Examining the Roles of Octopamine and Proctolin as Co-Transmitters in *Drosophila melanogaster*

By

Amanda Lynne Kornel, B.Sc. (Hons.)

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Abstract

The nervous system is a highly complex and intricate system that interacts with and controls nearly all the other body systems. The basic functions of nerve cells are conserved across most species and are very similar between vertebrates and invertebrates. Chemical transmitters (neurotransmitters) facilitate communication between nerve cells and their targets. The effects of these signals can be modified by co-transmitters that are released from neurons in conjunction with neurotransmitters, and by neuromodulators that are released as hormones. This thesis examines the effect of two neuromodulators on neuromuscular junctions of the fruit fly, *Drosophila melanogaster*. Two modulators, proctolin and octopamine, have been identified in motor nerve terminals and are thought to be released as co-transmitters to modify the effects of glutamate, the neurotransmitter that depolarizes muscle cells and triggers contraction.

The neuropeptide proctolin (Arg-Tyr-Leu-Pro-Thr) was found to increase the amplitude of body wall muscle contractions elicited by glutamate in the absence of nerve stimulation. Thus, proctolin appears to enhance contractions by acting postsynaptically. Previous work reported that increasing neural activity lowers the threshold and EC_{50} for proctolin's ability to enhance nerve-evoked contractions by two orders of magnitude. To determine whether such activity-dependence is caused by increased release of glutamate, effects of varying glutamate concentrations on the effectiveness of proctolin are examined here. The threshold for proctolin to increase body wall contractions decreased from 100 nM to 10 nM when glutamate concentration increased from 5 mM to 7 mM, but the threshold increased again to 100 nM for glutamate concentrations of 10-20 nM. Thus, although the effectiveness of proctolin shows

some dependence on glutamate concentration, alterations in glutamate levels do not appear to account entirely for the more substantial and more consistent changes in proctolin threshold that occur with increasing neural activity, reported elsewhere.

Since octopamine in known to be present in motor neurons innervating most of the body wall muscles of 3rd instar larvae, it was hypothesized that stimulating the motor neurons should release octopamine together with glutamate, and that increasing motor neuron activity should increase the release of both octopamine and glutamate. This hypothesis led to the prediction that an octopamine antagonist, phentolamine, should reduce the amplitude of nerve-evoked contractions, and that the antagonist should be more effective when the motor neurons are stimulated at higher frequencies. Phentolamine, however, did not alter the amplitude of body wall muscle contractions elicited by stimulating the motor axons using impulse bursts with intraburst stimulus frequencies of 5, 32 and 50 Hz. Surprisingly, exogenously applied octopamine did enhance the amplitude of nerve-evoked contractions, and, this effect was antagonized by phentolamine when contractions were elicited by impulse bursts with frequencies of 5 and 50 Hz. At a concentration of $1x10^{-6}$ M, octopamine did not induce contractions or alter the amplitude of glutamate-evoked contractions. These results do not support the hypothesis that endogenous octopamine is released onto muscle fibers as a co-transmitter to augment contraction amplitude.

One possible explanation for these findings is the octopamine may be released at higher concentrations at neuromuscular synapses, and the effects of octopamine on nerve-evoked contractions are mediated presynaptically, by increasing transmitter release. Overall, the results of this thesis indicate that both octopamine and proctolin modulate muscle contractions

in an activity-dependent manner; the level of external nerve-stimulus or exogenous glutamate concentration alter the effectiveness of the contransmitters. However, further work is needed to elucidate the mechanisms of such activity-dependence.

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Introduction

The nervous system, which consists of the central nervous system and the peripheral nervous system, is responsible for sensation, perception, learning, memory, behaviour and control of visceral organs (Nicholls et al., 2012). The functional unit of the nervous system is traditionally considered to be the neuron (nerve cell). There are many different shapes and types of neurons, all of which generate electrical and chemical signals. Most neurons are excitable, meaning that they generate electrical impulses that carry information over long distances. This information can be transmitted to other neurons electrically across gap junctions, by the release of chemical signals called neurotransmitters. These chemical signals are received or collected by the dendrites, integrated in the soma (cell body), and either generate or inhibit the production of impulses in the axon of the target cell. Neurotransmitters are released from the synaptic terminals at the distal end of the axon and diffuse across the synaptic cleft between the neuron and its target cell, a distance of approximately 20 – 40 nm (Nicholls, et al., 2012). The neurotransmitters elicit a response in the target cell by binding to receptors in the cell surface (Figure 1). Transmission of signals across these chemical synapses determines whether or not the postsynaptic cell is activated or inhibited, and which neuronal circuits are activated at any time. This transmission ultimately determines which muscles contract at any given time. Thus, events at chemical synapses play a key role behaviour as well as in learning, memory formation and memory recall (Nicholls et al., 2012).

Some neurotransmitters, such as dopamine, serotonin and glutamate, are small molecules, but larger molecules, such as oligopeptides, can also act as neurotransmitters (Nicholls et al., 2012). Small molecule transmitters are synthesized in the nerve terminal and

are packaged into small synaptic vesicles (SSVs) docked near the membrane. During neuronal excitation, electrical impulses leads to vesicle fusion to the plasma membrane, leading to the release of vesicular contents. The vesicles are recycled and refilled with neurotransmitters by chemical transporters that are embedded in the vesicle membrane (Nicholls et al., 2012). This local synthesis and recycling process means that there can be a nearly constant supply of neurotransmitters that are ready and available for release. Larger peptides and biogenic amines are synthesized on ribosomes in the cell body, packaged into large dense core vesicles (LDCVs) and then transported down the axon to the nerve terminal where they are stored prior to release (Wong, et al., 2015).

Some of the small neurotransmitters, such as acetylcholine and glutamate, can act on ionotropic receptors, which are ligand-gated ion channels that open when the transmitter binds to them due to a conformational change in the channel (Nicholls et al., 2012). Ion channels can be specific, allowing only a certain ion in, or they can be non-specific allowing a number of different ions to pass through. Non-specific ion channels can be charge-specific, in that they allow only cations or anions to pass through (Nicholls, et al., 2012). Opening the channel in response to neurotransmitter binding can depolarize the target cell and increase the probability of generating nerve impulses, or it can act to hyperpolarize the cell and decrease the probability of impulse generation (Figure 1). Ionotropic receptors are fast to respond to signalling molecules and have a short activation time.

Neurotransmitters can also bind to metabotropic receptors, whose activation can alter enzyme activity, leading to the production of second messengers and alteration of cellular activity. Many metabotropic receptors are G-protein coupled receptors (GPCRs) that act

through GTP-binding proteins. A third class of receptor involved in cell signalling includes the receptor tyrosine kinases, membrane-spanning proteins with an extracellular binding site and intracellular kinase activity. When a ligand binds, these receptors dimerize and autophosphorylate, which can in turn activate second messenger pathways, leading to changes in gene expression or protein synthesis (Trimmer, 1999). Receptor tyrosin-kinases can also directly phosphorylate ion channels, altering the charge on the channel and causing a conformational change (Trimmer, 1999).

Transmitters are said to be either excitatory or inhibitory, but their effects depend on the receptor to which they bind. Thus, the same transmitter molecule can be both inhibitory and excitatory based on receptor expression. An example of this in seen in *Drosophila*, where DPKQDFRRFamide inhibits the heart during early larval stages but has excitatory effects on larval body wall muscles (Clark, et al., 2008).

A neuron can contain more than one transmitter or neuropeptide, and at many synapses, a "co-transmitter" can be released at the same time as "primary" transmitters are being released (Katz, 1999). Co-transmitters can increase or decrease the response of the postsynaptic cell to the primary transmitter. In addition, some neurotransmitters released from the presynaptic terminal activate receptors on that same cell to modulate transmitter release. This is referred to as "autocrine signaling", which constitutes a feed-back loop that causes the presynaptic cell to release either more or less of the transmitter as required for proper signalling (Nicholls, et al., 2012).

This thesis examines co-transmission using *Drosophila* neuromuscular junction in the body wall muscles as a model system. The goal is to determine whether or not co-transmitters

can modulate the effects of activation of ionotropic receptors by a neurotransmitter. One strategy for achieving this goal is to elicit muscle contractions with the known excitatory neurotransmitter (L-glutamate) at larval neuromuscular junctions and to examine modulation of muscle contractions by proctolin, a neuropeptide thought to act as a co-transmitter and hormone in fruit flies and other arthropods. Another strategy is to stimulate the motor neurons at frequencies that should release both transmitter and co-transmitters, and to block the effects of a co-transmitter (octopamine) with pharmacological agents.

In this thesis, *Drosophila* is used as a model system to investigate how synapses are modulated. Many chemical messengers in invertebrates are also found present in the nervous systems of vertebrate animals, and they act on the same types of receptors, also, many, if not all, of the known second messenger pathways are the same in both invertebrates and vertebrates (Keshishian, 1996; Nicholls et al., 2012; Sanes et al., 2012). Understanding the fundamental principles of synaptic modulation can be achieved using organisms such as the fruit fly, which has fewer neurons than vertebrate animals (Johansen et al., 1989). In *Drosophila* larvae, each body wall muscle cell and its synaptic inputs have been identified and well characterized, making it easier to study synaptic modulation (Atwood et al., 1993; Hoang & Chiba, 2001; Keshishian, 1996). *Drosophila* has other advantages, including a completely sequenced genome and the existence of numerous mutant and transgenic strains that allow one to examine effects of up-regulating or down-regulating expression and/or function of neurotransmitters, their receptors and intracellular signalling pathways (Ormerod et al., 2018). Understanding synaptic modulation will further our knowledge of how the nervous system works and, ultimately, how behaviour is regulated.

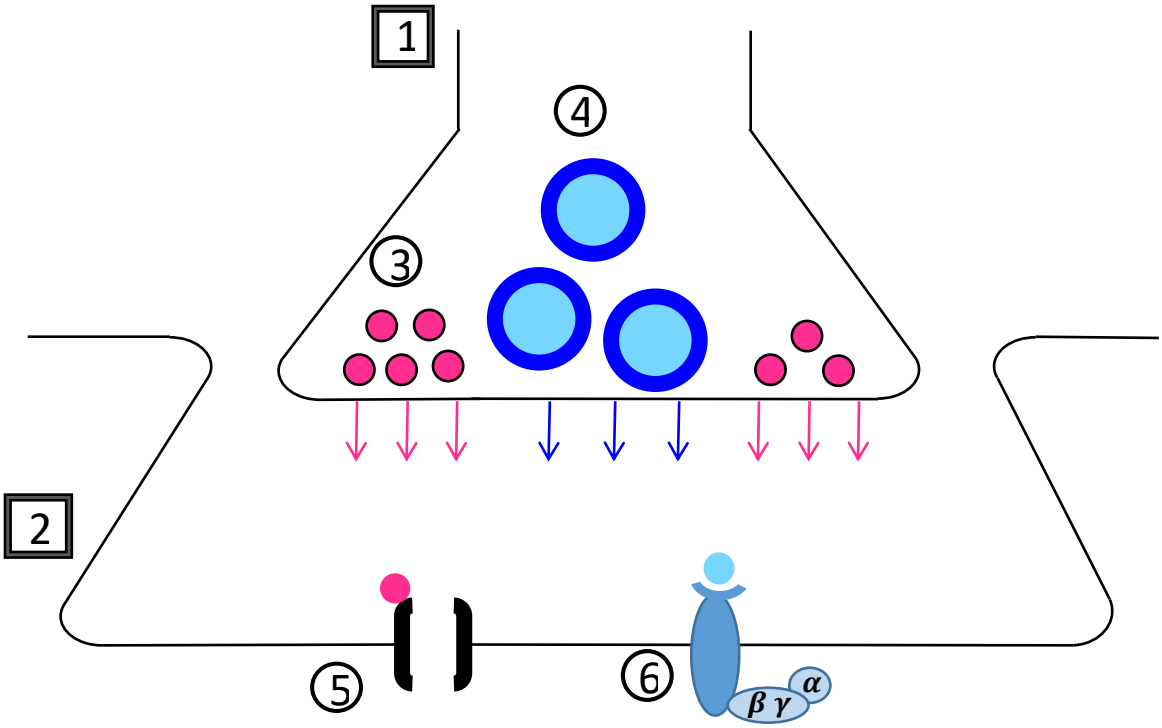


Figure 1: Schematic diagram of a “typical” synapse. In a typical synapse a presynaptic axon terminal (1) is in close proximity to a postsynaptic tissue (2). In the presynaptic terminal neurotransmitter is contained in small synaptic vesicles (3). Co-transmitters are contained in large dense-core vesicles (4). These chemical messengers act on receptors on the postsynaptic tissue, these receptors can be either ionotropic (5) or metabotropic, also known as, G-protein coupled (6) receptors.

