of interneuron populations that are activated as a result of our stimulations and their synaptic properties is essential in fully deciphering the role of modulators, including ACh, in modulating sensorimotor activity. However, the results presented here serve a useful purpose in providing assessment of activity changes within CNS circuits in the presence of cholinergic agonists and antagonist, which has proven a challenge for *Drosophila* neurophysiologists to date. Additional experimentation using techniques that allow for circuit mapping and identifying individual cholinergic interneuron connections and/or expression of cholinergic receptor subtypes in interneurons that may be manipulated by this sensory stimulation would be helpful in understanding how these circuits are modulated. Combining 'connectomics' and physiological approaches will be particularly powerful in assessing modulator actions, and the *Drosophila* model will likely soon be a feasible model for such analysis. Furthermore, identification of specific receptor subtypes regulating these circuits is necessary to address additional questions regarding their roles in circuit formation and maintenance. How might these receptors play a role in

activity-dependent circuit formation? What are the cellular mechanisms through which they impart this regulation? Are these mechanisms conserved across phyla? Given the immense developmental impact that nAChRs and mAChRs have on mammalian circuits, it would be intriguing to continue to promote the fly model as a model to study their role in regulating neural circuit connectivity. This serves a necessary step in allowing these questions to be addressed by providing insights into ACh modulation of intact circuits.
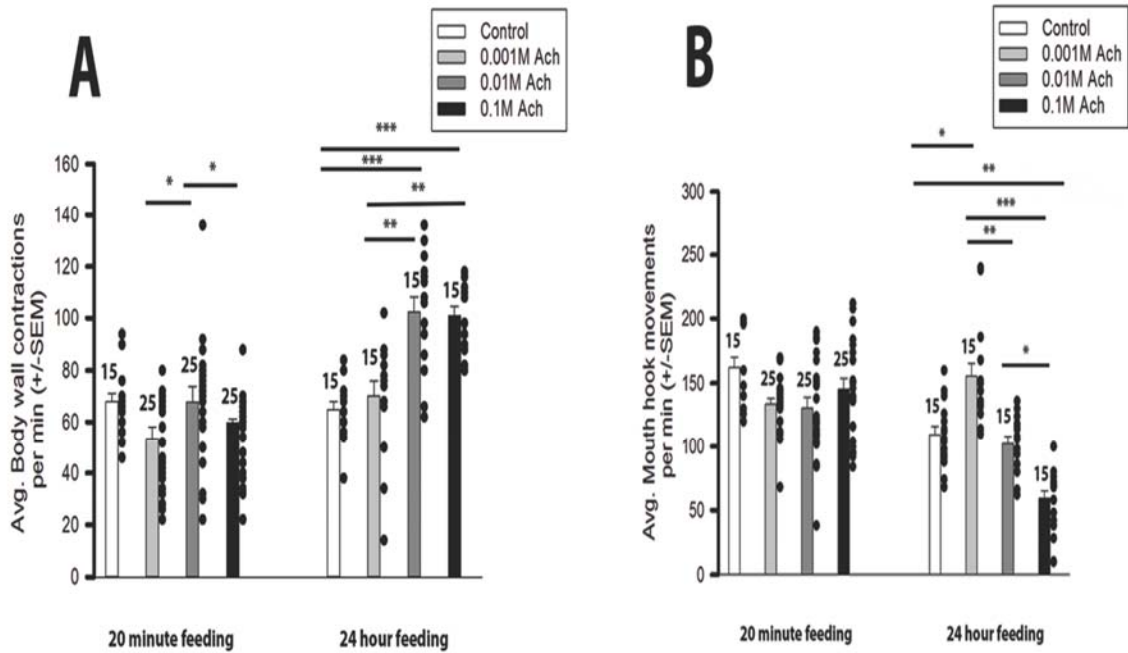
Figure 4.1. Oral supplementation of ACh and modulation of locomotion and feeding. A. Average number of body wall contractions for different concentrations of Ach over 20 min and 24 hr feeding. The sample size of each group is indicated by the number over its respective bar. Each point represents a single larvae. Feeding ACh over a 24 hr period generated a significant increase in locomotion B. Average number of mouth hook movements per minute for different concentrations of ACh over 20 min and 24 hr feedings. ACh supplementation for a 24 hr period induced a dose-dependent significant reduction In mouth hook movements upon presentation of yeast. Data is presented as average (+/-) SEM. One-Way ANOVA used for analysis. $P \leq 0.05$ (*), $P \leq 0.02$ (**), and $P \leq 0.001$ (***)
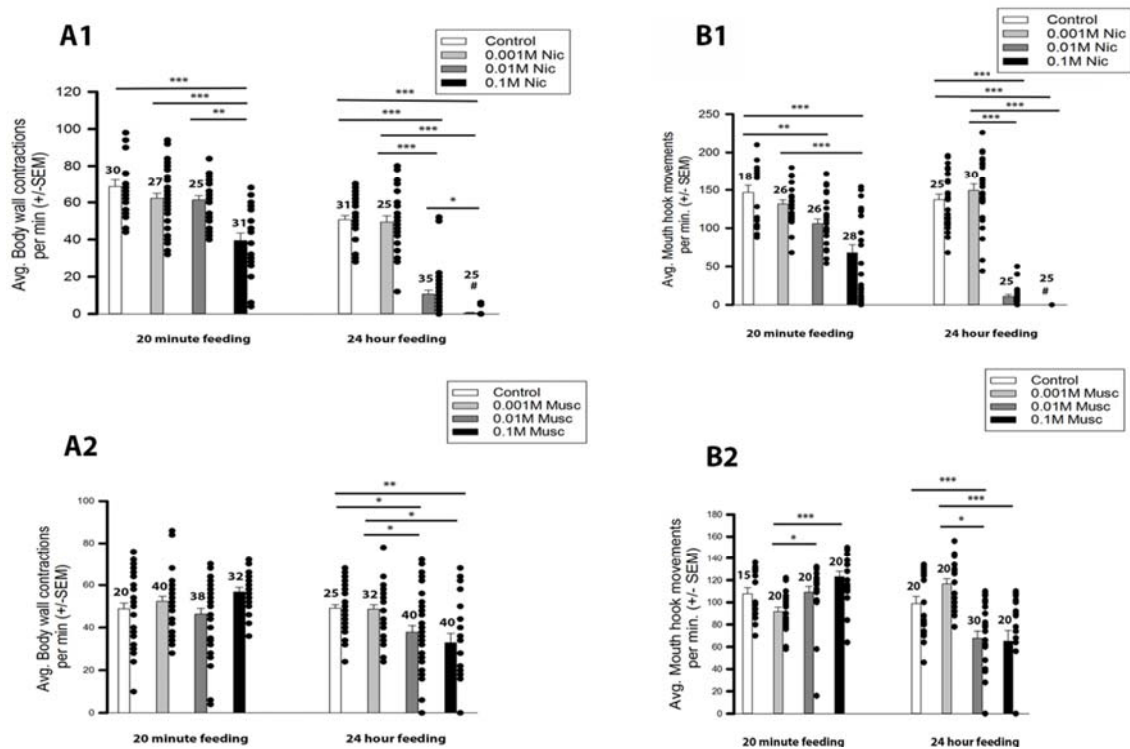
Figure 4.2. Oral supplementation of AChR agonist and modulation of locomotion and feeding. A column present locomotion results and B column presents feeding results. Sample size is incdicated above the bars. Each point represents an individual larva. A1. Average number of body wall contractions after exposure to nicotine over 20 min and 24 hr feeding. Feeding nicotine over a 20-minute and 24-hour generated a significant reduction in locomotion B1 Average number of mouth hook movements per minute after exposure to nicotine over 20 min and 24 hr feedings. Nicotine exposure induced a significant reduction in mouth hook movements after 20-minute and 24-hour feeding upon presentation of yeast. # Signifies lethal dosage. A2. Average number of body wall contractions after exposure to muscarine over 20 min and 24 hr feeding. Acute exposure to muscarine enhances locomotion while 24-hr exposure significantly reduces body wall contractions. B2. Average number of mouth hook movements after exposure to muscarine over 20 min and 24 hr feeding upon presentation of yeast. Acute exposure to muscarine enhances feeding while 24-hr exposure significantly reduces feeding behavior. Data is presented as average (+/-) SEM. One-Way ANOVA used for analysis. $P \leq 0.05$ (*), $P \leq 0.01$ (**), and $P \leq 0.001$ (***)
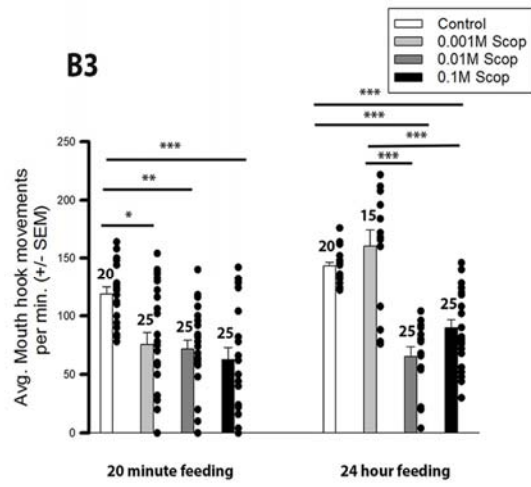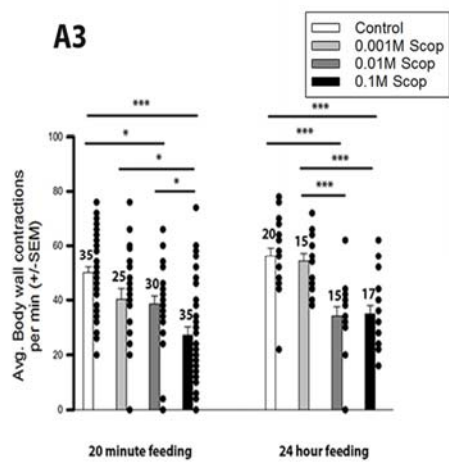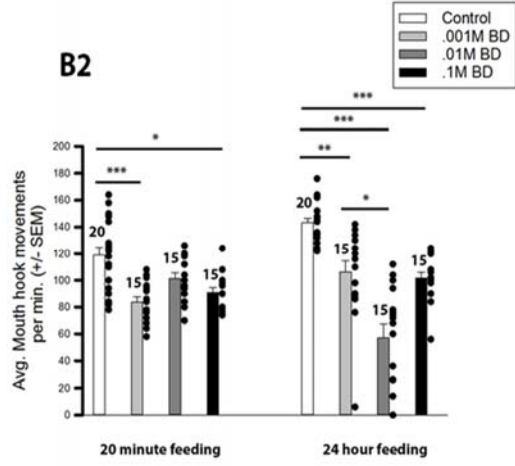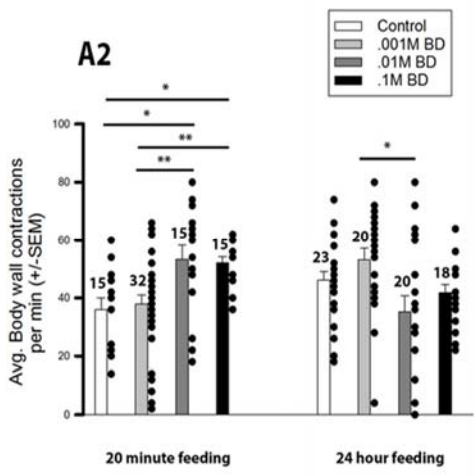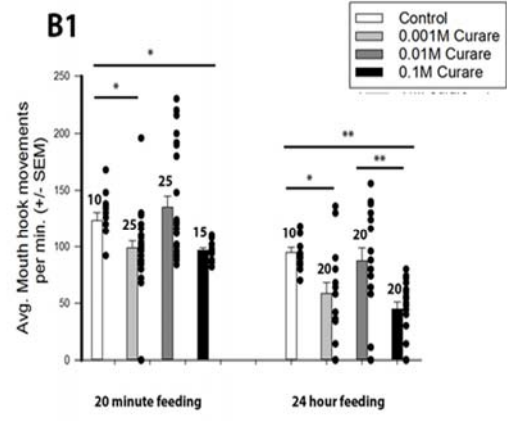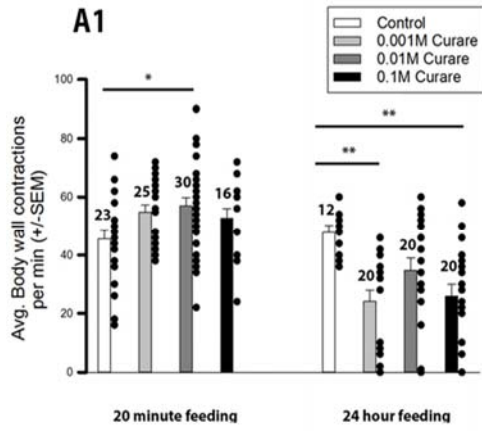
Figure 4.3. Oral supplementation of AchR agonist modulation and modulation of locomotion and feeding. A column present locomotion results and B column presents feeding results. Sample size is incdicated above the bars. Each point represents an individual larva**.** A1-B1. Average number of body wall contractions and mouth hook movements, respectively after exposure to Curare over 20 min and 24 hr feeding. Feeding curare over a 24-hour generated a significant reduction in locomotion and feeding. A2-B2. Average number of body wall contractions and mouth hook extensions after exposure to BD over 20-min and 24-hr feeding. Acute exposure to BD enhances locomotion while 24-hr exposure significantly reduces body wall contractions**.** Both acute and 24-hr feeding reduces mouth hook extensions A3-B3. Average number of body wall contractions and mouth hook movements, respectively after exposure to scopolamine over 20-min and 24-hr feeding. Acute and long-term exposure to scopolamine reduces both behaviors. Data is presented as average (+/-) SEM. One-way ANOVA with used for analysis. $P \leq 0.05$ (*), $P \leq 0.01$ (**), and $P \leq 0.001$ (***)
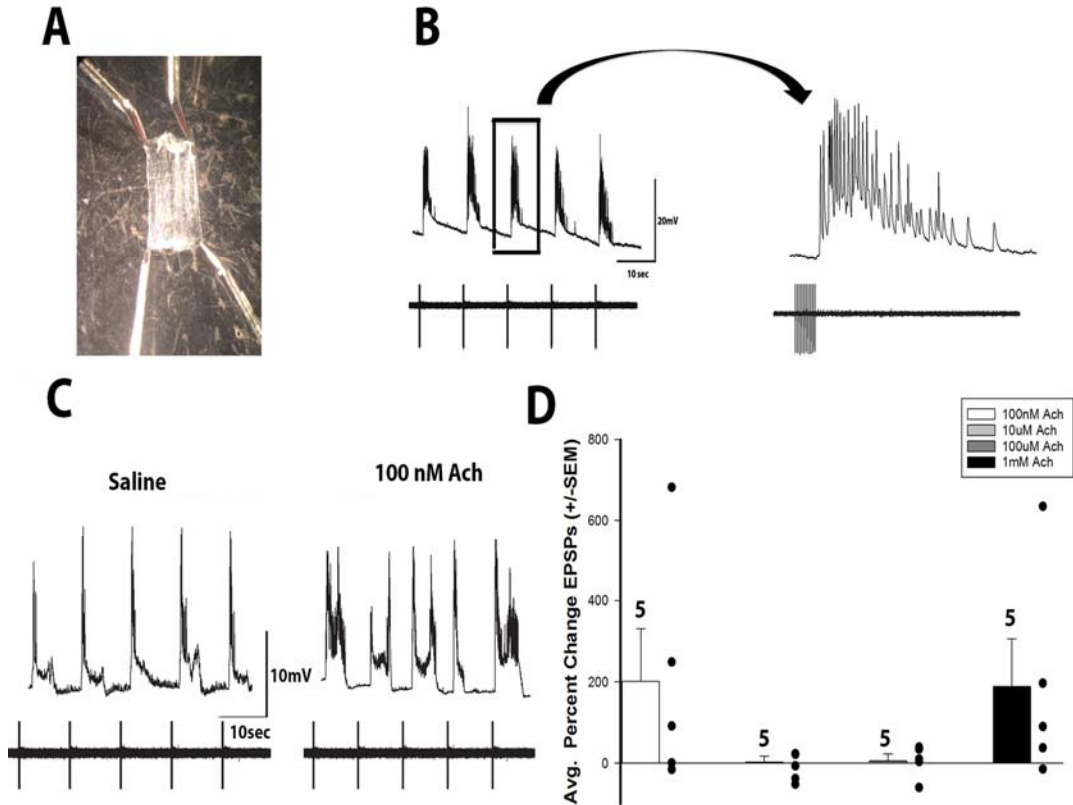
Figure 4.4. ACh modulation of sensorimotor circuit activity A. Longitudinal dissection of 3rd instar CS larvae. A suction electrode was used to stimulate the last two segmental nerves and sharp microelectrodes were used to record EPSPS in muscle fiber 6 (not shown) contralateral to the stimulus. B. Quantifying EPSP data. Each EPSP in muscle 6 was counted following a stimulus train stimulating sensory afferents, which are displayed below each trace. C. Sample trace displaying enhanced EPSP frequency in the presence of 100nM Ach. . The number of EPSPs was counted for 5 stimulus trains each for saline and each drug concentration. D. Average percent change in EPSPs to different concentrations of Ach. 5 larvae were tested for each concentration of Ach. The percent changes were calculated by comparing the average number of EPSPs when the larvae was exposed to saline to when the larvae was exposed to drug. 5 larvae produced 5 percent differences, which were then averaged. Each point represents the average percent change for a particular larval prep. The error bars were calculated using SEM. This procedure was repeated for Ach agonists and antagonists. Data is presented as Avg. percent change (+/-) SEM.

A1

Saline

100 nM Nic

20mV

10 sec

B1

Avg. Percent Change EPSPs (+/- SEM)

Legend:
- Control
- 0.001M Nic
- 0.01M Nic
- 0.1M Nic

5   5   5   5

A2

Saline   **Saline**

1 mM Nic

20mV

10 sec

B2

Legend:
- 100 nM Cloth
- 10 uM Cloth
- 1 mM Cloth

6   5   5

A3

Saline   **1mM Musc**

20mV

10 sec

B3

Legend:
- 100nM Musc
- 10uM Musc
- 100uM Musc
- 1mM Musc

6   6   7   7

C

1ppm pestanal

116
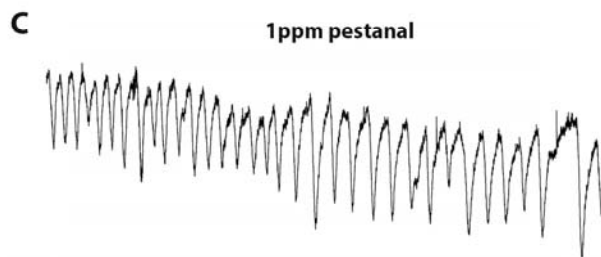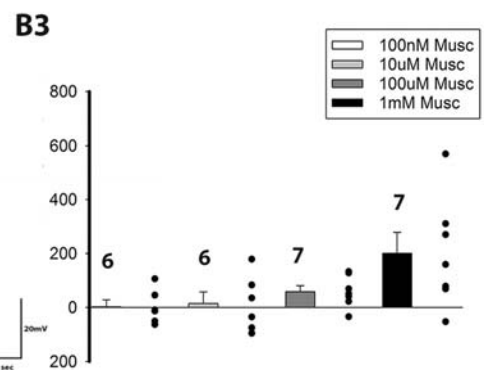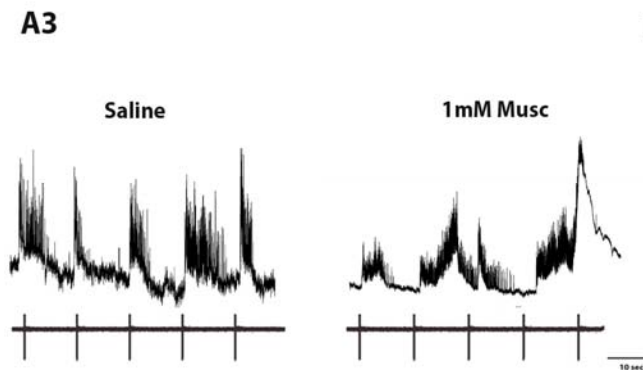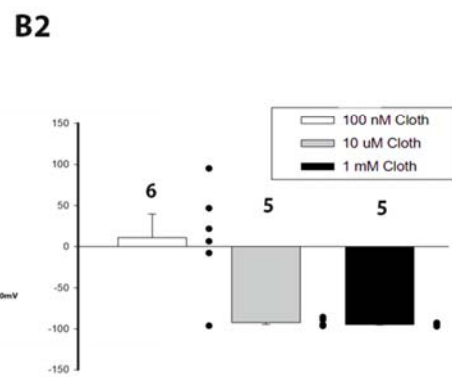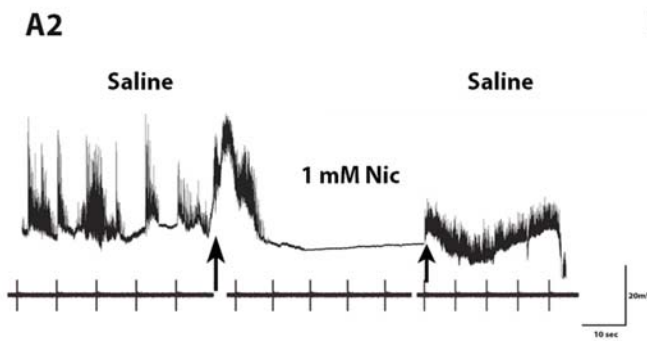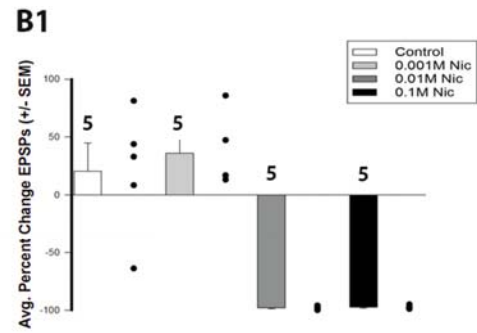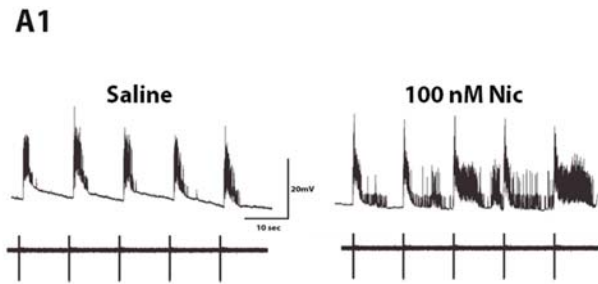
Figure 4.5. AChR agonist modulation of sensorimotor circuit activity. A column provides pertinent sample traces and B column depicts quantification of average percent change at each concentration. A1. Sample trace of 100 nM nicotine and saline. At 100 nM nicotine increased EPSP frequency. A2. Sample trace displaying response to 1mM nicotine exposure. 1 mM nicotine abolishes CNS activity. The first arrow indicates when nicotine was added. The second arrow shows when saline was used to wash out the nicotine. Upon the saline wash, activity returned. B1. Average percent change in EPSPs to different concentrations of nicotine. At higher doses, nicotine prevented EPSP response. B2. Average percent change in EPSPs for different concentrations of clothianidin. High dose of clothianidin also shut down activity similar to nicotine. (trace not shown). A3. Sample trace displaying response to 1mM muscarine. 1mM muscarine enhances sensorimotor activity. B3. Average percent change in EPSP frequency upon exposure to each concentration of muscarine. Muscarine enhances sensorimotor activity in a dose-dependent manner. C. Sample trace displaying response to 1ppm pentanal. High frequency spontaneous activity is observed. Oscillations in trace represent contraction of muscle. Activity in response to sensory stimulation could not be recorded due to consistent spontaneous activity.Data is presented as Avg. percent change (+/-) SEM.
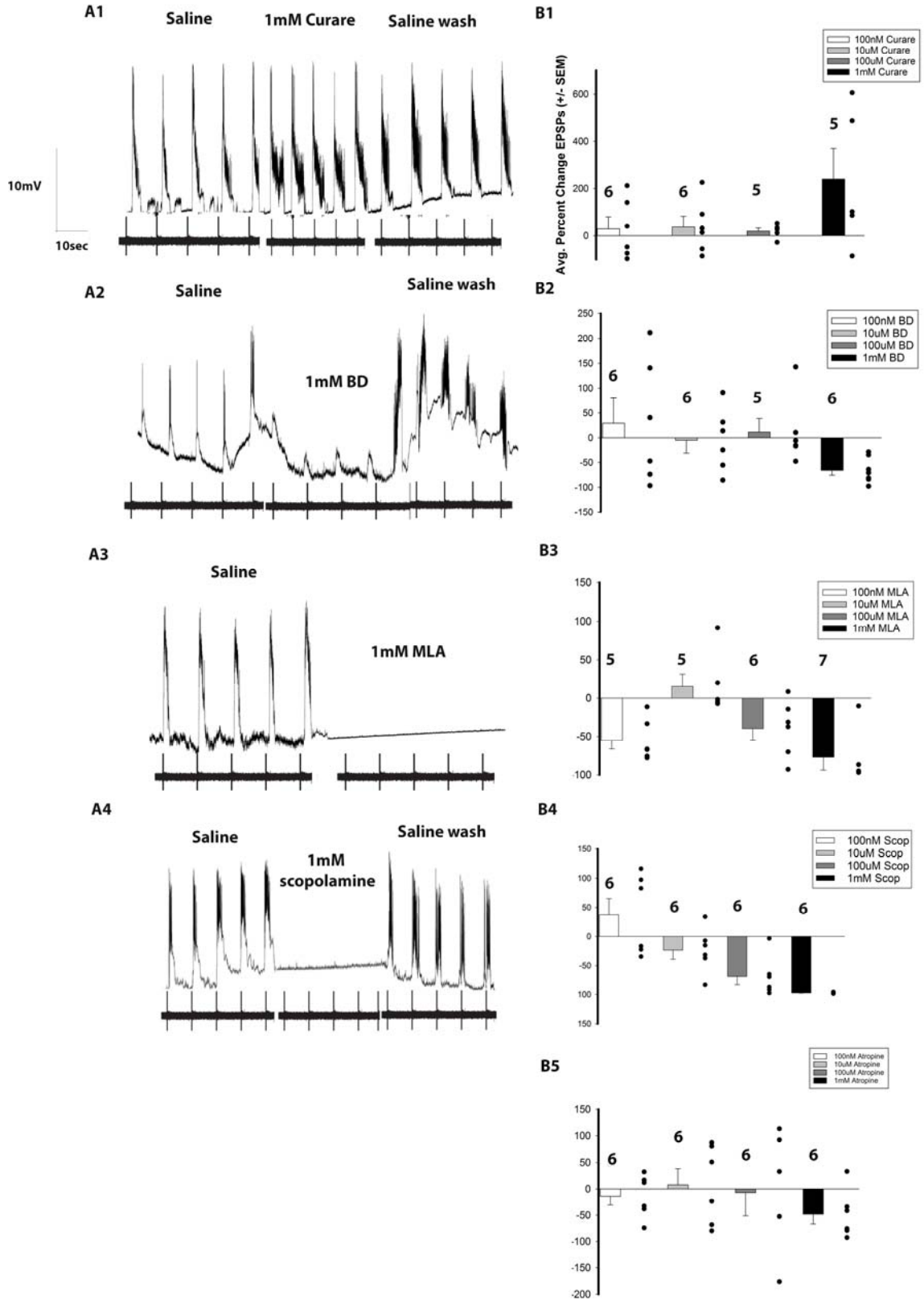
Figure 4.6. AChR antagonist modulation of sensorimotor circuit activity. A column provides pertinent sample traces and B column depicts quantification of average percent change at each concentration. A1. Sample trace of 1 mM curare and saline. At 1mM curare increased EPSP frequency. B1. Average percent change in EPSPs to different concentrations of curare. Curare increases EPSP frequency. B1. A2-B2. BD analysis with sample trace upon 1mM BD exposure and average percent change in EPSP frequency at each concentration. High dose BD reduces CNS activity. A3-B3. MLA analysis with sample trace upon 1mM MLA exposure and average percent change in EPSP frequency at each concentration. High dose MLA abolishes CNS activity. A4-B4. Scopolamine analysis with sample trace upon 1mM scopolamine exposure and average percent change in EPSP frequency at each concentration. High dose scopolamine abolishes CNS activity. B5. Average percent change in EPSP frequency in response to atropine application. Atropine induces variable responses at lower concentrations and a reduction in CNS activity at 1mM. Data is presented as Avg. percent change (+/-) SEM.

CHAPTER FIVE

Considerations in repetitive activation of light sensitive ion channels for long-term studies: Channel rhodopsin in the *Drosophila* model.

INTRODUCTION

The advent of optically stimulating exogenous ion channels and ion pumps, which can be expressed in specific neurons, allows one to augment neural circuits without altering non-specific neurons or introducing systemic agents (Banghart et al., 2004; Fiala, 2013; Klapoetke et al., 2014; Towne and Thompson, 2016;). The rapid growth and heightened attention in the experimental use of optogenetics in various animal models, worms, insects, rodents; Nagel et al., 2005; Hornstein et al., 2009; Titlow et al., 2015; Riemensperger et al., 2016; Giachello and Baines, 2017) and even humans (Scholl et al., 2016; Sengupta et al., 2016; Towne and Thompson, 2016) is demonstrating great promise for manipulating activity in various types of tissue (Quinn et al., 2016; Zhu et al., 2016; Malloy et al., 2017). In order to advance the field and uncover the potential therapeutic uses, the limitations, as well as the ability to finely tune the activation or silencing of optically sensitive ion channels must be understood (Bender et al., 2016; Blumberg et al., 2016). The ability to activate or inactivate ion channels rapidly and to control for specified cellular expression is an advantage of this technique (Gunaydin et al., 2010; Deisseroth, 2015). In addition, the ability to prod neurons deep within the brain with flexible optical fibers (Bass et al., 2010; Danjo et al., 2014) or the use of triggering channel rhodopsins by bioluminescence (Birkner et al., 2014) add to the tractability of optogenetics. However, there are some struggles researchers are facing with the use of these associated techniques (Dawydow et al., 2014; Deisseroth, 2014, 2015; Gradinaru et al., 2014; Grosenick et al., 2015; Lee et al.,

2014). With increased experimental investigations these issues will likely be resolved.

The complexity and accessibility of the central neural circuits complicates controlling some of the factors accounting for the variability in responses. It is also difficult to measure quantal events in intact CNS preparations at postsynaptic contacts. Thus, in examining activity dependent influences of synaptic transmission by optogenetic approaches we have focused on the larval *Drosophila* motor unit and obtaining synaptic measures at neuromuscular junctions (Pulver et al., 2009; Majeed et al., 2016). The larval *Drosophila* neuromuscular junction (NMJ) allows ease in measures of quantal events and evoked synaptic transmission under various experimental conditions. This animal model is excellent for investigating mechanism of synaptic development (Nose, 2012).

All trans retinal (ATR), a compound used to help in promoting the ion conductance and preventing the degradation of channel rhodopsin, is used in animal models in which the organisms does not naturally produce ATR; thus, it is supplemented in the diet (AzimiHashemi et al., 2014; Hegemann et al., 1991). The need to use ATR produces additional variables. The concentration used, the potential degradation over time in the presence of light, how the cell metabolizes the compound over time, and the impact on the channels rhodopsins themselves are all factors that need to be considered when utilizing optogenetics in experimentation. The ChR2-XXL variant is highly sensitive to blue light and does not require additional ATR supplementation in the diet for activation of cells. Therefore, we have tested the efficacy of responses with and without a controlled concentration of ATR to aid in understanding the impact on cellular and organismal function in order to refine this technique.

In this report, we highlight goals we are pursuing and experimental issues we have come across with the use of optogenetics in activating neurons throughout development in *Drosophila* larvae. We have uncovered reproducible but unexpected outcomes in particular experimental paradigms and here we share

these outcomes and discuss our interpretations and their implications for future experimentation. We illustrate with electrophysiological and behavioral approaches, that conditioning whole animals with the optogenetic technique might provide some misleading results if the physiology is not directly measured. Thus, if one is to alter neural circuits that may or may not be activated, electrophysiological measurement and stimulation paradigms are necessary to be experimentally determined.

Our long-term goal is to develop a means to repetitively and consistently activate neurons over the long term, throughout key developmental periods in neural development of animals. However, in pursuing this goal a number of novel experimental findings have awakened us to some issues in the larval *Drosophila* model. Thus, for the benefit of other researchers we highlight a few of the concerns we have had in repetitively exciting the channel rhodopsins in neurons in a matter of minutes to days and biophysical changes while electrically stimulating neurons during and after activation of channel rhodopsin.

MATERIALS AND METHODS

Drosophila lines

The filial 1 (F1) generations were obtained by crossing females of UAS-ChR2-XXL (BDSC stock # 58374) with males of D42-GAL4 (BDSC stock#8816). The parental lines were also examined for the effect of light sensitivity for behaviors and electrophysiological studies. *Drosophila* were raised on a mixture of cornmeal-agar-dextrose. The general maintenance is described in Campos-Ortega and Hartenstein (1985). The D42 strain was used as a proof of concept since it is known to be expressed highly in motor neurons (Yeh et al., 1995; Nitz et al., 2002) but also some expression in sensory neurons (Sanyal, 2009). When the ChR2-XXL expressing neurons are targeted, the result is the body wall muscle contraction, leaving the larvae in a state of paralysis.

Preparation of fly food supplemented with ATR

All trans retinal (ATR; Sigma-Aldrich, St. Louis, MO, USA) was diluted in 5 ml of standard fly food to a final concentration of 400µM and it was protected from light with aluminum foil. For control experiments, larvae were cultured in food that only contained the solvent (absolute ethanol) in fly food. All the animals were reared in vials with the same cornmeal–agar-dextrose-yeast medium (modified from Lewis, 1960). Food without added retinal is likely devoid of retinal as this food is cooked and made into a fly media for culturing the flies and larvae. Considering the food is boiled it is unlikely if whatever retinal did exist in the dried corn meal would be able to remain active since the compound is heat liable.

Larval behavior

Locomotion behavior was assessed by placing larvae on an apple-juice 1% agar plate (Majeed et al., 2016). The larvae were left for one minute to acclimate to the new environment. The body wall movements were recorded while being exposed to a dim white light and when exposed to diffuse blue light from an LED mounted in a soda can (see Titlow et al., 2014). The locomotion activities were recorded with a webcam (WEBCAM HD4110, Hewlett-Packard Company, Palo Alto, CA), which was connected to a computer, and the activity was recorded at 25 frames per second for various experimental paradigms (see Results).

Electrophysiology

The synaptic responses at the larval *Drosophila* NMJs were recorded by standard procedures (Lee et al., 2009) with stimulation at 0.5 Hz as described in the Results section. All the experiments were performed at room temperatures (20-21°C). The excitatory post synaptic potentials (EPSPs) were measured by intracellular recordings with a sharp glass electrode (3M KCl) and AxoClamp-2B amplifier (Molecular Devices, LLC. 1311 Orleans Drive, Sunnyvale CA, USA).

Stimulations were made with a Grass S88 dual stimulator (Natus Neurology Incorporated, Middleton, WI, USA). Preparations were used immediately after dissection. Electrical signals were recorded online to a computer via a PowerLab/4s interface (ADI Instruments, Colorado Springs, CO, USA). The larval *Drosophila* preparations were dissected as previously described (Li et al., 2001) for early 3rd instars. The CNS was left intact for studies as expression is likely high in the cell body and axons as compared to the isolated nerve terminal.