Literature Review

Modulation of Chemical Synapses

The basic explanation of how chemical transmission works seems simple and straight forward, but communication at chemical synapses in a living organism is far more complex. When an animal is engaged in activity, many neurons are activated, and multiple transmitters and receptor types are utilized. Synaptic transmission and neuromodulation occur simultaneously when neurons communicate with their target cells, and as a result postsynaptic potentials and physiological responses of postsynaptic cells vary in time, intensity and character (Katz, 1999). In addition, there are many different transmitters that work on many receptors to modify and modulate the electrical activity of any given target cell. The cumulative effect of the activities of various neurons ultimately determines behaviour.

Synaptic modulation has been defined to encompass any observed change in synaptic function (Shain, et al., 1981), and the following are some examples of different types of synaptic modulation. Biogenic amines and neuropeptides can increase the release of neurotransmitters at crustacean and insect neuromuscular junctions, and they can also act directly on muscle cells to induce contraction and/or increase the amplitude of nerve-evoked contractions (e.g. Adams & O'Shea, 1983; Clark et al., 2008; Jorge-Rivera & Marder, 1996; Klose et al., 2010; Kravitz et al., 1980). Biogenic amines and peptides also modulate synaptic transmission within neural networks. For example, biogenic amines can alter electrical coupling between neurons in the pyloric network of the lobster stomatogastric ganglion (Johnson et al., 1993a,b), which generates rhythmic movements of the pylorus during digestion. Some pairs of neurons in this network make both electical synapses and inhibitory chemical synapses with

each other. Dopamine and serotonin increase the strength of electrical coupling between some neuron pairs, increasing the tendancy of those neurons to fire impulses together, and the biogenic amines decrease coupling between other neuron pairs, allowing the inhibitory chemical synapses to dominate and decreasing the likelihood of neuron pairs to fire impulses simultaneously (Johnson, et al., 1993a,b). Thus, effects of the biogenic amines "shape" the output pattern of the pyloric neuron network.

Many examples of modulation have also been reported in studies of crustacean cardiac systems, where heartbeat and cardiac contractions are controlled by the cardiac ganglion and are modulated by effects of biogenic amines and neuropeptides at several levels (Cruz-Bermudez & Marder, 2007; Dickenson et al., 2015, 2019; Fort et al., 2004, 2007; Garcia-Crescioni et al., 2010, 2011; Stevens et al., 2009). These include effects: (a) on the cardiac ganglion itself, (b) on transmitter output from cardic motor neurons, (c) on the cardiac muscle cells, and (d) on peripheral feedback associated with the level of stretch of the myocardial cells. Like crustaceans insect cardiac systems also provide examples of modulation; cardiac rate in Drosophila is modulated by various biogenic amines and peptides throughout all stages of the life cycle (Zornik et al., 1999). These studies demonstrate that " central" modulatory effects on neural systems that generate motor output are coordinated with "peripheral" effects on muscle and neuromuscular synapses.

Modulatory effects can be activity-dependent. In fact, electrical activity, by itself, can modulate synaptic transmission. Different impulse frequencies and/or impulse patterns can elicti various forms of synaptic plasticity, such as facilitation, augmentation, potentiation, long-term potentiation, post-tetanic potentiation, synaptic depression and long-term depression, all

of which involve changes in synaptic transmission (Nicholls et al., 2012). Activity also can change the degree to which chemical modulators alter neurotransmitter release (Breen & Atwood, 1983) and nerve-evoked muscle contraction (Ormerod et al., 2013, 2016). Changes in impulse activity pattern also alters ionic conductances in isolated neurons (LeMasson, et al., 1993).

The term "modulation" can also refer to any intracellular effect caused by the release of a substance from a neuron that is not simply inhibitory or excitatory (Katz, 1999). An example of such modulation is the effect of octopamine on heart rate in the tobacco hawkmoth, *Manduca sexta*, (Prier et al., 1994). In this case octopamine alone at subthreshold levels (10^{-8} M) did not affect the heart, but this concentration potentiated effects of cardio-acceleratory peptides (CAPs; Prier, et al., 1994). The effects of octopamine and CAPs on *Manduca* hearts are synergistic and demonstrate that one modulator can "modulate" the effect of another modulator. It is thought that synaptic modulation occurs because it provides the animal with a gain in transmitter-mediated response but with little to no metabolic cost to the organism (Prier, et al., 1994). Having multiple forms of neuronal communication and modification provides a lot of flexibility to the nervous and neuromuscular systems.

Neuromodulatory effects can also be modulated. This process is referred to as second order modulation or "metamodulation" (Katz & Edwards, 1999). In this process, the effects of one modulatory substance onto target cell(s) represent "first order modulation", and the release of some other modulatory substance changes the release and/or effects of the first order modulator. Metamodulation can occur in at least two ways. In some cases, the second order modulator directly activates or inhibits neurons that release a first order modulator. This

is referred to as “serial modulation”. In other cases, the second order modulator may alter the effectiveness of first-order modulatory neurons by (a) altering the responsiveness of the postsynaptic cell to the first order modulator, (b) altering release of the first order modulator, or (c) altering their content of the modulatory transmitter. In such cases, metamodulation is achieved by “convergent” effects of the second order modulator onto the first order modulator (Katz & Edwards, 1999).

Serial modulation occurs in the teleost fish retina, where peptidergic axons from the olfactory bulb directly activate dopaminergic inner plexiform cells and, in doing so, increase the release of dopamine (Umino & Dowling, 1991; Zucker & Dowling , 1987; both cited by Katz & Edwards, 1991). In this system, dopamine is a first order modulator that alters responses of horizontal cells in the outer plexiform layer when the retina is stimulated with light, and the activity of the dopaminergic cells is modulated by gonadotrophin releaseing hormone (GnRH) released from centrifugal fibers in teleost fish such as the white perch (Umino & Dowling, 1991). Serial modulation also occurs in the locus coeruleus, where serotonin reduces impulse activity in neurons that release noradrenaline to modulate the activity of serotonergic neurons in the dorsal Raphe nucleus (Haddjeri et al., 1997). There is also evidence for serial modulation in insects (Mesce, K.A. 2002; Rauschenbach et al., 2007).

The cardioacceleratory effects of octopamine and CAPs on heart rate in *Manduca*, described above (Prier et al., 1994), provide an example of convergent modulation in which the sensitivity of the target cell to one modulator is altered by another modulator. In fact, subthreshold concentrations of either octopamine or serotonin potentiate the effects of CAPs, indicating synergistic effects of either amine with the cardioacceleratory peptides. The effects

of the two biogenic amines are mediated by cAMP and not by inositol-tris-phosphate (IP_3), and the effects of the CAPs are mediated by IP_3 but not cAMP (Prier et al., 1994). A similar pattern has been reported in other systems, where different agonists that act through distinct second messenger systems can exert synergistic effects, with one agonist enhancing effects of the other at what appear to be sub-threshold concentrations, and even shifting the dose-response curve of the other agonist (Coast, 1995; O'Donnell & Spring, 2000; Ohtomo et al., 1996).

There are reports of one modulator altering the release of another. Some examples include the enhancement of dopamine release by serotonin in the striatum and in ventral tegmental neurons (Brodie & Bunney, 1996; Ichikawa & Meltzer, 1995), and increased release of serotonin by noradrenaline in the hippocampus of the marine bivalve *Mytilus edulis* (Feuerstein et al., 1985; Barraco & Stefano, 1990). Artificially increasing acetylcholine levels in cholinergic *Aplysia* neurons increases the number of quanta of neurotransmitter released per nerve impulse, and artificially decreasing acetylcholine levels has the opposite effect (Poulain et al., 1986). Thus, it is possible to alter neurotransmitter release by modulating neurotransmitter synthesis and/or breakdown experimentally. The synthesis of norepinephrine in superior cervical ganglion cells is regulated by acetylcholine and vasoactive intestinal peptide (VIP), which modulate activity of the enzyme, tyrosine hydroxylase (Ip & Zigmond, 2000), and GPCRs for these modulators and several others do alter norepinephrine release (Kubista & Boehm, 2006).

Modulators in Invertebrates

There are many substances, including peptides and biogenic amines (Prier, et al., 1994), that function as modulators in invertebrates. Peptides and biogenic amines tend to be longer

acting than small molecule neurotransmitters, and so they are thought to have a modulatory role (Katz, 1999). Neuropeptides can act as neurotransmitters, neuromodulators or neurohormones. There is a vast number of neuropeptides in the animal kingdom. A database of proneuropeptides from the various animal phyla contains 6,225 distinct sequences, each of which can encode one or more distinct neuropeptides (Jekely, 2013). Transcriptome analyses of several arthropods have revealed 105-216 distinct peptides in any single species (Christie, et al., 2015).

In *Drosophila* there are 42 genes encoding neuropeptide precursors, and from these gene sequences 75 distinct neuropeptides are predicted (Nassel & Winther, 2010). These peptides can be grouped into families that share similar structure and function. Among these families are the allatostatins, a family of peptides that were first sequenced from the cockroach and found to inhibit synthesis of juvenile hormone (Marco, 2004). Since their discovery there have been three structural forms (A-, B- and C- types) found in insects. The A-allatostatins in insects have myoinhibitory effects and can modulate the activity of midgut enzymes (Marco, 2004). There are four allatostatin isoforms thought to be in *Drosophila melanogaster* and two receptors have been cloned (Lenz, et al., 2001). Another family of peptides in arthropods are the FMRFamides; members of this family generally share the carboxy terminal seqeunce –FMRFamide sequence, but there are some sequence variations. FMRFamide was first identified in the clam *Macrocallista nimbosa* and found to regulate cardiac activity (Price & Greenberg, 1977). In *Drosophila melanogaster* there have been eight FMRFamides identified and named dFMRFa-1 through dFMRFa-8 (Nassel & Winther, 2010). FMRFamides and similar peptides

(FLRFamides) affect a range of physiological processes in invertebrates, such as skeletal and visceral muscle actions, cardiac function and synaptic transmission (Mercier, et al., 2003).

Biogenic amines are a class of neuromodulators that are small, charged molecules synthesized from amino acids and containing an amino group ($R\text{-}NH_2$). In invertebrates this group includes dopamine, serotonin, histamine, octopamine and tyrosine (Widmaier et al., 2019; Monastirioti et al., 1999; Blenau & Baumann, 2001). Biogenic amines have a wide range of physiolgical and behavioural functions including cardiac regulation, circulation, reproduction (mating and egg laying), agression, locomotion, ion regulation, feeding and digestion.

Proctolin

Proctolin is a pentapeptide that appears to be found exclusively in arthropods. It was originally isolated from the cockroach hindgut (Brown, et al., 1975) and subsequently has been found in nearly all arthropod species (Schwartz, et al., 1980). Having only five amino acids (arginine-tyrosine-leucine-proline-threonine) it is a relatively small peptide. It is thought to be synthesized and packaged in to dense core vesicles in the cell bodies of neurons and then transported down the axon to the synaptic terminals (Bishop et al., 1987; Atwood et al., 1993). In arthropods, proctolin is thought to act as a co-transmitter and a neurohormone (Lange, 2002; Orchard et al., 1989; Sullivan & Newcomb, 1982).

In crustaceans, proctolin is localized in the pericardial organs, a major site for release of neurohormones into the circulation (Cooke & Sullivan, 1983; Schwarz et al., 1984; Siwicki et al., 1985), and it is also present throughout the central nervous system and is localized in motor neurons innervating superficial flexor muscles of the crayfish abdomen (Bishop et al., 1984). Bath application of proctolin to crayfish abdominal flexor muscles does not induce muscle

contraction and has no effect on the resting membrane potential, but it does enhance nerve-evoked muscle contractions without altering either excitatory junction potential (EJP) amplitude or contraction duration (Bishop et al., 1987). Stimulating the flexor motor neurons and the pericardial organs releases proctolin in sufficient amounts to be detected with bioassays and radioimmunoassays, respectively (Bishop et al., 1987; Schwarz et al., 1984). Thus there is fairly compelling evidence to support roles for proctolin as both a neurohormone and a neurotransmitter in crustaceans. Proctolin is also present in neurons supplying the crayfish hindgut (Mercier et al., 1997), and bath-applied proctolin increases the amplitude and frequency of hindgut contractions in the crayfish (Mercier & Lee, 2002).