The modified HL3 saline was used for physiological measures (Stewart et al., 1994) at a pH of 7.1 (de Castro et al., 2014). Saline solution (in mM): 1.0 $CaCl_2 \cdot 2H_2O$, 70 NaCl, 20 $MgCl_2$, 5 KCl, 10 $NaHCO_3$, 5 trehalose, 115 sucrose, 25 5*N,N*-bis(2-hydoxyethyl)-2-aminoethanesulfonic acid (BES), All chemicals were obtained from Sigma-Aldrich (St. Louis, MO). All measures were made in muscle 6 of segments 3, 4 or 5.

Blue light exposures

The blue light (470nm wavelength, LEDsupply, LXML-PB01-0040, 70 lm @ 700mA) was provided by a high intensity LED. The photon flux (number of photons per second per unit area) was measured with a LI-COR (model Li-1000 data Logger, LDL 3774; LI-COR from Lincoln, Nebraska, USA) which produced around 103 µMol s-1 m-2 per µA (or 22.24 µW mm-2) on the surface of the dish for the behaviors and on the dissected preparations around 133 µMol s-1 m-2 per uA (or 28.9 µW mm-2).

The exposures during the developmental conditions were around 50 µMol s-1 m-2 per µA (or 10.87 µW mm-2) at a distance of about 12 cm from the light source to the larvae. The larvae were distributed in the food so only a thin layer (about body thickness) would occur to continuously expose the larvae to the light. The food needed to be dampened with water about every 12 hours to keep it from drying out. The exposure during development occurred by taking early 2nd instar

larvae and either placing them in food with or without ATR and either exposing the dish to blue light or maintaining the dish in total darkness. The light was timed to provide 30 sec of continuous blue light followed by 30 min of darkness for 48 hrs. The ability to control the light stimulation was managed by Arduino system (2015 Arduino, LLC.); https://www.arduino.cc/).

Statistical analysis

All data are expressed as raw values or mean ± SEM. A paired Student's T-test (before and after) or ANOVA or a rank sum pairwise test was used to analyze changes in behavior or electrophysiological responses after changing bath conditions or stimulating with blue light. Since the groups are not normally distributed (i.e., a number of zeros in some groups) and having different sample sizes we used a Dunn's Test or a sign rank sum test for trends among preparations.

RESULTS

The initial approach to determine if intact larvae would show repetitive behavioral responses to pulses of blue light was performed by monitoring crawling behavior before, during, and after a series of repetitive light pulses. The light pulses consisted of 30 sec of blue light followed by 10 min of very dim white light which allowed the camera to monitor body movements. The time it took larvae to initiate a full body contraction after the blue light was turned off was used as an index in reproducibility of a behavioral response. The effect of supplementing the food with ATR was also examined by comparing larvae which were raised with and without food containing ATR (400 µM). In addition, parental lines (D42-GAL4 and ChRXXL-UAS) were also examined with and without exposure to ATR. The larvae were placed in the associated feeding conditions from early 2nd instar stage and left for 48hrs in the dark. The larvae reached an early 3rd instar stage by 48 hrs. The developmental time is slightly slowed in the presence of ETOH solvent in the

ATR mixture and. ETOH was added to food not containing ATR to control for the ETOH exposure.

The time to initiate a body wall contraction after 30 sec of blue light exposure and 10 min of dim white light (Figure 5.1A) indicated that ATR fed larvae were slower to initiate movements and over time the ability to move was sped up in larvae not exposed to ATR. This is illustrated by representative movement maps, which depict the first 10 min of dim white light exposure after blue light exposure for 3 larvae in each condition (Figure 5.1B). Upon blue light exposure, the larvae with and without ATR all showed strong body wall contractions creating a paralysis, which generally persisted the entire 30 sec of blue light exposure (Figure 5.1C; N=10, $P \leq 0.05$, T-test and ANOVA compared to with or without ATR as well as to parental lines). The parental lines with or without ATR showed no reduction in movement when exposed to blue light or white light. Thus, the time to initiate a movement is shown as time zero. To compare the effect of longer periods of blue light exposure, an entire hour was used followed with one hour of very dim white light. The blue light exposure was subsequently repeated a second time. The time to initiate a body wall movement was also measured and, as for the shorter blue light pulses, the ATR fed larvae took longer to move compared to the ones lacking ATR (Figure 5.1D, N=10, $P \leq 0.05$, T-test). The parental lines again showed no effects even to the longer blue light exposure (each group N=10).

In being able to manipulate a developing neural circuit, or one over long periods of time, it is of interest to determine if the optically activated responses are consistent in the outcome of the behavioral responses. Thus, we exposed larvae from 2nd to wandering 3rd instar to 30 sec of blue light and gave 30 min of time in the dark prior to repeating the blue light exposure. This paradigm was repeated for 48 hrs for larvae with and without ATR mixed with the food (Figure 5.2A1). We switched out the food after 24 hrs of feeding to control for the pulses of blue light possibly inactivating the ATR. A few preliminary trails indicated more pronounced responses in the larvae after 48 hrs to blue light when the food was replaced after 24 hrs but this phenomenon was not carefully documented; however, we kept with

standardizing the exchange to maintain consistency in the experimental conditions presented herein.

The behavioral experiments were performed to determine the time to initiate body wall contractions for the larvae exposed to blue light pulses during the previous 48 hrs. A comparison for larvae fed ATR and those without ATR was revealing. The same testing paradigm was used for the ones not exposed to the conditioning light pulses (Figure 5.2A2) The ones fed ATR did not move later within the 10 min in dim white light (labeled dark) after the 30 sec exposures of blue light (Figure 5.2B; N=10, P $\leq$ 0.05, T-test). When an hour of blue light exposure was used to assess behavioral responses, the larvae lacking ATR in their diet did not remain contracted the entire duration of the hour which is in contrast to those fed ATR (Figure 5.2C; N=10, P $\leq$ 0.05, T-test). In addition, the repetitive 30 sec blue light exposure and 10 min of dim white light indicated the larvae were able to recover quicker with subsequent light exposures. One might assume that the responses would have already plateaued from the previous 48 hrs of light pulse conditioning. The robust contractions with the first few blue light exposures during the behavioral test might be due to the fact that the conditioning blue light was at 50 µMol s-1 m-2 per µA (10.87 µW mm-2), whereas for the behavioral test on the agar dishes the blue light was around 133 µMol s-1 m-2 per µA (28.9 µW mm-2). For ease in comparing the treatments of blue light and the effect of feeding ATR the combined responses are shown in **F**igure 5.2D1 for the 30 sec light pulses and for the 1hr exposures in Figure 5.2D2. When the larvae were placed in complete darkness for a few hours, all the larvae move to new locations so even the hour exposure to blue light was not toxic to the larvae fed ATR and dark adapted (i.e. the conditions most sensitive to the blue light behavioral test).

To address the ability to repetitively and consistently activate channel rhodopsin proteins with blue light pulses, EPSPs in the muscles were monitored. The exposed filleted larvae bathed in physiological saline were exposed with 10 sec long periods of blue light with 10 min dark recover times and this was repeated 3 times. The effect of supplementing the food with ATR and prior exposures to blue

light pulses during the previous 48hrs was also addressed in the electrophysiological assays. Since we used the D42-GAL4 line some sensory neurons may also be activated in this assay.

Larvae raised with ATR (48 hrs) and kept in complete darkness had an unusual EPSP response to the light pulses. The majority of the initial exposures produced a strong burst of activity during the 10 sec exposure but would cease producing large EPSPs (which are due to action potentials within the motor neurons) within the 10 sec light stimulating period. Each of the 3 subsequent light exposures with 10 min dark periods is shown (see Figures 5.3A1, A2, A3). The subsequent pulses of blue light might or might not result in the evoked EPSPs ceasing within the 10 sec of blue light. Notice the 3rd subsequent 10 sec light pulse did not evoke a response in the axon to initiate action potential and only small miniature quantal events were observed (see Figure 5.3B). However, after the evoked EPSPs would stop, the motor nerve would remain inactivated for about 2 or 3 mins followed by a renewed burst of activity which would persist for 2 to 5 mins (Figure 5.3B). During the dark period after the blue light was shown, small quantal events could be observed which would dampen in frequency in the 10 min of dark exposure. The trend in the 10 sec blue light exposures produced 3 out 5 larvae to show this phenomenon of limiting evoked responses before the 10 sec of blue light exposure was over. In addressing if a longer exposure to ATR from 1st instar to 3rd (7 days) and a longer exposure to blue light while measuring evoked EPSPs (20 sec), in both larvae kept in dark as well as exposed to 10 sec blue light on and 30 min off for the full 7 days, was examined. A representative response shown in Figure 5.3C1 also illustrated the light induced evoked EPSPs stop before the light exposure is over, however many small quantal events continued (Figure 5.3C2). The frequency of these spontaneous quantal occurrences was not consistent from larvae to larvae or even within a series of the trials within a preparation. However, 6 out of 7 larvae raised in the dark demonstrated complete quiescent in evoked EPSPs (sign rank N=7, $P \leq 0.05$). In addition, these small quantal events would be masked by the larger evoked events when they occur.

Larvae exposure to the blue light conditioning for the 7 days all produce evoked EPSPs when tested and none showed a cessation of the evoked responses within the 20 sec blue light exposure (sign rank N=6, P $\leq$ 0.05).

The larvae without supplemented ATR for 48 hrs, but also raised in the dark, exhibited slightly different responses. The evoked EPSP would continue throughout the 10 sec blue light exposures and would sometimes continue for 1 to 2 mins after the blue light was turned off (Figure 5.4A1, A2, A3). Each of the 3 subsequent light exposures with 10 min dark periods is shown (Figure 5.4A1, A2, A3). To control for the possibility of the blue light itself stimulating the motor nerves or central neurons the D42-Gal4 parental line fed ATR for 48 hrs was also examined. The parental line did not show any response to the blue light exposures and the larvae appeared healthy as the spontaneous events occurred at a relatively consistent frequency with and without blue light exposures (Figure 5.4B1, sign rank N=6, P $\leq$ 0.05).). An enlargement of a quantal event within the trace shown in Figure 5.4B1 is shown in Figure 5.4B2.

The long-term 1 hr exposures of blue light for the larvae fed ATR and lacking ATR revealed a similar response; however, the neural activity would remain the full hour. The start of the 1 hr exposure is shown in Figure 5.4C1 and the end of the 1 hr is shown in 5.4C2. In this particular experimental paradigm, after 1 hr of blue light, an hour of dark was maintained followed by a second blue light exposure (Figure 5.4C3). The ATR fed larvae would also exhibit the initial burst and shut down followed by resumed firing. In one case the firing pattern was relatively constant at 10 Hz for the entire hour for a larva exposed to food without ATR. The larvae lacking ATR and exposed to the blue light for 1 hr did not display the initial refractory period of stopping the light induced responses (Figure 5.4D1) and were able to maintain the evoked EPSPs for the entire hour. The end of the hour of blue light exposure and subsequent dark exposure is shown in Figure 5.4D2.

We examined if the refractory period in the light induced evoked responses was related to the frequency of the evoked EPSPs. The maximum peak frequency of evoked EPSPs and the occurrence of a refractory period occurring within the 10

sec light exposure did not reveal a particular frequency at which the evoked responses would stop occurring. The peak frequencies would be as high as 60 Hz but there was no correlation with the cessation of the light evoked EPSPs due to the same high frequency being obtained in larvae which did not show a refractory period in light induced EPSPs.

The exposure to pulses of blue light (30 sec blue light, 30 min dark repetitively cycled) throughout development (early 2nd instar to 3rd) for 48 hr prior to electrophysiological testing with larvae fed ATR and without ATR also showed differences in the EPSPs when exposed to the 10 sec of blue light. The larvae exposed to blue lights for 48 hr did not show the rapid bursts and shut down within the 10 sec flashes of light (Figure 5.5A). The prevalence of this response was consistent in each of the 5 larvae examined. The larvae lacking ATR but conditioned for the 48 with light pulses did respond with evoked EPSPs to the tested 10 sec of blue light and maintained the evoked EPSPs for the 10 sec (5 out of 5) and would cease evoking EPSPs after the blue light was shut off. Two different larval responses are shown in Figure 5.5B1 and 5.5B2.

Larvae fed ATR and kept in the dark for 48 hr showed a burst of evoked EPSPs and then become quiescent within 10 sec of blue light exposure. Afterwards the EPSPs would spontaneously re-occur while in the dark, thus it appeared that the motor nerve was possibly in an electrical refractory period or that the nerve terminal was not able to provide evoked vesicle fusion. The time varied among each of the larvae but within the range of 50 to 90 seconds before spontaneous activity reappeared. Thus, we examined this by en passant stimulation of the segmental nerve roots to the segment in which the optically evoked EPSPs were being measured. The motor nerve was stimulated at 0.5 Hz and the evoked responses were monitored before, during and after the blue light pulse. In larvae fed ATR, but maintained in the dark, the electrically evoked EPSPs were able to be induced while the light evoked EPSPs were reduced in amplitude or stopped while still being exposed to the blue light or right after the blue light was turned off (Figure 5.5C). Thus, the nerve is still electrically active even though the

EPSPs were not maintained fully in amplitude during the blue light exposure. However, the responses were mixed. In one larva the electrically evoked EPSP grew smaller over the light pulse and afterwards the electrically evoked EPSPs became larger and regained the same amplitude as to pre-light exposure (Figure 5.5C). However, the very light sensitive larvae (i.e., fed ATR) demonstrated in 6 out of 8 larvae that the electrically evoked EPSPs stopped occurring when the light induced EPSPs also stopped. However, very small quantal events would still occur at a high frequency. After some time in the absence of the blue light, the electrically evoked EPSPs would start to appear sometimes gradually increasing in amplitude. The rise time of the electrically evoked responses would occur first with a slow rise and then a rapid rise time (Figure 5.4C2). The larvae lacking ATR but being electrically stimulated did not demonstrate the absolute refractory response but did demonstrate reduced electrically evoked EPSP responses initially. They would regain the evoked EPSP amplitude over time (Figure 5.4D). This is not because the muscle is not able to respond to glutamate release as the light induced EPSPs are still occurring and eliciting large EPSPs. In addition, the size of the light induced quantal events, after the light exposure, is robust. This would indicate that the postsynaptic receptors are not desensitized by the bursts of the EPSPs. Also, it appears the ChR2-induced electrical refractory period is ATR-dependent. Since the minis are occurring rapidly it would suggest there is a residual Ca^{2+} remaining within the nerve terminals.

DISCUSSION

In this study, we have clearly shown that repetitive short bursts (30 sec) of light activating the channel rhodopsin variant, ChR2XXL, expressed in neurons in intact larvae, can produce behavioral adaptation over time. When the larvae are fed ATR the responses are robust and even over an hour of exposure the behaviors showed little accommodation. Over a period of 48 hr with short bursts of light (30 sec on, 30 min off) there is less responsiveness of the larvae to remain

contracted when exposed to acute blue light pulses. The behavioral responses are mirrored in the electrophysiological measures at the NMJs with the presence of light evoked large EPSPs and smaller quantal sized EPSPs. A surprise novel finding was that the nerve would become electrically unexcitable when the nerve demonstrated a reduction in evoking large and synchronized EPSPs with light. Even though large EPSPs could not be electrically induced there were still spontaneous quantal events occurring. The inability to electrically stimulate the nerve during the optically induced refractory period, as well as the likelihood of the light inducing the refractory period, was related to the sensitivity of neuron to blue light. Larvae which were fed ATR showed more pronounced refractory periods relative to larvae lacking ATR in their diet or larvae exposed to light pulses over a 48 hr or 7 days of a conditioning window.

As demonstrated by Pulver et al., (2009) in acute studies of *Drosophila* larvae expressing channel rhodopsin in glutamatergic neurons (motor neurons and interneurons; OK371-Gal4 / UAS-ChR2 or UAS-H134RChR2 lines), pulses of blue light produces less accommodation than the constant blue light exposed over a few minutes for initiating nerve induced large EPSPs. In addition, Pulver et al., (2009) reported the more sensitive H134RChR2 variant as compared to ChR2 resulted in prolonged EPSP activity following light stimulation. We report similar findings with ChR2-XXL but we also examined the difference of feeding ATR or not on the sensitivity of the lines. Larvae maintained in the dark and fed ATR produced a very strong response in a burst of EPSPs but the EPSPs would stop occurring within the 10 sec period still being exposed to blue light or the first period of the hour exposure. Whereas the less sensitive larvae (not fed ATR) would generally remain active, producing light induced nerve evoked EPSPs throughout the 10 sec, and even hour-long, light exposure. The differences in sensitivities of channel rhodopsin strains gives the advantage of a range to use, but also opens many questions about differences in accommodation of the frequency of EPSPs and an apparent refractory period of the nerve which can occur for evoked nerve induced EPSPs. The high occurrences of small single quantal events, when the

nerve is in a light induced refractory period, provides evidence that the postsynaptic receptors are not desensitized, thus providing additional evidence that the abolishment of EPSPs is due to pre-synaptic absolute refractory. Also, since the amplitude of EPSPs gradually recovers fully over time and the rise time is shorter may suggest more about how the presynaptic vesicles being synchronized for electrical evoked voltage gated calcium channels. It is likely that the influx of Ca^{2+} through the ChR2 may indeed induce the voltage gated plasma membrane Ca^{2+} channels to open and even increase the Ca^{2+} load in the presynaptic terminal (Lin et al., 2009).

When the larvae are recovering from blue light pulses they have a distorted locomotion (i.e., wobbling from side to side while trying to crawl forward) at first and then followed by increased coordination in the wave of segmental contractions over the body. This may be a central effect or an indication of the functional recovery at the NMJs. One might expect when the electrophysiological responses of the light induced refractory in EPSPs occurs that the larvae would start moving and crawling instead of remaining in a contracted state. If we had not shown that motor neurons were also in an electrical refractory period from being stimulated the behaviors response might have been at odds with only measuring the light induced EPSPs. This result opens a new avenue of investigation into the biophysical properties of neurons being electrically excitable in functional neural circuits when inducing light sensitive channels. It is possible that cell bodies or even axons with different diameters may have varying threshold of being activated when channel rhodopsin are activated due to varied input resistance of the cells, and but also a larger surface area of the cell membrane which may provide for a higher density of channel rhodopsin proteins (Arlow et al., 2013). Fictive locomotion pattern measured in isolated larval *Drosophila* CNS, with genetically encoded Ca^{2+} indicators, demonstrated left-right asymmetry across segments (Pulver et al., 2015). This previous study suggested the asymmetry may arise to the larvae initiating a turning behavior. A follow up study would be to measure the Ca^{2+} flux and conditions presented in this study to determine if large alterations in

symmetry occur while some neurons are in electrical refractory and possibly during an uncoordinated crawling behavior.

Despite not being able to electrically excite the motor neuron during the light induced refractory period there is a high frequency of the quantal events in a non-synchronized manner. This suggests the channel rhodopsins are likely within the nerve terminal or pre-terminal membrane allowing Ca^{2+} to enter the terminal continuously while the blue light is on. The continuous occurrence of the quantal events with the nerve in an electrical refractory period is probably due to the nerve's inability to completely reset the $[Ca^{2+}]_i$ with pumps and exchangers in the plasma membrane and/or ER (Mattson et al., 2000). The reduction in the rate of the quantal events over time is also an indication that the terminal is able to reach the homeostatic level of a resting state. However, this is deceiving since in a minute after the light induced refractory period is over, the motor nerve starts to fire again with a barrage of nerve evoked EPSPs in larvae raised in the dark with supplemented ATR. This could be examined by blocking calcium membrane pumps and the Na^+-Ca^{2+} exchanger to determine there is a prolonged action resetting the refractory period after activating the channel rhodopsins. The intact larvae in the behavioral tests likely remain contracted during the light refractory period due to the fact that the quantal events are occurring at a high rate in many NMJs and can depolarize the muscle enough to keep a Ca^{2+} load within the skeletal muscles. A high rate of randomized spontaneous quantal events is able to induce muscle contractions in larval *Drosophila* (Majeed et al., 2015).

It appears there is more complexity occurring in respect to the properties of ion channels with a light induced refractory period and spontaneity of neural activity rather than just activating channel rhodopsins. The underlying causes to the light induced refractory remain unresolved. This finding compounds the difficulty in knowing how neural circuits within deep brain regions ladened with light sensitive channels are responding to synaptic events depending on the degree of light

sensitivity or timing of the light exposures. The activity within a circuit may also influence the response of the neurons to activating light sensitive channels (Adamantidis et al., 2011). Thus, it will be an exciting challenge as the field moves forward to assess the light sensitive channels to intrinsically changing neural activity under different conditions within behaving animals as not to dampen the neural activity when the goal is to excite the circuit and vice versa.

The habituation to the onset of movements over time from the repetitive light pulses might be explained by either a reduced sensitivity to the light or the possibility that the neurons regulated a compensatory mechanism to the repetitive activity. However, in measuring the evoked EPSPs with repetitive light pulses every 10 min with 10 sec light exposures revealed that the responses were less robust following each exposure. This was particularly evident for the larvae fed ATR. The larvae deprived of ATR also showed a reduced response over time but this reduction was not as pronounced. Thus, the electrophysiological responses would indicate that the behavioral habituation is due to the motor neurons not being as responsive to the blue light over time. As for mechanisms of the habituation, it would appear the ATR molecule itself or the associated channels result in some reduced response with repetitive optical stimulation. It is possible that the ATR or the ATR-channel complex undergoes a conformation change and the dark adaption time of 10 min is not long enough for new channels and ATR to be incorporated in the membrane. It is also possible that a less sensitive channel rhodopsin constructs or even ChR2-XXL expression without the addition of ATR in preparations which do not synthetize ATR would produce more consistent responses for repetitive light exposures. The use of rhodopsin constructs which are less sensitive to light or different wavelengths of light (Zhang et al., 2008) for better reproducibility in responses has been proposed by Grosenick et al., (2015) and Dawydow et al., (2014).

Maintaining consistent responses or levels of activation over time of the optically sensitive channels is important for developmental studies if one wishes to manipulate activity of neural circuits to address neuronal plasticity and factors involved in neuronal circuit formation or control (see review by Giachello and Baines, 2017). This is not only the case for neurons but also other tissues in which optically activated proteins are being used. It is of interest to understand the mechanisms behind the habituation of the responses as well as the biophysical properties underlying the light induced refractory period. Possibilities to be tested are the fact that over depolarization of the nerve by the high frequency of neural activity resulted in voltage gated sodium channels inactivating and thus produce a refractory period until the neurons could regain a resting state for some period of time. This phenomenon was reported by Lin et al., (2009) but without presenting data (data not shown, Lin et al., 2009). The possibility of the PMCA and other pumps, such as the Na-K pump, over compensating and lowering the resting membrane potential could reduce the basal voltage-gated sodium channel inactivation and result in a lower threshold (Nadim et al., 1995). Another possibility could be activation of calcium activated potassium channels keeping the cell hyperpolarized; thus, inhibiting the cell from firing when electrically evoked. Future investigations need to be conducted to determine the cellular mechanisms for the electrical refractory during channel rhodopsin activation. It is very likely that the long lasting sodium-dependent afterhyperpolarization driven by the electrogenic activity of Na^+/K^+ ATPase may be a contributing factor. The long lasting effect was shown in larval motor neurons and it was determined that number of spikes and not the short burst frequency is the contributing factor (Pulver & Griffith, 2010). This may well be the underlying mechanism to explain our observations with using this highly sensitive channel rhodopsin.

The possibility of the expressed proteins being targeted in other membranes besides the cell or being taken up in membrane recycling brings to light other phenomenon to consider. Could the synaptic vesicles themselves incorporate the ChR proteins due to their bilipid nature, and thus influence glutamate loading and

even fusion events? As far as we are aware this possibility has not been directly addressed. It is known that mast cells use calcium release activated Ca^{2+} (CRAC) channels that are mediated by IgE dependent activation through an internal inositol 1,4,5-triphosphate (IP3) system (Ashmole and Bradding, 2013) which would be similar to the ER within the nerve terminal during Ca^{2+} dumping. Thus, if ChR proteins were loaded into the ER membranes and activated in addition to the plasma membrane of the cell there would be various means of loading Ca^{2+} within the synaptic terminal resulting in vesicle fusion. A similar scenario of Ca^{2+} dumping could occur for mitochondria present within the nerve terminals. Even if the mitochondria were to be loaded with Ca^{2+}, due to the Ca^{2+} ions coming across the cell membrane and into the mitochondria, when light is present it could result in transit mitochondria damage which would result in Ca^{2+} dumping (Kislin et al., 2016). Likewise, if the lysosomes happen to incorporate ChR proteins and then activated by light the result would be a Ca^{2+} surge within the terminal (Brailoiu and Brailoiu, 2016). There is precedence for proteins to be expressed and targeted to the ER which appear to have no functional use in the membrane of the ER. There is evidence that nicotinic acetylcholine receptors (nAChRs) can occur on Endoplasmic Reticulum (ER) derived microsomes (Moonschi et al. 2015), which suggest cell trafficking of proteins can result in ion channels being directed to other localizations besides the cellular plasma membrane and be functional (Colombo et al., 2013). Possibly using a pharmacological approach with Brefeldin A would prevent the vesicle formation from the endosome (Park et al., 2016). Thus, vesicles would not contain the channel rhodopsin from this means but blocking this pathway would also alter recycling of vesicle for synaptic transmission and would not necessarily inhibit channel rhodopsin from being in the ER or even to the vesicles by other means such as lipophilic attraction.

Another issue that could probably have an impact on cellular function is high intensity LED lights may cause organelle damage (Chamorro et al., 2013). The expression systems induced high level of protein syntheses, such as channel rhodopsin, in cells which can interfere with native cellular process (Palomares et

al., 2004). In addition, the turnover rate of the channel rhodopsins may also be dependent on the frequency of activation and overall cellular activity (Ullrich et al., 2013). The amount of ATR in the diet or the endogenous production of ATR, depending on the animal model, is an aspect to consider in working out the effects on the channels sensitivity over longer period of time. These matters produce a changing target in order to deliver consistent optical responses in a cell. Despite some of the experimental problems with use of channel rhodopsins that we and others have encountered, there is promise that the use of light activated channels will provide technical breakthroughs for experimentation and potential use in therapeutics as well as long term manipulation during development of tissues, particularly of defined neural circuits (Iaccarino et al., 2016).

A

| 30 sec Light on | 10 min Light off | 30 sec Light on | 10 min Light off | 30 sec Light on | 10 min Light off |

B

Parental

ChR2-XXL with ATR

ChR2-XXL without ATR

C

30 Sec on / 10 min off

Time (min) until crawling vs Trials (1–5)

D

1 hr on / 1 hr off

Time (min) until crawling vs Trials (1)

Legend:
- ○ With ATR ChR2-XXL/D42
- △ Without ATR ChR2-XXL/D42
- □ Parental D42 with ATR
- ◇ Parental ChR2-XXL with ATR
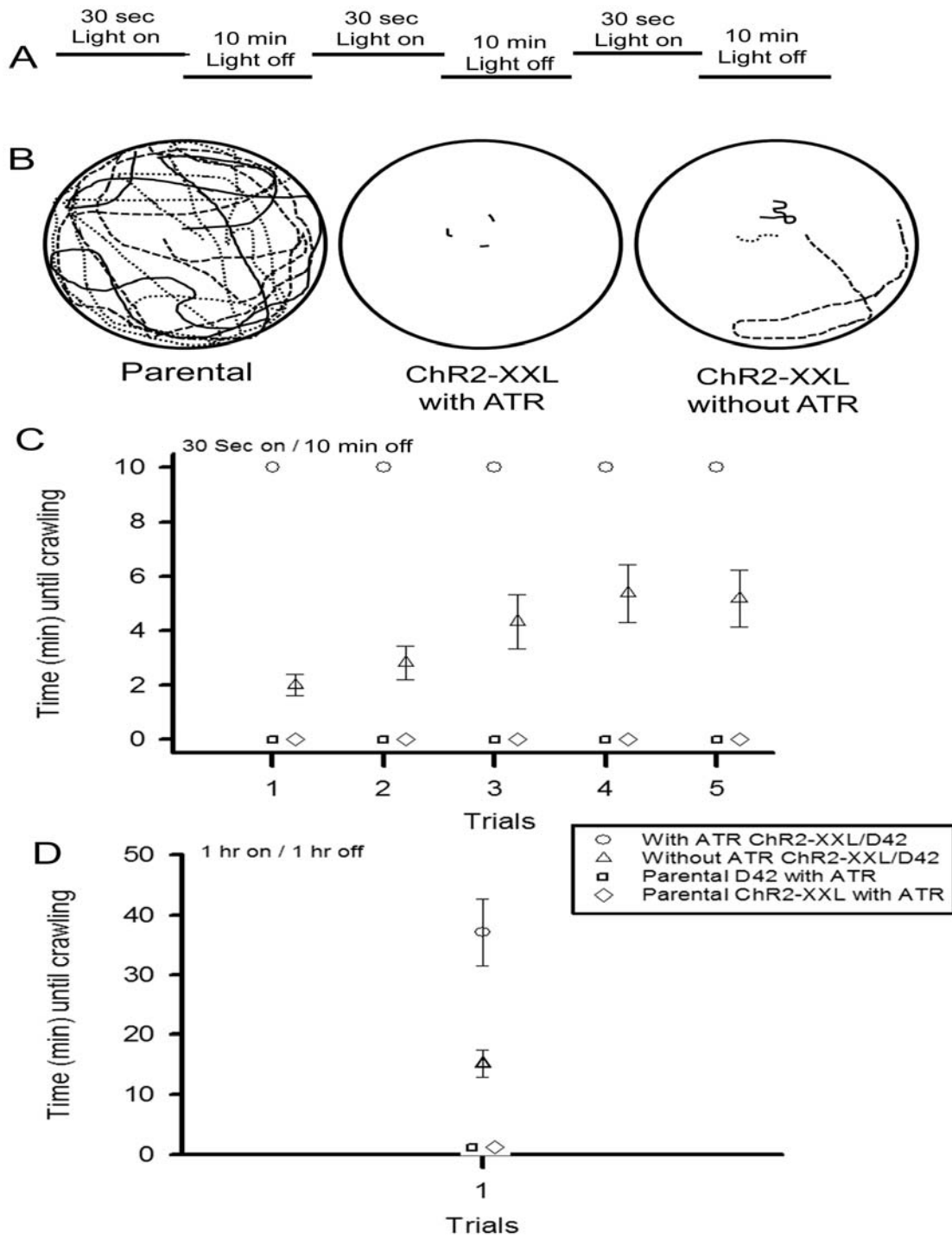
Figure 5.1. Locomotion of larvae after exposure to blue light to examine the effect of feeding ATR and expression of ChR2-XXL channels in motor neurons. (A) The acute exposure paradigm to blue light followed by very dim white light (i.e. labeled as dark) with just enough light to visualize the larvae with the camera. (B) The movement pattern of 3 larvae during the first 10 min dark period in the series of light/dark exposures. The larvae fed ATR and expressing the ChR2-XXL would not initiate a full body wall contraction within the 10 min. The larvae expressing the channel but not fed ATR only slightly moved in the same time period, whereas the parental strain of ChR2-XXL and fed ATR showed no inhibition to movement in blue light or the dark. (C) The time to initiate the first full body wall contraction is shown for the each of the 10 min dark periods in the light on/off series. The D42/ChR2-XXL larvae fed ATR remained paralyzed for each of the 10 min dark periods whereas the D42/ChR2-XXL larvae lacking ATR took a little longer to start moving with each exposure. The parental lines (D42 and ChR2-XXL) fed ATR showed no hesitation to keep moving after the blue light was turned off. (D) The effect of 1 hr exposure to blue light and then without the blue light resulted in the first occurrence of a complete body wall contraction having a longer lasting effect on D42/ChR-XXL larvae not fed ATR than for the 30 sec exposures. The D42/ChR-XXL larvae fed ATR took about 40 min as compared to 15 min for those lacking ATR to start moving. (N=10 mean+/-SEM).
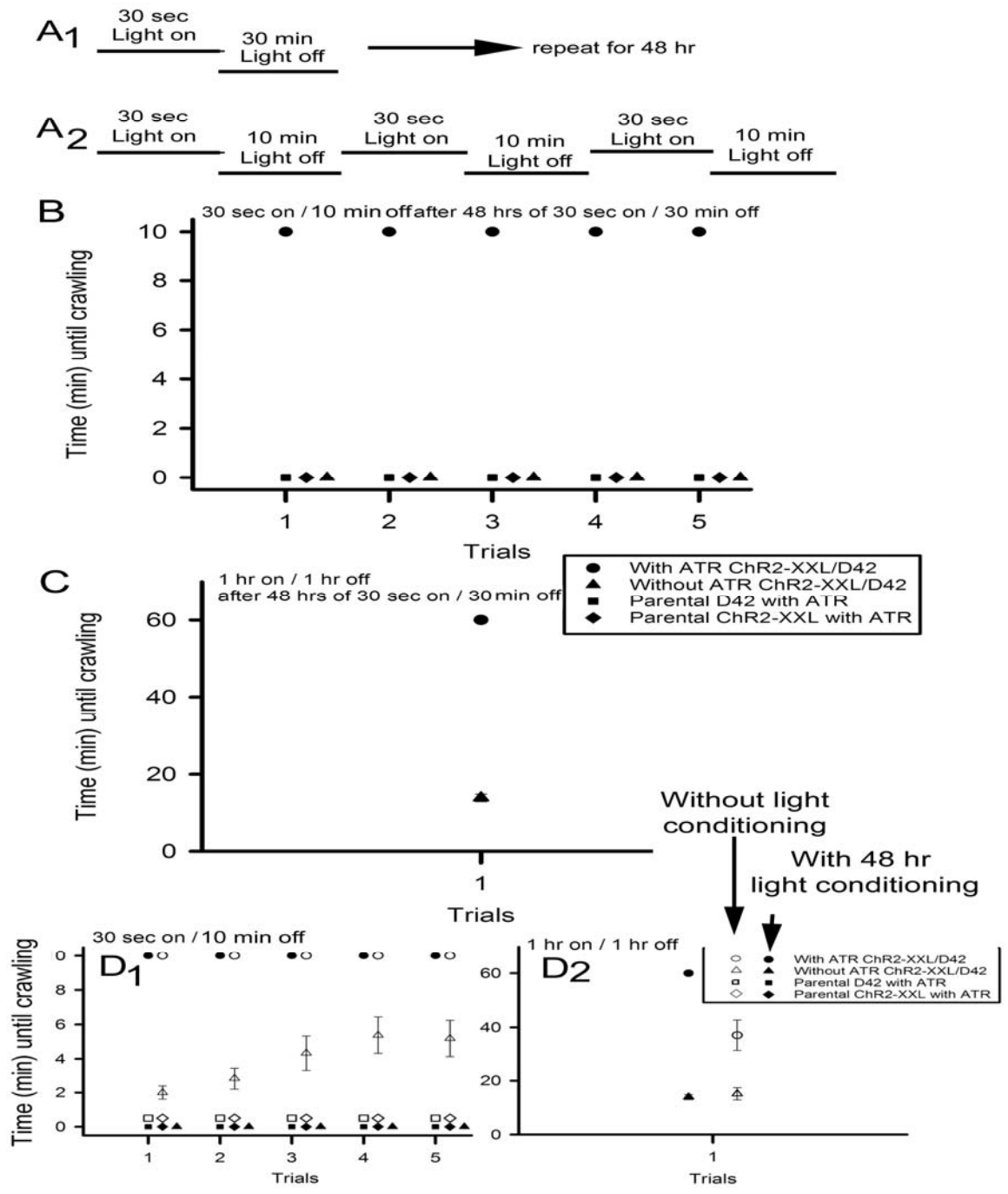
Figure 5.2. The effect of blue light pulses during larval development (early 2nd instar to 3rd instar) on the locomotion of larvae with subsequent acute exposures to blue light. This examines the effect of feeding ATR and expression of ChR2-XXL channels in motor neurons (D42) over time. (A1) The 48 hr conditioning paradigm followed by the acute exposure paradigm (A2) for the behavioral analysis conducted as for the acute only blue light exposures. (B) The time to initiate the first full body wall contraction is shown for the each of the 10 min dark periods in the light on/off series. Only D42/ChR2-XXL larvae fed ATR remained paralyzed for each of the 10 min dark periods; whereas, the D42/ChR2-XXL larvae not fed ATR and the parental lines (D42 and ChR2-XXL) fed ATR showed no hesitation to keep moving after the blue light was turned off. (D1) The comparison of the responses to initiate movement with and without the pre-conditioning over development to the acute blue light exposures of 30 sec on and 10 min off. (D2) The effect of 1 hr exposure to blue light and then with the blue light turned off for the first occurrence of a complete body wall contraction had longer lasting effect on D42/ChR-XXL larvae with the pre-light conditioning treatment of the 48 hr then the acute only light (N=10, $P \leq 0.05$, T-test). The D42/ChR2-XXL not fed ATR over the 48 hr showed no difference than larvae without the pre-conditioning treatment. (N=10, data expressed as mean +/-SEM).

Figure 5.3. The synaptic responses obtained in larval body wall muscles during acute blue light and dark exposures to examine differences in larvae expressing ChR2-XXL and feeding ATR. (A) D42/ChR2-XXL larvae fed ATR showed a pronounced response to blue light with a burst of excitatory postsynaptic potentials (EPSPs). However, the light induced action potential within the nerve was not maintained throughout the 10 sec light exposure. Even after the subsequent 10 min dark exposure with the 2nd or 3rd dark/ light series (see A1-1st, A2-2nd, A3-3rd blue light exposures) the action potential induced burst of EPSPs was not maintained for the 10 sec. In fact, in this representative example the 3rd light exposure (A3) did not evoked and large EPSPs. In this paradigm the light induced evoked responses would be turned off within the 10 sec light pulse and even during the dark period for 1 to 2 min before spontaneously starting up again (B). In examining for more robust responses, larvae raised from 1st instar to 3rd in ATR (7 days) and examined with 20 sec of blue light produce rapid EPSPs which lasted a few seconds before they ceased still while being exposed to blue light. This is illustrated in the period in during light exposure but a refractory period in evoking EPSPs during the light exposure (C1). Even though evoked EPSPs were not induced small quantal shaped synaptic events would rapidly occur (C2) during this period.

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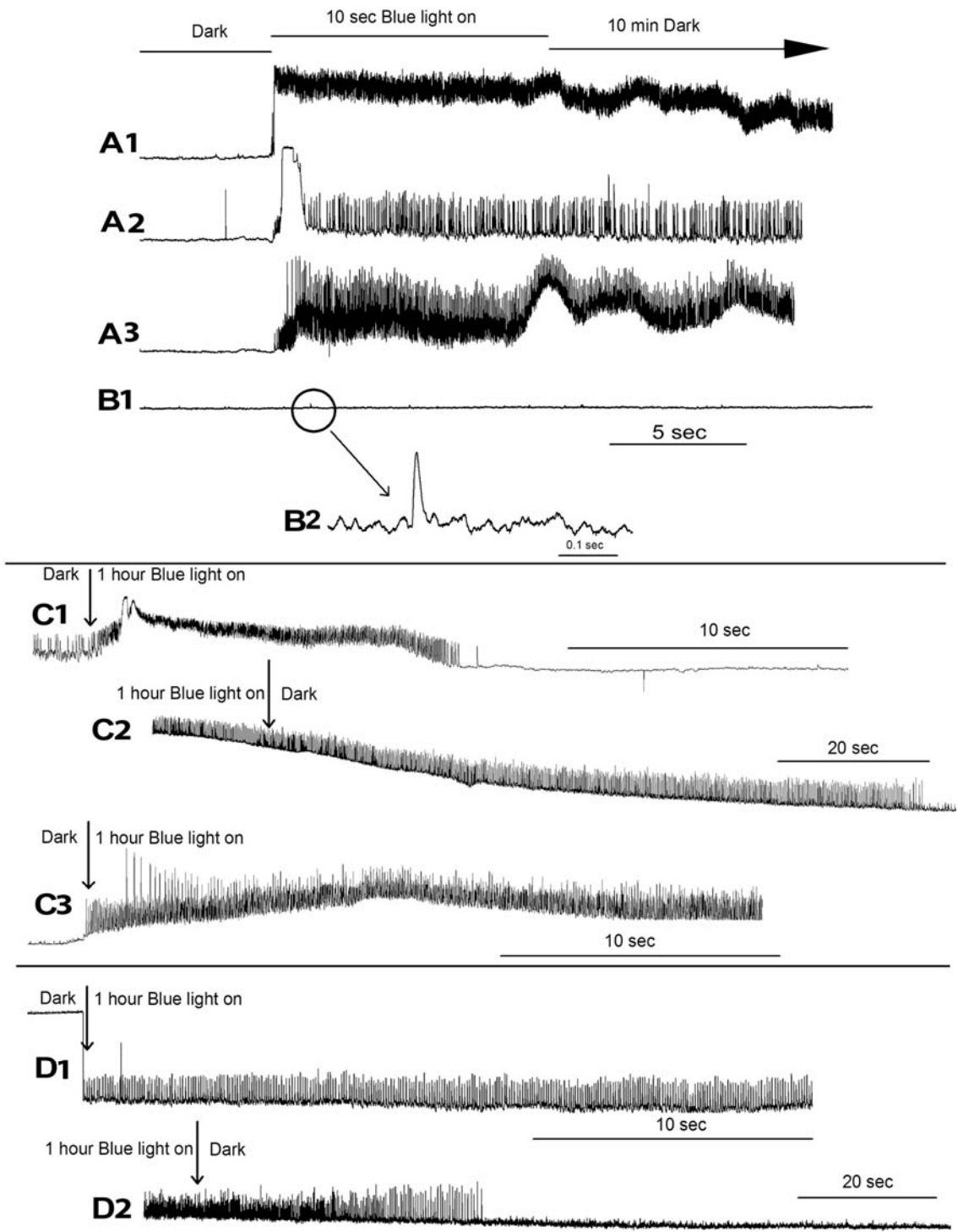
Figure 5.4. The synaptic responses obtaining in the larval body wall muscles during acute blue light and dark exposures to examine differences in larvae expressing ChR2-XXL without feeding ATR. (A) The D42/ChR2-XXL larvae lacking ATR would continue to demonstrate light induced action potential evoked EPSPs throughout the 10 sec blue light exposure. However with the series of 10 sec blue light and 10 min dark each of the 3 recorded subsequent trials varied (see A1, A2, and A3). (B) The D42 parental line fed ATR was used to examine the effect of the blue light on the preparation in the absence of the light sensitive rhodopsin channels. No light induced EPSPs were observed and the occasional spontaneous quantal event (B2) observed prior to blue light or with blue light exposures had no noticeable differences in the frequency of occurrences. (C) D42/ChR2-XXL larvae fed ATR and exposure to 1 hr of blue light while recording from the muscle with an intracellular electrode demonstrated the similar burst of EPSPs followed by a light induced refractory period with EPSPs resuming within the hr of constant blue light (C1). When the blue light is turned off (complete darkness) the EPSPs would remain firing for some time afterwards (C2). Notice after 1 hr of darkness and re-exposure to blue light the evoked EPSPs now remained present without demonstrating the light induced refractory period (C3). (D) The D42/ChR2-XXL lacking ATR over the 48 hr did not show the light induced refractory with exposure to blue light and would even maintain light induced evoked EPSP for the whole hour of blue light exposure (D1). Upon subsequent dark exposure the firing usually ceased relatively quickly (D2). Scale bars are shown in traces for C-D and these do not refer to light are dark exposure times.
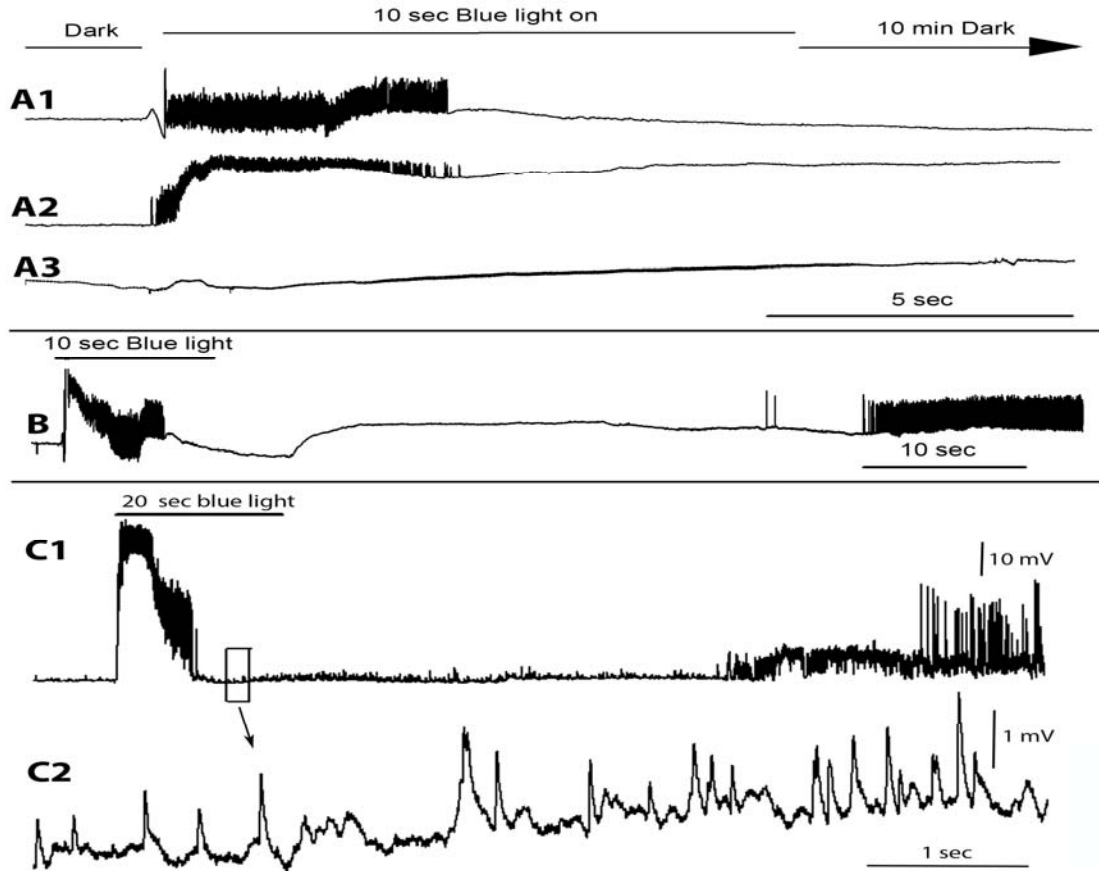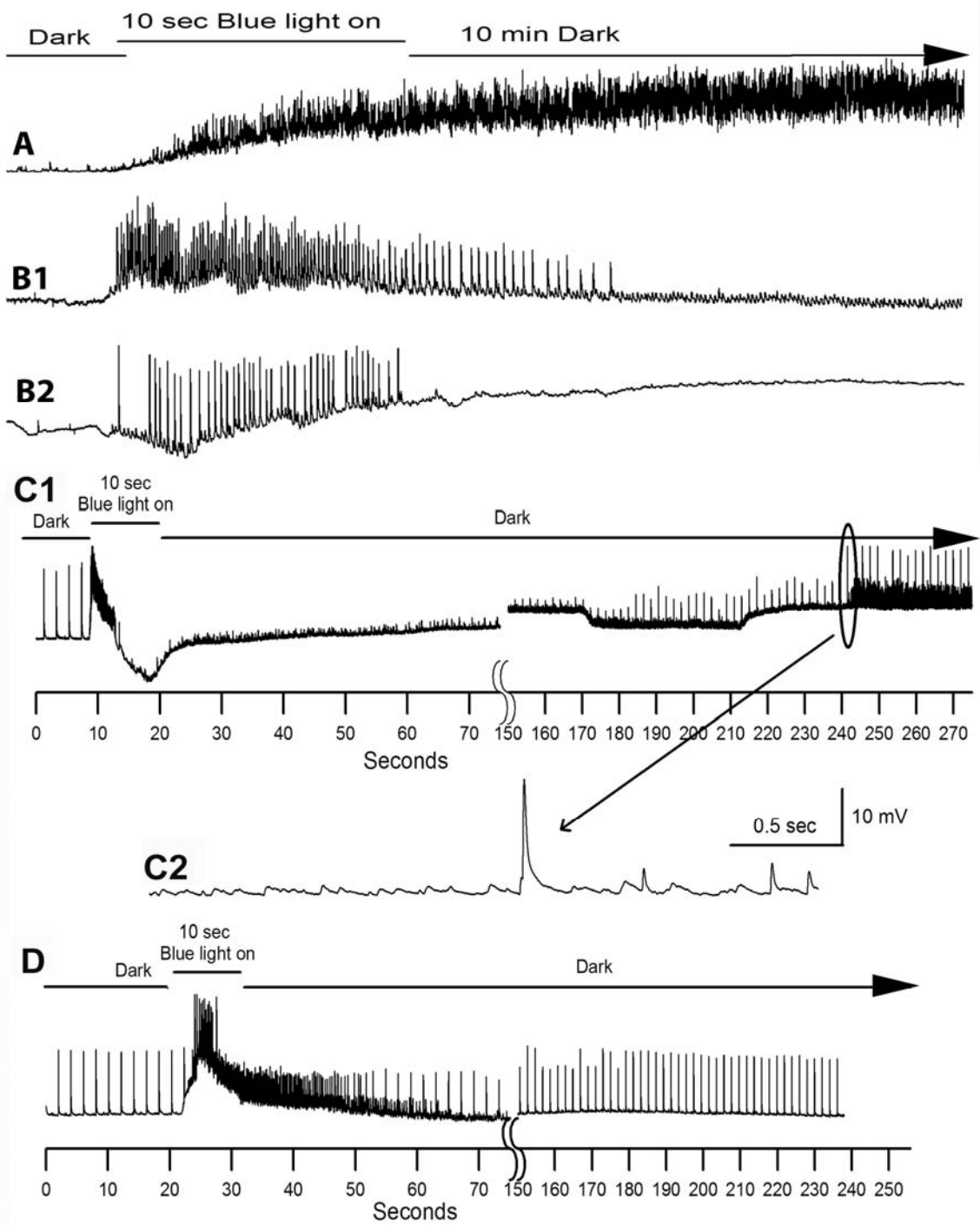
Figure 5.5. The synaptic responses obtaining in the larval body wall muscles during acute blue light and dark exposures to examine the effect of repetitive light pulses over development (48 hr early 2nd instar to 3rd instar) and the presence of ATR. (A) The D42/ChR2-XXL larvae fed ATR and pre-exposed to blue light pulses (30 sec blue light-30 min dark repeated for 48 hr) showed evoked EPSPs throughout the 10 sec blue light exposures and the evoked EPSPs were maintained for a short while after the blue light was turned off. (B)The D42/ChR2-XXL larvae lacking ATR but also pre-conditioned for 48 hours with blue light pulses would also show light induced evoked EPSPs which would cease quickly when the blue light was off. Two different preparations are shown (B1 and B2) for the relatively weaker response as compared to the larvae fed ATR. The light induced refractory period in activating ChR2-XXL for electrical stimulation is related to the sensitivity of the preparation. The segmental nerve is stimulated at 0.5 Hz prior, during and after the light and dark conditions in order to electrically evoked action potentials in the motor neurons to initiate EPSPs. (C) The D42/ChR2-XXL larvae fed ATR which demonstrated the light induced depression of evoked EPSPs also depressed electrically stimulated evoked EPSPs. The electrical evoked EPSP started to rise after almost 2 minutes of being in a relative refractory period (C1). The electrical evoked responses demonstrated rapid rise indicating the regaining of synchronized vesical fusion in the presynaptic terminal (C2). The D42/ChR2-XXL larvae lacking ATR which exhibited less of a response to blue light did not demonstrate as great of a relative refractory to the electrically evoked EPSPs although there was usually some reduction in the evoked EPSP amplitude (D).

CHAPTER SIX

Optogenetic analysis illuminates *Glutamic acid decarboxylase1* expression in *Drosophila* larval body wall muscle

* This work has been submitted for publication to **Journal of Insect Physiology** and is currently under review. Mr. Jacob Sifers, Ms. Angela Mikos, Mr. Eashwar Somasundaram, and Ms. Aya Omar collected data used to produce figures. Mr. Eashwar Somasundaram also helped analyze the data and produce the figures. I collected data, analyzed the data, helped produce the figures, and wrote the manuscript. Dr. Cooper edited the manuscript. Dr. Cooper and I conceived the experiments.

INTRODUCTION

The advent of optogenetics has revolutionized the ability to control the activity of excitable cells in a transient manner in model organisms, including *Drosophila melanogaster*. Recently, the optogenetic toolbox has grown as a number of light-sensitive proteins have been isolated, manipulated, and cloned for use in *Drosophila,* including hyperpolarizing opsins, like the yellow-light sensitive chloride pump, halorhodopsin, from the archaebacteria (*Natromonas pharaonis*)(*NpHR*) (Zhang et al. 2007; Inada et al. 2011) and multiple *ChR2* variants that have been engineered to increase expression, improve chromophore affinity, and ultimately enhance sensitivity to light (Dawydow et al. 2014). In order to target expression of these light-sensitive ion channels to specific tissue, the GAL4/UAS system is commonly used (Brand and Perrimon 1993). In the context of optogenetics, the goal of this system is to allow for precise targeting of expression of rhodopsins to specific cells for manipulation of activity; however, the enhanced sensitivity of engineered rhodopsins increase the potential for off-target effects. Specifically, this characteristic may allow for even low-level expression of the driver gene regulatory elements to induce production of enough protein to cause functional changes in the cell. A recent report (Dawydow et al. 2014) shed light on the sensitivity of a *ChR2* variant, *ChR2XXL,* and illustrates the enhanced expression of this variant relative to other commonly used channel-rhodopsins and, while there are substantial benefits for use of this

variant in *Drosophila*, its sensitivity increases the chance that it may induce off-target functional effects.

In order to assess the role of various neuromodulators in regulation of larval *Drosophila* behavior using optogenetics, we have employed the use of a host of opsins and have uncovered intriguing behavioral outcomes. In previous behavioral analysis, we used a *dGad1-Gal4* driver to drive expression of the sensitive channel-rhodopsin2-XXL (*ChR2XXL*). The goal was to excite GABAergic neurons; however, while conducting these studies we noted depolarization of the larval skeletal muscles and robust muscle contraction in our behavioral assessment of larval locomotion. Given that the motor neurons in larval *Drosophila* are glutamatergic and that GABA application on the larval neuromuscular junction (NMJ) has no physiological action, this was a surprising outcome. L-glutamic acid decarboxylase1 (*Gad1*) (CG14994) catalyzes the conversion of glutamic acid to gamma-aminobutyric acid and carbon dioxide. It is the rate-limiting enzyme in the synthesis of GABA, a primary inhibitory neurotransmitter in the nervous system of vertebrates and invertebrates alike. The *Drosophila* genome contains a single *dGad1* gene that is the primary enzyme involved in the conversion of glutamate to GABA and shares similar sequence similarity and function with the mammalian *Gad* (Kulkarni et al. 1994). A second *dGad* gene, *dGad2,* is also expressed in the fly nervous system; however, its functional role in GABA synthesis is minimal and it is thought to primarily function in glia (Phillips et al. 1993). Previous expression analysis in *Drosophila* suggests that *dGad1* is widespread in the adult CNS (Jackson et al. 1990). Furthermore, expression patterns of the specific promoter fusion (Gal4) construct utilized in this study show CNS-specific expression larval and adult *Drosophila* (see FlyBase; Gramates et al. 2017), overlapping previously identified native expression. Recently GABAergic neurons have been identified in regulation of motor programs underlying feeding and consumption in the subesophageal zone (SEZ) and ventral nerve cord (VNC) (Pool et al. 2014), in the mushroom bodies (MBs) regulating visual reversal learning (Ren et al. 2012)

as well as multiple subsets in the VNC innervating motor neurons and regulating larval locomotion (Fushiki et al. 2016). In each of these studies it was shown that GABAergic activation resulted in inhibition of the neural circuits they function within, supporting the notion that GABA serves as the primary inhibitory neurotransmitter in the *Drosophila* Central Nervous System (CNS). Additional pharmacological analysis has confirmed the modulatory effects of GABA on multiple motor programs (Leal et al. 2004). Given this extensive evidence, it is assumed that *dGad1* is expressed primarily in neurons within the CNS primarily to catalyze the synthesis of GABA with little expression in non-neuronal tissue in *Drosophila*. There is little evidence of functional native *dGad1* expression in *Drosophila* at the NMJ; however, studies performed in the 1960s and 1970s provide evidence of *Gad1* expression in the flight muscles of the flesh fly (Langcake et al. 1974) as well as in cardiac tissue, the Islets of Langerhans in the pancreas, and in kidneys in humans (Zachmann et al. 1966). Furthermore, the subcellular location of *dGad1* has been identified in the flesh fly flight muscle and has been found to be associated with mitochondria, where it maintains an integral metabolic role (Langcake et al. 1974). Likewise, an elegant study highlighted *dGad1* expression at the NMJ in *Drosophila*, where it appears to play a significant functional role in pre-synaptic control of post-synaptic glutamate receptor (GluR) level during larval development (Featherstone et al. 2000). Based on these analyses and the interesting behavioral and physiological phenotypes we have uncovered at the NMJ, we investigated the potential for *dGad1* expression through linked expression of light sensitive ion channels/pumps in skeletal and cardiac muscle in *Drosophila melanogaster* larvae. To follow up on previous behavioral analyses, we have utilized an electrophysiological approach coupled with optic stimulation of opsins driven by *dGAD1-Gal4,* to detect rhodopsin-mediated excitation directly in larval body wall muscle. Here, we provide evidence of *dGad1-Gal4* -driven activation opsins in larval body wall muscles and provide confirmation of expression in motor neurons innervating muscles integral in larval locomotion. We also confirm

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dGAD1-Gal4-driven expression using confocal imaging and address potential expression in larval cardiac tissue using an established optogenetic pacing approach. While we are aware of the potential for promoter fusion constructs to induce expression that may not match native expression with 100% reliability, it is important to illuminate potential off-target expression that may arise utilizing the GAL4/UAS system, especially in the context of optogenetics, where, as we show here, even low-level expression of proteins that alter neuronal excitability may produce alterations in behavior and physiology.   The *dGAD1-Gal4* driver was used in previous analysis to drive expression in the larval CNS (see Flybase Gramates et al. 2017), and it is essential to uncover details regarding expression profiles of this, and other, Gal4 driver lines. Therefore, the goal of this study is to provide important insights into the use of optogenetic tools in larval *Drosophila in vivo* in concert with electrophysiological approaches and to illuminate a previously undefined expression pattern of *dGAD1-Gal4*, which is commonly used as a driver for selective expression within the *Drosophila* CNS.

MATERIAL AND METHODS

Fly rearing

All flies used for electrophysiological and heart rate (HR) analyses were held for a few days at 22°C in a 12-hour light/dark incubator before being tested. All animals were maintained in vials partially filled with a cornmeal-agar-dextrose-yeast medium. The general maintenance is described in (Hartenstein and Campos-Ortega 1984).

Transgenic fly lines

The filial 1 (F1) generations were obtained by crossing females of the recently created ChR2 line (which is very sensitive to light) called y^1 w^{1118}; *PBac{UAS-ChR2.XXL}VK00018* (BDSC stock # 58374)(Dawydow et al. 2014) , a less sensitive ChR2 line, *w*; P{UAS-H134R-ChR2}2* (BDSC stock # 289950

(Pulver et al. 2011), and a halorhodopsin line, w*;P{*UAS-eNpHR-YFP*}attP (BDSC# 41752) with male w*;P{*24B-Gal4 (III)*}/Cyo} (BDSC stock # 1767) to drive expression in mesodermal-derived tissue (particularly skeletal muscle) and w*;P{*D42-Gal4*} (BDSC # 8816) to drive *Chr2XXL* in motor neurons. In addition, females from these UAS effector lines were crossed with males from a *P{Gad1-Gal4.3.098}2/CyO* (BDSC stock # 51630) for assessment of the activity of the opsins in *dGad1*-expressing tissue. For confocal imaging of the *Drosophila* larval NMJ, males from the *dGad1-GAL4* line and a positive control, muscle-specific driver line, *Mef2-Gal4* were crossed with virgin females from a *UAS-GFP* (kindly provided by Dr. Doug Harrison, University of Kentucky) to analyze cytoplasmic/membrane GFP in *dGAD1* expressing neurons. The parental *UAS-ChR2XXL* and *UAS-eNpHR* lines were used as controls for the electrophysiological analysis and the *UAS-GFP* parental line were used as controls to test for leaky GFP expression for the and imaging assays in addition to the lines used in the absence of ATR supplementation.

Preparation of fly food supplemented with ATR

All trans-retinal (ATR; Sigma-Aldrich, St. Louis, MO, USA) was diluted in standard fly food to a final concentration of 1 mM (for ChR2 use) or 10 mM (for *eNpHr* use) and protected from light with aluminum foil. For control experiments, larvae were cultured in food that only contained the solvent (absolute ethanol in the same volume used for the ATR mixtures in the fly food). The ATR or ethanol food mixtures were left alone for 48 hours prior to adding larvae in order to allow some evaporation of the alcohol solvent from the mixture. Adult flies from the driver (Gal4) lines and the *UAS-ChR2* and *UAS-eNpHR* effector lines were crossed on standard fly food. Approximately 3 days following the cross, 2nd instar larvae were removed from standard food vials and placed in ATR-food mixtures and left for 48 hours prior to testing. It has been noted that larval development

slows in the presence of ethanol, so precautions were taken to limit its developmental influence.

Intracellular recordings from the neuromuscular junction

Larval dissections were performed as described previously (Ruffner et al. 1999; Stewart et al. 1994; Stewart et al. 1996). In brief, the preparations were "fileted" along the mid-dorsal longitudinal axis and pinned flat. Excitatory postsynaptic potentials (EPSPs) were evoked by exposing the dissected preparations with blue light (470nm wavelength, LEDsupply, LXML-PB01-0040) or yellow-lime light (567.5 nM wavelength, LEDsupply, LXML-PM02-0000) from a high intensity LED that was focused on the specimen through a 10x ocular objective while the EPSPs were measured (Titlow et al. 2014). Intracellular recordings from muscle 6 were made with microelectrodes filled with 3M KCl having a resistance of 30–60 MΩ. Responses were recorded with a 1X LU head stage and an Axoclamp 2A amplifier. Electrical signals were recorded to a computer A/D interface (ADInstruments). All events were measured and calibrated with the LabChart7 software (ADInstruments). All experiments were performed at room temperature (21-22°C). The larval preparations were dissected, fileted, and bathed in a physiological saline and the responses in the presence of dim white light, followed by a 1-second blue light or yellow light pulse to detect the response in the muscle following development in complete darkness. To rule out activity from higher centers in the larval brain, in some paradigms, the CNS was removed by transecting the segmental nerves from the brain as noted. In this case only spontaneous vesicle fusion events would be recorded in the muscle fibers. However, to rule out any stimulator action on the motor nerve terminals and masking any possible channel action of halorhodospin or channel-rhodopsin-2 directly on the muscle fibers, glutamate (10 mM) was added to the saline to desensitize the glutamate receptors on the muscle.

Monitoring heart rate in the dissected larva

A detailed description of the dissection protocol is shown in video format (Cooper et al. 2009). In brief, the third instar larvae were opened by an incision in the ventral midline and the internal organs were washed aside by saline in order to expose the intact heart to various solutions. The brain was removed by cutting the segmental roots. The movement of the trachea is commonly used to monitor *Drosophila* larval HR because of the clear contrast of the structures (Dasari et al. 2006; White et al. 1992). With the heart exposed, fresh saline was applied and the preparation was left alone for 2 minutes to allow the HR to stabilize. Following a 2-minute waiting period, contraction rate was counted at the most caudal end of the heart under dim white light. Following white light exposure, HR was counted while under exposure to a blue light (470nm wavelength, LEDsupply, LXML-PB01-0040) and beats were counted through a 10x ocular objective. Responses of each preparation were recorded in saline with a 1mM Ca^{2+} concentration and a 2mM Ca^{2+}. The 1mM Ca^{2+} solution was exchanged with the 2mM Ca^{2+} solution and a 2-minute resting period was used to allow the heart to stabilize following solution exchange. The light paradigm was then repeated in the 2mM Ca^{2+} solution.

The saline utilized was a modified Hemolymph-like 3 (HL3) containing: (in mmol/L) 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 1 $CaCl_2$, 5 trehalose, 115 sucrose, 25 N,N-Bis-(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) at a pH 7.1. Because heart performance is very sensitive to pH change, the pH was tightly regulated and adjusted as needed (de Castro et al. 2014).

Dissection and Imaging

The *dGAD1-Gal4* line aforementioned was used to drive expression of a fluorescent protein (GFP) fluorescent reporter and analyzed for expression at the larval (NMJ). 3rd instar larvae were dissected in modified HL-3 saline and mounted on depression slide. In short, a longitudinal dorsal midline cut was made in 3rd

instar larvae, and the CNS with efferent segmental roots was left intact to detect fluorescence at the NMJ. The ventral body wall muscles were also left intact. Imaging was performed with a laser-scanning Leica microsystems confocal SP8 (10X objective) microscope and recorded with LasX software. Confocal stacks were taken from the dorsal to ventral side of the animal. The muscle innervation in segments 3 and 4 on muscles 6 and 7 were of interest since this was the site of intracellular recordings. Z stacks were made to capture the thickness of the muscles in question or the planes of motor nerve terminal along the surface of the muscles to generate composite photos.

Statistical analysis

In order to associate each fly line and the probability in inducing light-evoked EPSPs or membrane potential change in larval skeletal muscle, a Fisher's exact test was used in our electrophysiological analysis. Since responses are highly varied from preparation to preparation, analysis was centered on the probability of inducing synaptic responses in each line in response to light stimuli and multiple comparisons were directly compared. For heart rate counts, Student's t-test was used to compare the mean percent changes from control to our experimental lines. We compared responses in two different Ca2+ concentrations (1mM Ca^{2+} and 2mM Ca^{2+}) from each line relative to a control; thus, an unpaired t-test was used for analysis. A Tukey's test was used as a post hoc test following the t-test to compare the relative changes of HRs for all the compounds within an experimental paradigm to determine significant differences. This analysis was performed with Sigma Stat software. P of $\leq$ 0.05 is considered as statistically significant. The number of asterisks are considered as $P \leq 0.05$ (*), $P \leq 0.01$ (**), and $P \leq 0.001$ (***). Data for heart rate percent change is presented in means +/- standard error of the mean (SEM).

RESULTS

Our initial interest in investigating *dGad1* expression in muscle was spurred in our behavioral analysis using the *dGad1-Gal4* driver. We noted an interesting phenotype: robust full body-wall contractions upon acute activation of GABAergic neurons using optogenetic and thermogenetic approaches. While we did not discount the role of central GABA signaling in driving excitation of inputs to motor neurons through potential disinhibition, we sought to assess potential activity at the NMJ nonetheless. We utilized intracellular electrophysiological recordings at the larval NMJ to directly assess *dGad1*-driven opsin regulation of membrane potential in body wall muscle fibers. Following evaluation of activity in body wall muscles, confocal imaging was utilized to assess the expression of a GFP reporter at the NMJ. Additionally, cardiac pace making via optogenetic drive of cardiomyocytes was examined to investigate d*Gad1-Gal4* expression in cardiac muscle.

Effect of blue light stimulation on NMJ activity with intact nervous system

Following our behavioral assessment, we sought to address how *dGad1* expressing cellular activation was exciting a motor circuit.   Excitatory post-synaptic potentials (EPSPs) were measured via intracellular electrodes in skeletal muscle 6, which is innervated by a single segmental nerve branch with two individual neurons (type Ib and Is) (Atwood et al. 1993). The larval preparations were dissected, fileted, and bathed in a physiological saline and the responses in the presence of dim white light, followed by a 1-second blue light pulse to detect the response in the muscle following development in complete darkness.   The pulses were repeated 4 times per preparation with a resting period of 3 minutes in between each pulse to avoid channel desensitization and/or synaptic depression due to repetitive channel rhodopsin activation. Each line was divided into two cohorts: one having been fed a diet supplemented with 1mM all–trans-retinal (ATR) (represented in Figure 1 with simply an associated letter in the left column) and one having been fed a diet without ATR

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(represented in the left column of Figure 1 and indicated with (') in order to assess the efficacy of ATR supplementation. Representative traces from the electrophysiological recordings are displayed in the figures with scale bars for reference (Fig 1B-F) and cumulative analysis comparing the relationship of genotypes vs, the probability of inducing EPSP and/or membrane potential change are displayed (Fig 1G).

Upon initial blue light stimulation, F1 larvae from the *dGad1-Gal4 > ChR2XXL* +ATR cross exhibited strong depolarization in muscle 6 and enhanced EPSP frequency (often exceeding 30 Hz)(Fig 1B).   This strong depolarization was repeated in 4 subsequent 1-second pulses. We noted that the EPSP amplitude was maintained with each pulse and that little facilitation or depression occurred and the activity did not shut down in response to additional pulses. We identified this response in 7 out 7 larval preparations and 4 out of 4 pulses per preparation, for a total of 28 out of 28 light pulses (Fig 1G). The amplitudes and frequencies of EPSPs as well as the duration of excitation varied from preparation to preparation and even from pulse to pulse within an individual preparation, but an enhancement of EPSPs and strong depolarization arose in response to each pulse of blue light indicating activity in the CNS evoked action potentials in motor neurons innervating larval muscle 6 (Fig 1B) with 100% reliability (Fig 1G). The presence of high (Is) and low-output (Ib) synaptic terminals innervating this muscle produces highly varied responses, so this variation was not surprising (Atwood et al. 1993). The resting membrane potentials were monitored and generally recorded between -65 and -45 millivolts (mV) prior to blue light pulses being initiated in order to maintain Na^+/Ca^{2+} driving gradients. Miniature EPSPS (mEPSPs) were present throughout the recording indicating the repetitive pulses of blue light did not result in desensitization of post-synaptic glutamate receptors at the NMJ. Following the blue light stimulus, evoked potentials were observed on average for about 30 second post-pulse, but, as mentioned, this varied from preparation to preparation and even from

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pulse to pulse. The time scale and voltage scale is provided in the figures for reference.

Likewise, the -ATR cohort exhibited strong depolarization as well (Fig 1B'). In 7 out of 7 preparations and a total of 28 light pulses, we observed strong membrane depolarization and enhanced EPSP frequency immediately following a 1-second blue light pulse (Fig 1G'). Again, the amplitudes, frequency and duration of responses varied from preparation to preparation, but we found a consistent development of EPSPs following the blue light stimulus in each trial (Fig 1B').   Following the blue light stimulus, evoked potentials were observed for an average of around 35 seconds, but individual pulses were shown to elicit EPSPs for as long as 1.5 minutes following a 1-second pulse.