In cockroaches, proctolin is present in the corpus cardiacum, a major neuroendocrine organ (O'Shea et al., 1984) and in motor neurons innervating leg muscles that release proctolin as a co-transmitter to modulate contractions elicited by glutamate (Adams & O'Shea, 1983). Proctolin plays physiological roles in reproduction, as demonstrated by studies of the locust oviduct, where it acts as both a co-transmitter and neurohormone (Lange, 2002; Orchard & Lange, 1986). In the locust ovipositor muscle, proctolin increases the amplitude of contractions and appears to be required to maintain the digging behaviour associated with egg-laying (Belanger & Orchard, 1993).

Because proctolin is able to enhance nerve-evoked contractions but doesn't alter the size or shape of excitatory post-synaptic potentials (EPSP's) in muscle cells (Bishop et al., 1987; Wilkens et al., 1985), it has been inferred that proctolin does not alter neurotransmitter release from nerve terminals but acts on receptors on the post-synaptic muscle cell. Genes encoding proctolin receptors have been identified in *Drosophila* (Egerod et al., 2003; Johnson et al., 2003)

and lobster (Christie, et al., 2015). Due to the receptors' amino acid sequence and predicted structure, which includes a domain with seven membrane-spanning sequences, they are classified as metabotropic G-protein coupled receptors (GPCRs). Based on crystal structure studies of the *Drosophila* proctolin receptor, it has been shown that, in what is most likely the biologically active conformation of proctolin, each of the five amino acids in the peptide is available to bind to or interact with the receptor, and therefore can contribute to its bioactivity (Howard et al., 2010). Proctolin analogs that have been modified at either the 1, 2, or 5 position show changes in threshold and EC_{50} in both the hindgut and foregut of the cockroach *Blaberus craniifer* (Mazzocco-Manneval, et al., 1998). Although the lobster (*Homarus americanus*) has two proctolin receptors (Chrisite et al., 2015), only one appears to be present in *Drosophila* (Egerod, et al., 2003; Johnson, et al., 2003).

In *Drosophila* 3rd instar larvae, proctolin-like immunoreactivity is localized in cell bodies of the central nervous system, nerve-endings on the hind gut and nerve endings on the body wall muscles of the abdominal segments (Anderson et al., 1988). The gene for the *Drosophila* proctolin receptor, which is a GPCR, was identified in 2003 (Johnson, et al., 2003; Egerod, et al., 2003). Through studies using Chinese hamster ovary (CHO) cells and studies with human embryonic kidney (HEK) cells, the identified receptor was found to be selective for proctolin over other insect neuropeptides (Johnson, et al., 2003). Northern blots of the mRNA showed expression of the identified receptor in the CNS, heart, hindgut and malphighian tubules (the excretory and osmoregulatory system in insects) (Egerod, et al., 2003; Johnson, et al., 2003). Although the receptor has been identified as a GPCR, the intracellular signals that mediate protolin's effects in *Drosophila* have not yet been established. There is some evidence in the

foregut and hindgut of cockroach, *Blaberus craniifer*, that the phospholipase C pathway is involved (Mazzocco-Manneval, et al., 1998; Singer et al., 2002), and there is also some evidence that inositol phospholipid hydrolysis is a component of the proctolin signalling pathway in locust oviducts (Lange, 1988).

Proctolin applied in the bathing solution is able to elicit contractions in body wall muscles of 3rd instar *Drosophila* larvae (Ormerod et al., 2016). This effect occurs after removing the central nervous system and in the absence of nerve stimulation, and the concentrations of proctolin needed to elicit this effect are relatively high (10^{-7} to 10^{-5} M) (Ormerod, et al., 2016). Lower concentrations of proctolin (10^{-10} to 10^{-7} M) increase the amplitude of nerve-evoked contractions in these muscles, and this effect is probably physiologically relevant. Due mainly to the small size of *Drosophila*, it has not been possible to estimate the amount of proctolin release.

Octopamine and Tyramine

Octopamine (OA) is a biogenic amine and is considered to be the invertebrate analog of norepinephrine due to its similar structure and involvement in the stereotypical "fight or flight" responses (Roeder., 1995, 1999; Verlinden et al., 2010). In humans, octopamine is considered a stimulant, so much so that it has been banned in professional sports by the World Anti-Doping Association (WADA) (https://www.wada-ama.org/en/content/what-is-prohibited/prohibited-in-competition/stimulants). Octopamine has been found in trace amounts in the mammalian central nervous system and acts on the trace amine receptor TA1 (Borowsky, et al., 2001). In invertebrates, octopamine is synthesized via a two-step process, in which tyrosine is first converted to tyramine by the enzyme tyrosine decarboxylase, and tyramine is then converted

to octopamine by the enzyme tyramine-ß-hydroxylase (Cooke & Sullivan, 1982; Cole, et al., 2005). In invertebrates octopamine has been established as a neurotransmitter, a neurohormone and a neuromodulator. Like proctolin and many other modulators, octopamine is found in neurosecretory cells in crustacean pericardial organs (Cooke & Sulllivan, 1982), and it modulates chemical synaptic transmission at neuromuscular synapses and enhances muscular force in crustacean muscles (Breen & Atwood, 1983; Harris-Warrick & Kravitz, 1984; Kravitz et al., 1980). In insects, octopamine modulates visceral muscles and exoskeletal muscles and is released both locally and as a neurohormone (Evens, 1984; Evans & O'Shea, 1978; Monastirioti et al., 1999; Orchard, 1990; Orchard & Lange, 1985, 1987; Ormerod et al., 1993).

Octopamine plays important roles in insect social behaviors. In honey bees octopamine helps developing worker bees transition from nursing to foraging behaviours (Barron et al., 2002; Schulz et al., 2002). This effect involves modulation of olfactory inputs in the mushroom bodies (Schulz et al., 2001), a region in the insect CNS with extensive neuropil. The mushroom bodies have very high levels of octopamine receptors (Han et al., 1998) and are involved in olfactory memory (Hammer, 1993). In *Drosophila,* octopamine is important in fighting (Dierick, 2008; Hoyer et al., 2008), courtship (Certel et al., 2007, 2010), egg laying (Monastirioti et al., 1996; Rezeval et al., 2014), the initiation and maintenance of flight (Brembs et al., 2007) and larval locomotion (Sarawati et al., 2004).

When octopamine is released from the peripheral nervous system it has been found to have an effect on muscle contractions, altering both the basal tonus and the peak force of contractions (Koon et al., 2011; Ormerod, et al., 2013). It has been shown that octopamine enhances the amplitude of excitatory junction potentials without changing the amplitude of

spontaneous miniature excitatory junction potentials, suggesting octopamine is increasing the number of quanta being released per nerve impulse (Koon et al., 2011).

There are four known octopamine receptors in *Drosophila* larva, which are: OAMB, Octβ1R/OA2, Octβ2R and Octβ3R, all of which are GPCRs (El-Kholy, et al., 2015). The receptor Octβ2R shows strong expression in the body wall muscles of the third in-star larva and is expressed in both dorsal and ventral regions in all muscle types including oblique, acute, transverse and longitudinal muscles (El-Kholy, et al., 2015). This expression pattern carries over to the adult fly. There is weak expression of the Octβ2R in the larval central nervous system.

Tyramine is a precursor to octopamine, and it was once thought that this was its only purpose in invertebrate neurons (Roeder, 1999). It is now known that tyramine can itself act as a neurotransmitter and a neuromodulator in invertebrates (Alkema et al., 2005; Donini & Lange, 2004). In staining studies tyramines expression patterns do not match those of octopamine (Saudou, et al., 1990). Interestingly it often has been found to have opposite effects to octopamine due to it acting on different receptors that are tyramine specific receptors. *Drosophila* have three tyramine receptors that are GPCRs, and these receptors have different expression patterns, some distinct from octopamine expression and some that overlap such as in the brain (El-Kholy, et al., 2015). Tyramine has inhibitory effects in some tissues examined. For example, tyramine applied to the oviduct of the locust lowers the tonus of the muscle in a dose-dependent manner (Donini & Lange, 2004). In *Caenorhabditis elegans* tyramine inhibits egg laying; it is released from tyraminergic UV1 cells and acts in a paracrine manner on egg-laying muscles to inhibit egg laying behaviour (Alkema, et al., 2005).

Drosophila melanogaster as a model system to study modulation

Drosophila melanogaster has been a common model animal used in biology labs for more than a century (Kohler, 1994). It has been found to be useful for investigations of neurotransmission and synaptic plasticity research as well as behaviour and even human genetic diseases (e.g. Keshishian et al., 1996; Metpally & Sowdhamini, 2005; Nässel, 2018; Ormerod et al., 2018). There are many things that make *D. melanogaster* a good model species for research. Some advantages of this species include: (a) the presence of a fully sequenced genome, (b) life cycle of approximately 10 days from egg to adult, which facilitates studies of development and production of mutant and transgenic lines, (c) easy care and low maintenance costs.

The *Drosophila* life cycle includes three larval stages referred to as instars. The first instar is the smallest and is when the larva has just emerged from the egg. Second instar larvae are getting larger but are still living within the food source. Third instar larva are referred to as the wandering stage because at this stage they leave the food source and begin looking for a place to pupate. All larval stages require approximately four days of progression. The third instar is the most common larval stage used for experiments because it is the largest. A full grown *Drosophila* larva is about 3-4 mm in length (Jan, et al., 1976). The larval body can be divided into segments, there are three thoracic and eight abdominal segments (Nusslein-Volhard & Wieschaus, 1980). Each of the abdominal segments contains a repeated pattern of muscle cells making up the body wall muscle; each hemi-segment contains 30 muscles. Forward locomotion is achieved by sequential contraction of muscles from posterior to anterior segments (Berrigan & Pepin, 1995; Matsunaga et al., 2017).

Glutamate is an excitatory neurotransmitter found in both vertebrates and invertebrates. Most motor neurons in arthropods are glutamatergic (Atwood, 1982; Takeuchi & Takeuchi, 1964; Usherwood, 1967, 1968; Walker et al., 1996). It is the primary excitatory transmitter in the *Drosophila* neuromuscular junction (Jan & Jan, 1976) as demonstrated by combining electrophysiological recordings and iontophoresis techniques. Glutamate applied iotophoretically near synaptic terminals produces depolarizations that closely mimic the amplitude and time course of excitatory junction potentials (ELPs). When the application was moved further from the axon terminal the amplitude of EJPs decreases, suggesting that glutamate acts specifically at the active zone (Jan & Jan, 1976). The reversal potentials of glutamate-evoked potentials and EJPs are identical, and bath application of glutamate at concentrations that desensitize glutamate receptors decreases the amplitude of (a) depolarizations elicited by iontophoretic application of glutamate, (b) EJPs, and (c) spontaneous miniature EJPs (Jan & Jan, 1976).

The glutamate receptors are localized in muscle cells at the *Drosophila* neuromuscular junctions, directly under the presynaptic terminals, and these receptors are homologous to the AMPA receptor found in vertebrates (Marrus, et al., 2004). The ionotropic glutamate receptor in the *Drosophila* body wall muscles are comprised of three possible subunits, each receptor contains the subunit DGluRIII and either DGluRIIA or DGluRIIB (Marrus et al., 2004). Synapses with lower levels of DGluRIIA than DGluRIIB have lower quantal size, suggesting that there are subtle differences in responsiveness between glutamate receptors in *Drosophila* muscle (Petersen et al., 1997). *Drosophila* motor neurons have distinct axon terminal (bouton) types that can be identified based on size, location and the type of transmitters they release (Atwood

et al., 1993; Gorczyca et al., 1993; Jia et al., 1993; Keshishian et al, 1993). Type I terminals contain primarily small synaptic vesicles and include “small” boutons (type Is) and “large” boutons (Ib). Type II and Type III boutons contain large numbers of small synaptic vesicles and larger, dense core vesicles. Glutamate is present in the small synaptic vesicles; the dense core vesicles contain octopamine and neuropeptides in type II terminals (Gramates & Budnik, 1999; Monastirioti et al., 1995) and insulin-like peptides in type III terminals (Gorczyca et al, 1993). Peptides present in type II terminals include proctolin (Anderson et al., 1988; Taylor et al, 2004), pituitary adenylate cyclase activating peptide (PACAP; Zhong & Peña, 1995) and leucokinin (Cantera & Nässel, 1992). Interestingly, glutamate receptors expressed below 1b and 1s terminals have differenct proportions of GluRIIA and GluRIIB (Marrus et al., 2004), suggesting some matching of receptor type with nerve terminal type.