We reasoned that this was likely the result of excitatory input from pre-motor interneuron populations to motor neurons and causing initiation of action potentials, or direct activation of motor neurons. We followed this by testing the response of blue light activation of motor neurons, directly, via *ChR2XXL*. A driver known to target motor neurons, *D42-Gal4*, was used to drive *ChR2XXL* expression in motor neurons. Upon blue light stimulation of motor neurons, F1 larvae from the *D42-Gal4>ChR2XXL* cross exhibited a robust burst of EPSPs, which was identified in 6 out of 6 preparations tested and in each pulse given (Fig 1D) for a total of 24 pulses, again with 100% reliability (Fig 1G).   The observed EPSPs in this larval population were similar in amplitudes, frequency, and duration in relation to the *dGad1-Gal4>ChR2XXL* line and often caused strong muscle contractions following evoked responses. Upon subsequent pulses the amplitudes of the EPSPs remained constant with little depression following the 3-minute resting period. Resting membrane potentials (RP) were carefully monitored and generally ranged from -60mV to -45mV.   The responses in the *D42-Gal4>ChR2XXL* –ATR cohort were also observed and, surprisingly, only half of the pulses (12 out of 24) elicited similar evoked responses in the muscle (Fig 1D'). In each case the larvae that exhibited strong EPSPs in the first

pulse showed similar evoked response in subsequent pulses. Responses in 3 out of the 6 larvae tested in the –ATR cohort did not show any response to blue light stimulus. This indicates *ChR2XXL* is activated in the absence of ATR supplementation when driven by these drivers with the CNS intact when driven by *dGad1*; however, interestingly, the *D42* driver did not induce enhancement of EPSPs as reliably as *dGad1-Gal4* in the absence of ATR.

Additionally, for proof of concept and to observe comparative responses when *ChR2XXL* was directly activated in muscle, we drove opsin expression with a mesodermal driver that expresses in larval body wall muscles, *24B-Gal4 (III)>ChR2-XXL.* Upon blue light stimulation we observed strong depolarization in 5 out of 5 preps (20 out of 20 pulses) tested in the +ATR cohort (Fig 1C). As can be seen in Fig 1C, we noticed occasional evoked responses as the membrane depolarized, which we suspect to be the result of muscle contraction and activation of sensory neurons tiling the body wall, inducing feedback through the CNS. However, the strong enhancement of EPSPs observed in the *D42-Gal4>ChR2XXL* and *dGad1-Gal4>ChR2XXL* were typically absent in the *24B-Gal4(III)>ChR2XXL* larvae and only a slower rise in membrane potential occurred, suggesting this was due to cation influx directly into the muscle and not from the result of action potentials initiated in the motor neurons innervating muscle 6. This was also observed in the –ATR cohort.   In 5 out 5 preparations, and 4 out of 4 light pulses, for a total of 20 pulses, a similar rise in membrane potential was observed upon blue light stimulation (Fig 1C',G'). The average amplitudes of responses in the muscle varied from preparation to preparation, but generally were found to depolarize 12-15 mV from RP (Fig 1C). Additionally, the time with which the membrane potential returned to rest varied, but averaged 45 seconds to 1.5 minutes across all preparations.

It is known that *Drosophila* larvae display negative phototaxis and initiate avoidance behavioral responses to light (Jennings 1904; Mast 1911; Sawin et al. 1994; Xiang et al. 2010). Thus, we tested the potential for blue light, alone, in inducing excitatory responses in muscle 6. As a control, the parental *UAS-*

ChR2XXL line was analyzed. Using the same paradigm, we found no depolarization in muscle 6 in any preparation, with 5 out of 5 preparations displaying no response to blue light, showing blue light alone is unable to evoke EPSPs in body wall muscles (Fig 1F, G).   Likewise, the –ATR cohort displayed no depolarization upon blue light stimulation (Fig 1F', G').

In hope of describing this phenomenon with a less sensitive rhodopsin, we crossed the *dGad1* driver line with an additional ChR2 variant, *UAS-ChR2-H134RII-mcherry (dGad1-Gal4>ChR2-H134RII-mcherry)*. The use of multiple channel rhodopsin, in addition to altering the presence of ATR, offers a range of to elucidate properties of these different variants in vivo.   Surprisingly, the F1 larvae from this cross did not display the same EPSP responses identified in *dGad1-Gal4> ChR2XXL* group. While we did identify the appearance of EPSPs in 4 out of 6 preparations tested, the probability of inducing depolarization in response to the blue light stimulus was significantly reduced relative to the *dGad1>ChR2XXL* line (Fig 1E, G). Also, interestingly, in previous trials using the *ChR2XXL* variant, we identified EPSPs in each pulse; however, in two preparations expressing *ChR2-H134RII-mcherry*, initial pulses induced EPSPs while subsequent pulses did not, suggesting potential accommodation in these preparations. In total, 12 out of 24 pulses elicited EPSPs upon blue light exposure (Fig 1G). While the presence of EPSPs upon blue light exposure was predictable in the XXL expressing larvae, the *ChR2-H134RII-mcherry* variant was not as sensitive. In this case, the blue light stimulus did not induce a strong depolarization of body wall muscles as previously shown (Figure 1 E). Likewise, the –ATR cohort did not display any change in membrane potential with the blue light stimulus (6 out of 6 preparations) (Fig 1 E', G').

Application of 10mM glutamate

We deduced that the responses observed in *dGad1-Gal4>ChR2XXL* larvae with the CNS intact were the result of excitatory input on motor neurons; however, because we noted gradual depolarization in the muscle akin to the

response in the *24B(III)-Gal4>ChR2XXL* larvae, we sought to address the potential that *dGad1* may be driving opsin expression directly in muscle. To investigate this, 10mM glutamate was added to the saline bathed on the larval preparations. Invertebrate NMJs, including *Drosophila melanogaster*, consist of post-synaptic non-NMDA-like glutamate receptors (GluRs) that are known to rapidly desensitize in the presence of 0.1-10mM glutamate (Anderson et al. 1976; Patlak et al. 1979; Gration et al. 1981; Shinozaki et al. 1981; Cull-Candy and Parker 1983; Dudel et al. 1992). Thus, the addition of glutamate to the saline served to block excitatory responses in muscle produced through glutamate release from innervating motor neurons. Following a 2-minute waiting period to allow post-synaptic GluRs to desensitize, we used the same experimental paradigm to address if depolarization occurred in the presence of blue light. In F1 larvae from the *dGAD1-Gal4> ChR2-XXL* cross +ATR cohort, strong membrane depolarization was still observed in 7 out of 7 preparations in each pulse elicited (Fig 2 A, D). The presence of spontaneous mEPSPs, which were present with the CNS intact, were absent in these larvae 2 minutes post-glutamate application, suggesting the glutamate receptors were desensitized. In addition, in the larvae generated from the *D42-Gal4 > ChR2XXL* cross, no depolarization was observed following glutamate exposure and miniature EPSPs (mEPSPs) were abolished (Fig 2C). In all 5 preparations tested, initial bursts of EPSPs were identified in normal saline, but the application of 10mM glutamate shut down this activity, suggesting this is sufficient to desensitize post-synaptic GluRs (Fig 2C, D). Thus, the responses observed in the *dGAD1-Gal4>ChR2XXL* appear to be directly from the muscle. To confirm this, we utilized the *24B(III)-Gal4>ChR2XXL* line in the presence of 10mM glutamate. Larvae from the *24B-Gal4>ChR2-XXL* cross also showed continued membrane depolarization upon blue light stimulation in the presence of 10mM glutamate (Fig 2B). The *dGad1-Gal4>UAS-ChR2XXL* –ATR cohort did not show muscle depolarization in response to blue light in the presence of 10mM glutamate and only the *24B(III)-*

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Gal4>UAS-CHR2XXL line displayed consistent responses in the absence of ATR (4 out of 5 preparations; 16 out of 20 responses) (Fig 2 D).

Effect of Blue light stimulation on NMJ activity with motor nerves transected

To continue to address whether the membrane depolarization in *dGad1Gal4>ChR2XXL* larvae was generated through *ChR2XXL* directly in muscle fibers, we removed the CNS from 6 preparations and subjected them to the same light paradigm described previously.   The removal of the CNS abolishes evoked responses in the muscle and only mEPSPs are observed as a result of the motor nerve terminals remaining intact with the muscle (Fig 3A-C). In the *dGad1-Gal4>ChR2XXL* larvae, 6 out of 6 preparations displayed a rise in membrane depolarization and an increase in the frequency of mEPSPs (Fig 3A, D). In each preparation, each pulse induced a rise in membrane potential, although the amplitudes and duration of depolarization varied. Again, the mesodermal driver, *24B(III)* was used to drive *ChR2XXL* in body wall muscles for comparison. On average, the peak amplitude of responses observed in the *dGad1-Gal4 >ChR2XXL* cross were approximately 60% of the total of *24B(III)-Gal4* larvae, or around 8 mV, whereas the average peak amplitudes before repolarization of depolarization in the *24B-Gal4>ChR2XXL* was approximately 12-15mV (Fig 3A-B).   In this case, in contrast to observations with an intact CNS, evoked EPSPS were absent, suggesting a lack of sensory-CNS-motor induced activation of muscle.   Consistent with our findings following the application of glutamate, this suggests that the depolarization observed is through direct cation influx via opsins expressed in muscle. An interesting observation in this condition was the appearance of increased mEPSP frequency in the *dGad1-Gal4>ChR2XXL* line upon blue light exposure, which brings to light *dGAD1*-driven presence of *ChR2XXL* in the motor nerve terminals as well. No responses were observes in a control *UAS-ChR2XXL* parental line (Fig 3C). As we observed in the GluR desensitization paradigm, the –ATR cohorts did not

display similar depolarization, with only *24B(III)-Gal4>UAS-ChR2XXL* larvae displaying responses. This cohort was omitted from Figure 3.

eNpHR (halorhodpsin)-mediated hyperpolarization in larval skeletal muscle

To rule out the notion that our observations were not a phenomenon of blue light exposure on the semi-intact preparations, we tested the possibility of driving chloride-induced hyperpolarization directly in larval body wall muscles (Fig 4A-C). Halorhodospin is a yellow-light sensitive chloride pump from the archaebacteria (*Natromonas pharaonis*)(*NpHR*) (Zhang et al. 2007). We reasoned that a yellow light stimulus might induce a hyperpolarizing chloride current through *dGad1-eNpHR* linked expression in body wall muscle 6. We focused this analysis on preparations in which the CNS was transected in order to assess the role of expression in muscle, directly and utilized the light paradigm as explained previously, with an important distinction being the duration of time between pulses. We noted a reduced time to RP return, and thus, in some trials initiated more frequent pulses (Fig 4A-C). In this paradigm, we used 10mM ATR, as previous analysis has suggested the use of more concentrated ATR solution for proper halorhodopsin function (Zhang et al. 2007).   In F1 larvae generated from a *dGad1-Gal4 > UAS-eNpHR* cross, we found a small hyperpolarization in 3 out of 6 preparations tested. In these 3 preparations, in 4 out of 4 light pulses induced hyperpolarization; however, the average degree with which the membrane hyperpolarized averaged between 1-2mV depending on RP, representing a slight hyperpolarization from resting potential (Fig 4A). Additionally, we noticed that membrane potential returned to RP quickly (Fig 4A-B), whereas in previous analysis utilizing ChR2XXL, the membrane remained depolarized following a 1-second light pulse for as long as 1.5 minutes. In –ATR larvae, no such hyperpolarization was observed (6 out of 6 preps) (Fig 4D). Thus, out of a total of 11 out of 24 pulses elicited 1-2mV membrane hyperpolarization from an RP that fluctuated between -65 to -45 mV (Fig 4A, D).

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For comparison, we used *24B(III)-Gal4> UAS-eNpHR larvae.* In the +ATR cohort, 5 preparations were tested and a total of 20 pulses elicited hyperpolarization in the muscle membrane (Fig 4B, D). The hyperpolarization in these larvae ranged from as high as 9mV in amplitude to as low as 4, with an average of 5 mV in amplitude, but this varied depending on RP. Interestingly, like in the *dGad1-Gal4>eNpHR* larvae without ATR supplementation, the –ATR cohort in this line did not show any hyperpolarization in response to yellow light pulsing. Thus, the *24B(III)-Gal4>eNpHR* line was much more sensitive to yellow light pulses than the *dGad1>eNpHR* larvae with 5 out of 5 preparations (+ATR) showing hyperpolarization. The parental control line, *UAS-eNpHR*, did not hyperpolarize in response to the yellow light stimulus in either the +ATR or –ATR environment (Fig 4C).

dGad1-Gal4 driven fluorescent reporter in larval body wall muscles

Additionally, we drove expression of a GFP reporter using the Gal4/UAS system to assess *dGad1* expression at the neuromuscular junction. Because we identified potential *dGad* expression in both muscle and motor nerve terminals, we focused on imaging the NMJ at muscle 6 and muscle 7, which were the focus of our intracellular recordings. We utilized a myoctye enhancer factor-2 driver (*Mef2-Gal4>UAS-GFP*) known to express strongly in muscle for means of comparison (Fig 5). We noticed reliable fluorescence in larval motor neurons, with readily distinct synaptic bouton fluorescence (Fig 5). We also noticed body wall muscle fluorescence in each preparation tested (5 out of 5). However, there was variability in the fluorescence pattern. While some muscles in a single preparation fluoresced strongly, others did not (Fig 5). Additionally, the consistency of a given muscle fluorescing from preparation to preparation was quite low, as a given muscle would fluoresce in one preparation but would be difficult to detect in the next. This was quite surprising and warrants further investigation, but suggests stochastic alteration in expression in body wall

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muscles. Nonetheless, we noted GFP fluorescence in larval body wall muscles in each preparation imaged. We utilized a parental *UAS-GFP* transgenic line to assess potential leaky expression and observed no fluorescence in this line.

dGad1-Gal4 mediated cardiac pace making in the larval heart

In addition to evidenced expression of *dGad1* in skeletal muscle in insects previously alluded to; it has also been shown that *Gad* is expressed in human cardiac tissue. Given this previous finding, we sought to address the potential for *dGAD1* expression in the larval heart through similar linked expression analysis. Optogenetic pace making has recently been introduced in model organisms, including *Drosophila* (Alex et al. 2015; Zhu et al. 2016). Thus, we utilized an optogenetic approach to assess the ability of the *dGad1* driver to drive *ChR2XXL* expression directly in cardiac tissue in order to observe the potential for light-induced pace making (Fig 6A-C). As a positive control, we used the same mesodermal driver *24B(III)-Gal4*, described previously, which has been shown to drive expression in larval heart (Zhu et al. 2016). We compared these responses to a parental control line, *UAS-ChR2XXL*, and assessed the change in HR in response to blue light exposure under two conditions: 1mM Ca^{2+} in HL-3 bathing saline and 2mM $Ca^{2+.}$ The data is presented as raw changes in heart rate (HR) as well as percent change from white light to blue light exposure in both conditions (Fig 6 A-C; A'-C') with the left panel showing responses in the 1mM Ca^{2+} condition and right panel (') displaying responses in the 2mM Ca^{2+} condition. While we found that exposing the *24B-Gal4>ChR2-XXL* larvae to blue light induced an average positive percent change of 11.84 (+/- 6.45 SEM) beats per minute (BPM) in the 1mM Ca^{2+} condition, and a positive percent change of 7.29 (+/- 5.84 SEM) in the 2mM Ca^{2+} there was no such increase in HR in the *dGad1-Gal4>ChR2XXL* larvae (Fig. 6B-C) In fact, in the 1mM Ca^{2+} condition, we noticed a decrease in HR upon blue light exposure (-31.37 %; +/- 21.7 SEM) (Fig. 6 C) and an average percent change of %-0.235 (+/- 3.83 SEM) in the 2mM Ca^{2+} condition (Fig. 6C'). Likewise, in the control line, we found a negative

percent change of 4.49 (+/-2.56 SEM) in the 2mM Ca $^{2+}$ condition, with 4 out of 7 preparations displaying a negative percent change (Fig. 6A'), but noticed a small increase in HR of 2.82% (+/- 5.99 SEM) with 4 out of 7 increasing in the 1mM Ca^{2+} condition (Fig. 6C). The comparisons between the average percent changes in HR in each condition between the lines tested did not elicit statistically significant differences (unpaired t-test; p>0.05; control percent change vs. experimental line percent change) (Fig. 6D). This suggests that the *dGad1* driver does not drive *ChR2XXL* expression in larval tissue enough to induce cardiac stimulation upon blue light exposure.

DISCUSSION

Here we show that the exposure of a semi-intact preparation expressing the recently developed *ChR2XXL* opsin driven by *dGad1-Gal4* displays robust EPSPs in body wall muscle. Initial behavioral phenotypes that were uncovered utilizing optogenetic and thermogenetic techniques were of great interest given the inhibitory role of this neuromodulator during larval development. The behavioral phenotypes suggested the role of GABA release in exciting motor neurons innervating longitudinal body wall muscles causing full body wall contractions, much like what is observed when exciting excitatory neurons within the CNS, or through activation of motor neurons, directly. The electrophysiological analysis performed here corroborates the behavioral findings showing robust EPSP responses in the muscles controlling body wall length and dives deeper into the expression profile of the *dGad1-Gal4* while providing important insights into the possibility of off-target effects in utilization of optogenetic tools in *Drosophila*.

dGad1-driven Chr2XXL-mediated induces bursts of evoked responses in body wall muscle 6 and simultaneous slow muscle depolarization

Our previous optogenetic and thermogenetic behavioral findings begs the question whether these responses were a result of excitatory GABA modulation

within the CNS or if this response is directly related to activity at the NMJ. The observations provided in (Featherstone et al. 2000) give credence to our findings, which uncovered remarkably similar EPSP responses in muscle 6 following blue-light activation of *dGad1*-expressing cells and motor neurons. However, we also did not rule out potential disinhibition in the CNS. All previous findings regarding GABA signaling in the *Drosophila* CNS suggest the role of this neuromodulator as inhibitory. As it relates, more specifically, to a motor program underlying contraction of the muscles that were analyzed in this study, recent findings suggest the primary role of GABA as inhibitory in a locomotive central pattern circuit. A recent study identified a group of segmentally repeated interneurons in the ventral nerve cord (VNC) of *Drosophila* larvae that are important in regulating larval muscle contraction during larval locomotion (Fushiki et al. 2016). Specifically, these neurons (GDL) are part of a circuit, which work in concert with excitatory input to inhibit larval segments in a temporal manner ahead of body wall muscle contraction to enable rhythmic, coordinated locomotion. Suppression of these neurons and genetic knockdown of *dGad1* in this subset of cells disrupts locomotion; suggesting GABA transmission is crucial in regulation of body wall contraction/relaxation (Fushiki et al. 2016). Likewise, pharmacological assessment, through increasing systemic GABA tone by inhibiting GABA transport has also implicated GABA as an inhibitory modulator of larval locomotion (Leal et al. 2004). Thus, the overwhelming evidence to date points to GABA transmission, through the required action of *dGad1*, as inhibitory in regulating motor neuron output in neurons innervating muscles 6. Therefore, our prediction using the *dGad1* driver in driving channel-rhodopsin, targeting a broader network of GABAergic neurons, was to observe impairment in larval locomotion with a relaxed body wall phenotype given the previously described expression profile of the transgene (FlyBase Gramates et al. 2017). Instead, a peculiar tetanus-like full-body contraction was observed using the *ChR2XXL* variant. Interestingly, in utilization of a less sensitive ChR2 variant, *ChR2-H134RII-mcherry*, blue light activation prompted reduced locomotion; however,

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full-body paralysis is not observed, and the locomotion defect is primarily the result of increased duration of body wall 'wave' propagation. This finding matches more of our predicted outcome given previous analyses. The lower sensitivity of this rhodopsin variant likely promotes circumvention of the effects of direct activation of motor neurons, or is not as robust in activation of all motor neurons guiding body wall contraction. Thus, our analysis provided here with the CNS intact is most likely explained by direct activation of motor neurons through *ChR2XXL*.

      While the strong bursts of evoked potentials observed here might not be surprising, we also noticed a slow rise in membrane potential in the muscle. We thus decided to investigate the potential for *dGad1* expression directly in body wall muscle fibers. The techniques utilized here provides strong evidence that *dGad1-Gal4,* not only is expressed in pre-synaptic motor neurons at the NMJ, but also is expressed in larval body wall muscles. Specifically, larval body wall muscle 6, which is amenable for intracellular electrophysiological recording and is important in larval locomotion, displayed continued, dependable depolarization following glutamate application and removal of the larval CNS. The application of 10mM glutamate, which has been shown to be a reliable method for post-synaptic GluR desensitization in a host of invertebrate preparations (Anderson et al. 1976; Patlak et al. 1979; Gration et al. 1981; Shinozaki et al. 1981Cull-Candy and Parker 1983; Dudel et al. 1992), quickly abolished spontaneous EPSPs as well as mEPSPs in each preparation tested, confirming desensitization. The responses following glutamate application in the *dGad1-Gal4>ChR2XXL* larvae mimicked the responses in the *24B(III)-Gal4>ChR2XXL* larvae, with the notable exception being a reduced peak amplitude of depolarization. The duration of repolarization and reliability in inducing responses in the +ATR cohort was indistinguishable. The application of glutamate was performed on preparations subjected to previous pulses with the CNS intact bathed in normal HL-3 saline, so the individual preparations served as their own controls. The likelihood of additional confounds affecting the results, including muscle damage and/or

preparation viability, were minimal, and we can dependably conclude that the observations were through *ChR2XXL*-driven cation influx directly in the muscle. Additionally, separate larval preparations were tested with the CNS removed as a means to validate these findings. Removal of the CNS prevents evoked EPSP responses and only mEPSPs are identified. Upon blue light stimulation, evoked responses are not observed; rather, in *dGad1-Gal4>ChR2XXL* larvae only a slow rise in membrane potential is observed, again mimicking what is observed in *24B(III) Gal4-ChR2XXL* larvae. This strengthens our finding utilizing the glutamate desensitization paradigm and suggests the depolarization is regulated via *ChR2XXL* directly in muscle. We did not find any response to blue light stimulation in our parental control line, showing that blue light stimulus alone fails to elicit activation of central circuits that may drive excitation of motor output. Thus, while we cannot definitively rule out the contribution of disinhibition in the CNS in our analysis with the CNS intact, we suggest that the excitatory responses observed when using *ChR2XXL* driven by *dGad1-Gal4* are the result of activation at the NMJ.

Reduced responses in less-sensitive opsins suggests low-level dGAD1-driven transgene expression in larval muscle

In order to use our optogenetic paradigm to assess the activity level of *dGad1*-driven *ChR2XXL* in larval body wall muscle, we divided our larvae into +ATR and –ATR cohorts. In addition, we tested responses using opsins that are known to be less sensitive to blue light stimulation. This allowed us, not only to detect the possible level of expression of *dGad1-Gal4* in the muscle, but also served to provide important insights into the usefulness of these tools in analyzing neural circuit function and muscle response to light in association with electrophysiological recordings. In our analysis, we found the expression of *ChR2XXL* to be efficient in inducing membrane potential changes in muscle. Specifically, in paradigms with the CNS intact, in preparations where *ChR2XXL* was driven by *dGad1-Gal4*, robust EPSPs were observed in response to blue

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light. These were similar in frequency, duration, and amplitudes to the *D42-Gal4>ChR2XXL* larvae, and we presume this is mainly the result of *dGAD1-Gal4* expression directly in motor neurons. What was surprising was the reliability in responses in the –ATR group in the *dGAD1-Gal4>ChR2XXL* larvae. While it has been shown that *ChR2XXL* is still functional without ATR supplementation, conductance does increase in the presence of this cofactor (Dawydow et al. 2014). The fact we observed 100% reliability in response to blue light in the *dGad1-Gal4>ChR2XXL* and only 50% in the *D42-Gal4>ChR2XXL* was surprising as it is known *D42* drives expression directly in motor neurons. This implies either that there is influence from CNS circuits, or that *dGAD1-Gal4* drives abundant expression in motor neurons. In paradigms with the CNS removed, we again observed a 100% reliability in muscle depolarization in the *dGad1-Gal4>ChR2XXL* +ATR larvae; however the -ATR group did not show a similar increase in membrane depolarization. The *24B-Gal4>ChR2XXL*, as expected, showed robust depolarization in response to blue light with greater average amplitudes of depolarization in both groups (+/-ATR), suggesting a higher level of expression of 24B in muscles. The lack of responses in the –ATR cohort is likely explained by the ability of ATR supplementation to increase *ChrR2XXL* abundance in the cell membrane (Dawydow et al. 2014).

As noted, in behavioral analysis, the use of a less-sensitive channel-rhodopsin, *ChR2-H134RII-mcherry,* did not induce the violent tetanus-like full body wall contractions upon blue light stimulation. The fact that we don't observe such robust responses was a bit surprising but the contribution of central circuits confounds these behavioral responses. Thus, we tested the electrophysiological response in *dGad1-Gal4>ChR2-H134RII-mcherry* upon blue light stimulation. Even with the CNS intact, EPSPs in only half the total blue light pulses were observed. Likewise, no EPSPs were observed in a –ATR cohort, suggesting this rhodopsin variant is more sensitive to the removal of ATR. Therefore, when driven by *dGad1*, only the expression of *ChR2XXL* reliably evokes responses in both behavioral and electrophysiological analysis. This illustrates that even in

tissue where *ChR2XXL* is driven by the regulatory elements of a gene that may be expressed at low levels, optic stimulation can induce physiological effects. Additionally, in the *dGad1-Gal4>ChR2-H134RII-mcherry* larvae, when glutamate was applied to the preparation, no additional muscle depolarization was observed in the larvae that showed enhanced EPSP frequencies sans glutamate, suggesting the lower sensitivity and lower expression levels were not sufficient to induce physiological responses in muscle, directly. Additionally, the mosaic nature of expression illuminated in our confocal analysis suggests the possibility that fewer muscles may express the transgene, or express it at reduced levels. Because of this, we did not continue to use this variant in experiments with the CNS removed. While we were surprised to see such reduced responsiveness in *dGad1-Gal4>ChR2-H134RII-mcherry* larvae with the CNS intact, the inability to produce a photo response in the presence of glutamate is likely explained by the low-level *dGad1-Gal4* expression in muscle.

We also noted an interesting result when using *ChR2-H134RII-mcherry*. In multiple preparations, we noticed an abolishment of EPSPs following initial blue light pulses, which elicited responses, in subsequent pulses. This suggests that there was accommodation and it is possible that the lack of continued responses is the result of rhodopsin desensitization following the initial blue light stimulations. We did not notice this characteristic in any of the preparations expressing *ChR2XXL*, which suggests differing stimulus adaptions between these variants.

Additionally, we utilized the yellow light sensitive rhodopsin, halorhodopsin (*eNpHR*), in testing Cl⁻ mediated hyperpolarization directly in muscle. We employed this in the experiments with the CNS removed to avoid any confounding influence from the CNS. While we noticed slight hyperpolarization in some *dGad1-Gal4>eNpHRL* larvae in response to yellow light, this response was not consistent. In the trials that generated hyperpolarization, the change in membrane potential was much less robust than in the *24B(III)-Gal4>eNpHR* larvae, consistent with analysis with other opsins. While the expression

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differences under various experimental conditions using halorhodopsin have not been as thoroughly examined as the channel-rhodopsins, we have noted in our behavioral studies that the sensitivity is reduced relative to *ChR2XXL*. For instance, in *dGad1-Gal4>eNpHR* larvae, a yellow light stimulus does not cease larval crawling, which would be expected if this would silence motor neurons or muscle. Rather, an impairment in larval crawling is noted, but this abnormality is not as evident as the response in the *dGad1-Gal4>ChR2XXL* larvae. Likewise, in using halorhodopsin, a 10-fold greater ATR concentration required for reliable function (Zhang et al. 2007). Thus, the reduced responsiveness to a yellow light stimulus as well as the reduced amplitudes of hyperpolarization relative to the *24B(III)-Gal4>eNpHR* larvae suggest that this opsin is being driven at low levels in the *dGad1-Gal4>eNpHR* larvae.

Therefore, the evidence points to low-level *dGad1-Gal4* expression in larval body wall muscle. We've shown this through supplementing ATR in larval diets and also through utilization of less-sensitive rhodopsins that are known to require more abundant expression to produce similar photo responses.

Imaging illuminates interesting muscle phenotypes and potential GAL4/UAS variegation

We sought to provide additional evidence of potential low-level *dGad1-Gal4* expression in larval body wall muscle using imaging techniques. We drove a fluorescent reporter in *dGad1* expressing tissue (*dGad1-Gal4>UAS-GFP*) and imaged 3rd instar larvae with confocal microscopy following development under standard developmental conditions. We had predicted to observe low-level fluorescence in body wall muscles due to our optogenetic analysis. We focused primarily on muscles 6 and 7 since our electrophysiological recordings were restricted to those muscles. Our imaging revealed several interesting outcomes. We first noticed strong, consistent fluorescence in motor neurons innervating our muscles of interest, with synaptic boutons readily identifiable. This is consistent with the findings from (Featherstone et al. 2000), and, again, strengthens the

notion of strong *dGad1* expression in motor nerve terminals. In contrast, the fluorescence body wall muscles were, in relation, weaker than in segmental nerves and motor nerve terminals; however in every preparation tested we noted fluorescence in body wall muscles. What was remarkably striking was that we noticed a high degree of variability in our imaging results. While we saw consistent fluorescence in muscle, we noticed that there is a high degree of inconsistency in this fluorescence. Specifically, fluorescence in various muscles in a single preparation was highly variable. While some body wall muscles fluoresced brightly others were much more difficult to detect. Likewise, there was discrepancy from preparation to preparation as far as which muscles fluoresced, with mixed consistency. While some muscles fluoresced quite reliably, others were identified in only a single preparation tested. Specifically, we noted reliably strong fluorescence (4 out of 5 preparations) in muscles 21 and 23. The stochastic nature of GFP expression is a bit puzzling, particularly since we suspect similar *dGad1-Gal4* expression in muscles. It has been suggested in previous work that *Gad* in the fleshy fly is utilized in providing necessary cellular fuel in the Citric Acid Cycle (CAC) (Langcake et al. 1974). It is possible that the energetic needs for body wall muscles may be distinct, although we don't suspect this to be drastically different. A more likely explanation may be the result of a phenomenon observed in numerous other instances utilizing the GAL/UAS system in *Drosophila*. It has been shown that there is a high degree of variability in expression of effector genes when driven by a given Gal4 construct. This was demonstrated by (Skora and Spradlin 2010) in a stable follicle stem cell line, where GFP showed differential expression in cell lineages when driven by a multitude of *Gal4* drivers. This phenomenon was also observed in a number of additional similar studies (see Skora and Spradlin 2010). While the basis of this variegation is not fully understood, such a scenario may explain the outcomes described here, and it may be amplified by the low-level expression of *dGad-Gal4*. In comparison we used a myocyte enhancer factor-2 driver (*Mef2-Gal4>UAS-GFP*) line and did not recognize this variability, likely due to the strong

expression of this gene in muscle. While small variations in expression in *Mef2* would not be detected, we suspect low-level expression of *dGad1* in muscle makes small changes in expression is quite noticeable. Thus we suggest that low-level of expression of the *dGad1-Gal4* transgene and potential variegation that has been documented previously using the GAL4/UAS system is the reason for this surprising result. This further supports our hypothesis that low-level expression of *dGad1-Gal4* in muscle is the likely culprit inducing responses in our electrophysiological analysis.

ChR2XXL activation under dGAD1 control does not stimulate larval HR

The use of optogenetics in optically stimulating cardiac tissue has been employed in larval *Drosophila* (Alex et al. 2015; Zhu et al. 2016) and is becoming a more established method with the goal of introducing this technique as an alternative to electrical pace making in additional models. We utilized this approach to investigate the potential for *dGad1-Gal4* expression in the larval heart. The bases for this assessment stems from previous work identifying a *GAD* isoform expressed in mammalian heart, with the heart *GAD* a separate entity from the neuronal and/or glial *Gad*. Since only a single copy of *dGad*(*Gad1*) is found in the *Drosophila* genome and the *dGad2* gene is suggested to exclusively express in glia (Phillips et al. 1993), we considered the potential for conserved function and expression in *Drosophila* larval heart. The sensitivity of ATR supplementation in optogenetic stimulation of larval HR has been shown previously (Zhu et al. 2016), so we focused on larvae that were exposed to 48 hours of ATR feeding prior to testing in order to generate the most robust responses. Since the *24B(III)-Gal4* driver has been shown to drive expression in larval cardiac tissue (Zhu et al. 2016), our finding that HR increased in response to blue light exposure in larvae expressing *ChR2XXL* in the heart was not surprising. Likewise, the exposure to saline with increased Ca^{2+} induced higher baseline HR rates, so the decreased percent change in response to blue light stimulus in this environment was also not surprising. The

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phenomenon of increasing HR with increased extracellular Ca^{2+} has been shown previously (Zhu et al. 2016). The increased driving gradient in the 2mM Ca^{2+} saline enhanced pacemaker activity as evidenced by the increased HR at baseline, but the additional cation influx via direct or indirect, via voltage-gated Ca^{2+} channels, likely dampens the stimulatory response to Ca^{2+} influx as the pacemaker cells begin to fill with calcium. The precise regulation of Ca^{2+} handling required for normal cardiac pace making is likely perturbed with increased Ca^{2+} conductance, therefore disrupting normal Ca^{2+} buffering. In comparison, the *dGad1-Gal4>ChR2XXL* larvae did not display an increase in HR upon blue light exposure. Instead a reduction in HR was observed in both environments. The reduction in HR observed can most likely be explained by the continued delay following saline-to-saline exchange prior to observation in response to blue light exposure. It has been shown on many occasions that mechanical stimulation induces a slight increase in HR, through presumed activation of stretch-activated ion channels (Titlow et al. 2013; Majeed et al. 2014; Malloy et al. 2016). Generally, a small decrease in HR occurs following a waiting period post-saline exchange, so these findings suggest no physiological change in these larvae in response to blue light. This also strengthens the notion that the increased HR observed with blue light stimulation in *24B(III)-Gal4>ChR2XXL* is mediated by *ChR2XXL* activation. Nonetheless, the inability to stimulate HR upon blue light exposure in an environment most conducive to *ChR2XXL*-mediated enhancement (+ATR and both Ca^{2+} concentrations) suggests low-level or no expression of *dGAD1-Gal4* in cardiac tissue.

Unexpected dGAD1-Gal4 expression brings to light intrigue of underlying function

This unexpected finding has brought to light the potential for a functional role for *dGad1* in muscles in *Drosophila* larvae.   To our knowledge no one has identified expression of this enzyme in body wall muscle in *Drosophila* larvae. Only in the flesh fly has *Gad* been shown to express (Langcake et al. 1974).

Our analysis suggests low-level expression of the *dGad1-Gal4* transgene and confirmation of native *dGad1* expression through immunohistochemical or *in-situ* hybridization approaches would be an intriguing follow-up. Again, the possibility of that this promoter fusion construct may not match native expression is certainly plausible; however, it is important to illuminate Gal4 transgene expression patterns especially in the context of optogenetics, where precise spatial and temporal regulation of cellular activity is paramount. In so doing, adjustments in experimental procedures can be made in order to avoid potential confounding results. With this noted, our combined behavioral, electrophysiological, and imaging analysis provided here suggest that the promoter fusion construct utilized in this analysis drives expression where native *dGAD1* expression has been previously described, with an intriguing identification in motor nerve terminals, where expression was, unexpectedly, identified previously (Featherstone et al. 2000). Furthermore, previous documentation of expression in flesh fly muscle supports the idea of an evolutionarily conserved expression profile of *dGAD1* in insects (Langcake et al. 1974). Therefore, we suspect that driver line utilized here matches closely with native expression.

Observations of *dGad1* expression in pre-synaptic motor neurons have been limited. Through an unbiased genetic screen, (Featherstone et al. 2000) identified a previously unknown functional role of *dGad1* in motor neurons. They found *dGad1*to be integral in regulation of post-synaptic GluR abundance and clustering in synaptogenesis during embryonic and larval development. This was the first study identifying a crucial regulatory role for *dGad1* at the larval NMJ. Given the primary role of *dGad1* in the conversion of the neurotransmitter glutamate to GABA, the question regarding the potential for GABA signaling at the NMJ comes to the forefront. GABA is thought to play a role as trophic factor in development of vertebrate neural circuits (Messersmith et al. 1993; LoTurco et al. 1995). Unlike in other arthropods, including crustaceans, there is no evidence of GABA signaling at the NMJ in larval *Drosophila*. In utilization of application of

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GABA at the NMJ in our lab and additionally in immunohistological and electrophysiological analysis performed by (Featherstone et al. 2000), no evidence that would support a functional role for GABA transmission at the NMJ was identified. Thus, it is unlikely that the role of *dGad1* in regulation of post-synaptic GluRs is through GABA transmission-mediated processes. The alternative hypothesis focuses more closely on the potential for *dGad1* regulation of glutamate tone at the NMJ. Given the delicate balance between activity and synaptogenesis and synaptic maintenance, it is reasonable to assert that the role of *dGad1* pre-synaptically may be in managing extracellular glutamate concentration through mechanisms that could range from vesicular release, non-vesicular leak or uptake (Featherstone et al. 2000). The mechanisms through which this may occur remain uncertain; however, the importance of retrograde regulation of synaptic differentiation at the NMJ calls in to the question a potential parallel function in muscle.

The second important function observed in previous analysis is the role of *dGad1* in non-neuronal tissue focuses on its role in providing fuel in the CAC cycle. The energetic demands of larval skeletal muscle are great, as this serves as a time of rapid development. The consistent movements exhibited by *Drosophila* larvae and adults, through constant feeding and rapid growth and development place high demands on the production of cellular fuel. It is possible, where there is a metabolic demand, *Gad* would be expressed considering its association with the metabolic pathways (Langcake et al. 1974).

CONCLUSION AND FUTURE STUDIES

Our behavioral and electrophysiological analysis, as mentioned, is quite intriguing. Given our findings of the close association of the *dGad1-Gal4* transgene and previously uncovered native expression profile we suspect the driver line to mimic closely the native expression of *dGAD1*. It would, therefore, be a worthy endeavor to investigate the function of *dGad1* in larval muscle. Moreover, the action of GABA in the CNS and it's role in exciting motor programs

involved in locomotion require further investigation, but we have provided evidence that this response may be dose-dependent, with the supplementation of ATR producing enhanced release of GABA. Potentially, the increase synaptic concentration of GABA may desensitize receptors involved in modulation of the central pattern generator regulating locomotion. The potential selective regulation in expression of *dGad1* based on metabolic activity is of interest. It would be particularly interesting to determine if there is a correlation with other proteins related to mitochondrial activity as compare to activity in the cytoplasm excluding the mitochondria. Since it is known that GABA is important in circadian patterns and potentially the sleep cycle, the likelihood of the cyclic nature in the expression of *GAD* may be phase locked and regulated in the CNS within subsets of neurons also tied to circadian patterns. We examined the action in larval body wall muscles but have yet to examine adult body wall muscles and associated motor neurons. Some muscles in adults are highly active in relation to flight while others are less active and the relative expression profiles could help shed more light on the correlation with activity. The likelihood of GABA being produced in body wall muscles and the potential action of GABA not acting as a transmitter is a topic worthy of some pursuit as novel biochemical process maybe revealed. It is known that GABA can provide important excitatory input during development of neural circuits through stabilization of synapses. Whether there are important time points where GABA signaling at the NMJ may be active have not been fully addressed, although (Featherstone et al. 2000) provides important developmental insights. Whether this may parallel in muscle and then down-regulate during larval development remains to be addressed.
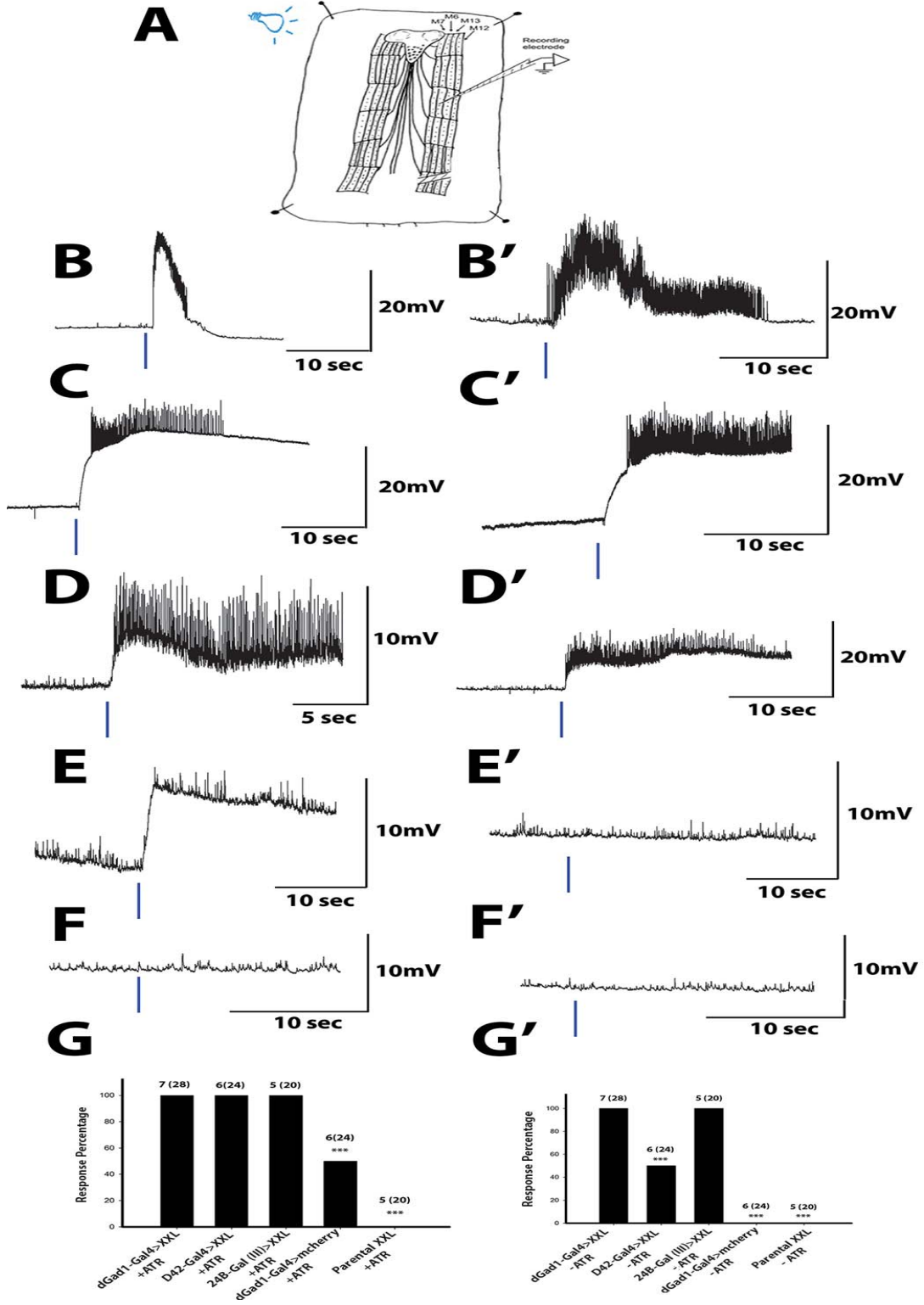
Figure 6.1. Muscle depolarization in response to blue light with CNS intact. A) Representative figure displaying fileted larval preparation. A blue light stimulus illuminated the preparation and synaptic responses were recorded with a sharp intracellular electrode in muscle 6. B) *dGad1-Gal4>UAS-ChR2XXL* larvae with the +ATR cohort in the left panel (B) and –ATR in the right panel (B'). Strong muscle depolarization upon blue light exposure-indicated by the blue tick- and embedded EPSPs. Both cohorts displayed 100% reliability in depolarization upon blue-light exposure (28 out of 28 pulses; n=7). C) *24B(III)-Gal4>UAS-ChR2XXL* larvae. Strong muscle depolarization upon blue light exposure-indicated by the blue tick- and embedded EPSPs. Both cohorts displayed 100% reliability in depolarization upon blue-light exposure. Note continued identification of EPSPs as a result of sensory feedback (20 out of 20 pulses; n=5). D) *D42-Gal4>UAS-ChR2XXL* larvae. Enhanced frequency of EPSPs observed upon blue light stimulation in the +ATR cohort (24 out of 24 pulses; n=6). Less reliable EPSP frequency observed in the –ATR cohort with only 50% reliability observed (12 out of 24 pulses; n=6) E) *dGad1-Gal4>UAS-ChR2-H134RII-mcherry* larvae. The less sensitive rhodopsin line exhibited only 50% reliability in responses to blue light. The sample trace shows one such response. –ATR cohort did not show any response to blue light (12 out of 24 pulses; n=6). F) *UAS-ChR2XXL* parental control. No change in muscle membrane potential was observed in the control line 20 out of 20 pulses; n=5). G) Representation of multiple comparisons between lines in percentage responding. Fisher's exact test used for analysis with preparation sample sizes and total number of pulses in parentheses indicated above bars. (***= p<0.001)

Figure 6.2. Muscle depolarization in response to blue light upon 10mM glutamate application. A) *dGad1-Gal4 larvae* +ATR. Depolarization with embedded EPSPs was reliably evoked upon blue light exposure. 10mM glutamate was applied with a sample response indicated by middle the middle trace displaying initial depolarization upon application and gradual decay. A second round of blue light pulses were administered following glutamate application indicated by the third trace. 100% reliability in continued responses was observed with EPSP and mEPSP responses abolished (28 out of 28 pulses; n=7). B) *24B(III)-Gal4>UAS-ChR2XXL* larvae +ATR. 100% reliability in continued responses were observed with EPSP and mEPSP responses abolished (20 out of 20 pulses; n=5). C) *D42-Gal4>UAS-ChR2XXL* larvae +ATR. Prior to glutamate application, 100% reliability in evoked EPSPs were elicited but these responses were abolished following glutamate application (20 out of 20 pulses; n=5)   -ATR cohorts were omitted in this analysis as only *24B(III)-Gal4>UAS-ChR2XXL* larvae displayed consistent membrane depolarization. D) Representation of multiple comparisons between lines in percentage responding in the presence of 10mM glutamate. Fisher's exact test used for analysis with preparation sample sizes and total number of pulses in parentheses indicated above bars. (***= $p < 0.001$)
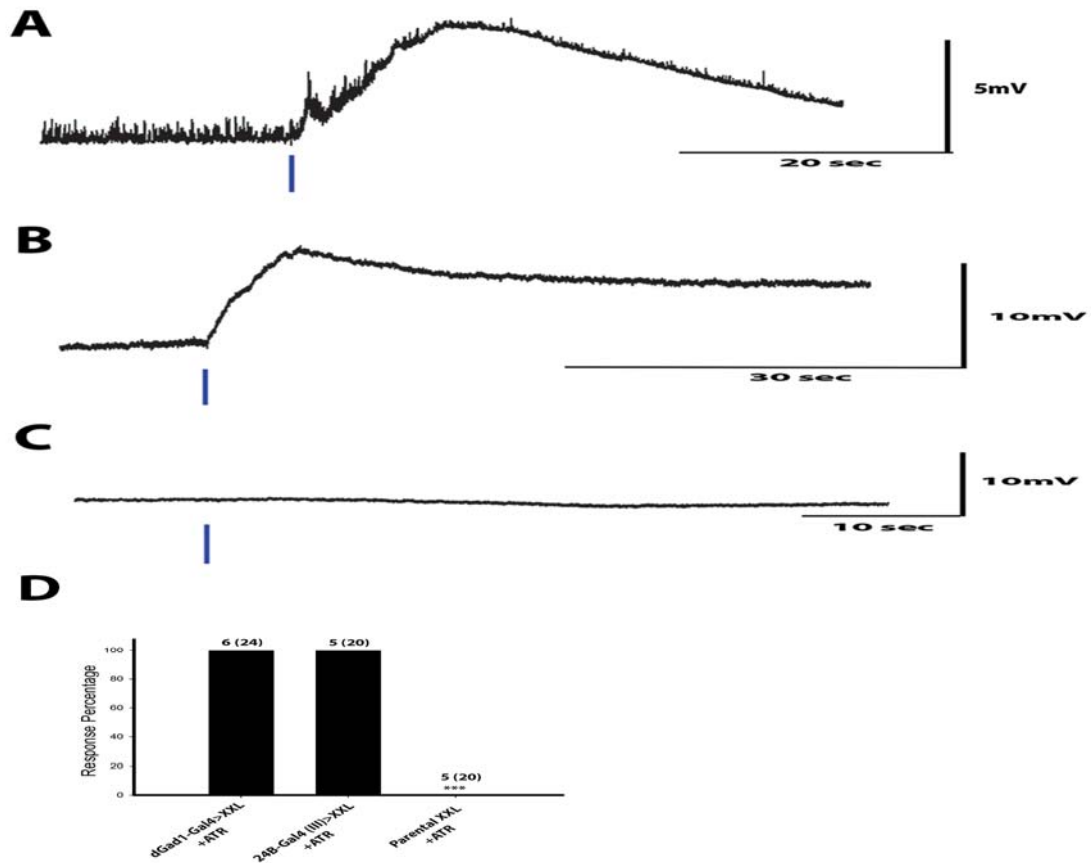
Figure 6.3. Muscle depolarization in response to blue light in the absence of the CNS. A) *dGad1-Gal4>UAS-ChR2XXL* +ATR larvae exhibited gradual depolarization (24 out of 24 pulses; n=6) in response to blue light stimulus (indicated by blue tick). The CNS was transected in order to corroborate findings following GluR desensitization. EPSPs observed with an intact CNS were abolished in these larvae and only mEPSPs could be identified B) *24B(III)-Gal4>UAS-ChR2XXL* exhibited reliable depolarization in response to blue light (20 out of 20 pulses; n=5). The amplitudes of responses were, on average greater than in *dGad1-Gal4>ChR2XXL* larvae. C) *UAS-ChR2XXL* parental line does not exhibit depolarization upon blue light stimulus (20 out of 20 pulses; n=5 no response). -ATR cohorts were omitted in this analysis as only *24B(III)-Gal4>UAS-ChR2XXL* larvae displayed consistent membrane depolarization. D) Representation of multiple comparisons between lines in percentage responding. –ATR cohorts were ommitted herein due to lack of response. Fisher's exact test used for analysis with preparation sample sizes and total number of pulses in parentheses indicated above bars. (***= p<0.001)
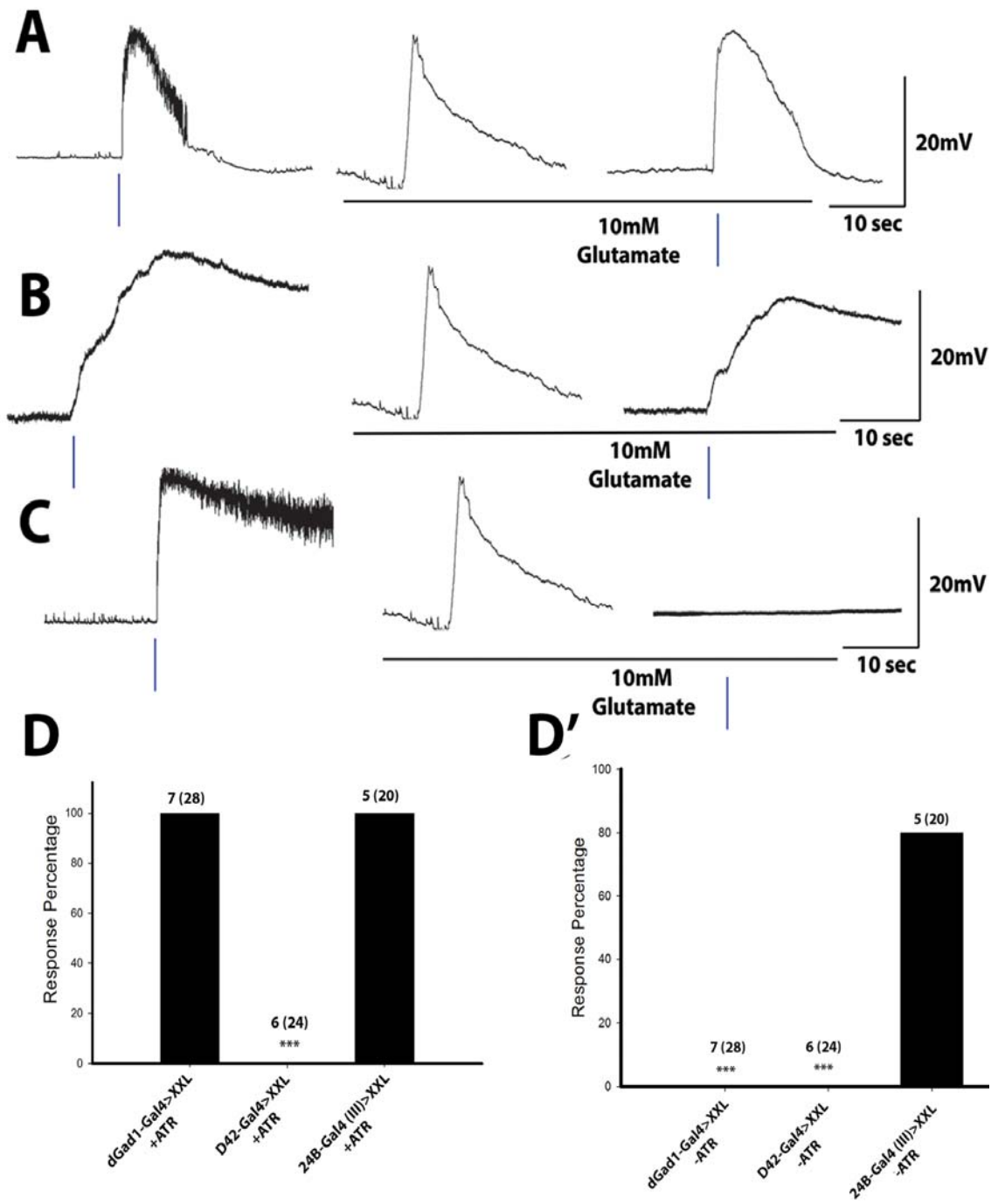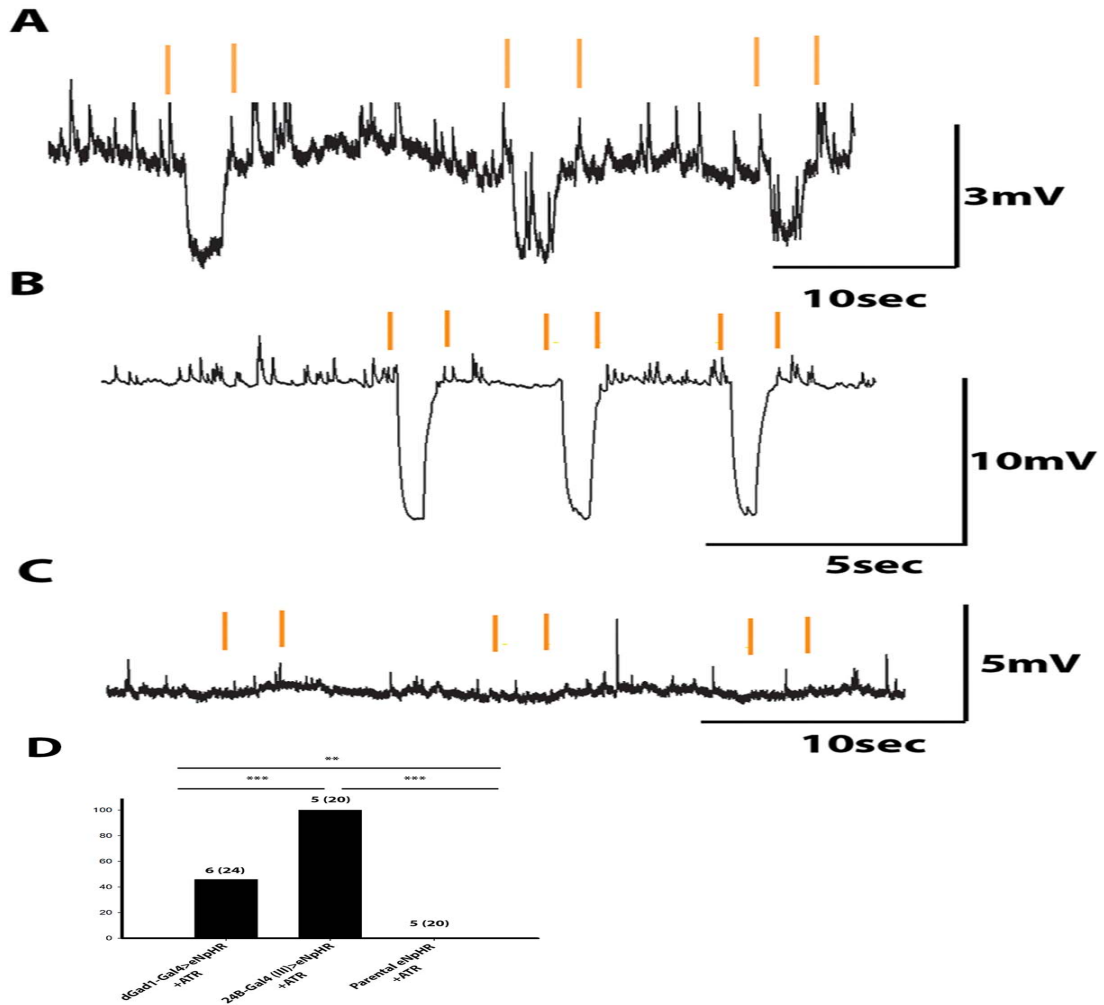
Figure 6.4. Muscle hyperpolarization in response to yellow light in the absence of the CNS. A) *dGad1-Gal4>UAS-ChR2XXL* larvae exhibited slight hyperpolarization in approximately half of the preparations tested (11 out of 24 pulses; n=6) in response to yellow-light stimulus (indicated by yellow/orange tick). The CNS was removed in these preparations to avoid influence from CNS circuits. B) *24B(III)-Gal4>UAS-ChR2XXL* exhibited reliable hyperpolarization in response to yellow light (20 out of 20 pulses; n=5). The amplitudes of responses were, on average greater than in *dGad1-Gal4>ChR2XXL* larvae. C) *UAS-eNpHR* control larvae did not display any hyperpolarization to the yellow-light stimulus (20 out of 20 pulses; n=5 no response) -ATR cohorts were omitted in this analysis as no line displayed consistent membrane hyperpolarization. D) Representation of multiple comparisons between lines in percentage responding. Fisher's exact test used for analysis with preparation sample sizes and total number of pulses in parentheses indicated above bars (**= p<0.01; ***=p<.001).

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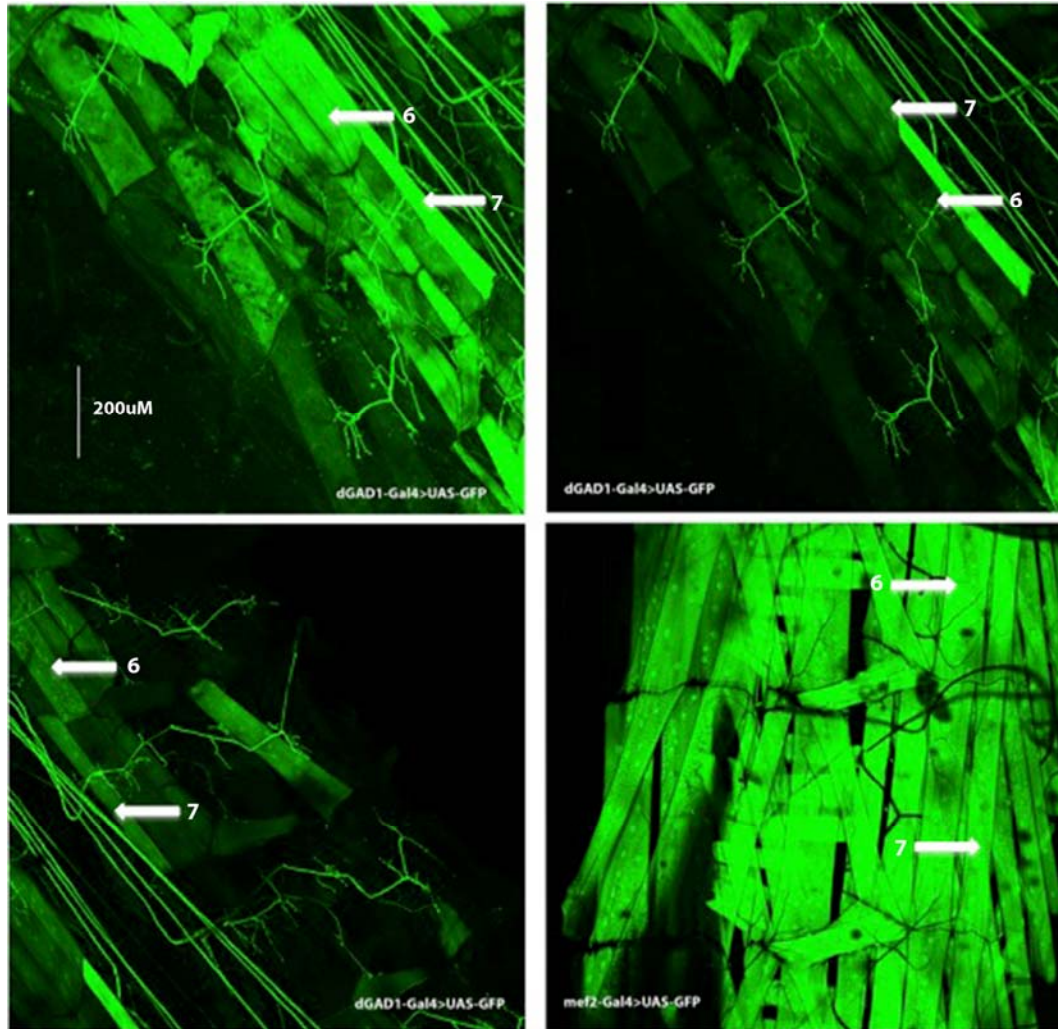
Figure 6.5. Confocal imaging of larval body wall musculature. GFP expression patterns observed under the control of *dGad1-Gal4* and a muscle-specific *mef2-Gal4* driver were analyzed. The top two images show the same section with the left image at a higher gain. The bottom left image shows a different preparation at a region contralateral to the section displayed in the top panel. A high degree of variation was observed in GFP expression patterns in body wall muscles. Nerve branches are illuminated with individual synaptic boutons readily observable in the three *dGad1-Gal4* panels. GFP driven by *mef2* did not show the same stochastic expression patterns-indicated in the bottom right image. White arrows indicate location of muscle 6 and 7 in each image. Leica microsystems confocal SP8 (10X objective) microscope and recorded with LasX software.
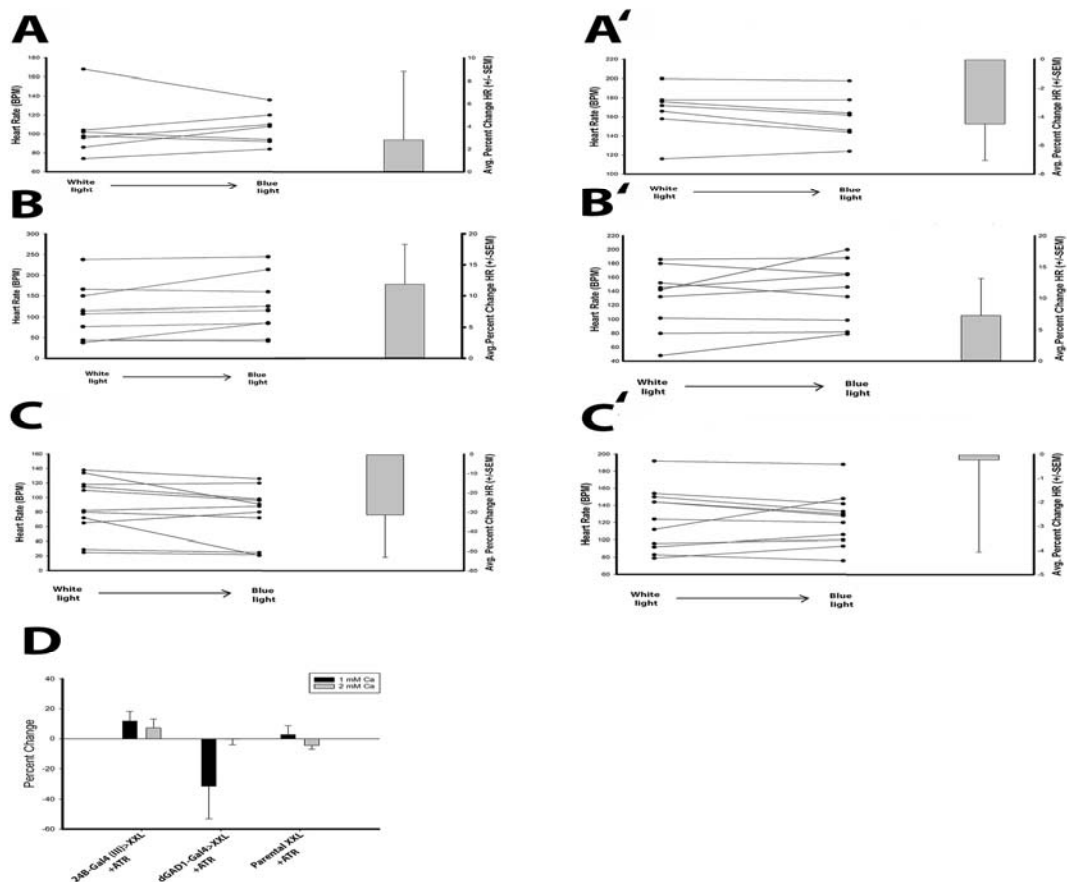
Figure 6.6. Optogenetic pacing of larval heart. Optogenetic stimulation of larval heart rate (HR) at room temperature. HR was counted in the presence of white light and again following the exposure of a blue-light stimulus. Raw changes in HR and average percent change are presented in each panel. HRs were measured in 1mM Ca2+ bath (indicated in left column) and in 2mM Ca2+ (indicated in right column by (')). A) *UAS-ChR2XXL* parental control line did not display a consistent change in HR upon blue light exposure in either 1m or 2mM environment. B) *24B(III)-Gal4>UAS-ChR2XXL* larvae displayed a positive percent change in HR in both the 1mM and 2mM Ca2+ environments; however, this did not represent a statistically significant change relative to control. C) *dGad1-Gal4>UAS-ChR2XXL* larvae displayed a negative percent change in response to blue light in each environment; however, this did not represent a statistically significant change relative to control. No significant difference was observed between either line vs the parental control as indicated by Student's t-test with Tukey's post-hoc analysis (p>0.05). D) Cumulative representation of percent changes for each tested line in 1mM Ca^{2+} and 2mM Ca^{2+} saline.

CHAPTER SEVEN

The effects of potassium and muscle homogenate on proprioceptive responses in crayfish and crab

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INTRODUCTION

The treatment of tissue injury by health care providers is complex depending on the type of injury, tissue type and location. Treatment and care for healing goes beyond focusing on the injured site itself since other body systems and healthy tissue can be indirectly affected (Brancaccio et al. 2010; Cintra-Francischinelli et al. 2010). This is particularly an issue with large amounts of tissue injury due to the spillage of intracellular constituents into extracellular fluid (ECF) and entrance into the blood stream or in the hemolymph in the case of invertebrates. Compartmentation of dense tissue can reduce the effect to the rest of the body but may have an increased effect on the neighboring cells within the compartment. The acute and long term effects on healthy tissue, which is exposed to cellular debris, is varied. The initial tramatome can have a mild to large effect on surrounding tissue depending on the amount of tissue initially damaged, degree of compartmentation, amount of ionic spillage, CO_2 accumulation and resultant alterations in pH (Astrup et al., '77; Dreier et al. 2016).

Much of the focus in the acute effects of damaged tissue on healthy cells is the raised extracellular $[K^+]$ and the rapid depolarization of surrounding cells as a result. In addition high *proteinemia* is a consideration. The associated depolarization of most cells with varied extracellular $[K^+]_o$ is due to the delicate balance in the permeability of Na+ and K+ across the membrane at rest (Bernstein, '02) and the activity of the Na-K ATPase pump (Skou, '65,'98). Feng, in the 1930's, recognized the effects of $[K^+]_o$ on sensory neurons and the consequences of raised $[K^+]_o$ has been a key factor to focus on for the systemic and direct effects on non-damaged cells following an injury. The uncontrolled excitation of cardiac and skeletal muscle as well as neurons can result in rapid death of an animal. However, even under physiological conditions, with heightened electrical activity and efflux of K^+, depolarization in the surrounding cells can result from small changes in $[K+]_o$ (Frankenhaeuser and Hodgkin, '56; Orkand '66; Baylor and Nicholls, '69; Astrup et al., '77).

Since muscle and neurons have a relatively high permeability, through leak channels to K^+, an increase in extracellular $[K^+]_o$ will depolarize the membrane. A slow depolarization can lead to increasing number of voltage gated Na^+ channels opening and then inactivation of the voltage gated Na^+ channels, thus raising the threshold of initiating an action potential. If action potentials are initiated along with the $[K^+]_o$ maintaining a depolarized state, then the cells cannot repolarize and the voltage gated Na^+ channels will remain inactivated (Hodgkin and Huxley, 1952). The electrogenic Na^+/K^+ pump is more active in a depolarized state and would try to regain homeostatic ionic regulation of cells. Neurons with voltage gated Ca^{2+} channels, which are opened by the maintained depolarization, can be sensitive to loading of Ca^{2+} ions and trigger cellular processes leading to cellular damage and cell death (Kuo et al., 2005). A maintained depolarized cell tends to alter membrane properties and causes the membrane to become leakier and then irreversible damage to the cell occurs due to osmotic shock as well as damage to organelles within the cell which can release toxic substances

(Kristensen, '94). The K^+ and enzymatic as well as protein spillage from damaged cells then impacts neighboring cells which can enlarge the tramatome. The associated cells, such as muscle spindles, Golgi tendon organs, pain endings or neurons within a nerve next to or located within the fascia of localized damaged skeletal muscle can be effected. If systemic level of K^+ and protein rise this can effect tissues throughout an entire animal.

In addition to K^+ efflux, other intracellular constituents (i.e., amino acids, enzymes), as well as substances contained within intracellular organelles, can also promote more indirect tissue damage from the initial injury. Depending on the tissue in question, particular constituents within the cytoplasm of cells will have different effects. For example, the amino acid glutamate can bind to glutamate receptors on synaptic sites within the CNS and result in glutamate induced toxicity. If an injury was in the vicinity of the brain or spinal cord, or even more in a more distant location, the glutamate serge can be transported from the blood to the CNS (Camien et al., '51; Simpson et al., '59; Abdel-Salam, 2014). Free glutamate can also rise in the blood from a substantial amount of skeletal muscle damage. Thus, glutamate can travel to distant sites, including the CNS, to cause alterations in physiological function.

The ability for an animal to have coordinated locomotion is in part due to the sensory feedback from proprioceptive neurons. In mammals, muscle spindles (i.e., intrafusal muscles), which are embedded within the much larger extrafusal muscle fibers, provide limb proprioception. Thus, one would predict that damage to a subset of extrafusal muscle fibers within a muscle would alter the function of the healthy neighboring muscle fibers and associated sensory neurons monitoring the muscle spindles. The muscle receptor organ (MRO) is analogous to the mammalian muscle spindle and is found within the crayfish abdomen (Kuffler, '54; Rydqvist et al. 2007). We utilized the model crayfish MRO to examine both the effects of raised $[K^+]_o$ and a saline mixed with a homogenate of

crayfish skeletal muscle on the function of the MRO. The sensory endings are embedded within the thick skeletal muscle fibers, which are neural innervated, for the MRO.