In the early studies of *Drosophila* neuromuscular physiology, neuromuscular preparations from third instar larvae were bathed in “Solution A” (Jan & Jan., 1976), which was considered a “standard” solution. Later work showed that in this solution, muscle fibers showed vacuolation, the formation of small holes in the tissue, within the first minute of exposure and a gradual loss of the resting membrane potential (Stewart et al., 1994). Schneider’s medium was used as an alternative bathing solution, and this reduced the formation of vacuoles and made the nerve terminals easy to identify. In Schneider’s medium the preparation could be maintained over several hours but synaptic transmission was blocked, so this solution could not be used for electrophysiological studies (Stewart, et al., 1994). To overcome these problems, natural hemolymph contents were re-measured, and the results were used to develop a better physiological saline to ensure the health of the preparation over long time periods. One such

solution, haemolymph-like-3 (HL-3) saline was considered reliable for physiological studies because the muscle fibers formed fewer vacuoles and EJP amplitudes remained unchanged over two hours (Stewart, et al., 1994). The first version of HL-3 developed had a high concentration of magnesium (20 mM), which reduced membrane excitability. Reducing the magnesium level to 4 mM restored membrane excitability (Feng, et al., 2004). Since normal HL-3 solution contained sucrose but lacked any amino acids, another saline was developed to more closely resemble *Drosophila* hemolymph. This solution, Haemolymph-like-6 (HL-6), contains ten amino acids and a substantially higher concentration of the naturally found blood sugar for *Drosophila*, trehalose (Macleod, et al., 2002). This saline allowed the production of more consistent muscle contractions compared to HL-3, and it was suitable for experiments lasting 20-30 minutes.

Some recent studies of synaptic modulation in *Drosophila* 3rd instar larvae have focused on effects of proctolin and octopamine on neuromuscular synapses and muscle contraction (e.g. Koon et al., 2011; Ormerod et al., 2013, 2016). Both of these modulators are present in motor axons innervating the body wall muscles and, thus, are thought to act as co-transmitters (Anderson et al., 1988; Monastirioti et al., 1995; Taylor et al., 2004). Both proctolin and octopamine induce contractions of the body wall muscles and increase the amplitude of nerve-evoked contractions.

Proctolin was able to enhance nerve-evoked contractions and the threshold and EC_{50} for this effect was activity-dependent. As the frequency and number of impulses given was increased from 2 Hz (1 impulse) to 32 Hz (9 impulses) the threshold for proctolin to enhance contractions dropped by two orders of magnitude (Ormerod et al., 2016). This suggests that

proctolin's ability to enhance nerve-evoked contractions is activity-dependent. This activity-dependence of proctolin could be due to presynaptic or postsynaptic mechanisms. Increased presynaptic activity may be releasing more of the excitatory transmitter glutamate from motor neurons which subsequently increases the level of depolarization in muscle cells, leading to higher intracellular calcium concentrations postsynaptically. Since calcium is a ubiquitous second messenger, an increase in calcium in muscle cells might activate intracellular signaling pathways that ultimately decrease the threshold for proctolin to increase contraction amplitude. Another possible mechanism could involve increased release of proctolin and/or other co-transmitters from the axon terminals with increased activity. It is possible that endogenously released proctolin (or other cotransmitters) might act in an additive manner with bath-applied proctolin to increase contractions.

The nerve-evoked stimulation protocol involves the suction of as many of the cut nerves as possible into a suction electrode and stimulation of all these nerves at once. Using this method, it can be assumed that many, if not all, the nerves innervating the body wall muscles are being stimulated and therefore releasing their contents. As the stimulation intensity increases more of the small synaptic vesicles as well as the dense core vesicles release their transmitters and modulators onto the muscles.

Depolarizing the *Drosophila* body wall muscle cells directly by applying glutamate in the bathing solution is a way of examining the postsynaptic mechanism without the interference of other co-transmitters being released. Glutamate-evoked contractions can be used to examine whether there is a postsynaptic mechanism responsible for the drop in threshold for proctolin to enhance nerve-evoked contractions, as seen by Ormerod et al (2016). Octopamine is

reported to have both a pre- and a postsynaptic effect and therefore possibly both a pre- and postsynaptic mechanism may be involved in the reported effects seen with application of octopamine.

Hypothesis and Predictions

Communication at synapses involves the release of multiple substances and modulation by multiple substances. An animal's physiological and therefore behavioral outcome depends on how all of these substances work in concert. The overall goal of this thesis is to better understand how various co-transmitters interact and modulate chemical synapses. Towards this aim, some specific questions are addressed. The question is whether increased glutamate release alters the responsiveness of muscle cells to proctolin. This question is intended to address the hypothesis that the ability of neuronal activity to reduce threshold and EC_{50} for proctolin's ability to enhance nerve-evoked contractions (Ormerod at al., 2016) results from increased release of glutamate from the motor neurons. This hypothesis leads to the prediction that increasing the concentration of glutamate in the bath solution should lower the threshold for proctolin to increase glutamate-evoked contractions by up to two orders of magnitude (Ormerod et al., 2016).

The second question addressed in this thesis is whether or not octopamine is released from motor neurons and acts directly on the muscle cells to modulate muscle contractions. This question addresses the hypothesis that octopamine is released by motor neurons onto muscle cells as a co-transmitter with glutamate in order to enhance contractions. This hypothesis leads to three predictions that can be tested, which are: (a) that octopamine should increase contractions elicited by applying glutamate directly to larval muscles, (b) that adding an

antagonist for octopamine, such as phentolamine, should decrease octopamine's ability to enhance glutamate-evoked contractions, and (c) that adding an octopamine antagonist, such as phentolamine, should decrease the amplitude of nerve-evoked contractions even when octopamine is not added to the bathing solution.

Materials and Methods

Fly culture and dissection

All experiments were performed using naive wandering stage third instar larvae (male and female) of Canton S *Drosophila melanogaster* originally obtained from Bloomington *Drosophila* Stock Center (Bloomington, Indiana). Animals were housed in small groups at room temperature and kept on a 12-hour light/dark cycle. They had a constant supply of commercial fly food, Formula 424, purchased from Carolina Biological Supply Company (Burlington, NC, USA).

Larvae were dissected in the same manner for all the experiments. Each third-instar larva was carefully removed from the vial in which it was housed and was placed in a dissection dish. The dish had a one milliliter well in the center, surrounded by a magnetic lining to support the dissection pins. The animal was pinned dorsal side up with a pin on the most anterior segment and a second pin on the most posterior segment. Physiological saline (Table 1) was added to the dish to replace the hemolymph lost when the animal was subsequently cut. A cut was made along the dorsal central line using fine dissecting spring scissors, beginning approximately 2 segments rostral to the tail and ending about 2 segments behind the head. The central nervous system and visceral organs were carefully removed, and the hemolymph was replaced with approximately 1 mL fresh saline. Every trial was performed on a fresh larvae, only one solution was used per animal.

Glutamate-evoked contractions

In all trials involving glutamate-evoked contractions, the posterior end of the larva was attached to a force transducer by a fine hook inserted into the larva, and the head remained

pinned down (Figure 2). A catheter was placed between the flaps of cut cuticle in order to continuously pump the solutions directly onto the body wall muscles. Excess liquid was removed with a suction catheter near the edge of the dish. The resting muscle tonus was established by gently stretching the larva until a flat, steady tonus was established in the recording. Having the resting tonus established it was used as the baseline tonus for measuring the contraction amplitude in all future experiments.

Recordings were made first in saline to establish resting tension. After five minutes the solution was changed to either glutamate or to one containing a combination of glutamate and either proctolin or octopamine for one minute. The larva was then bathed in saline for five minutes to allow the tension to return to resting level. At the end of every trial 300 mM KCl was applied to elicit maximal contraction. This procedure ensured that the larva was viable and provided a standard for comparing contraction amplitude between different larvae.

Tonus experiments

Octopamine's ability to induce contractions was examined by measuring changes in muscle tonus. The larvae were dissected in the same manner and attached to the force transducer. They were gently stretched to create a relatively flat baseline muscle tonus recording. Saline (HL-6) containing 0.5 mM calcium was applied for five minutes to establish the baseline, then the solution containing octopamine or phentolamine was applied for five minutes. The solution was washed out with saline for another five minutes followed by application of 300 mM KCl to induce the maximum contraction. A change in tonus was measured by subtracting the tonus in the presence of neurotransmitter (nine minute time point) from the tonus in the presence of saline (four minute time point):

$$Tonus_{transmitter} - Tonus_{saline} = \text{change in muscle tonus}$$

The results were reported in millinewtons (mN).

Nerve-evoked contractions

Nerve-evoked trials were performed on larvae that were dissected in the same way as for experiments examining glutamate-evoked contractions. However, for this set of experiments a suction electrode was used to suck up as many of the cut segmental nerves as possible. Electrical stimuli were applied to the nerves using a Grass electronic stimulator (Model S88) and a Grass Stimulus Isolation Unit. Bursts of stimuli were required to elicit muscle contractions. Individual stimuli were 0.5 ms in duration, and the stimulus intensity was maintained above threshold during each trial.

Chemicals

The majority of experiments were performed using *Drosophila* hemolymph-like 6 (HL-6) saline (Table 1). The pH of the saline was adjusted to 7.2 using NaOH or HCl. The components of the saline were purchased from BioShop. Glutamate evoked trials were performed using saline that had 0.5 mM calcium added while nerve-evoked trials used 1.5 mM calcium containing saline. In one set of experiments a modified *Drosophila* hemolymph-like 3 (HL-3) saline, containing 0.5 mM calcium, was used. It was made in the lab in the same manner as HL-6 and adjusted to a pH of 7.2.

Proctolin (Abbiotec, LLC, San Diego, USA) and octopamine (Sigma-Aldrich, Oakville, Canada) were dissolved in distilled water to make a stock solution. These stocks were frozen and stored at -20^{o}C and were thawed before use at room temperature and diluted with saline as required. Solutions for trials were made fresh daily.

Data analyses

All experiments were recorded on a PC-compatable computer using a Model DI-145 DATAQ acquisition system and software (Akron, OH). Statistical analysis was performed using GraphPad Prism software. ANOVA was used for statistical comparisons unless otherwise stipulated in the results.

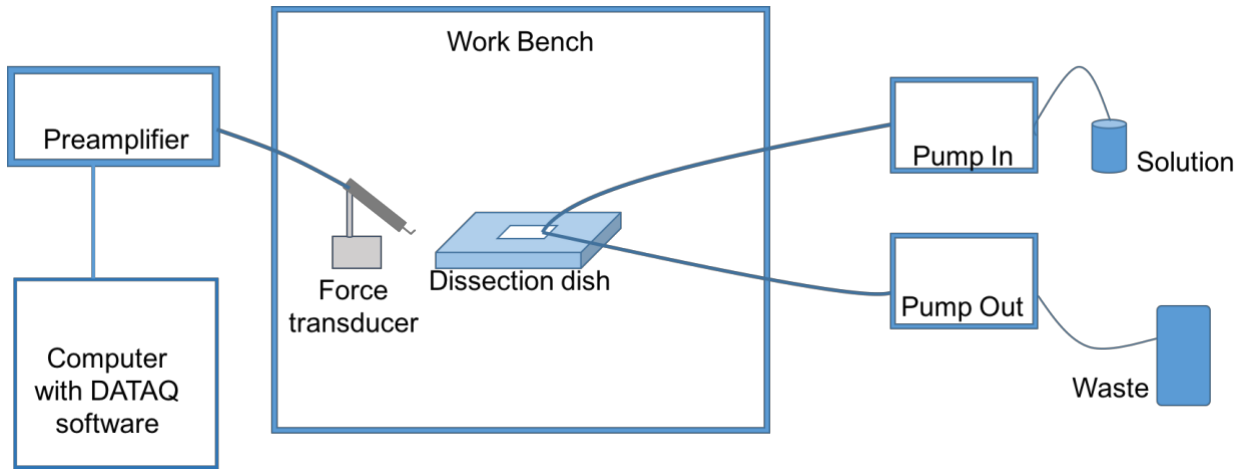
In most trials contractions were expressed as a percentage of the maximal contraction elicited by 300 mM KCl in that preparation. This compensated for differences in larval size and degree of damage during dissection. Although every effort was made to use animals of similar size and age, slight differences could have altered contraction amplitude.

To quantify proctolin's ability to enhance contractions, the baseline tonus was subtracted from the peak of the glutamate-evoked contraction. The same procedure of subtracting baseline from the peak was performed for the KCl-induced contraction at the end of each trial.

Effects of modulators on nerve-evoked contractions were expressed as a percentage change. Four contractions immediately before the application of the modulator ($Contraction_0$) were averaged and were compared to the average of the last four contractions recorded in the presence of the modulator ($Contraction_M$). Percentage change was calculated as:

$$[(Contraction_M - Contraction_0)/(Contraction_0)] \times 100.$$

A



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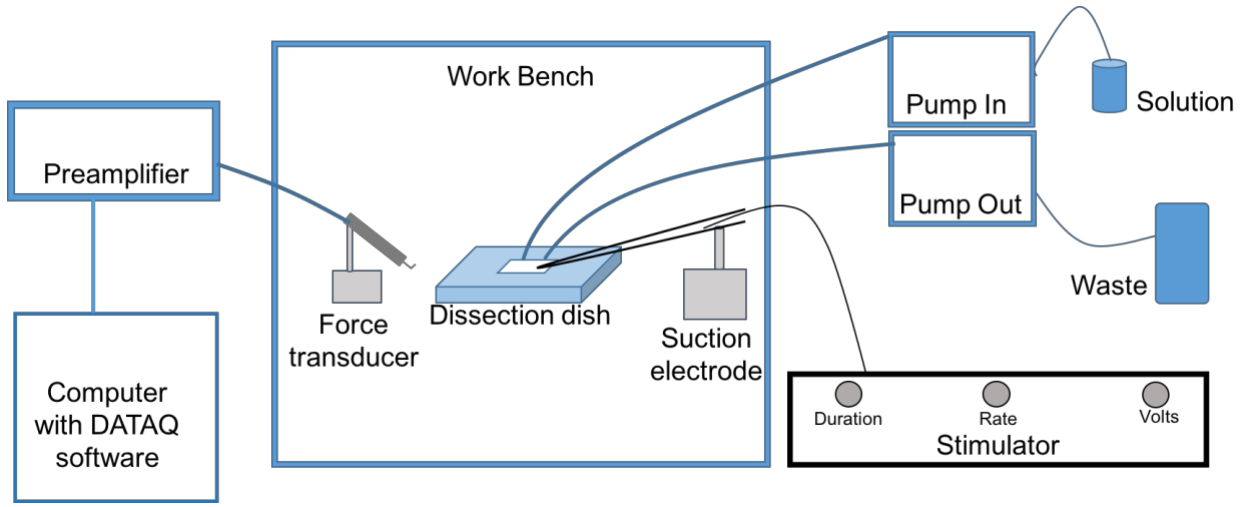


Figure 2: Schematic diagram of the work bench set up. A) Glutamate-evoked contractions set up B) Nerve-evoked contraction. A glass pipette suction electrode and a stimulator were added to the second set up in order to "suck up" the cut nerve ends and stimulate the nerves.

Table 1: Saline components and concentrations.

Reagent	HL-6 Concentration (mM)	Modified - HL-3 Concentration (mM)
$CaCl_2$-$2H_2O$	0.5 or 1.5	0.5
NaCl	23.7	70
KCl	24.8	5
$MgCl_2$-$6H_2O$	15	20
$NaHCO_3$	10	10
Trehalose	80	5
Sucrose	-	115
Isethionic Acid	20	-
BES	5	-
HEPES	-	5
Alanine	5.7	-
Arginine	2	-
Glycine	14.5	-
Histidine	11	-
Methionine	1.7	-
Proline	13	-
Serine	2.3	-
Threonine	2.5	-
Tyrosine	1.4	-
Valine	1	-
pH	7.2	7.2

Results

Increasing the frequency and number of impulses generated in the motor neurons was shown previously to decrease the threshold for proctolin to increase the amplitude of nerve-evoked muscle contractions of *Drosophila* body wall muscles (Ormerod et al., 2016). Since glutamate is the excitatory transmitter released onto these muscles, (Jan & Jan., 1976), the reduction in threshold might be due to increased release of glutamate with increased neural activity. This possibility was examined by evoking muscle contractions with glutamate and determining whether increasing glutamate concentration decreases the threshold for proctolin to enhance muscle contractions. Bath application of L-glutamate for I min elicited contractions at glutamate concentrations of 3-60 mM (Figure 3). To compensate for possible size differences between larvae and damage to muscle fibers during dissection, contractions were expressed as a percentage of the maximal contraction elicited by 300 mM KCl solution (Figure 3). Increasing the concentration of glutamate increased the amplitude of the contractions from approximately 2% of maximum with 3 mM glutamate to approximately 33 % of maximum with 30 mM glutamate. A concentration of 60 mM glutamate caused no further increase in contraction amplitude (Figure 4).

To examine the effects of proctolin on glutamate-evoked contractions, proctolin and L-glutamate were applied together for 1 min, and the resulting contractions were compared to control trials in which L-glutamate was applied alone. This procedure avoided effects that might alter the response to glutamate, such as desensitization of glutamate receptors (Jan & Jan., 1976) and potential activation of second messenger systems in response to a rise in intracellular calcium levels during the muscle contraction. Proctolin increased the amplitude of

glutamate-evoked contractions reliably at a concentration of 100 nM (Figure 7). Since this effect occurred in the absence of nerve stimulation, it most likely involves a postsynaptic mechanism. This is consistent with previous observations in *Drosophila* and other species showing that proctolin modulates muscles postsynaptically without altering membrane potential or EJP amplitude (e.g. Bishop et al., 1987; Lange, 2002; Mercier & Wilkens., 1985; Ormerod et al., 2016). Figure 6 shows sample recordings of proctolins ability to enhance glutamate-evoked contractions. Because 7 mM glutamate elicited consistent contractions that were large enough to easily and reliably measure, but still small enough to show enhancement by proctolin, and other co-transmitters, this concentration was selected to show the sample recordings.

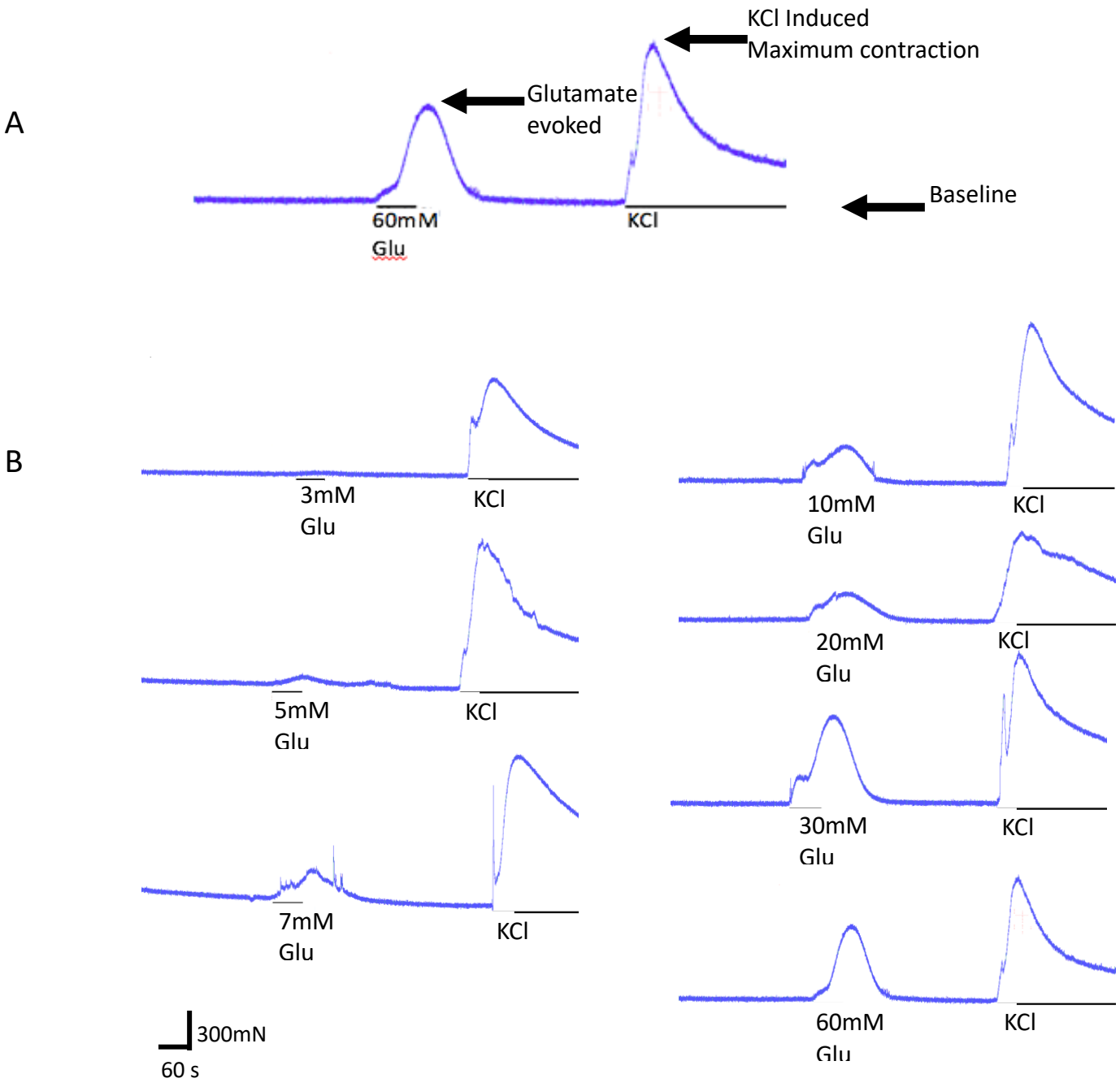


Figure 3: Representative force recordings of glutamate-evoked contractions. (A) An example of where each measurement was taken. (B) Each recording started with five minutes of exposure to HL-6 saline (0.5 mM calcium) to establish a baseline for comparisons. Glutamate was applied for one minute at the times indicated by the black bars under each recording. Following glutamate application, the preparation was washed in saline to re-establish baseline tone. Every trial was ended with a five-minute application of 300 mM KCl to provide a maximal contraction for that preparation.

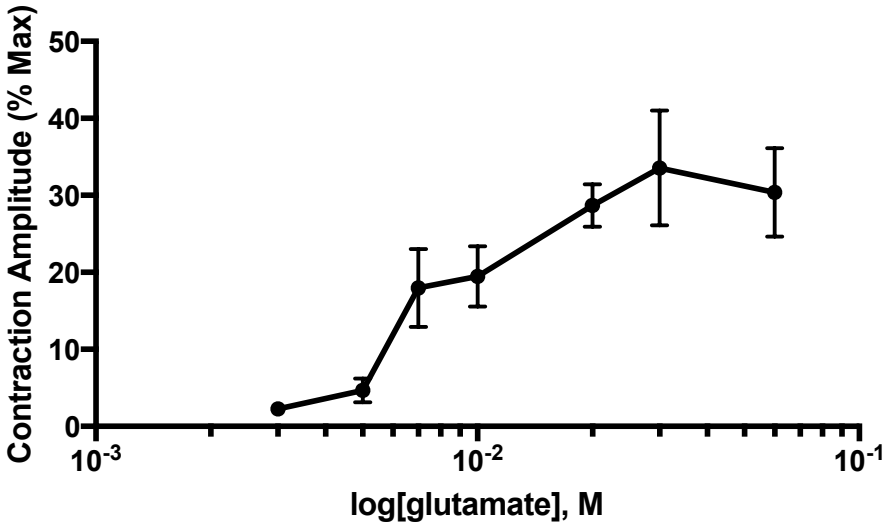


Figure 4: Dose-response curve for glutamate to elicit contractions of *Drosophila* body wall muscles. Contractions were elicited with glutamate in HL-6 saline and are expressed as a percentage of the maximal contraction as elicited by 300 mM KCl. With increasing concentrations of glutamate, the amplitude of the contractions increased up to a concentration of 30 mM glutamate. At 60 mM glutamate there was no further increase in amplitude. (Number of trials: 3 mM n=14, 5 mM n=12, 7 mM n=18, 10 mM n=17, 20 mM n=15, 30 mM n=8 & 60 mM n=11. In this and all other cases, each trial was performed on one larval preparation in 0.5 mM Ca^{2+} HL-6 saline.)