For comparative purposes, we also investigated the effects of raised $[K^+]_o$ and a crab muscle homogenate on the joint proprioceptor in the crab walking leg.The joint proprioceptors in the limbs of crustaceans are similar to those in all arthropods. These joint receptors are a type of mechanoreceptor with sensory endings embedded within chordotonal organs (COs). The COs are comprised of an elastic strand, which monitors the joint movements. The sensory endings of neurons monitoring this movement are embedded within the elastic strand. The neurons detect the direction and rate of joint movement as well as static positions of the joint (Wiersma, '59; Bush, '65a; Cooper and Hartman, '99; Cooper, 2008). Alexandrowicz ('67) named the COs by the joint they are monitoring (i.e., PD is a CO between the propodus and dactylus). Alexandrowicz ('58, '67, '72) described the gross anatomy of the limb proprioceptive organs in the limbs of a variety of crustaceans and Whitear (1962, 1965) as well as others (Lowe et al., '73; Mill and Lowe, '73; Mill, '76) described the fine structure of the COs. In this study, the PD organ in the walking leg of a blue crab (*Callinectes sapidus*) was used to measure functional changes, over a range of movements, for altered levels of $[K^+]_o$ and the influence of a homogenate of skeletal muscle from the same species of crab. The PD organ was chosen to use for these experiments as it is one of the better described COs in Crustacea (Burke, '54; Hartman and Boettiger, '67; Cooper and Govind, '91; Hartman and Cooper, '94; Cooper and Hartman, '99; Cooper, 2008). The PD organ preparation is devoid of muscle directly associated with the sensory ending. Thus, the direct actions on the activity of PD organ can be assessed by changing the bathing environment.

The contribution of this study is that it serves as a model for teaching purposes as well as fundamental research in the influence of raised $[K^+]_o$ and to relate the effects of a known $[K^+]_o$ to that of a diluted muscle homogenate for

drawing parallels to conditions which arise for other animals with tissue injury. The use of muscle homogenate serves to provide analyses on the role of intracellular constituents on sensory nerve function in addition to K^+ alone. The effect of raising $[K^+]_o$ on the resting membrane potential for teaching purposes is commonly demonstrated and a classical student physiology laboratory exercise (Atwood and Parnas, '68; Baierleinet al., 2011). However the novelty of this study is addressing the effects of raising $[K^+]_o$ and cellular homogenate on the function of proprioceptors in two organisms which serve as basic models in addressing neurobiological principles.

MATERIALS AND METHODS

Crab

Blue crabs, *Callinectes sapidus*, were obtained from a local supermarket in Lexington, KY, USA which were delivered from a distribution center in Atlanta, Georgia, USA. They were bought and maintained in a sea water aquarium for several days prior to use in order to assess their health. The crabs were adults and in the range of 10 to 15 cm in carapace width (from point to point). All crabs used were alive and were very active upon autotomizing a leg for experimentation. While holding the crab with a net or large tongs across the carapace from behind, and avoiding the claws, a pinch across the merus of the walking leg with a pair of pliers would induce the leg to be autotomized. The leg was then placed in the Sylgard-lined dissecting dish and covered with crab saline at room temperature (21°C).

The chordotonal organ in the propodite-dactylopodite joint (PD) of the first or second walking legs of the crab was used. The details of the dissection and procedures are described in video and text by Majeed et al. (2013). After exposing the PD nerve and pulling the nerve into a suction electrode for recording the nerve activity, the dactyl was moved throughout the extended and flexed positions for several cycles with the aid of a wooden probe to ensure the

nerve was not pulling on the chordotonal strand. A length of the nerve was left out of the suction electrode to provide slack.

The experimental conditions consisted of moving the dactyl from a flexed 90 degree angle from the propus to a full 0 degrees in an extended position (or open position) and then released. When the dactyl was released the joint would obtain a partial flexed position. Prior to the next displacement the joint was flexed to the same starting position. The rates of movements from a 90 degree angle to 0 degrees were performed within 0.5 sec (rate of 180 degrees/sec) and 4 sec (rate of 22.5 degrees/sec) with 5 sec between displacements. In other studies with crab CO's (*Cancer magister)* reproducibility in repetitive movements at 1Hz gave very high reproducibility in the firing rates as indexed by an eta^2 value (Cooper and Hartman, 1999). Thus, a 5 second interval we assumed to be sufficient to avoid any habituation for the PD neuron in blue crab (*Callinectes sapidus*) but this was not explicitly examined. The analysis consisted of counting the number of spikes of the nerve within the periods of displacement. The joint was also extended in 1 sec (rate of 90 degrees/sec) and held in the extended position for another 9 sec to assess static responses of the neurons in an extended state of the joint. The physical movements performed are described below in section "*To ensure reproducibility in experimentation"*.

Crayfish

Crayfish (*Procambarus clarkii*), measuring 6–10 cm in body length, were used throughout this study (Atchafalaya Biological Supply Co., Raceland, LA). They were housed individually in indoor tanks. The details of the dissection and procedures are described in video and text (Leksrisawat et al. 2010). The MRO nerve to either abdominal segment 2 or 3 was used in this study. The displacements used were from a relaxed position (similar to an extended abdomen in the intact animal) to a stretched position (similar to a flexed abdomen in the intact animal). The displacement rates were 0.5 sec and 4 sec. In addition, a 1 sec stretch and hold was used to obtain the static position sensitive response. The

same electrode and signal recording technique was used as for the crab PD. The physical movements performed are described below in section "*To insure reproducibility in experimentation*".

Saline and pharmacology

The salines used are the normal salines described previously (Majeed et al. 2013; Leksrisawat et al. 2010) with slight exceptions in the use of varied $[K^+]_o$ and saline containing homogenized skeletal muscle. Dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (in mM: 205 NaCl, 5.3 KCl, 13.5 $CaCl_2 \cdot 2H_2O$, 2.45 $MgCl_2 \cdot 6H_2O$, and 5 HEPES adjusted to pH 7.4). All bathing and experimental solutions were kept at the experimental room temperature of 21°C. All chemical compounds were obtained from Sigma (St. Louis, MO, USA). Skeletal muscle was diluted with the species-specific saline with 1 part muscle to 3 parts saline by volume. The muscle was then homogenized and let to settle for 5 to 10 minutes before using. The supernatant of the homogenized skeletal muscle was from the same species as the proprioceptors examined. For the crayfish, skeletal muscle was taken from the claws as well as the abdomen and for the crab, muscle were taken from both claws which consisted mostly of closer muscle.

The procedure used for the various bathing environments was to first obtain recordings in normal saline and the replace the bathing medium with 3 exchanges from the recording dish with 20mM K^+ and let the saline stand for 2 minutes before recording the neural activity to displacements. The media was then replaced to one containing 40mM with 3 more bath exchanges and waiting 2 minutes again before recording. The supernatant of the homogenized skeletal muscle were performed on fresh preparations, which were only exposed prior to normal saline. The normal saline was removed and the diluted supernatant of the homogenized skeletal muscle was introduced to the bath with gently swishing it around in the bath to ensure exposure to the PD or the MRO tissue. Afterwards the bathing

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media was replaced with 3 to 5 exchanges of normal saline and the responses displaces recorded again.

Electrophysiology

Suction electrodes made from glass pipettes fitted with plastic tips were used to record extracelluar signals from the cut nerves (details of making the suction electrodes is provided in Baierlein et al. 2011). A P-15 amplifier (Grass Instruments) in conjunction with a PowerLab/4s A/D converter and Lab Chart 7 software (ADI Instruments, Colorado Springs, CO, USA) obtained the signals to be recorded on a computer at a 10 or 20 kHz sampling rate.

Statistical analysis

All data are expressed as a mean ($\pm$ SEM). The rank sum pairwise test was used to compare the difference of frequency of neural activity after exchanging solution with saline containing each varied [K^+] or saline with homogenized muscle. This analysis was performed with Sigma Stat software. P of $\leq$ 0.05 is considered as statistically significant.

Experimental paradigm for displacements

The crab PD organ is used to model the effects on neurons directly since the skeletal muscle associated with the organ is removed (Figure 7.1A). The neuronal sensory endings are embedded within the elastic strand to detect the movement of the strand. However, the crayfish MRO is closer to modeling the mammalian muscle spindle as the terminals of motor neurons are still attached to the muscle and any force exerted on the sensory endings will result in inducing activity of the SACs (Figure 7.1B). Within the sensory endings of the PD organ and the MRO are the SACs, which initiate ionic flux and depolarization of the neuron when they are deformed by the mechanical forces placed on them. The neurons within the PD organ and MRO respond differently depending on the rate and direction of movement as well as the static position of the joint. Schematic

diagrams of the movements used in this study are shown along with the representative neural activity recorded from the whole nerve (Figure 7.1). The PD joint was displaced from 90º to 0 at various rates (0.5 and 4.0 seconds). The same rates of movements were used for the MRO to also provide a fast and slow displacement. However, the anatomical arrangement is different so a direct correlation in firing rates of the neurons cannot be made between the two preparations. The general responses to the same environmental conditions can be compared. The displacement for the MRO was to a set position that mimicked flexion of the abdomen. A static position of flexion (stretching of the MRO) or extension of the PD, which was held for 10 seconds, was used to index the neural activity and the effects of changing the bathing [K^+] or exposure to skeletal muscle homogenate.

To ensure reproducibility in experimentation

The data collected in the classroom with all the students using 8 different physiological rigs was preliminary data in order to obtain an idea of what to expect for the different experimental conditions. The students made the recordings and analyzed the data. In addition, all the students contributed to compiling information and content for the manuscripts. For standardizing the rate of the movements and analysis all the data presented in the manuscript was obtained by 2 people (one conducting the movements and one marking the files on the computer. One individual (V.D.) analyzed all the data sets so analysis would be consistent. The movements of the joints were performed by the same individual (R.C) for all trails. The movement were made by physically moving the joint and counting out loud: one- Miss (0.5 sec), one- Mississippi (1 sec), two- Mississippi (for the 2nd sec), etc… We timed the counting on a stopwatch several trials to be consistent in the speed of counting. Each time a movement was started or stopped, a mark on the file with a tap on the key pad would be recorded. To be sure the static holds were correctly measured, a set time of 10 seconds were analyzed as indicated by a time stamp on the acquisition software.

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The velocity throughout the movement was kept as constant as possible by manual movement from the starting position to the end position for the crab PD and the crayfish MRO. Each movement was performed 1 time and repeated in each the various adjusted saline conditions.

RESULTS

The effect of [K$^+$]$_o$

Three concentrations of bathing K$^+$ were examined in relation to the neuronal activity for the displacements of the joints related to the PD and MRO preparations. The normal physiological saline used for the crab preparations is 10.8mM K$^+$ so exchanging the bath with 20 and 40mM K$^+$ represented a doubling in concentration for each exposure. Representative responses from a crab PD preparation is shown (Figure 7.2) for each of the displacements in normal saline at a half sec (Figure 7.2A1), 4 seconds (Figure 7.2B1) and stretch and hold for 10 seconds (Figure 7.2C1). After changing the bathing media to a saline containing 20mM K$^+$ the activity generally increased for each displacement (half sec, Figure 7.2A2; four seconds, Figure 7.2B2; and stretch and hold for 10 seconds, Figure 7.2C2). For the four second displacement there was an increase of activity for all six PD preparations, although one preparation only slightly decreased in activity. However, exchanging the media to one containing 40mM K$^+$ the activity was drastically reduced for the same displacements (half sec, Figure 7.2A3; four seconds, Figure 7.2B3; and stretch and hold for 10 seconds, Figure 7.2C3). To ensure the preparations were not permanently damaged from the high [K$^+$] exposure the bathing saline was returned to the normal physiological saline with 3 complete rinses of the saline bath. All six preparations showed some recovery upon replacing the bathing media to normal saline and continued to respond to a range of displacements (see Figure 7.2 A4, B4, C4).

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The same experimental paradigm was also conducted for six crayfish MRO preparations; however, the joint in between the abdominal segments to mimic flexion was bent. The A1, B1, C1 series of Figure 7.3 is the activity which occurs with the normal physiological saline for crayfish at a [K$^+$] of 5.4mM. The crayfish saline was adjusted to 20mM and 40mM [K$^+$] and used as bathing media for examining the effects on the MRO activity. At 20mM the crayfish MRO activity varied with some preparations increasing activity (see the A2, B2, C2 series in Figure 7.3) and others decreasing in activity. The exposure of 20mM K$^+$ for the crayfish preparations is almost four times the normal [K$^+$] in crayfish saline while for the crab the concentration was only doubled at 20mM. The majority of the preparations (four out of six) did decrease in the frequency of spiking with the displacements; however, two preparations increased in neural activity with displacements. Similar to the crab is that the activity was nearly silenced at 40mM K$^+$ in all six preparations (see the A3, B3, C3 series in Figure 7.3). As for the crab PD, a saline rinse was performed to examine if the preparations were still viable. All six preparations responded well in exchanging back to normal saline although the activity did not fully recover (see the A4, B4, C4 series in Figure 7.3).

Effects of Muscle Homogenate

To simulate an authentic situation of a skeletal muscle injury on the effects of joint proprioception, a homogenate of skeletal muscle was applied to the exposed crab PD and crayfish MRO preparations. The species-specific homogenate was used for each preparation. Since it is unlikely that a 100% muscle homogenate would occur in an injury to expose the healthy neighboring tissues, a dilution of the injured cells was modeled with 1 part muscle homogenate to 3 parts species-specific saline. Thus, the cellular constituents would be diluted approximately to 1/4 of the value within the cells. The muscle homogenate solution was made and used immediately on the preparations (less than 2 hours for all preparations).

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A representative crab PD preparation trace is shown in Figure 7.4 with exposure to saline, followed by muscle homogenate and then back to normal saline after several rinses in normal saline. The same displacement rates were used for examining the effect of the muscle homogenate as for examining the effects of varied [K$^+$]. The half second (Figure 7.4A series), four second (Figure 7.4B series) and 10 second stretch and hold (Figure 7.4C series) is shown for normal saline and then exposure to muscle homogenate and after returning to normal saline. Note that the muscle homogenate silenced the PD organ activity except for some very small spikes in the recording. These small spikes might arise from the very small axons of the static position sensitive neurons. However, the majority of the signal is completely absent with exposure to muscle homogenate. The muscle homogenate did not damage the preparations within the 5- minute exposure as all six preparations returned to higher activity than for the muscle homogenate upon rinsing out the muscle homogenate with normal physiological saline.

The crayfish MRO preparation showed a similar trend with the diluted muscle homogenate exposure. The preparations did not become completely silenced in neural activity with the displacements but in all six experiments the activity was drastically reduced (Figure 7.5). As for the crab PD, rinsing of the preparations 4 to 5 times with fresh normal saline the activity returned back to normal levels or to even a higher level of activity for each of the displacements. The same displacement rates were used for examining the effects of muscle homogenate as were used for examining the effects of the varied [K$^+$].

To compare the overall effects of 20mM [K+], 40mM [K$^+$], and muscle homogenate on the activity of the proprioceptors for the crab and crayfish, the average percent change from the initial saline exposure was determined and is shown in Figure 7.6 for each of the displacement rates. Neuronal activity was reduced when both 40mM [K$^+$] and muscle homogenate were applied (N=6, p<0.05 Non-parametric sign rank sum) for a half second, four seconds and 10 second hold displacements for the crab PD organ (Figure 7.6A). The same is

true for the crayfish MRO preparation in that neuronal activity by 40mM [K$^+$] as well as muscle homogenate is a statistically significant in a reduction of activity (N=6, $p<0.05$ Non-parametric rank sum) for the half second, four seconds and 10 second hold displacements (Figure 7.6B). The activity profile for the 20mM [K$^+$] exposure produced the greatest variability among the other exposure conditions. The same amount of time was provided for the experimental exposure; however, the activity increased in some preparations while it decreased in others. To better illustrate the changes in activity for the 20mM K$^+$, the number of spikes counted within each displacement paradigm for saline and for 20mM K$^+$ exposure is shown in Figure 7.7. This was determined for the crab PD (Figure 7.7A) as well as for the crayfish MRO (Figure 7.7B).

The change in activity profiles was not consistent for all the preparations, for any one displacement, or for the different displacements. Only one out of six crab PD decreased in activity for the half-second displacement. In addition, only one out of six for the four second displacement as well as for the 10 second displacement, decreased in activity (Figure 7.7A). The pattern for the crayfish MRO was more erratic in that 4 out of the 6 preparations decreased for each displacement rate (Figure 7.7B). The 40mM K$^+$ exposure and muscle homogenate all showed the same trends in decreasing activity after 2 to 5 minutes of exposure for both the crab PD organ as well as the crayfish MRO. However, the MRO preparation showed an initial difference upon exchanging saline for muscle homogenate, whereas as the crab PD organ did not. The activity right after the initial bath exchanges to muscle homogenate first increased activity, within the 1st minute (6 out 6 preparations, $p<0.05$ Non-parametric sign rank sum). The activity then rapidly decreased the activity for the time point of the 5 minutes of exposure as shown for the average responses (Figure 7.6, 6 out 6 preparations, $p<0.05$ Non-parametric sign rank sum).

DISCUSSION

In this study we demonstrated the proprioceptive neurons associated with the PD organ in the walking leg in the crab and the crayfish MRO preparation are viable for examining the effects of raised [K$^+$] in the bathing media as well as the effects of muscle damage (or other tissue) on healthy proprioceptive function. The preparations can help serve as models for these effects, which may be observed in other invertebrate species as well as mammalian species, including humans. The rise in [K$^+$]$_o$ to 20mM for both preparations resulted in mixed results with some preparations showing an increase in activity while others a decrease with the displacements. However, the higher [K$^+$]$_o$ of 40mM drastically decreased activity in all preparations, which was similar for exposure to the diluted muscle homogenate.

The consequences of raised extracellular K$^+$ ions on the resting membrane potential of cells is well established, but how the activity of the neurons within a unit such as a mammalian muscle spindle or an intact proprioceptive organ is altered is still under investigation. Earlier studies reported that [K$^+$]$_o$ had an effect on membrane potential (Bernstein, '02) but an interest in the effects of cellular leakage on surrounding tissue arose much later. The classical studies on the activity of sensory neurons in the skin of frogs with tissue damage, induced by scratching the skin, brought to the forefront an understanding and intrigue surrounding the implications of tissue damage on healthy cells. These studies showed that the decrease in tactile responses resulting from tissue damage could also be reproduced by raised [K$^+$]$_o$ (Feng, '33). This early report is likely the first instance of explaining a mechanism to account for the increase in tissue damage to healthy cells within an initial site of a tramatome (Astrup et al., '77; Dreier et al. 2016). How concentration gradients of charged ions resulted in potential differences was being addressed as early as the late 1800's (Nernst, 1888). When it was realized that cells were permeable to K$^+$ at rest and that slight alterations in the [K$^+$]$_o$ had an impact on resting membrane potential as well as axon excitability, the understanding regarding

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sensitivity of cell to $[K^+]_o$ (Goldman, '43; Hodgkin and Katz, '49; Hodgkin and Huxley, '52; see review Atwood, '82). It was not until about two decades later in the leech nervous system that it was shown neural activity in one cell could depolarize resting neighboring cells and that this was due to a rise in $[K^+]_o$ (Orkand et al., '66; Baylor and Nicholls, '69). We are not aware of any studies addressing the heightened activity of cells within envelopes of mammalian muscle spindles influencing each other. This would be of interest to address, as the potential for this to occur in diseased states certainly exists (i.e., neuronal and muscle spasticity, fibromyalgia, ion channel pathologies).

The depolarization induced by raising the saline to 20mM K^+ is substantial considering $[K^+]$ in normal crayfish saline is 5.4mM (van Harreveld, '36; Fatt and Katz, '53; Cooper and Cooper 2009). Interestingly this nearly four times increase compared to the physiological level did not result in a persistent desensitization of voltage-gated Na^+ channels in the neurons. The nearly four times increase in the K^+ for crab saline from 10.8mM (normal) to 40mM resulted in a decrease of activity for all six PD preparations. One preparation only decreased a slight amount in activity. In this one case the 40mM solution was exchanged with a quick exposure to 60mM K^+ and the decrease in activity was very substantial. The recovery of activity for this one preparation, which was exposed to 60mM K^+ was rapid with a return to normal saline as the exposure was only for 2 minutes. The doubling of K^+ to 20mM from 10.8mM also resulted in most crab PD (five out six) preparations increasing in activity. Given that the species of crayfish used in this study is found in fresh water to estuarine water, perhaps this particular species is not as sensitive to fluctuations in $[K^+]$ within the hemolymph compared to exclusively freshwater crayfish. Additionally, the blue crab used in this study ranges from the Chesapeake Bay (fresh water) to the open ocean. It would be of interest to know how well these animals can regulate $[K^+]_o$ in their hemolymph when exposed to varying salinities. A range for K^+ in the CSF for non-diseased state humans in one study was found to be 2.4 to 3.0mM/l and 3.5 to 4.70mM/l in

plasma (Pye and Aber, '82). A two fold increase in plasma $[K^+]$ to 7 or 8mM/l in humans will likely lead to death unless rapidly reduced (Gennari, '98; Nyirenda et al. 2009; Conway et al. 2015). A recent study measured the $[K^+]$ in the abscesses in the human brain and found the levels to have an average 10.6 ± 4.8 mmol/L (Dahlberg et al. 2015). The leakage of such an abscess would severely detrimental for the surrounding healthy tissue. In the referenced study, it was implied the K^+ within the abscess was a result of tissue breakdown contained in the location of the abscess. Given the slight varied responses of excitation and depression of activity of the PD and MRO preparations and considering the activity was not totally abolished, it could be suggested that the depolarization by 20mM K^+ was not likely sustained long enough and to a large enough degree to result in Na^+ channel inactivation induced by depolarization. The 40mM K^+ exposure may well have resulted in the neurons ceasing to fire due to Na^+ channel inactivating from a sustained depolarized state. Future studies with intracellular recordings of the neurons within the crayfish MRO and crab PD organ will be able to address this matter. However, in the exciter motor neuron of the opener muscle in the walking leg of crayfish it was determined that at 23mM of K^+, the axon will stop firing due to voltage-gated Na^+ channel inactivation (Smith, '83). This is likely a similar concentration at which the sensory neurons of the MRO ceased firing. We doubled the $[K^+]$ from 20 to 40mM so as to exceed what was previously determined to result in voltage-gated Na^+ channel inactivation (Smith, '83) and we used the same 20mM and 40mM extracelluar concentrations to compare the effects between the crayfish and crab preparations. It would be of interest to know if a different $[K^+]_o$ is required to have the same effect for the neurons in the crab PD organ.

Alterations in activity for intact proprioceptors within the animal may be a result of raised $[K^+]_o$ directly on sensory neurons, skeletal muscles, and/or motor neurons innervating the muscle. The activity of motor neurons may lead to contractions of skeletal muscle, which would have an impact on the firing frequency of the proprioceptors. Muscle contraction itself can occur with raised

$[K^+]_o$ (Prosser, '40; Hodgkin and Horowicz, '60a). The individual tissues (motor nerve terminal, synaptic responses, muscle and sensory neurons) as well as intact preparation could be assessed in these models preparations to understand the integrative nature of proprioception by alterations in $[K^+]_o$ as well as factors resulting from tissue injury. The crayfish MRO is similar to an isolated mammalian skeletal muscle spindle since the motor nerve terminals remained on the muscles associated with the sensory neurons. However, the crayfish neuromuscular junctions are unique in that glutamate is the transmitter for the excitatory motor neuron and inhibitory GABA-ergic motor neuron innervation is also present (Kuffler '54; Elekes and Florey, '87a,b; Swerup and Rydqvist, '92).

The experiments with the diluted muscle homogenate were intriguing as the estimated $[K^+]_o$ is approximately 30mM considering the crayfish skeletal muscle maintains a $[K]_i$ of close to 120mM. As far as we know the $[K^+]_i$ has not been determined *precisely* for the skeletal muscle of crayfish or crab; however, the giant axons within the ventral nerve cord of the crayfish maintained a $[K]_i$ of 233mM for an upper limit (Strickholm and Wallin, '67). So if this concentration is used as the $[K^+]_i$ for muscle then the estimated level would be around 59mM for the diluted muscle homogenate (1 part muscle to 3 parts saline). Considering the activity profile for the crayfish MRO with 40mM K^+ exposure and muscle homogenate is quite similar, the free K^+ levels with the diluted muscle homogenate might be fitting for the observed effects. However for the crab PD, the muscle homogenate generally shut down activity. This may likely be due to the fact that marine invertebrates are known to contain a higher $[K^+]_i$. Estimates are in the range of 370mM for $[K^+]_i$ in squid axons (Caldwell and Keynes, '60). If the same were true for the skeletal muscle of the seawater blue crab then a diluted muscle homogenate would be close to 90mM K^+. This high concentration would result in maintaining inactivation of voltage-gated Na channels after they opened (Hodgkin and Horowicz, '59,'60a,b). As for humans, skeletal muscle contains about 80% of the body's total potassium which is not surprising considering muscle makes up the majority of mass for a healthy human

(Sjøgaard et al. '85; Cheng et al. 2013). The $[K^+]_i$ is around 160mM which would mean about 40mM if the same dilution of skeletal muscle to saline was used. Human plasma is normally within a range of 3.5 to 5 mM (Cheng et al. 2013). In considering damage to a large muscle in a human this could raise plasma $[K^+]$ substantially.

Other constituents within the muscle cytoplasm, besides K^+, could also have an impact on the function of the proprioceptors. The free amino acids may impact some ion channels directly on the sensory neurons. The MRO may have more involved potential targets given the glutamate and GABA receptors are on the muscles embedded with the sensory endings (Robbins, '59; Thieffry, '84). Glutamate is known to be present in the homogenized crustacean skeletal muscle (Camien et al., '51; Simpson et al., '59; Abdel-Salam, 2014). The observed initial increase in activity upon exposure to muscle homogenate in the MRO could be the result of glutamate receptor activation and associated muscle contraction. This muscle contraction may stimulate SACs within the proprioceptors and enhance firing. Lowered responses over time are consistent with the hypothesis that continued depolarization likely induce Na^+ channel inactivation. The relative isolation of sensory endings in the crab PD preparation allows the circumvention of muscle-derived influence of SAC activation and an initial increase in activity.

Unlike these crustacean preparations, the muscle spindle in mammals would likely behave differently with exposure to free amino acids since the motor neurons are cholinergic. The effects on neurons in the CNS by local tissue damage has heavily focused on the toxicity induced by free glutamate by activation of glutamate receptors (Yamamoto et al., '99; Doyle et al. 2008) or K^+ depolarization of neurons with little attention given to other amino acids such as cysteine, homocysteine, glycine as well as many others which are contained in cells (Eaglig et al., '61; Boldyrev and Johnson, 2007). The osmotic shock of cytoplasmic fluid, which has high protein content compared to the ECF, may also have an impact on the function of ion channels. In addition, an alteration in

cytoplasmic pH in healthy cells surrounding tissue damage may arise, as cytoplasm is slightly more basic than ECF in general (Galler and Moser, '86). It is noted that organelles often maintain a relatively acidic environment, so leakage of organelle-derived H^+ may influence ECF acidity and subsequently the cytoplasmic pH in healthy cells (Moody, '81; Bevensee and Boron, '98). This alteration in cytoplasmic pH may have a number of influences on synaptic transmission. Body wall muscle in crustaceans is known to be able to buffer intracellular pH relatively rapidly by ion exchange mechanisms (Galler and Moser, '86). We did not address the osmotic effects with the application of the muscle homogenate but this could indeed impact neuronal excitability. The effect of osmotic shock, free amino acids and duration in exposure to raised $[K^+]_i$ on primary neurons can readily be addressed in these model invertebrate preparations which may provide some insight into addressing similar consequences in mammalian systems.

The compact CNS in vertebrates would likely amplify the effects of neighboring cellular damage on healthy cells. If swelling is present, which can dampen vascular flow, the damaging effects on healthy cells may even be more pronounced due to osmotic shock, changes in pH, ionic/amino acid spillage, and CO_2 accumulation (Dreier et al. 2016; Hartings et al., 2016). Slight imbalances in ionic state, specifically $[K^+]$ and pH/CO_2, may contribute to the onset of epilepsy and other neurological diseases (Nedergaard et al. '91; Kaila and Ransom, '98; Mellergard et al., '98; Mahad et al. 2015; Tregub et al. 2015; Andrianopoulos et al. 2016). In such individuals, additional insults may have an additive or synergistic effect. It would be interesting to assess the susceptibility to changes in neural activity in response to hypokalemia or hyperkalemia in individuals with *Chronic Obstructive Pulmonary Disease (COPD),* who experience systemic reduction of plasma pH (Adrouge and Madias, '81). Both signaling within the CNS as well in sensory neurons, including those found in muscle spindles (Bewick and Banks, 2015), are likely affected (Meves and Volkner, '58). A firm understanding of the role of intracellular constituents released from tissue

damage on healthy cells is in an early stage, and these model preparations can be used to spur additional analyses that can be translated to mammalian models.
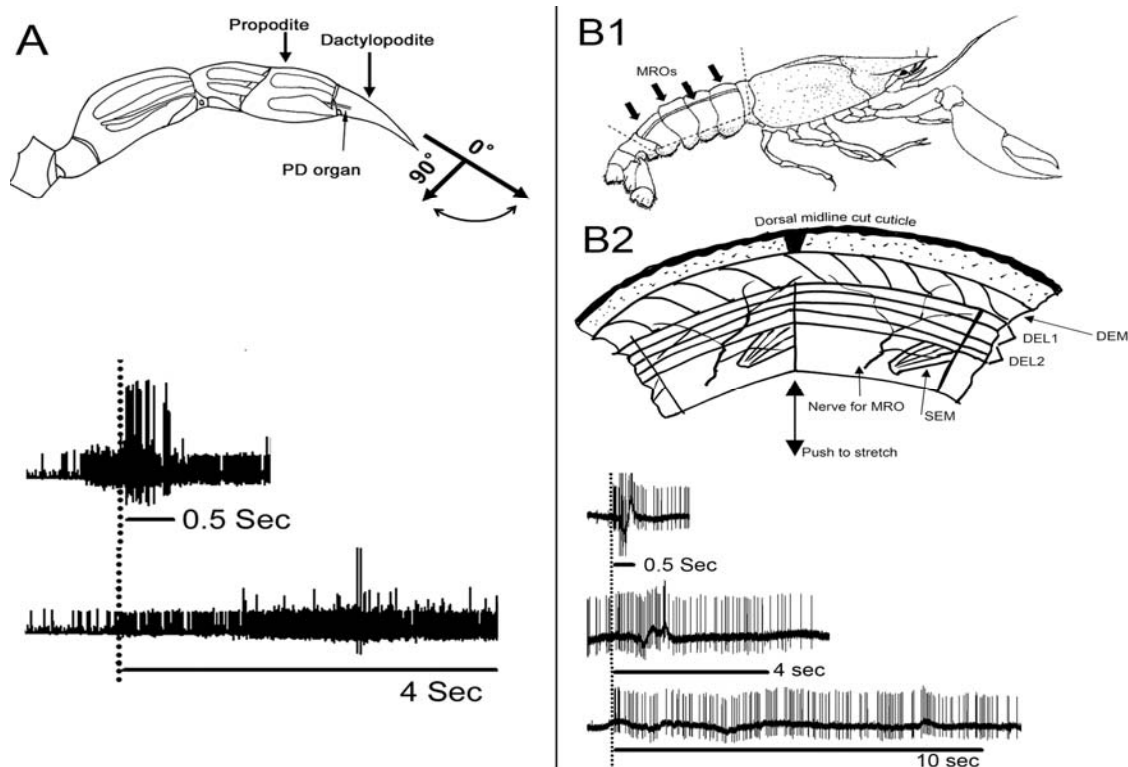
Figure 7.1. Anatomical arrangement of the displacements used for the PD organ of the crab walking leg (A) and the MRO of the crayfish abdomen (B). Either a stop pin or an anatomical position was used for consistency in the displacements. Rates of displacement for the crab joint were 0.5 and 4 seconds from 90^0 to fully extended (0°). (B1) The MROs are located on the dorsal aspect of the abdomen. Movements for the MRO consisted of bending a joint in the hemi-longitudinal segment of the abdomen to a set location at a rate of 0.5 or 4 seconds as well as stretched and held for 10 seconds. (B2) Two abdominal segments are illustrated. A schematic view of the deep extensor muscles (looking from ventral to dorsal) is provided (see Sohn et al., 2000). The crab limb is shown from the side with the position of the tendons and PD organ as they would be in transparency (A1; Whitear, '60; Majeed et al., 2013). The particular muscles identified are: deep extensor medial (DEM) muscles, which have a spiral fiber pattern; DEL1, which is the first lateral group followed by the DEL2 muscles; the superficial extensor medial muscle (SEM), which lies directly dorsal to DEL2, and the two MRO muscles, which are more dorsal to the DEL1. The joint between the abdominal segments would be displaced at various rates to a set position while recording from the MRO nerve (the double arrow indicates where the joint between segments is located). Typical firing activity of the nerves is shown for a PD and a MRO preparation at each of the displacement rates.
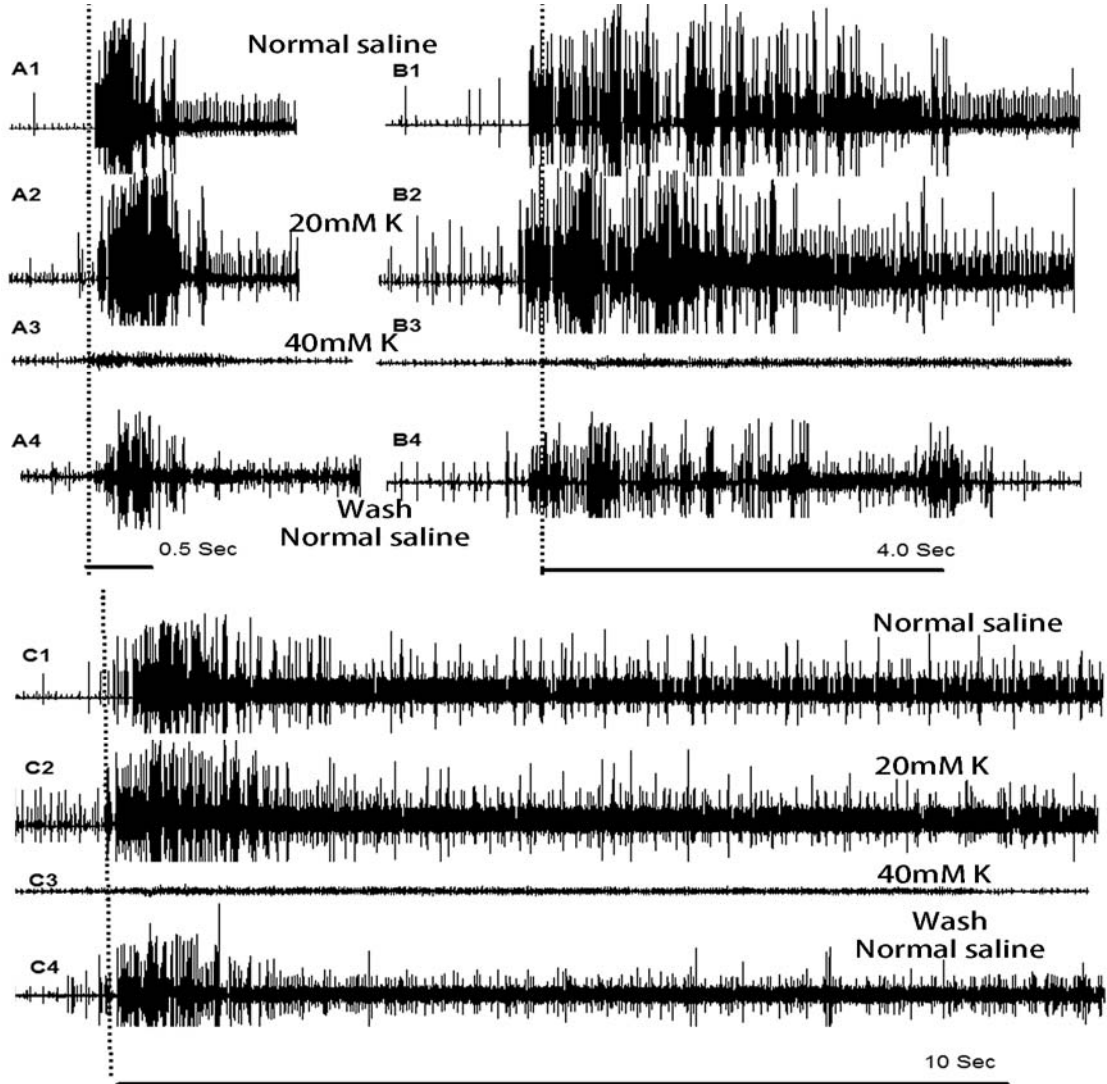
Figure 7.2. Representative traces in neuronal spiking for the different displacement rates and response to varying exposures in [K$^+$] for the crab PD organ. The half second displacement is shown in A, while the four second is shown in B and the static held displacement of 10 seconds in shown in C. The responses in normal saline (A1, B1, C1), during exposure to 20mM K$^+$ (A2, B2, C2) and during exposure to 40mM K$^+$ (A3, B3, C3) as well as wash out with a return to normal saline (A4, B4, C4) are shown. The y-axis scale is the same throughout.