Since different physiological salines have been used in experiments on *Drosophila* neuromuscular preparations (McLeod et al., 2002; Stewart et al., 1994), proctolin's effect on glutamate-evoked contractions was examined using two physiological salines, a modified hemolymph-like 3 (HL-3) and hemolymph-like 6 (HL-6), which differ substantially in composition (Table 1). Some of the key differences are as follows. Normal HL-6 contains 0.5 mM calcium, 80 mM trehalose and ten amino acids. The modified HL-3 saline used here contained 5 mM trehalose and no amino acids, but the calcium concentration was 0.5 mM as in HL-6, in order to compare just the effects of changing trehalose and amino acids. A concentration of 10 nM proctolin was selected in these trials because it is just below the threshold for proctolin to enhance glutamate-evoked contractions (Figure 7), which should allow enhancement differences between the two salines to be more easily detected. Contractions evoked by 5 mM Glu were significantly larger in HL-3 saline than in HL-6, (Two-way ANOVA; Sidak's multiple comparisons test; $p=0.028$, $F(1,39)$), but this was not true for contractions elicited with 7 mM Glu (Two-way ANOVA; Sidak's multiple comparisons test; $p=0.404$, $F(1,46)$, Figure 5). In HL-6 saline, 10 nM proctolin enhanced contractions elicited by 7 mM glutamate ($p=0.009$; two-way ANOVA, Sidak's multiple comparisons test) but this concentration of proctolin failed to cause a significant increase in amplitude of contractions elicited by both 5 mM and 7 mM glutamate in HL-3 saline ($p=0.7943$ & $p=0.9872$ respectively; 2way ANOVA; Figure 5). Thus, HL-6 saline appeared to be more suitable for investigating the effects of proctolin on muscle contraction, and this saline was used for the remainder of the experiments.

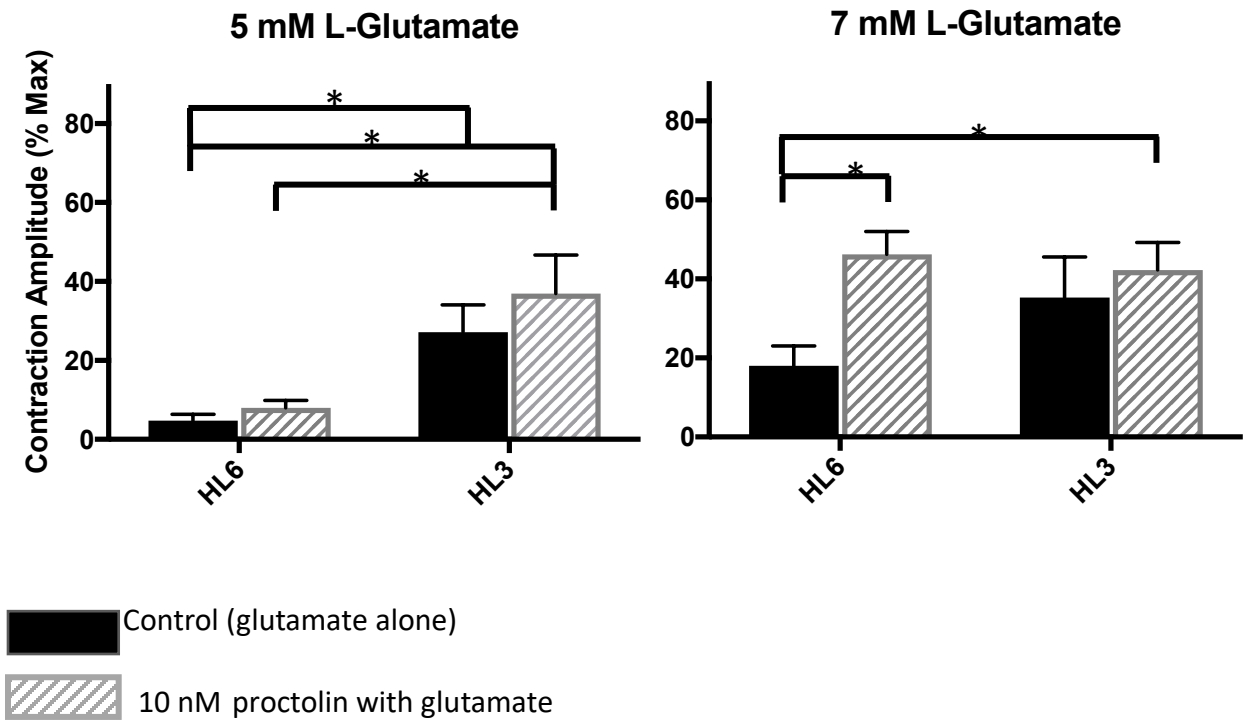


Figure 5: Effect of saline compositions on *Drosophila* body wall muscle contractions. Effects of glutamate and proctolin on muscle contractions were compared between two physiological salines (HL-3 and HL-6) to examine if there is a difference in proctolin's ability to enhance contractions with the different saline compositions (Table 1). Glutamate elicited larger contractions in HL-3 saline than in HL-6 saline. At a concentration of 10 nM, proctolin increased the amplitude of contractions elicited by 7 mM glutamate. Statistical comparisons were made using a two-way ANOVA; * indicates $p < 0.05$. (Numbers of trials: for 5 mM Glu HL-6 control n=12, 10 nM proc n=12; HL-3 control n=10, 10nM proc n=9; for 7 mM Glu HL-6 control n=18, 10 nM proc n=13; HL-3 control n=10, 10 nM proc n=11)

To characterize the relationship between glutamate concentration and proctolin's threshold more completely, thresholds were estimated over a range that includes the lowest glutamate concentration to reliably elicit contractions (3 mM) and a concentration very close to saturation of glutamate's effect (20 mM; Figure 4). Thresholds were estimated by determining the minimum proctolin concentration that caused a statistically significant increase in the amplitude of glutamate-evoked contractions compared to control trials with no proctolin. A one-way ANOVA was used for each glutamate concentration. Significant enhancement of contraction was achieved with 100 nM proctolin for each of the glutamate concentrations tested (Figure 7; $p<0.05$ in each case; 3 mM, $F(4,78)=3.46$; 5 mM $F(4,65)=4.39$; 7 mM $F(4,66)=8.92$; 10 mM $F(4,57)=3.76$; 20 mM $F(4,61)=7.71$). For the lowest concentrations of glutamate, (3 and 5 mM) the threshold for proctolin to enhance contractions was between 10 and 100 nM, but for 7mm glutamate the threshold for proctolin decreased to between 3 and 10 nM. At the two highest concentrations of glutamate tested, 10 and 20 mM, however, the threshold for proctolin rose again to between 10 and 100 nM proctolin. Thus, glutamate concentration did alter the threshold for proctolin to enhance contractions, and these results suggest that there could be an "optimal" glutamate concentration, around 7 mM, at which proctolin enhances contractions.

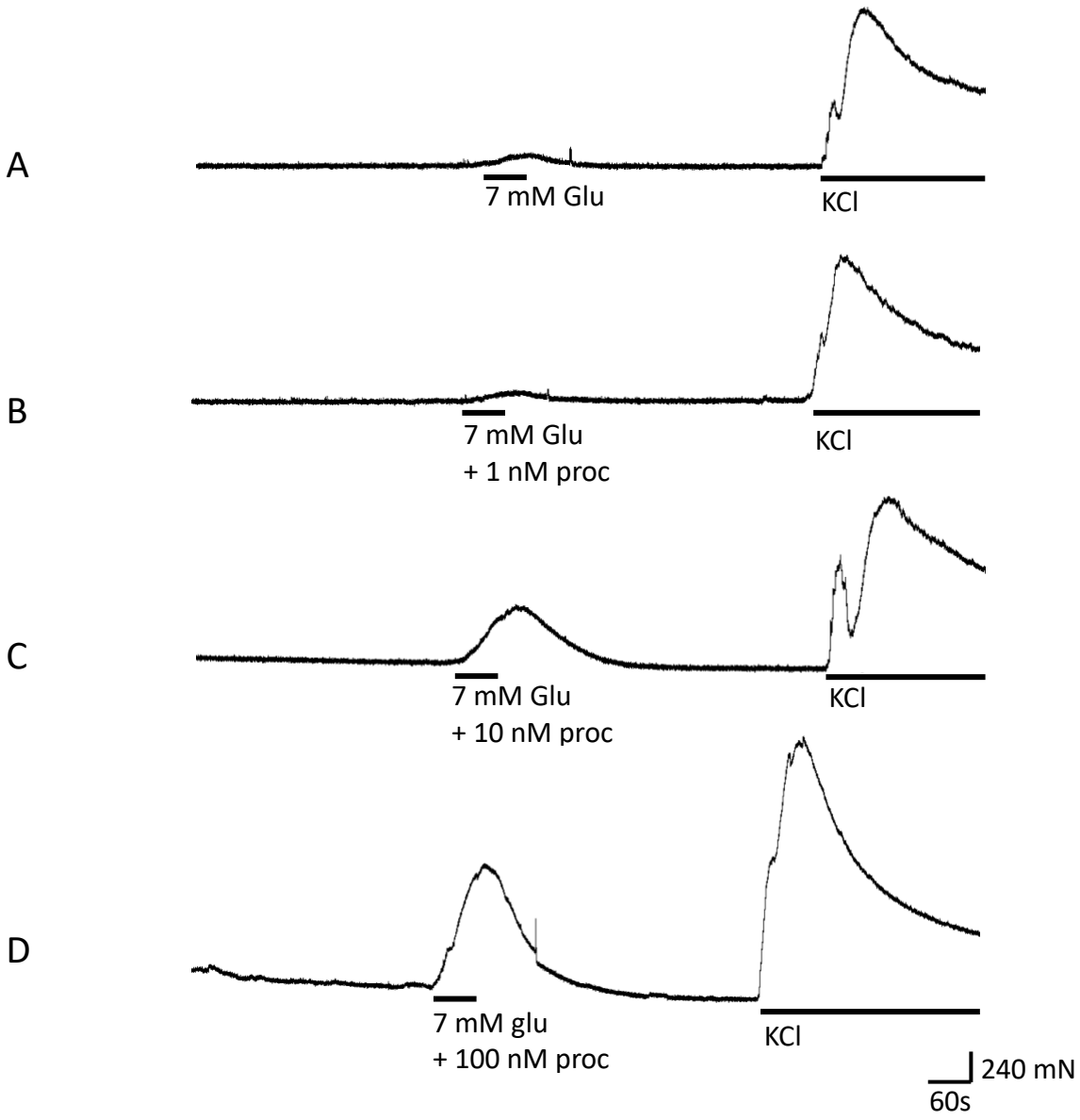


Figure 6: Proctolin enhances glutamate-evoked contractions of *Drosophila* body wall muscles. Representative recordings, from three separate preparations, of 7 mM glutamate-evoked contractions demonstrating that as the concentration of proctolin increased, so did the amplitude of the contractions. These trials were performed in HL-6 saline containing 0.5 mM calcium, each trace represents one trial. A concentration of 7 mM glutamate was selected as it highlights the enhancement effect of proctolin on glutamate-evoked contraction amplitude. (A) In control trials glutamate was applied for one minute. (B-D) Increasing concentrations of proctolin were added to the glutamate to determine the threshold concentration for proctolin to enhance glutamate-evoked contractions; 1 nM proctolin (B), 10 nM proctolin (C), and 100 nM (D).

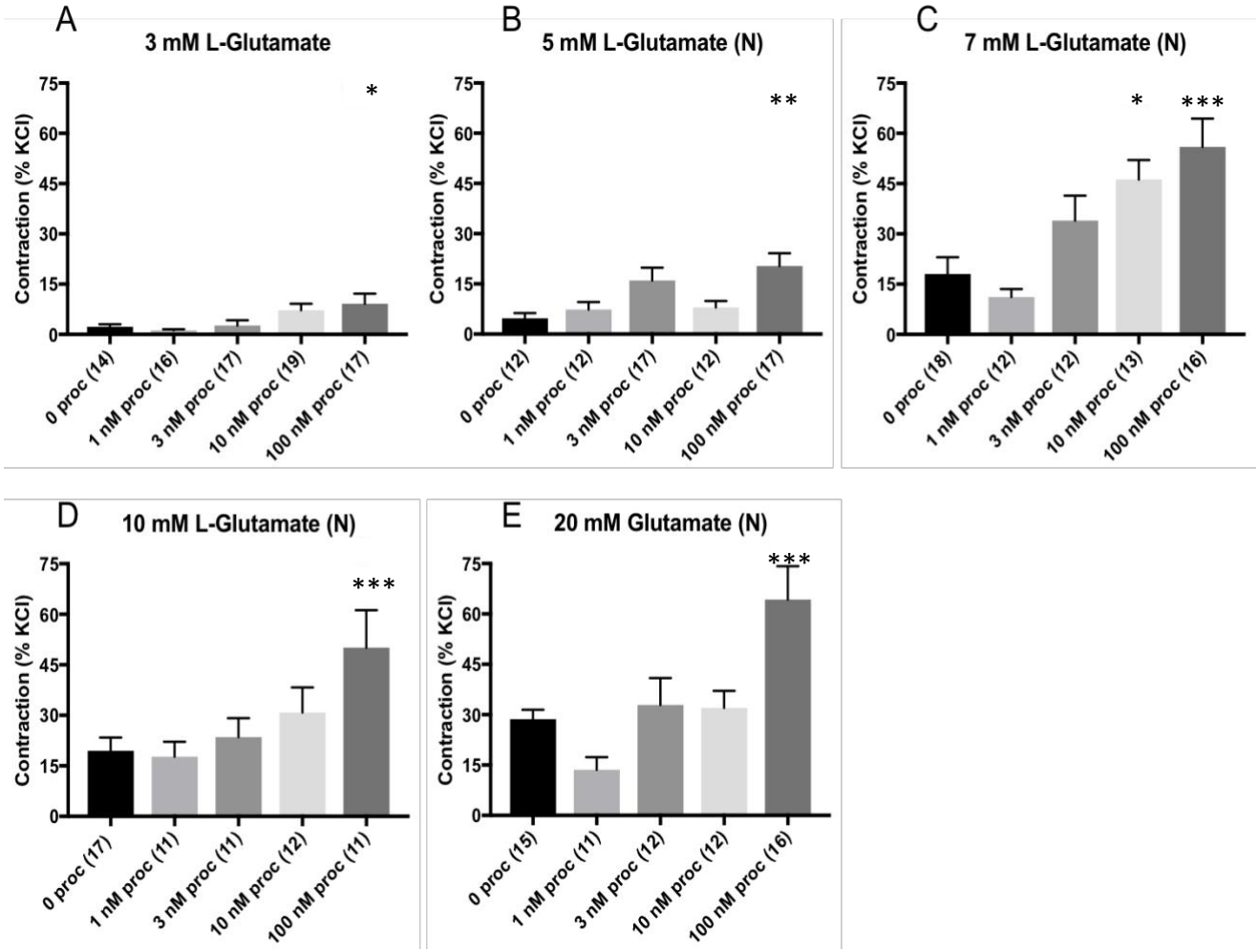


Figure 7: Thresholds for proctolin to enhance glutamate-evoked contractions of *Drosophila* body wall muscles. Statistical comparisons were made using a one-way ANOVA for each glutamate concentration and Tukey's multiple comparisons test. * indicates a statistically significant difference compared to control trials ("0" proc) in each case (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$). Numbers of trial replicates are indicated in parentheses.

Since proctolin acts via a GPCR (Egerod et al., 2003; Johnson et al., 2003), it seems possible that its effects might require longer than the one-minute treatments used in the experiments described above. One-minute treatments with glutamate were sufficient to elicit measurable and consistent contractions, and longer treatment with glutamate was avoided to minimize secondary effects such as receptor desensitization that might influence the results. To investigate whether more time was needed for second messenger pathways to develop and be fully activated, proctolin was applied alone for four minutes, and then a solution containing both 7 mM glutamate and proctolin solution was applied for one minute (Figure 8). Under these conditions, proctolin, at concentrations up to 100 nM, did not cause a significant change in contraction amplitude (one-way ANOVA, Tukey's multiple comparisons test). Interestingly, 10 nM and 100 nM proctolin increased contractions elicited by 7 mM glutamate when exposure to both substances began at the same time, but not when proctolin was applied first, even though both substances were subsequently applied together for one minute (Figure 8). This suggests that the effects of proctolin were "time-sensitive" and could only enhance contractions elicited by glutamate within the first few minutes of proctolin exposure, at least at the concentrations used here.

Taken together these results support the hypothesis that proctolin acts as a co-transmitter to enhance *Drosophila* larval muscle contractions. The original hypothesis predicted that increased concentrations of glutamate would reduce the threshold for proctolin to enhance contractions by up to two orders of magnitude. While this original prediction was not met, there was a drop in threshold for proctolin to enhance glutamate-evoked contractions with 7 mM glutamate.

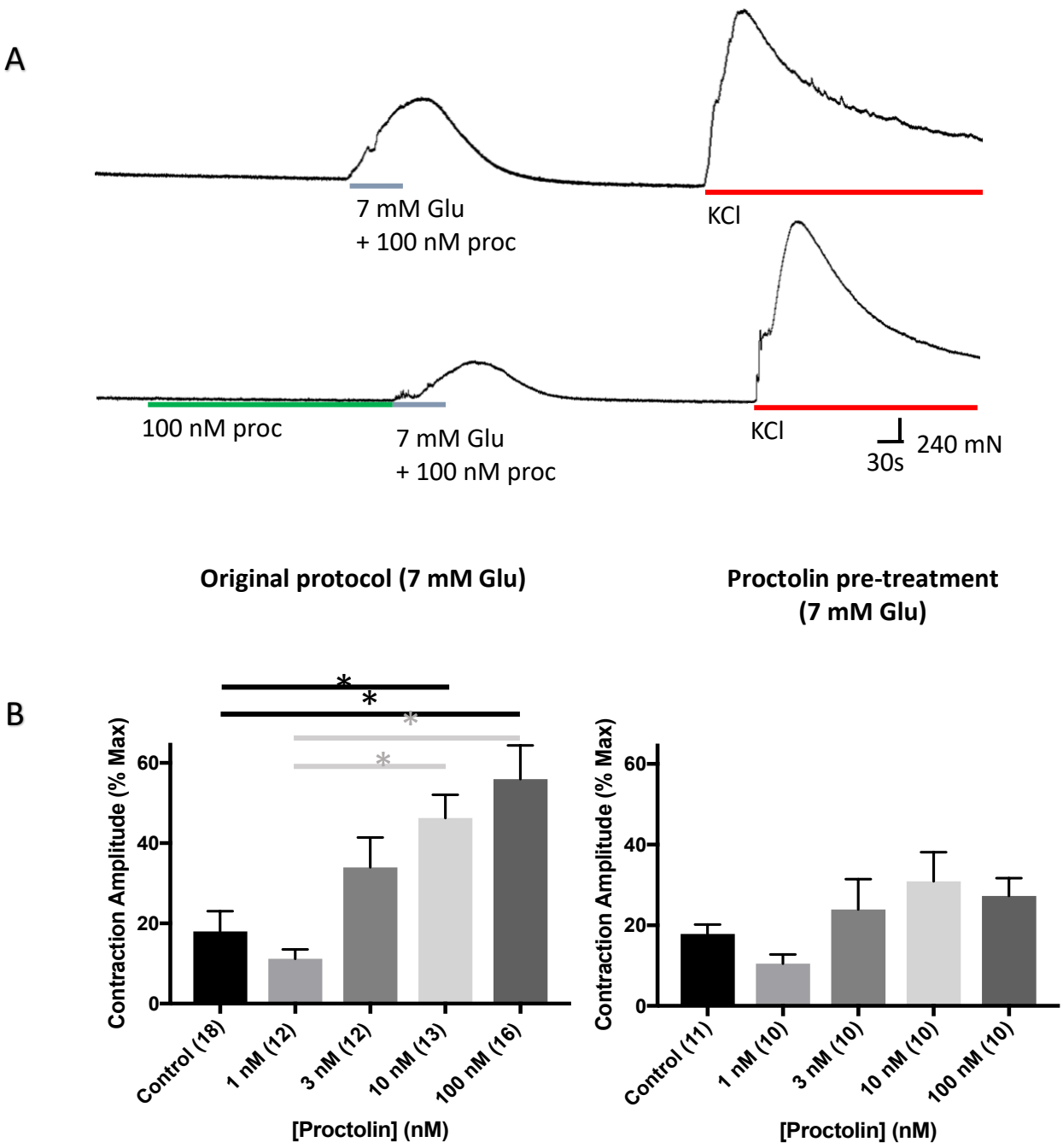


Figure 8: Time-dependence of proctolin's ability to enhance glutamate-evoked muscle contractions. (A) Sample recordings of time-dependence experiment. In the top trace 100nM proctolin and glutamate were applied together for 1 min represented by the blue bar under the trace. The bottom trace represents the proctolin pre-treatment; 100nM proctolin was applied alone first for four minutes (green portion), and then applied with glutamate for 1 min (blue portion). These traces highlight the finding that proctolin applied before glutamate did not enhance the glutamate contraction above that of proctolin applied with glutamate. (B) Graphs of the results comparing the two protocols used. Contraction amplitude is expressed as a percent of the maximum. Numbers in brackets represent number of replicates.

Octopamine

Like proctolin, octopamine is present in motor neurons of third instar *Drosophila* larvae and is thought to be released as a co-transmitter on the body wall muscles (Koon et al., 2011; Monastirioti et al., 1995; Ormerod et al., 2013). The octopamine receptor, Octß2R, is expressed in nearly all the body wall muscles (El-Kholy et al., 2015), and octopamine is reported to induce muscle contractions and to enhance nerve-evoked muscle contractions at concentrations of 0.1-100 µM (Ormerod et al., 2013). The latter effect involves an increase in neurotransmitter release that increases EJP amplitude (Koon et al., 2011) and might also involve a direct modulatory effect on muscle cells. To investigate the possibility that octopamine acts on muscle cells as a co-transmitter, experiments were performed to determine whether octopamine enhances contractions elicited by glutamate in larval preparations from which the central nervous system was removed.

The first series of trials was performed using the same experimental protocol as for examining effects of proctolin on glutamate-evoked contractions; a resting baseline was established in HL-6, 0.5 mM calcium saline for five minutes, glutamate or glutamate plus proctolin was applied for one minute and 300 mM KCl was applied after a five minute saline washout. The glutamate concentration selected for these trials was 7 mM as it had previously been found to elicit consistent and easily measured contractions while being small enough to allow for enhancement. At a concentration of 1 µM, octopamine failed to increase the amplitude of contractions elicited by 7 mM L-glutamate (Figure 9) ($p=0.121$; one-way ANOVA, Tukey's multiple comparisons test; Figure 10). In fact, 1 µM octopamine actually appeared to decrease the amplitude of glutamate-evoked contractions, but the effect was not statistically

significant (p=0.121). The octopamine antagonist, phentolamine was added with the glutamate at a concentration of 10 µM, (Donini & Lange, 2004), in separate trials to determine whether it had any effect on glutamate-evoked contractions, either by itself or in combination with octopamine. Phentolamine did not alter contractions in either case (One-way ANOVA control vs 10 µM phen p=0.192; control vs [1 µM octopamine + 10 µM phen] p=0.150). These results do not support the hypothesis that octopamine acts on muscle cells as a co-transmitter. If octopamine was released from the presynaptic cell during stimulation and acted on the muscle fibers as a co-transmitter, then application of exogenous octopamine would be predicted to enhance the glutamate-evoked contractions. However, these results found no difference in the contraction amplitude (when expressed as a percent of the maximum contraction), between glutamate alone controls and the addition of either octopamine or its antagonist phentolamine.