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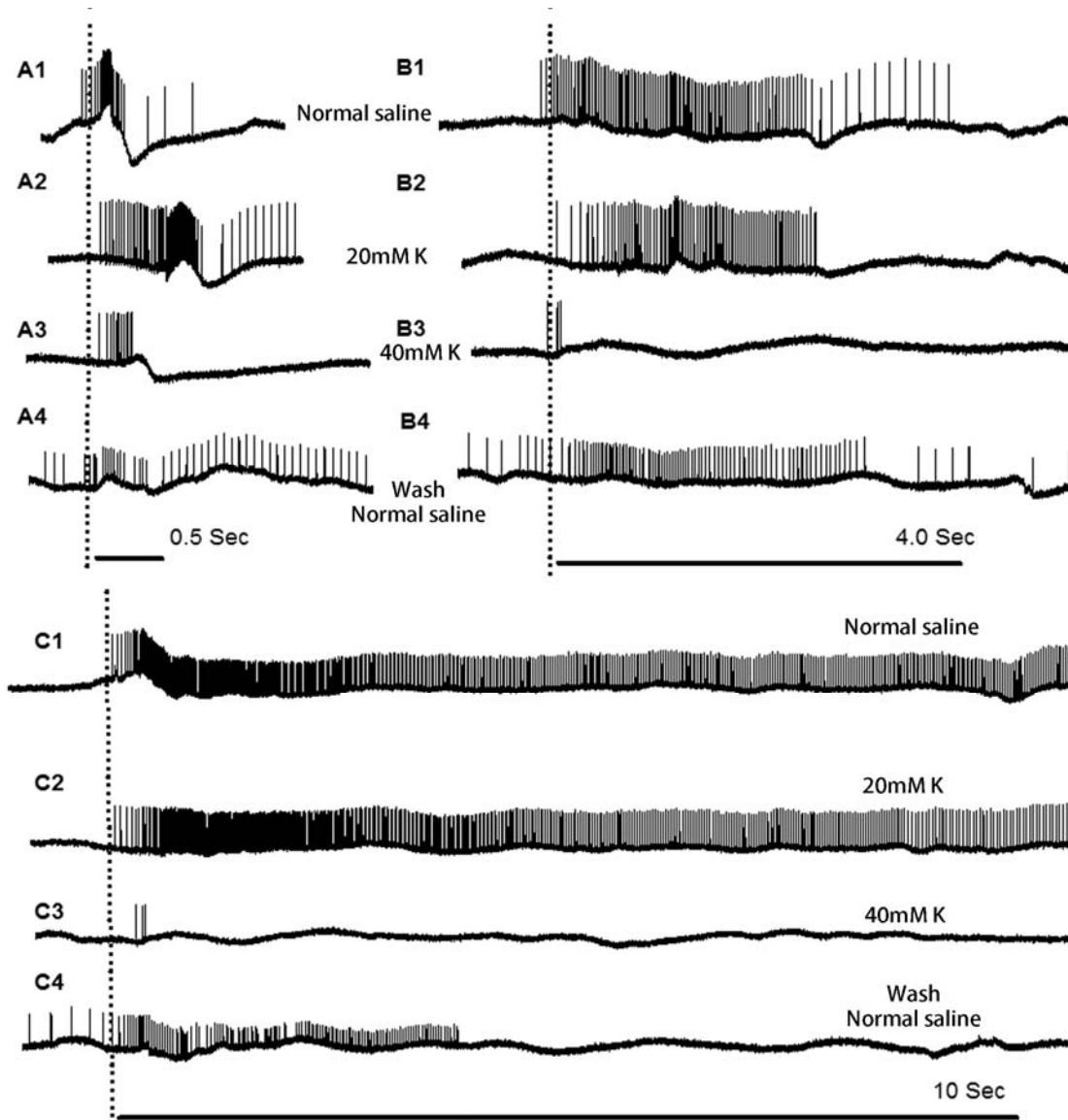
Figure 7.3. Representative traces in neuronal spiking for the different displacement rates and response to varying exposures in [K+] for the crayfish MRO. The half second displacement is shown in A, while the four second is shown in B and the static held displacement of 10 seconds in shown in C. The responses in normal saline (A1, B1, C1), during exposure to 20mM K+ (A2, B2, C2) and during exposure to 40mM K+ (A3, B3, C3) as well as wash out with a return to normal saline (A4, B4, C4) are shown. The y-axis scale is the same throughout.
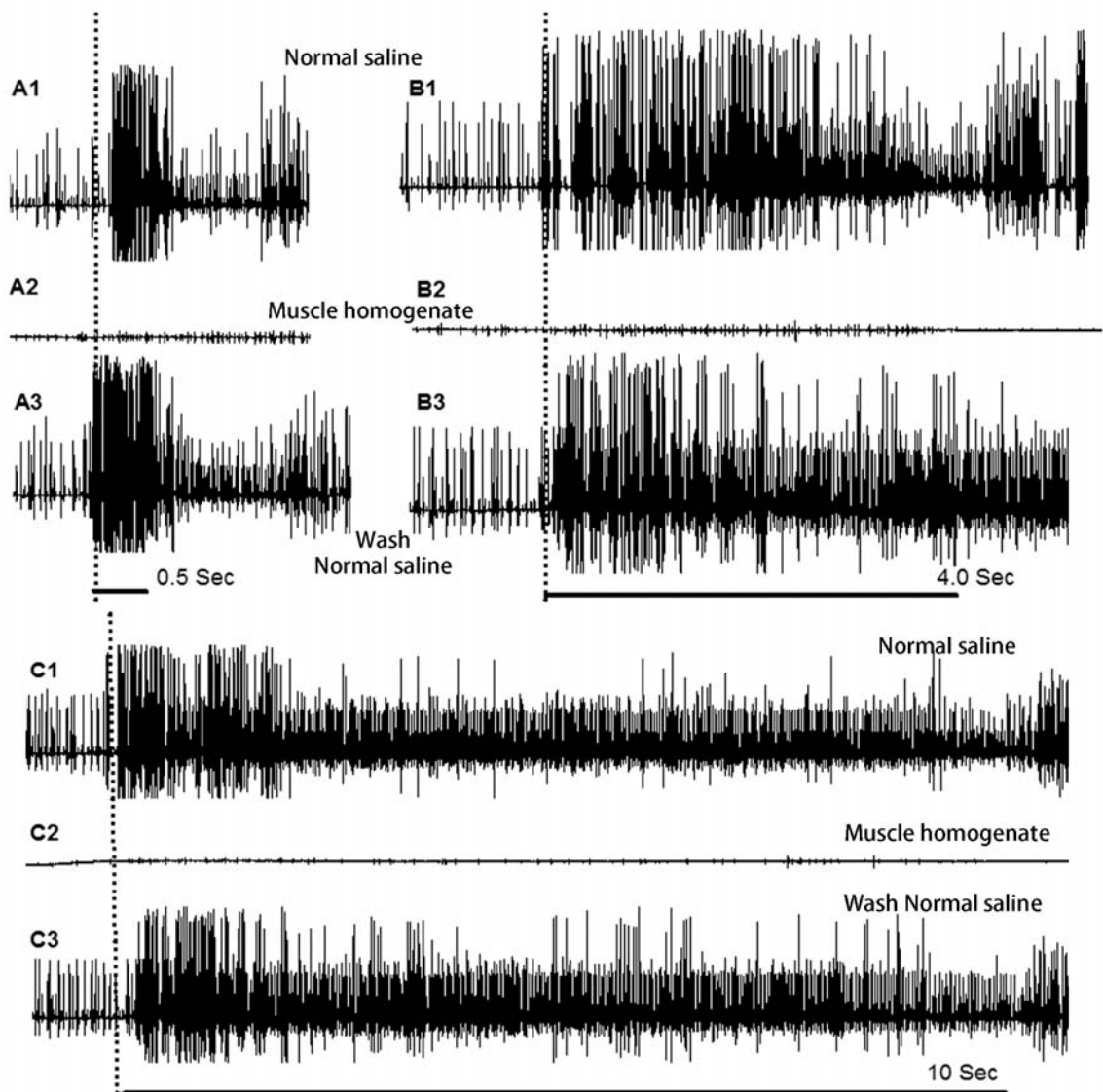
Figure 7.4. Representative traces in neuronal spiking for the different displacement rates and response to normal saline and muscle homogenate for the crab PD organ. The half second displacement is shown in A, while the four second is shown in B and the static held displacement of 10 seconds in shown in C. The responses in normal saline (A1, B1, C1), during exposure to diluted muscle homogenate (A2, B2, C2) as well as wash out with a return to normal saline (A3, B3, C3) are shown. The y-axis scale is the same throughout.
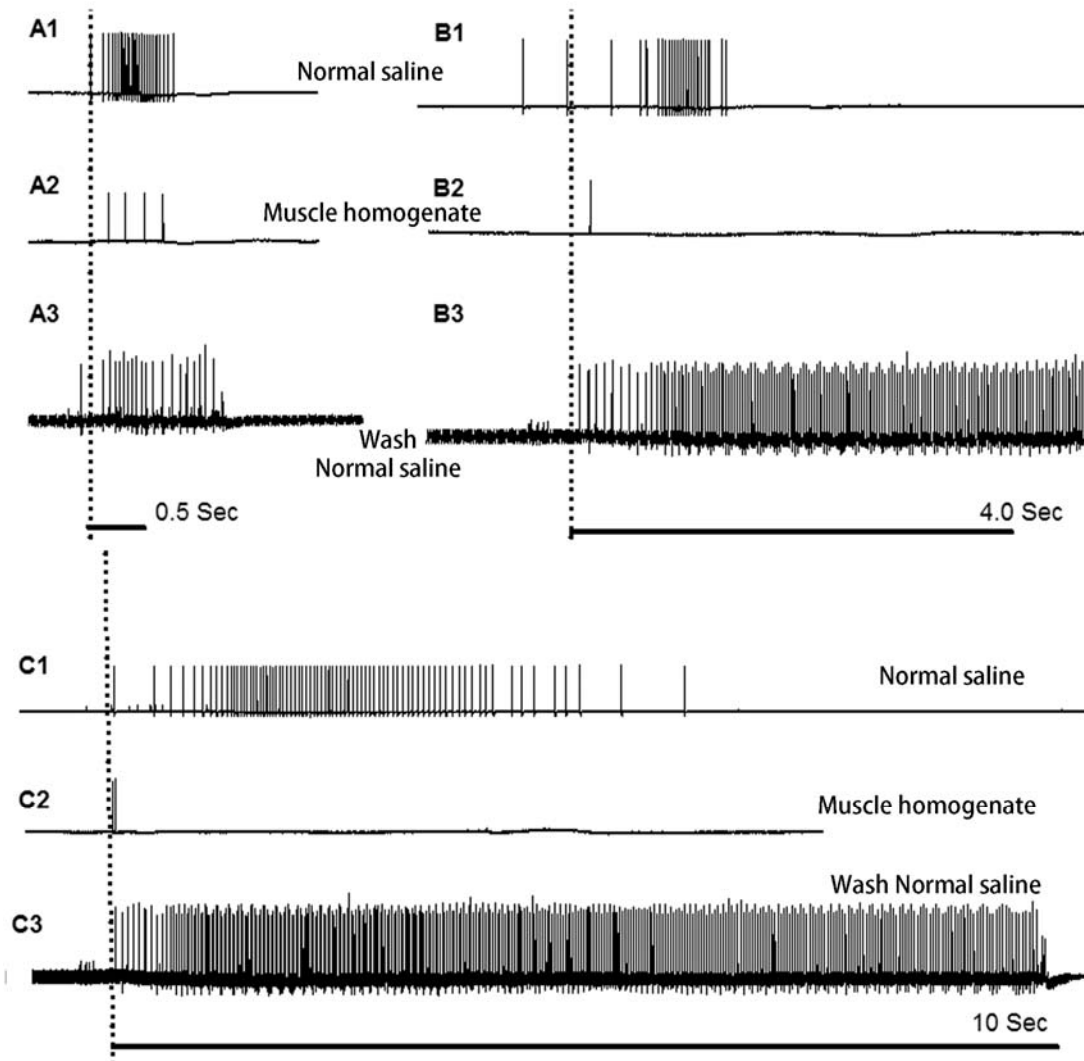
Figure 7.5. Representative traces in neuronal spiking for the different displacement rates and response to normal saline and muscle homogenate for the crayfish MRO. The half second displacement is shown in A, while the four second is shown in B and the static held displacement of 10 seconds in shown in C. The responses in normal saline (A1, B1, C1), during exposure to diluted muscle homogenate (A2, B2, C2) as well as wash out with a return to normal saline (A3, B3, C3) are shown. The y-axis scale is the same throughout.
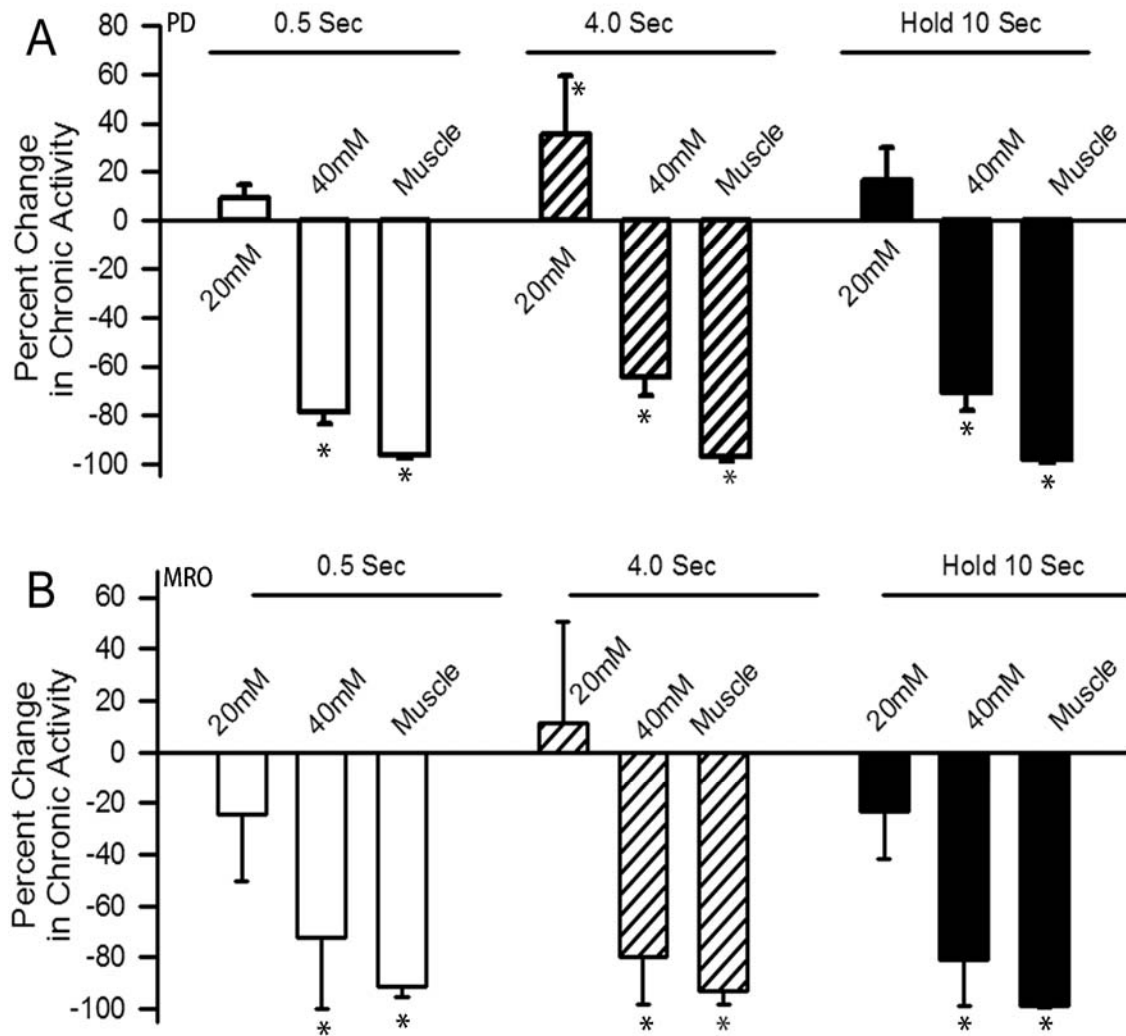
Figure 7.6. A percent change from saline was used to compare among the PD (A) and MRO (B) preparations for the effects of K+ exposure (20mM and 40mM) as well as the diluted muscle homogenate for the various displacement rates and static held position. * refers to 6 out 6 preparations illustrated the same trend (p<0.05 Non-parametric sign rank sum).
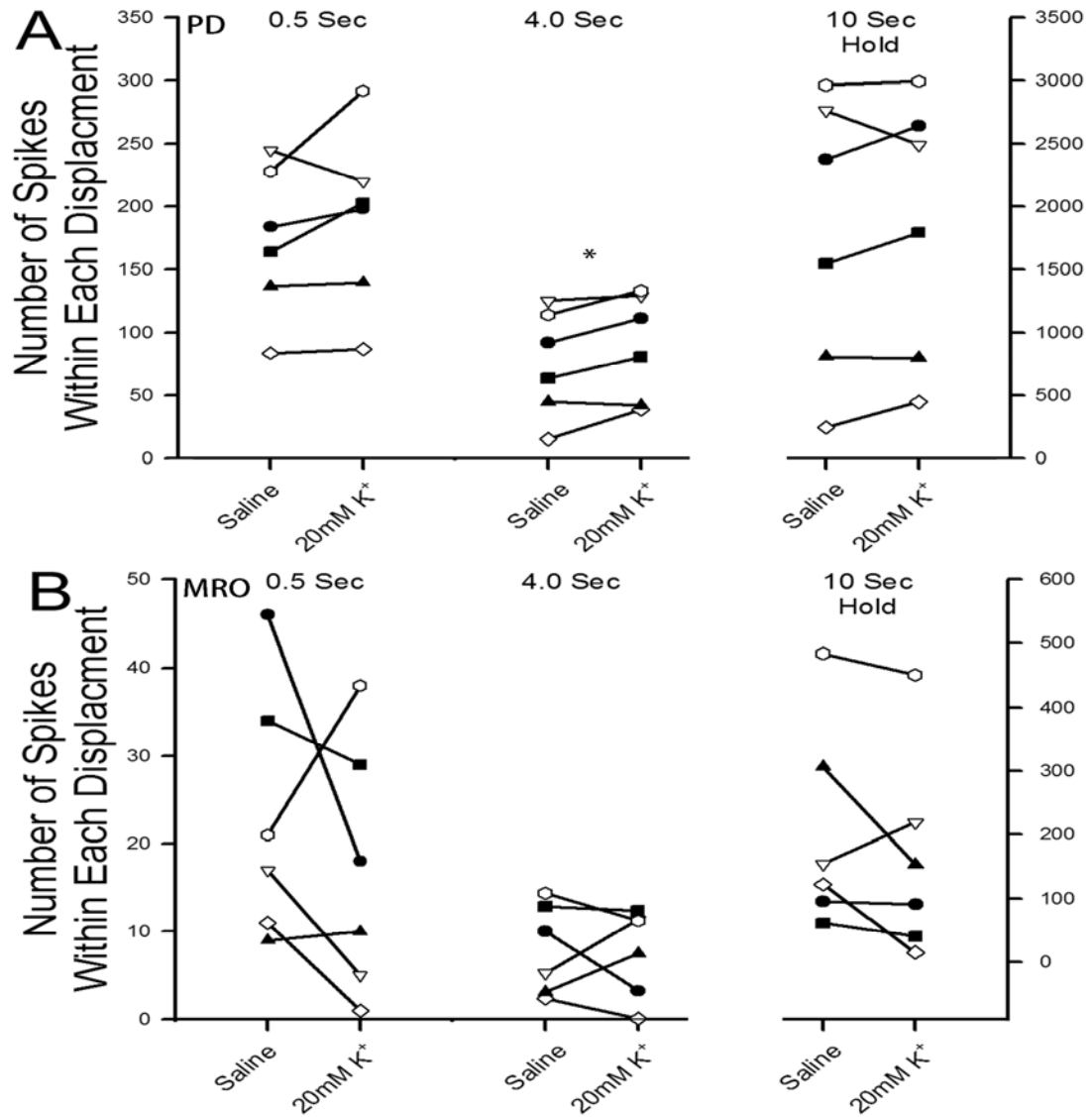
Figure 7.7. The number neuronal spikes measured for each preparation for each displacements condition before and during exposure to 20mM K^+. The measures for the crab PD organ (A) and the crayfish MRO (B) for 0.5sec and 4.0sec displacements are shown on the left where as for the 10sec static held positions are shown on the right ordinate. Only the PD for the 4sec displacement had a consistent effect. (*6 out 6 preparations illustrated the same trend, $p < 0.05$ Non-parametric sign rank sum).

CHAPTER EIGHT

Research Overview, On-going Projects and Future Directions

Impact on the field of Drosophila neurobiology and cardiac physiology

In this research, I set out to describe the role of the vital neurotransmitter, ACh, in regulating cardiac and neural circuit function in a model organism suitable for translational studies. I've utilized a powerful combination of pharmacology, molecular genetics, behavioral analysis, and electrophysiology to assess the physiological and behavioral outcomes that arise as a result of manipulating cholinergic activity, and progress our understanding of its role in cardiac pace making as well. While previous literature has illuminated the role of ACh as a prominent neurotransmitter in the *Drosophila* CNS (Lee and O'Dowd 1999; Yasuyama and Salvaterra 1999; Su and O'Dowd 2003) its role in behavior has not been fully addressed. The complexity of this system has provided many challenges and, as a result the functional classification of receptor subtypess and their influence on select neural circuits have progressed relatively slowly. As noted in Chapter 4, the functional reconstitution of ACh receptor subtypes, particularly nAChRs, in heterologous expression systems has achieved limited success in providing a means for fully describing receptor subtypes (Lansdell et al. 2012). These techniques have proven fruitful in characterizing various ion channels and receptors in a host of model organisms but have not provided as much insight into *Drosophila* AChRs as one may predict given its integral role in the *Drosophila* nervous system. Therefore, our baseline understanding of the properties of AChRs in the fruit fly is somewhat limited and, as a result, interpreting their influence in behavior and circuit function is exponentially more complicated. It is however, essential to continue to promote the fly model as amenable in investigating neural circuit function, with particular focus on AChR contribution. The fly nervous system offers the unique ability to investigate neural circuit properties in a fully intact system. I detailed a number of studies that

highlight conserved mechanisms involved in ACh-meditated circuit function in the context of nicotine dependence and plasticity in Chapter 1. I expanded on these studies and provided insights into pharmacological agents that can be used to alter cholinergic signaling in the *Drosophila* brain, and described a novel electrophysiological technique to assess the role of specific receptors in these processes, and in development of neural circuits. The analysis I provided in Chapter 4 serves as a foundational framework in this endeavor. Furthermore, I used the *Drosophila* larval heart as a means to provide additional insights into AChR pharmacological properties.

Acetlycholine displays a prominent role in modulating larval heart rate

The *Drosophila* larval heart has rapidly become a vital model for investigating physiological mechanisms regulating cardiac function. Many genes regulating heart development and regulation in the fly are conserved, which make it useful in translational research (Bodmer et al. 1998). Additionally, hearts are functionally assessed by comparable physiological measurements, such as, cardiac output, rate and time in systole or diastole (Choma et al. 2011). Studies have used the *Drosophila* heart to identify proteins that are crucial in regulating cardiac muscle contraction and ion transport (Bier and Bodmer, 2004; Wolf et al. 2006; Ocorr et al. 2007; Cammarato et al. 2011). Thus, an increasing number of research groups around the world have utilized this model to enhance our understanding of cardiac physiology. The concerted effort to assess the genes that regulate cardiac function serve as important in identifying proteins that may be altered in disease states. Our lab has been at the forefront of this endeavor. Our focus has been on identifying the role of modulators on regulation of larval heart rate and the intracellular signaling cascades they modulate. We've developed a technique that enables direct investigation of the role of these modulators of the role of cardioactive modulators in cardiac pace making. From this, we've uncovered significant influence from dopamine (Titlow et al. 2013),

serotonin (5-HT) (Majeed et al. 2014), and octopamine (de Castro unpublished). Furthermore, we've provided insights into the role of mechanical disturbance through activation of stretch-activated ion channels that may serve crucial in regulating heart rate (de Castro unpublished), adding to knowledge that has been provided from other labs (Sénatore et al. 2010).

I have provided a comprehensive analysis of the role of ACh in modulating larval heart rate. Specifically, I identified a role for mAChRs in regulation of cardiac function in the larval *Drosophila* model. While the action of ACh in mammalian cardiac regulation is through inhibitory mAChRs, I identified an excitatory function for mAChRs in larval cardiac pacemaking, suggesting a distinct functional role for these receptors in cardiac physiology. While ACh was implicated in modulation of heart rate in this model, prior work centered on the pupal stage, which is a highly dynamic stage in the fly life cycle (Zornik et al. 1999). Furthermore, previous techniques utilizing injection procedures do not isolate individual modulators for direct examination of their influence and may stress the animal. It is known that cardiac regulation by the parasympathetic nervous system is mediated primarily by ACh through activation of the M_2 muscarinic ACh receptor (M_2-AChR) in many vertebrates (Gavioli et al. 2014). However, an increasing body of evidence supports the presence of other mAChR subtypes in mammalian cardiac tissue, and a number of cardiac pathologies are implicated in failure of parasympathetic regulation of heart function (see Roy et al. 2014). These dysfunctions are potentially mediated by an alteration in ACh tone, which may directly alter activity through mAChRs (Roy et al. 2014). The influence of additional mAChRs is possible, including through excitatory subtypes, which would more suggest a conserved functional role for homologous receptors expressed in *Drosophila*. Therefore, my analysis suggests the mAChR receptors and the intracellular signaling cascades they regulate may be a useful target for investigation into the underlying mechanisms contributing to disease.

I also provided additional analysis of the role of ACh in modulation of larval heart rate. The use of the semi-intact, *in-situ*, approach is advantageous in

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a number of ways, chief among them the ability to control the influence of a single cardioactive substance without confounding variables. The obvious detriment to this is that these modulators are not isolated in normal physiological conditions. The larval circulatory system is an open system and a cocktail of cardioactive substances traverse the hemolymph, interacting with cardiac tissue. Thus, a synergy exists among these modulators, and it is of interest to investigate the role of neuroendocrine modulation of heart rate in an intact system. I utilized an optogenetic approach, which served to augment the concentration of circulating compounds to observe alteration of heart rate in an intact animal. I targeted cholinergic neurons and additional neural opulations, which release modulators that have been shown to alter heart rate using a semi-intact approach (Titlow et al. 2013; Majeed et al. 2014, 2016). I found a striking similarity in the ability of these modulators to increase heart rate upon initial release, both in room temperature and at 10° C. This strengthens previous findings and also provides insights into the neural basis of cardiac regulation. Anatomical and behavioral studies of a potential autonomic system in invertebrates were started back in the 1920s and 40s by Ju. Orlov and A.A. Zavarzin (Orloz et al. 1926,1927,1929; Zavarzin 1941).   *Drosophila* larvae may utilize neuroendocrine modulation of the heart and other organs through autonomic regulation. Moreover, movements of a larvae, which does not possess neural stimulation of the cardiac tube, may require humoral factors to increase HR for distribution of endocrine factors and nutrient supply to activate the skeletal muscles to maintain active escape responses. In addition, environmental factors such as cold may require the cardiac system to remain functional so that response to stimuli is maintained and appropriate nutrient dispersal for regulation through transitional stages, such as with cold hardening or conditioning for longer-term cold survival, are conserved. It is possible that neuroendocrine hormones help to maintain cardiac function during an environmental transition (Zhu et al. 2016b).

Future directions for larval cardiac projects

Follow up analysis on these projects is feasible and simple experiments can be performed that will likely be informative in detecting specific receptor subtype involvement in larval pacemaking. There are additional questions that should be re-visited, including the peculiar action of mAChR and nAChR action on larval heart rate. We noted that both nAChR antagonists increased heart rate upon exposure, which was surprising given their role as competitive antagonists. Furthermore, scopolamine, a competitive antagonist for mAChRs, also exhibited agonist-like characteristics. While scopolamine acts on mammalian hearts to increase heart rate through blockade of M2 mAChRs, the action observed here was not expected to alter heart rate in a similar manner given the isolation of the heart and implication of excitatory mAChR influence in larvae. The action of nicotine and the identification of a direct influence of nAChR antagonists on heart rate was interesting given the absence of these receptors in mammalian cardiac tissue. We could not definitively rule out the role of these receptors in mediating heart rate and screening of additional antagonists may be necessary as the assayed antagonists also display interesting affects in the larval CNS.

In addition to our heart rate counting techniques described in Chapter 2 and 3, it would also be interesting to measure membrane potential changes in cardiomyocytes via electrophysiological techniques in order to assess, more directly, the role of modulators in manipulating membrane potential changes. While recording from larval muscles, I occasionally pick up field potentials from adjacent cardiomyoctyes and it would be interesting to test if we could recapitulate our heart rate analysis through more direct measure of oscillation in membrane potential change in response to bathing of cholinergic agonists and antagonists. Either field potential changes and/or intracellular recordings in cardiomyocytes is feasible, and this could shed more light on ionotropic and chronotropic actions of modulators, with measurements of membrane potential amplitude changes particularly useful in detecting the an influence that may alter

strength of contraction in addition to alteration in heart rate. However, a more feasible follow-up to addressing specific receptor subtypes involved in heart regulation would be to repeat the experiments, using a semi-intact approach, in concert with receptor mutants. It is quite feasible to drive RNAi knockdown directly in cardiac tissue using a mesodermal driver that we've identified is expressed in cardiac tissue and assess responses to applied agonists. We can assess the alteration in heart rate in response to the applied agonists to identify which of these receptors is prominent in enhancing rate.   Based on our analysis, I would focus on identifying the exact mAChR involved. Although we detected nicotinic modulation of HR, we could not recapitulate this with an additional agonist, clothianidin, and neither non-selective antagonist tested exhibited the ability to block nicotine action.   Furthermore, I did not directly assay the second messenger cascades through which mAChRs act to regulate heart rate. In heterologous expression systems, it has been shown that mAChR-A and mAChR-C display very similar pharmacological properties and both act through excitatory signaling pathways (Collin et al. 2013; Xia et al. 2016). While the A-type has been shown to act through the $G_{q/11}$, PLCβ, IP_3 pathway (Ren et al. 2015), the pathway through which the C-type acts is uncertain. In utilization of receptor knockdowns in concert with pharmacology, I could identify, more specifically, which receptor subtype is involved while also potentially analyzing a novel pathway through which the C-type may exert its influence. Nonetheless, it would be interesting to assess the cellular processes regulated by these receptors, *in vivo*.

Insights into the impact of cholinergic transmission in the Drosophila CNS

In addition to the difficulties in reconstituting functional AChRs in heterologous expression systems, an additional obstacle preventing our full understanding of the role of neuromodulators in the *Drosophila* CNS is the relative difficulty in measuring synaptic responses within the CNS. As a result,

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much of what we know about neurotransmission in the larval *Drosophila* model centers on work at the NMJ (Kawasaki et al. 2000; Koh et al. 1999; Marek et al. 2002; Renger et al. 2000).   Additionally, our insight into cholinergic transmission has centered on the use of neurons in culture, which may mask the influence of other intact circuits (Baines and Bate 1998; Baines et al. 1999, 2001; Lee and O'Dowd 1999; Yao et al. 2000). There have been few studies that have investigated alterations in synaptic efficacy in CNS circuits *in vivo*, with a notable study provided by Rohrbough and Broadie (2002), who showed enhanced excitability of motor neurons in the presence of Ach. While this served informative as a seminal *in vivo* approach, they did not address the receptor subtypes that were involved in Ach-mediated excitation. Thus, I set out to provide significant information regarding the role of AChRs in altering circuit efficacy. Our lab has developed a unique methodology that enables reliable investigation of the role of neuromodulators in regulating activity in the larval CNS. Through activation of sensory afferents and measurement of EPSPs in muscle contralateral to the stimulus, identification of circuit changes within the CNS are readily identifiable. I used this approach to address cholinergic modulation of sensorimotor circuit physiology. I illuminated roles for both receptor subtypes in modulating this defined circuit and provided more insight in to the pharmacological properties of AChRs *in vivo*. In concert with the behavioral analysis I discussed in Chapter 4, I feel this is a powerful approach to address basic questions regarding the role of these receptors in modulating neural circuits.

A focus on mAChR receptor modulation of larval neural circuits

As shown in Chapter 4, it is evident that muscarine modulates feeding, locomotion, and sensorimotor circuit efficacy. Thus, it is apparent that mAChRs are prominent in the larval CNS and are important in regulating circuits underlying these behaviors. However, this analysis does not address the specific

receptor involved. Additionally, I feel in order to support these findings, it is necessary to continue to address their roles through a combined genetic and pharmacological approach. This will provide a powerful combination in support of all of the pharmacological analysis I've performed to date. From a feasibility standpoint, manipulating expression of nAChRs is a laborious task. The relative reduction in receptor number in genome make the mAChRs a much more feasible target for broad scale investigation of AChR roles in behavior. Thus, I have directed a project that is currently underway to focus on muscarinic cholinergic signaling involvement in these behaviors. Based on my pharmacological findings, I've centered my focus on the A-type receptor and the C-type receptor, which have shown to be responsive to the drugs I assayed in the CNS project (Collin et al. 2014). I've begun by addressing alterations in behavior in RNAi knockdown lines, whereby I'm directing knockdown in multiple neurons. I am assessing behaviors as described in Chapter 4 following normal development. In order to enhance knockdown, I am also raising RNAi mutants at increased temperature (27 C). I am utilizing the GAL4/UAS system (Brand and Perrimon 1993) to direct knockdown with spatial precision, and it is known that GAL4 expression is temperature sensitive (Duffy 2002). Thus, raising larvae at increased temperatures is suspected to enhance mAChR-A and mAChR-C knockdown. I've collected behavioral data on the lines and preliminary analysis suggests an enhancement in feeding behavior (mouth hook extensions) in both knockdown lines, with a significant increase in larvae with the C-type receptor knocked down in all neurons (Elav-Gal4/UAS-mAChR-C-RNAi). Effects on locomotion have proven less efficacious. This analysis is being performed currently on larvae raised at elevated temperatures and comparisons between the lines under both conditions will be assessed. Furthermore, behaviors are being assessed in an additional line, which drives knockdown in the pars intercerabalis (protocerebrum) region of the larval brain to assess mAChR regulation of feeding through regulation of satiation (Schlegel et al. 2016) and previous analysis has shown abundant expression of mAChRs in this region

(Schlegel et al. 2016). An additional means for measuring food consumption is through identification of colored food in the gastrointestinal tract of the larvae after a period of larval feeding. Observation of food intake in mAChR receptor mutants relative to control larvae via quantification of food ingested over a period of time would be useful to analyze in addition to the rate of mouth hook movements in order to address questions regarding mAChR role in satiation.

In addition to performing these behavioral analyses, a number of additional experiments are necessary. To support the findings using the pharmacological feedings approach discussed in Chapter 4, I will test receptor mutant larval responses to agonist feeding, in order to identify whether the behavioral changes observed in wild type flies are recapitulated. I predict, based on the robust nature of muscarine-induced reduction of behavior, that the mutants will display a lessened sensitivity to agonist feeding and this will illuminated more prominently in the feeding circuit, as mAChRs have been implicated in regulation of larval feeding in additional studies (Schoofs et al. 2014; Schlegel et al. 2016). Furthermore, I will test the responses of these mutants using the electrophysiological approach discussed, to assess each individual recetor's role in modulation sensorimotor circuit efficacy. Again, I can direct knockdown in targeted neurons to identify where in the sensorimotor circuit these receptors may be playing a prominent role.   This will be informative in illuminating the strength of my findings utilizing the pharmacological approach, alone. Additionally because the receptor knockdown experiments performed to date do not reveal significant differences in locomotion, I reason that there is a potential for drug off-target effects and/or compensation in neurocircuitry as a result of receptor knockdown throughout development. Thus, I plan to utilize a genetic approach for temporal control of receptor knockdown. To do this, I can add a GAL80 construct expressed ubiquitously to block receptor knockdown until temperature is elevated. Elevation of temperature permits Gal4-UAS interaction directing receptor knockdown at that time, therefore allowing for a specific time frame for alteration of expression. I've begun to develop the lines that will enable

this testing. Furthermore, I plan to do developmental pupation rate assays in the mutants and in wild-type flies developed on food supplemented the assayed drugs to identify potential development alterations upon manipulating muscarinic cholinergic activity.   A comparison can be made on developmental rates in these flies relative to controls. This will help corroborate our pharmacological findings, and will illuminate, more prominently, the role of mAChRs in regulating behavior and circuit function.

Refining the sensorimotor electrophysiological approach

The electrophysiological approach I have described serves useful in addressing modulator influence in neural circuits in the *Drosophila* CNS. It is remarkably difficult to perform intracellular recordings within the larval CNS, and the approach described in Chapter 4 proves beneficial in addressing broad scale changes in CNS circuit efficacy. However, there are some drawbacks with this approach. It is difficult to know exactly how many synaptic connections are altered as a result of the sensory stimulations. It is also challenging to determine which interneurons are altered as a result of our sensory stimulations. The nerves stimulated contain a collection of sensory neuronal axons that project to various parts of the CNS, so it is likely that an abundant collection of neurons are altered in response to nerve stimulation. To fully understand how ACh, for instance, modulates this circuit, it is imperative to investigate the properties of the individual components that make up the circuit. This can be done using imaging approaches in concert with electrophysiological stimulation, but this process is remarkably arduous. This would require one to drive expression of an activity-dependent genomic calcium indicator (GcAMP)(Akerboom et al. 2012) in select neurons. Utilization of the GAL4/UAS system allows for targeting of specific interneurons, and this is increasingly feasible with the development of a host of interneuron GAL4 driver lines (Jenett et al. 2012). However, this would necessitate abundant trial and error.   To pare down the task of identifying the

host of interneurons that are likely involved in altering efficacy in this circuit, a simpler approach would be to activate a smaller subset of sensory neurons. This is feasible using an optogenetic approach whereby light sensitive opsins can be expressed differentially in select sensory neurons and activated through optic stimulation. In this approach, electrical stimulation is replaced by optic stimulation, allowing for alteration of activity of a subset of neurons as opposed to a broader collection. Additional techniques can be used in concert to assess the interneruons that may be altered by select sensory stimulations. Because an individual subset of neurons can be isolated and targeted through the use of a binary expression system, it is feasible to use techniques including GFP reconstitution across synaptic partners (GRASP) (Feinberg et al. 2008) to identify synaptic connections that are made by these sensory neurons. This would still require a relatively laborious screen, but the exact second order components of the sensorimotor circuit can be identified. Furthermore, once these are isolated, the same approach can be used to screen third order components that synapse with the neurons identified in the initial screen. This would allow for mapping of neural connections. Moreover, instead of using genomic calcium, or voltage-sensitive constructs, which may involve complicated genetics in concert with the optogenetic approach, cell-permeable, AM ester form indicator dyes can be used in association with light stimulation of subets of sensory neurons, which would identify which parts of the nervous system are innervated and/or activated as a result of sensory neuron activation. The resolution wouldn't be as clear as the previous approaches, but would be much less arduous and would be a useful first step in analyzing which region of the CNS may be activated by the targeted sensory neurons. There are a number of additional approaches that could be used, but ultimately the most straightforward approach in assessing a sensory to motor circuit activated by a given subset of sensory neurons, is to optically stimulate and record in muscle. I've utilized this approach in order to begin to address the properties of a more defined sensorimotor circuitry. I have begun to utilize this method to address cholinergic modulation of a distinct somatosensory

circuit regulated by a specific class of multidendritic (md) sensory neurons: class IV md neurons. The class IV dendritic arborization (da) neurons represent the polymodal nociceptors in *Drosophila* larvae (Hwang et al. 2007). They detect intense mechanical forces, noxious heat, harmful short-wave light, and dry-surface environments, as well as harmful hydrogen peroxide (Hwang et al., 2007, Johnson and Carder, 2012, Kim et al., 2013, Kim and Johnson, 2014 and Xiang et al., 2010). Upon activation of these neurons, EPSPs are identified in larval body wall muscle 6. Thus, I can use the pharmacological and/or genetic approaches to identify cholinergic modulation of this more defined circuit. I have already begun to screen some the drugs I've tested using the electrical stimulation paradigm, and have identified a role for the Dalpha7 nAChR in regulation of this circuit. Because I noticed robust shutdown of activity using the alpha7 specific drug methyllycacotinine (MLA), I was not surprised that it abolished activity in this circuit in response to class IV sensory stimulation. However, this provides a means for identifying a target that may be screened for its involvement in neural circuit development, and the alpha7 in this manner intriguing for a multitude of reasons.

Additionally, the variability in responses to given sensory stimulations are quite high. Even in the presence of a physiological saline, the activity in response to a given stiumulus train changes from preparation to preparation and even from stimulus to stimulus. While spontaneous activity within the CNS may alter the activity in response to afferent stimulation, a number of additional factors are likely at play. For instance, the degree that the segmental nerves are pulled, or stretched when sucked into the suction electrode may pull on the CNS and alter the ability of drugs to get across the glial sheath that encompasses the nervous system. This may change the responses to application of solutions. Moreover, the location where the intracellular electrode is inserted may alter activity at the NMJ. It is possible to insert into motor nerve terminals or into organelles in the muscle where potential changes may be altered or injury discharge may occur, thus altering the spontaneous activity in the recording.

Furthermore, the dissection may damage nerves in varying manners, affecting the ability to drive activity across the CNS. While the influence of receptor subtypes that display differing affinities for assayed compounds is likely a primary influence in altering sensitivies, the variation in response to a controlled saline is of note and it is important to continue to refine the technique to ensure reproducibility.   Large experimental sample sizes are necessary to control for this variation.

Assessing activity-dependent plasticity in a somatosensory circuit

I have curtailed the idea put forth in the previous section and am utilizing this approach to chronically manipulate activity of a sensorimotor circuit throughout fly development. Previous analysis has been performed investigating the consequences of sensory deprivation on motor circuit development (Fushiki et al. 2013); however, I have been manipulating activity throughout larval development, after critical periods, identified during embryonic stages, when neural connections have formed. The rationale for this is that the larval brain continues to increase in size and neurogenesis and synaptic remodeling persist until pupal stage. What are the consequences of altering activity of a select somatosensory circuit during this period? While previous analysis focused on broader circuitry at earlier developmental time points, the utilization of an optogenetic approach to manipulate activity in a more defined circuit during larval development is completely novel. I've chosen this particular subset of neurons because their role in behavior has been well characterized (Hwang et al. 2007). Thus, it is feasible to assess behavioral consequences that may occur following chronic manipulation of activity throughout development. Specifically, because this subset of neurons is primarily involved in integrating information regarding nociceptive mechanical stimulation (Hwang et al. 2007), I can use a technique to deliver a tactile nociceptive stimulation to investigate their response to tactile touch. This behavioral work has already been performed and I have observed

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significant enhancement of touch sensitivity in light-treated larvae. To associate behavioral changes with structural changes that potentially arise, I plan to utilize confocal imaging to assess alterations in axonal projections of these neurons to illuminate possible alterations in sensory input. Moreover, because I've identified activity at muscle 6 upon optic stimulation of this subset of neurons, morphological analysis can be performed to assess whether motor neuron innervation of this muscle changes in response to repetitively altering the activity of class IV multidendritic neurons.   Antibody staining using horseradish peroxidase (HRP) stains the larval NMJ reliably, so larvae can be staged at 3rd instar following light treatment and synaptic bouton number can be quantified in light-treated larvae relative to controls. In doing so, I may illuminate structural changes that result following manipulation in activity within an entire sensory-CNS-motor circuit. The quantification of synaptic bouton number at this NMJ is feasible and associated synaptic response can be recorded in addition to correlate morphological changes with circuit excitability changes.

How might manipulation of specific cholinergic receptor subtypes alter neural plasticity in the larval model? The foundation set forth through the pharmacological assessment provided in this work allows for the utilization of pharmacological agents as a means to reduce or enhance cholinergic activity. In association with this technique, I can address the role of specific receptor subtypes that may be targeted for activity-dependent modulation. This can be used in concert with genetic approaches, for instance using a Dalpha7 receptor mutant, or through pharmacological supplementation of nicotine or MLA to alter receptor activity in light-treated larvae. It has already been shown in that this receptor expression is altered in response to exposure to nicotine (Velazquez-Ulloa 2017) and curare (Ping and Tsunoda 2012). Is the Dalpha7 receptor expression altered in response to alteration of activity within this circuit? I've shown in this work that this receptor is integral in regulating sensorimotor circuit activity through utilization of pharmacological approaches, and particularly integral in regulating this defined circuit.   How may this play a role in regulating

circuit development? Its known function in synaptic plasticity in the mammalian hippocampus makes it a particularly intriguing target for investigation, and the previous work in the fly support a conserved role in circuit modulation (Ping and Tsuonda 2012; Halff et al. 2014). The parallel expression of mAChRs in the hippocampus also point to their potential to be integral in classical mechanisms underlying plasticity, and their involvement in this circuit can be assayed using genetic or pharmacological approaches. Furthermore, I've provided important experimental insights into the consequences of repetitively activating light-sensitive rhodopsin on synaptic physiology. There are important properties that should be addressed using optogenetic tools to repetitively stimulate opsins and I have outlined these points in Chapter 5. This served as a prelude to this work so the potential confounds that may be present in this analysis have been addressed.

Conclusion

This work provides important insights into the role of ACh in larval neural circuits. From a developmental perspective to acute modulation, I have enhanced understanding of the role of this vital transmitter in regulating circuit performance and cardiac physiology. I provide foundational analysis that I think will serve as instrumental in progressing the field of neuromodulation in this model organism*.*

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CHAPTER ONE

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CHAPTER FOUR

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CHAPTER EIGHT

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PEER-REVIEWED PUBLICATIONS

1. **Malloy, C.,** Ritter, K., Robinson, J., English, C., and Cooper, R.L. (2016). Pharmacological identification of cholinergic receptor subtypes on *Drosophila melanogaster* larval heart. (***Journal of Comparative Physiology-B***).

2. de Castro, C., Titlow, J.S., Majeed, Z.R., **Malloy, C**., King, K.E., and Cooper, R.L. (2016) Chemical and mechanical factors required for maintaining cardiac rhythm in *Drosophila melanogaster* larvae. (***In Review***).

3. Dayaram,V., **Malloy, C.,** Martha, S., Alvarez, B., Chukwudolue, I., Dabbain, N., D.mahmood, D., Goleva, S., Hickey, T., Ho, A., King, M.,Kington, P., Mattingly, M., Potter, S., Simpson, L., Spence, A.,Uradu, H., Van Doorn, J.L., and Cooper, R.L. (2016). Stretch activated channels in proprioceptive chordotonal organs of crab and crayfish are sensitive to Gd3+ but not

amiloride, ruthenium red or low pH. (*In Press-IMPULSE Undergraduate Journal of Neuroscience*)

4. **Malloy, C.,** Dayaram,V., Martha, S., Alvarez, B., Chukwudolue, I., Dabbain, N., D.mahmood, D., Goleva, S., Hickey, T., Ho, A., King, M., Kington, P., Mattingly, M., Potter, S., Simpson, L., Spence, A., Uradu, H., Van Doorn, J.L., and Cooper, R.L. (2016). The effects of neighboring muscle injury on proprioception responses in crayfish and crab. (*In Revision*)

5. Dayaram,V., **Malloy, C.,** Martha, S., Alvarez, B., Chukwudolue, I., Dabbain, N., D.mahmood, D., Goleva, S., Hickey, T., Ho, A., King, M., Kington, P., Mattingly, M., Potter, S., Simpson, L., Spence, A., Uradu, H., Van Doorn, J.L., and Cooper, R.L. (2016). The effect of CO2, intracellular and extracellular pH on mechanosensory proprioceptor responses in crayfish and crab. (*In Review*)

6. **Malloy, C.,** Sifers, J., Mikos, A., Samadi, A., Omar, A., Hermanns, C. and Cooper, R.L. (2016) Using optogenetics to assess neuroendocrine modulation of heart rate in *Drosophila melanogaster* larvae. *(Epub ahead of print- Press-Journal of Comparative Physiology-A)*

7. **Malloy, C.**, Sifers, J., Mikos, A., Somasundaram, E., Omar, A., and Cooper, R.L. (2017) Optogenetic analysis illuminates *Glutamic acid decarboxylase1* expression in *Drosophila* larval body wall muscle. (*In Review*)

8. **Malloy, C.,** Omar, A., Somasundaram, E, and Cooper, R. L. (2017) Pharmacological identification of cholinergic receptor subtypes: modulation of behavior and neural circuits. (*In Manuscript)*

9. Higgins, J., Hermanns, C., **Malloy, C.**, and Cooper, R.L. (2017) Considerations in repetitive activation of light sensitive ion channels for long term studies: Channel rhodopsin in the *Drosophila* model. (*In Press-Neuroscience Research*)

10. **Malloy, C.**, Somasundaram, E., and Cooper, R.L. (2017). Alteration of sensory activity illuminates structural and synaptic plasticity within a nociceptive sensory-CNS-motor neural circuit. (*In Manuscript*)

GRANTS AND FELLOWSHIPS RECEIVED

1. **May 2015.** Gertrude Flora Ribble Research Fellowship. University of KY Dept. of Biology. **Title**: The role of cholinergic system activity in the development of axonal projections of multi-dendritic arborization neurons in *Drosophila melanogaster*.

2. **May 2016.** Gertrude Flora Ribble Research Fellowship. University of KY Dept. of Biology. **Title**: Modulatory action of the cholinergic system in behavior and sensory-motor circuit physiology in *Drosophila melanogaster*.

3. **July 2016.** The Research and Development Excellence Program managed by the Kentucky Science and Engineering Foundation (KSEF) at the Kentucky Science and Technology Corporation. **Title**: "The influence in the development of the central nervous system with limiting sensory gravitational input in a fast developing animal model." PI: Robin Cooper; Student: **Cole Malloy**

CONFERENCE ABSTRACTS AND PRESENTATIONS

1. **Malloy, C.,** Wang, C., Hill, J., Wu, W.-H and Cooper, R.L. (2014) The pharmacological profile and actions of cholinergic system in larval Drosophila: Behavior, development, CNS activity, and heart. Bluegrass Chapter of Society for Neuroscience annual meeting. Lexington, KY. March, 27.

2. **Malloy, C.,** Wang, C., Hill, J., Wu, W.-H and Cooper, R.L. (2014). The pharmacological profile and actions of cholinergic system in larval *Drosophila*: Behavior, development, CNS activity, and heart. 2nd Annual meeting of the Ky chapter of the American Physiological Society, Univ. of Louisville, Ky. March 31, 2014.

3. **Malloy, C.** Hill, J., Wu, W.-H. and Cooper, R.L. (2014). The pharmacological profile and actions of cholinergic system in larval *Drosophila*: Behavior, development, CNS activity, and heart. Annual meeting of Society for Neuroscience.Washington, D.C., USA.

4. Cooper, R.L., Majeed, Z.R., **Malloy, C**., Blümich, S.L.E. and Putnam, R.W. (2014). Synaptic transmission: Effects of intracellular and intravacuolar pH. Annual meeting of Society for Neuroscience. Washington, D.C, USA.

5. Cooper, R.L., Majeed, Z.R., **Malloy, C.**, Potts, D., Zeidler-Watters, K., Krall, R.M., Johnson, D., Mayo, S., Zwanzig, G., Anderson, H., Colgan III, W., Chung, W.-Y., Megighian, A. and Dupont-Versteegden, E.E. (2014). Citizen science with high school students and adults from around the world participating in analysis of synaptic transmission. Annual meeting of Society for Neuroscience. Washington, D.C., USA.

6. **Malloy, C.** Hill, J., Ritter, K., Robinson, J., Wu, W.-H. and Cooper, R.L. (2014). The pharmacological profile and actions of cholinergic system in larval *Drosophila*: Behavior, development, CNS activity, and heart. Annual meeting of the Kentucky Academy of Sciences. Nov. 14-16, 2014 at Lexington, KY.

7. Uradu, H., **Malloy, C**. Hill, J., Ritter, K., Robinson, J., Wu, W.-H. and Cooper, R.L. (2014).

The pharmacological profile of the cholinergic system on larval *Drosophila* heart. Annual meeting of the Kentucky Academy of Sciences. Nov. 14-16, 2014 at Lexington, KY.

8. Cooper, R.L., Majeed, Z.R., **Malloy, C.**, Blümich, S.L.E., Chung, W.-Y., and Putnam, R.W. (2015). Effects of intracellular pH on synaptic transmission: Differences in evoked and spontaneous release. Poster. Society for Integrative and Comparative Biology. Annual Meeting. January 3-7, 2015 West Palm Beach, FL.

9. **Malloy, C.,** English, C., Cooper, R.L. (2015). The role of acetylcholine in neural circuit modulation, behavior and development in *Drosophila melanogaster*. Annual meeting of Society for Neuroscience. Oct. 16-21, 2015. Chicago, IL

10. deCastro, C., Titlow, J., Majeed, Z.R., **Malloy, C.** Zhu, Y.-C.,Vaughn, M., King, K. and Cooper, R.L. (2015). Maintaining the *Drosophila* larval heart in situ: Modulators and stretch activated channels. Annual meeting of the Kentucky Academy of Sciences. Nov. 13-14, 2015 at Northern Kentucky University.

11. **Malloy, C.,** English, C., Cooper, R.L. (2015). The role of acetylcholine in neural circuit modulation, behavior and development in *Drosophila melanogaster*. Annual meeting of Society for Neuroscience. Oct. 16-21, 2015. Chicago, IL. USA.

12. **Malloy, C.,** English, C., Cooper, R.L. (2015). The role of acetylcholine in neural circuit modulation, behavior and development in *Drosophila melanogaster*. Annual meeting of the Kentucky Academy of Sciences. Nov. 13-14, 2015 at Northern Kentucky University.

13. Martha, S.R., **Malloy C**., DMahmood, D., Dabbain, N., Van Doorn, J., Uradu, H.S., Spence, A.E., Simpson, L. C., Potter, S.J., Mattingly, M.X., Kington, P.D., King, M., Ho, A., Hickey, T.N., Goleva, S.B., Chukwudolue, I.M., Alvarez, B.A., Cooper, R.L. (2016) The effect of CO2, intracellular and extracellular pH on mechanosensory proprioceptor responses in crayfish and crab. Annual Meeting of the Kentucky Chapter of the American Physiological Society, March 24, 2016, Univ of KY., Lexington, KY (all the students in the neurophysiology class Bio446/Bio650). * All equal authors.

14. Hickey, T.N., Thenappan, A., Martha S.R., **Malloy C**., DMahmood, D., Dabbain, N., Van Doorn, J., Uradu H.S., Spence, A.E., Simpson, L. C., Potter, S.J., Mattingly, M.X., Kington, P.D., King, M., Ho, A., Goleva, S.B., Chukwudolue, I.M., Alvarez, B.A., Cooper, R.L. (2016) An undergraduate education module based on a research question: The effects of neighboring muscle injury on proprioception responses in crayfish and crab. Annual Meeting of the Kentucky Chapter of the American Physiological Society, March 24, 2016, Univ of KY., Lexington, KY (all the students in the neurophysiology class Bio446/Bio650). * All equal authors.

15. Simpson, L. C., Martha S.R., **Malloy C.,** DMahmood, D., Dabbain, N., Van Doorn, J., Uradu, H.S., Spence, A.E., Potter, S.J., Mattingly, M.X., Kington, P.D., King, M., Ho, A., Hickey, T.N., Goleva, S.B., Chukwudolue, I.M., Alvarez, B.A., Cooper, R.L. (2016) Examining the pharmacology of stretch activated ion channels on mechanosensory proprioceptor responses in crayfish, crab and *Drosophila*. Annual Meeting of the Kentucky Chapter of the American PhysiologicalSociety, March 24, 2016, Univ of KY., Lexington, KY (all the students in the neurophysiology class Bio446/Bio650). * All equal authors.

16. Spence, A., Pallotti, A., Gosser, S., Allen, K., Hall, K., Perrotti, Z., Fritz, M., Rama, S., Ho, A., **Malloy, C.,** Goff, P., Poeppelman, S., McNall Krall, R., Capilouto, G.J., and Cooper, R.L. (2016) Middle and High school distance learning with college student mentors: On line blogging and video communication. Annual Meeting of the Kentucky Chapter of the American Physiological Society, March 24, 2016, Univ of KY., Lexington, KY.

17. Somasundaram, E., **Malloy, C.A.,** Omar, A., Cooper, R.L. (2016) Modulatory action of acetylcholine in mechanosensory processing in *Drosophila melanogaster*: behavior, development, and sensory-motor circuit physiology. Annual Meeting of the Kentucky Chapter of the American Physiological Society, March 24, 2016, Univ of KY., Lexington, KY. Won a cash prize for best undergraduate poster presentation.

18. Malloy, C.A., Omar, A., Somasundaram, E., Cooper, R.L. (2016) Pharmacological identification of cholinergic receptor subtypes in modulation of a *Drosophila melanogaster* sensory-motor circuit. Annual Meeting of the Kentucky Chapter of the American Physiological Society, March 24, 2016, Univ of KY., Lexington, KY.

19. Somasundaram, E., **Malloy, C**., English, C., Omar, A., Cooper, R.L. (2016). Modulatory action of acetylcholine in somatosensory processing in *Drosophila melanogaster*: behavior, development, and sensory-motorcircuit physiology. National Conference on Undergraduate Research. University of North Carolina, Asheville. April 7-9, 2016

20. Omar, A., **Malloy, C.**, English, C., Somasundaram, E., Cooper, R.L. (2016). Pharmacological identification of cholinergic receptor subtypes in modulation of a *Drosophila melanogaster* sensory-motor circuit.
National Conference on Undergraduate Research. University of North Carolina, Asheville. April 7-9, 2016.

21. Somasundaram, E., **Malloy, C.**, Omar, A., Cooper, R.L. (2016). Modulatory action of acetylcholine in mechanotransduction in *Drosophila melanogaster*: behavior, development, and sensory-motor circuit physiology. Bluegrass Chapter of Society for Neuroscience annual meeting. Lexington, KY. April 21, 2016.

22. Omar, A., **Malloy, C.,** Somasundaram, E., Cooper, R.L. (2016). Pharmacological identification of cholinergic receptor subtypes in modulation of a *Drosophila* sensory-motor circuit. Bluegrass Chapter of Society for Neuroscience annual meeting. Lexington, KY. April 21, 2016. Won a cash prize for best undergraduate poster.

23. Martha, S.R., **Malloy, C.**, DMahmood, D., Dabbain, N., Van Doorn, J., Uradu, H.S., Spence, A.E., Simpson, L.C., Potter, S.J., Mattingly, M.X., Kington, P. D., King, M., Ho, A., Hickey, T.N., Goleva, S.B., Chukwudole, I.M., Alvarez, B.A., Cooper, R.L. (2016). The effect of CO2, intracellular and extracellular pH on mechanosensory proprioceptor responses in crayfish and crab. Bluegrass Chapter of Society for Neuroscience annual meeting. Lexington, KY. April 21, 2016.

24. Simpson, L.C., **Malloy, C.,** Martha, S.R., DMahmood, D., Dabbain, N., Van Doorn, J., Uradu, H.S.,   Spence, A.E., Mattingly, M.X., Kington, P.D., King, M., Ho, A., Hickey, T.N., Goleva, S.B., Chukwudole, I.M., Alvarez, B.A., Cooper, R.L. (2016). Examining the pharmacology of stretch activated ion channels in mechanosensory proprioceptor responses in crayfish, crab, and *Drosophila*. Bluegrass Chapter of Society for Neuroscience annual meeting. Lexington, KY. April 21, 2016.

25. **Malloy, C.A.,** Omar, A., Somasundaram, E., Cooper, R.L. (2016) Modulatory action of the cholinergic   system in locomotion and feeding behavior in *Drosophila melanogaster* larvae. Bluegrass Chapter of Society for Neuroscience annual meeting. Lexington, KY. April 21, 2016. Won a cash prize for best graduate poster.

26. **Malloy, C**, Omar, A., Somasundaram, E., Cooper, R.L. (2016) Pharmacological identification of cholinergic receptor subtypes in modulation of *Drosophila melanogaster* sensory-motor circuits. Kentucky Academy of Sciences Annual Meeting. November 4-5, 2016, Louisville, KY.

27. Somasundaram, E. Omar, A., **Malloy, C.** Cooper, R.L.(2016). Activity-dependent modulation of somatosensory processing in *Drosophila melanogaster*: behavior, development, and sensory-motor circuit physiology. Kentucky Academy of Sciences Annual Meeting. November 4-5, 2016, Louisville, KY. Won a cash prize for best undergraduate poster presentation.

28. Mikos, A,, **Malloy, C.**, Samadi, A., Hermanns, C., Sifers, J., Omar, A., Cooper, R.L. Using optogenetics to assess neural influence on heart rate in *Drosophila melanogaster* larvae. Kentucky Academy of Sciences Annual Meeting. November 4-5, 2016, Louisville, KY.

29. Sifers, J., **Malloy, C.**, Mikos, A., Omar, A., Cooper, R.L. Optogenetic expression and activation of rhodopsins in glutamic acid decarboxylase (GAD) containing skeletal and cardiac muscle. Kentucky Academy of Science Annual Meeting. November 4-5, 2016, Louisville, KY.

30. Malloy, C.A., Omar, A., Somasundaram, E. Cooper, R.L. (2016) Pharmacological identification of cholinergic receptor subtypes in modulation of a sensory-motor circuit in *Drosophila melanogaster*. Annual Meeting of Society for Neuroscience. November 12-16, San Diego, CA, USA.

31. Higgins, J., Hermanns, C., **Malloy, C.**, and Cooper, R.L. (2017) Considerations in repetitive activation of light sensitive ionchannels for long-term studies: Channel rhodopsin in the Drosophila model. Annual Meeting of the Kentucky Chapter of the American Physiological Society, Western Kentucky University, March 18, 2017.

32. Somasundaram, E., Omar, A., **Malloy, C.,** and Cooper, R.L. (2017) Activity-dependent modulation of somatosensory processing in Drosophila melanogaster: behavior, development, and sensory-motor circuit physiology. National Conference on Undergraduate Research (NCUR). University of Memphis, Memphis, TN, April 6-8, 2017.

33. Malloy CA, Sifers J, Mikos A, Omar A, Samadi, A, Cooper RL. (2017) Optogenetic analysis illuminates Glutamic acid decarboxylase1 expression in Drosophila larval NMJ. Annual Meeting of the Bluegrass Chapter of the Society for Neuroscience. Lexington, KY. April 21, 2017.

34. Hermanns, C., Higgins, J., **Malloy, C.**, and Cooper, R.L. (2017) Considerations in repetitive activation of light sensitive ion channels for long-term studies: Channel rhodopsin in the Drosophila model. Annual Meeting of the Kentucky Chapter, Bluegrass Society for Neuroscience, Lexington, KY April 21, 2017.

35. Somasundaram, E., Omar, A., **Malloy, C.**, and Cooper, R.L. (2017) Activity-dependent modulation of somatosensory processing in Drosophila melanogaster: behavior, development, and sensory-motor circuit physiology. Annual Meeting of the Kentucky Chapter, Bluegrass Society for Neuroscience, Lexington, KY April 21, 2017.

36. Malloy, C., Sifers, J., Mikos, A., Samadi, A., Omar, A. and Cooper,R.L. (2017) Optogenetic expression and activation of rhodopsins in glutamic acid decarboxylase (GAD) containing skeletal and cardiac muscle. Annual Meeting of the Kentucky Chapter, Bluegrass Society for Neuroscience, Lexington, KY April 21, 2017.

37. Omar, A., **Malloy, C.**, Somasundaram, E., and Cooper, R.L. (2017) Pharmacological identification of cholinergic receptor subtypes in modulation of Drosophila melanogaster sensory-motor circuits. Annual Meeting of the Kentucky Chapter, Bluegrass Society for Neuroscience, Lexington, KY April 21, 2017. Won a cash prize for best undergraduate poster presentation.

38. Cooper, R.L., **Malloy, C.**, Majeed, Z.R., Titlow, J., D.Mahmood, D., Somasundaram, E., Omar, A., Hermanns, C., Higgins, J., Hall, K. (2017)

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Effects in altering activity of sensory systems, motor output, skeletal muscle and neurons containing ACH, dopamine, 5-HT and GABA in Drosophila melanogaster. American Physiological Society. Annual meeting, Chicago, IL. April 22-26.

39. de Castro, C., Titlow, J., Majeed, Z.R., **Malloy, C.**, King, K., and Cooper, R.L. (2017) Mechanical and chemical factors required for maintaining cardiac rhythm in Drosophila melanogaster larva. American Physiological Society. Annual meeting, Chicago, IL. April 22-26.

40. Hermanns, C., Higgins, J., **Malloy, C.**, and Cooper, R.L. (2017) Considerations in repetitive activation of light sensitive ion channels for long-term studies: Channel rhodopsin in the Drosophila model. Undergraduate Showcase of Scholars at the University of KY. April 26, 2017.

41. Omar, A., **Malloy, C.**, Somasundaram, E., and Cooper, R.L. (2017) Pharmacological identification of cholinergic receptor subtypes in modulation of Drosophila melanogaster sensory-motor circuits. Undergraduate Showcase of Scholars at the University of KY. April 26, 2017.

42. Mattingly, M., Dayaram, V., **Malloy, C.**, Zhu, Y.-C., McNall-Krall, R., and Cooper, R.L. (2017) Experiences with course-based undergraduate research experience (CURE) to address authentic research questions for a neurophysiology laboratory class. Society for Neuroscience, Washington, DC. Nov. 11-15 2017.

MANUSCRIPT REVIEW

Reviewed manuscripts/chapters from the following journals or publishers since 2014.

- Journal of Experimental Biology (2014)
- Journal of Insect Physiology (2016)
- Journal of Comparative Physiology B (2016)
- PLOS ONE (2016)

PROFESSIONAL AFFILIATIONS

- 2014-current Kentucky Academy of Science
- 2014-current Society for Neuroscience
- 2014-current American Physiological Society
- 2014-current Bluegrass Society for Neuroscience

TEACHING EXPERIENCE

- **Teaching Assistant,** Department of Biology, University of Kentucky, Lexington, KY
  - Fall (08/2013-12/2013) BIO155 (General Biology Lab) Section 011 & 012

- Spring (01/2014-05/2014) BIO155 (General Biology Lab) Section 012 & 016
- Fall (08/2014-12/2014) BIO 304 (Principles of Genetics Lab) Section 005 & 008
- Spring (01/2015-05/2015) BIO 304 (Principles of Genetics Lab) Section 007 & 008
- Fall (08/2015-12/2015) BIO 350 (Animal Physiology) Section 001 & 002
- Spring (01/2016-05/2016) BIO 446/650 (Neurophysiology Lab) Section 001
- Fall (08/2016-12/2016) BIO 350 (Animal Physiology) Section 001 & 003
- Spring (01/2017-05/2017) BIO 350 (Animal Physiology) Section 001 & 002

OUTREACH

2014

- July 30, 2014. Kentucky Girls STEM Collaborative to host a Girls STEM Day. Presenting lab projects to middle school students from across KY. 3 sets of 10 students. Contact Dr. Carol D. Hanley, University of Kentucky.

- Personal Tutor- Principles of Biology II (BIO 152)

2015

- Leestown Middle School Science Day---Display Participant.

- STEM Summer Camp University of Kentucky---Volunteer

- Lexmark Science Camp- Display Participant

2016-17

- Present a life science activity, "Modulators effect on crayfish fly behaviors," to 3 classes at Sayre High School. Lexington, KY. Contact: R. Holsinger.

- Morton Middle School Science Fair- Judge Glendover Elementary Science Fair- Judge

- Present a life science activity. "Optogenetics and Arduino coding" to classes at Pulaski County High school, KY. Contact: Jennifer Wilson. All day event to drive there to present to the various classes and drive back to Lexington.

- Biotechnology class visit from Pulaski High School. 9 AM to 2 PM visit. Showed lab activities and tour of campus. Contact: Jennifer Wilson.

- STEM Blue Summer Camp session organizer. Presented and lead classes on Optogenetics and fruit fly behaviors.

- National Neurotrauma Society annual meeting. Conference volunteer. Helped set up and take down poster boards for poster presentations.

- Mentor for with course-based undergraduate research experience (CURE) to address authentic research questions for a neurophysiology laboratory class

- May 29, 2016. Presentation to the public. Health aspects and help present high school students' research. A public booth at the Somernites Cruse event in Somerset, KY.

  http://www.somernitescruise.com/ (6 Biotechnology students from Pulaski High School, Cole Malloy, Robin Cooper)

- Presentation to elementary students at Red Oak Elementary for "Career Day." A booth at the elementary school in Nicholasville, KY. May 12, 2017.

UNDERGRADUATES MENTORED

Kyle Ritter, Centre College (KBRIN Summer Research Fellowship)
Jonathan Robinson, Morehead State University (KBRIN Summer Research Fellowship)
Connor English, BS, University of Kentucky
Jacob Sifers, Alice Lloyd College (KBRIN Summer Research Fellowship)
Aya Omar, University of Kentucky
Eashwar Somasundaram, University of Kentucky
Angie Mikos, Georgetown College (KBRIN Summer Research Fellowship)

STUDENT COMMENTS

"Cole was very kind and considerate during lab. Yet he challenged us to work to our very best to get the best results possible."
-BIO 446 Spring 2016
"Great TA!"
-BIO 446 Spring 2016
"Honestly, Cole is the best TA I have ever had. He is a great TA, very understanding of lab issues, very knowledgeable of content and made the 4 hour lab much more enjoyable. He makes his expectations very clear for lab write ups and provides quality feedback."
-BIO 350 Fall 2016

REFERENCES

Dr. Robin Cooper (Advisor)
contact: robinlewiscooper1@gmail.com

Dr. Douglas Harrison (Intradepartmental Committee Member)
contact: dough@email.uky.edu

Dr. Bruce O'Hara (Intradepartmental Committee Member)
contact: bohara@uky.edu

Dr. Wayne Cass (Extra Departmental Committee Member**)**
contact: **wayne.cass@uky.edu**