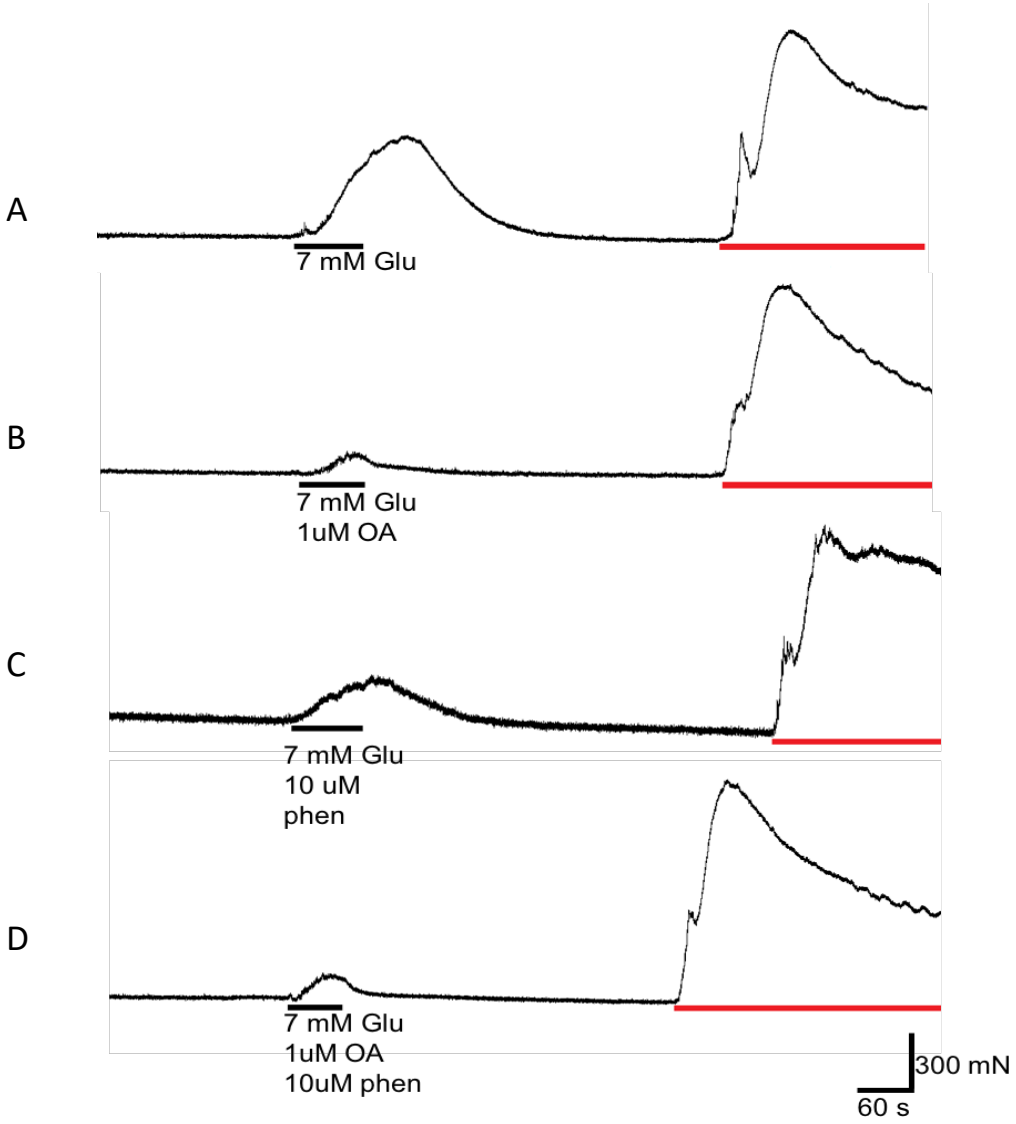


Figure 9: Sample recordings from larval preperations during application of octopamine and phentolamine. 7 mM glutamate was added: (A) without octopamine (OA), (B) with 1µM OA, (C) with 10µM phentolamine (phen), and (D) with the combination of OA and phen. Solutions were added for one min as indicated by the black bar under the graph. 300 mM KCl solution was applied at the end of each trial as indicated by the red bar.

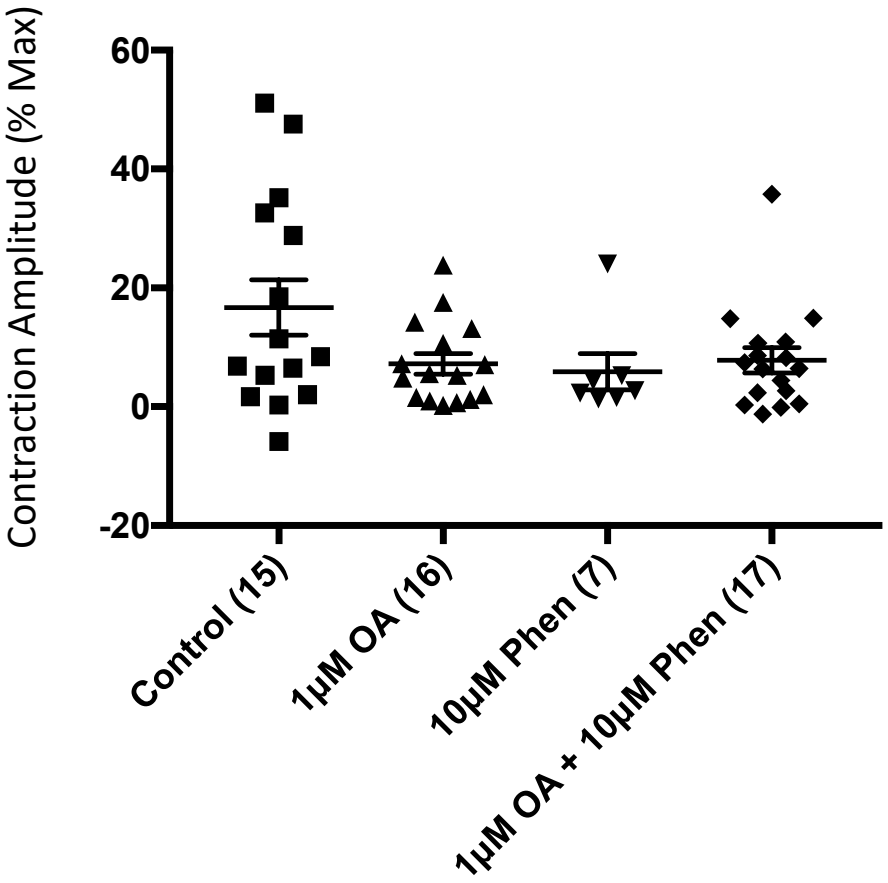


Figure 10: Effects of octopamine and its antagonist, phentolamine, on glutamate-evoked contractions. Contraction amplitude data were averaged for each treatment and expressed as a percent of the contraction amplitude elicited by 300 mM KCl solution. 7 mM glutamate alone had a mean contraction amplitude of 16.7% (of maximal contraction induced by 300 mM KCl). There were no significant differences between groups (one-way ANOVA with Tukey's multiple comparison test; $p > 0.05$).

Since octopamine failed to alter glutamate-evoked contractions in these trials, the reported ability of octopamine (1µM to 100µM) to induce muscle contractions (Ormerod et al., 2013) was re-examined. As in all other trials, the central nervous system was removed, and in these trials the effects of octopamine and phentolamine were assessed as changes in muscle tonus. A baseline muscle tonus was established for the larva in saline for five minutes, then octopamine or phentolamine was applied to the preparation and the change in tonus was calculated (materials and methods). Octopamine did not show any effect on the tonus at concentrations of 0.01 µM and 1µM, and neither did phentolamine at concentrations of 10 µM and 100 µM (Figure 11). All experimental trials reported in this thesis exhibited a small decrease in muscle tonus over the time course of the trial, which might reflect gradual relaxation of elastic elements in the stretched larval preparation or electronic drift in the recording amplifier. The drop in tonus was not significantly different between control trials (saline) and trials with octopamine or phentolamine (Figure 13; one-way ANOVA; $F= 1.751$; $p>0.197$). Thus, when applied alone, neither octopamine nor its antagonist, phentolamine, induced contractions in body wall muscles, at least under the conditions employed here (HL-6 saline containing 0.5 mM Ca^{2+}). The lack of an effect of octopamine was surprising, given its reported ability to induce contractions of body wall muscles at concentrations as low as 0.1 µM (Ormerod et al., 2013).

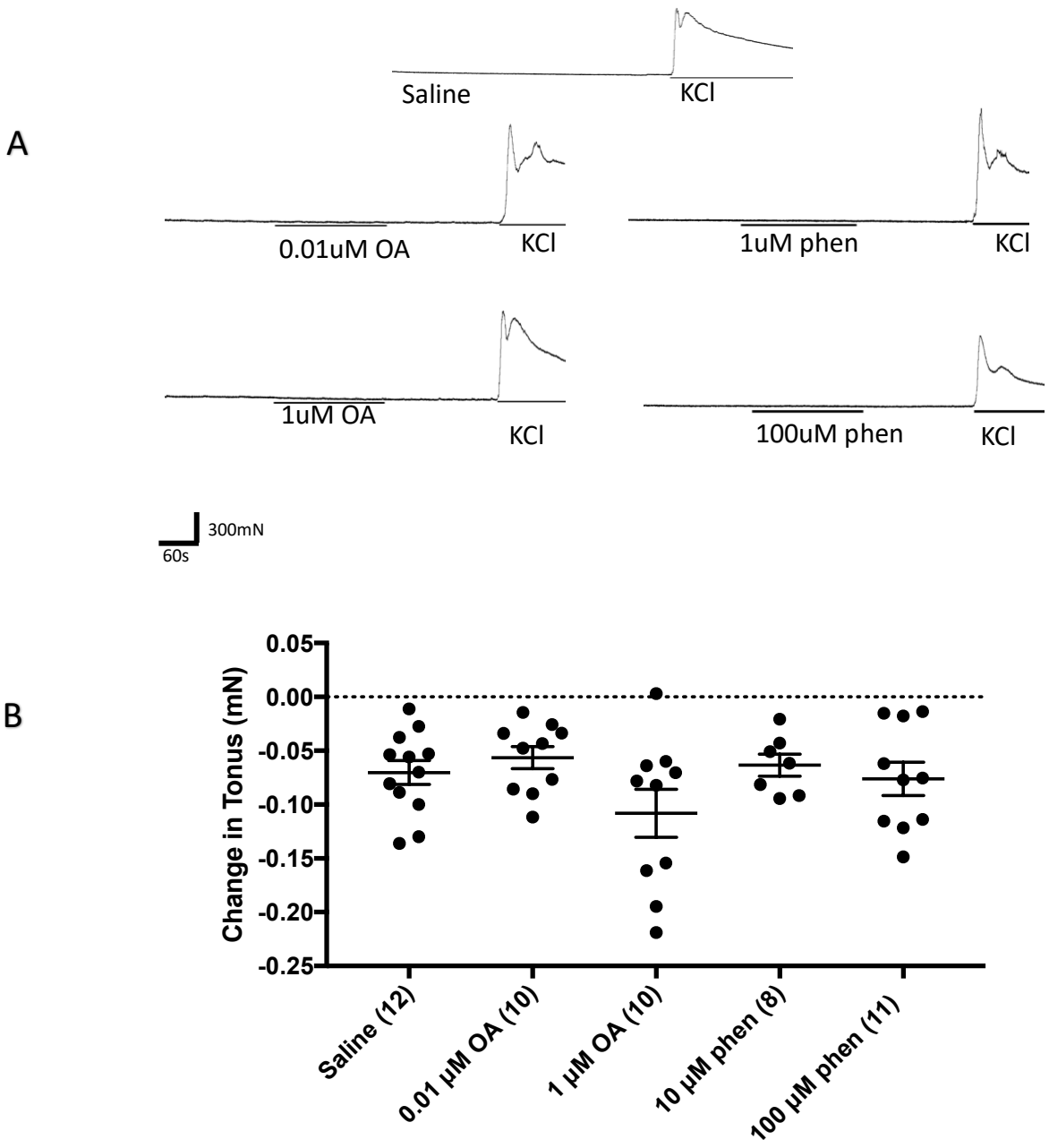


Figure 11: Effects of octopamine and phentolamine on muscle tonus of *Drosophila* body wall muscles. (A) Sample recordings from larval preparation during applications of octopamine and phentolamine. Neither substance altered muscle tonus that was established as the baseline with saline (HL-6, 0.5 mM calcium). (B) Data expressed as a change in tonus [OA tonus – saline tonus]. At 0.01 µM and 1 µM concentrations, octopamine failed to elicit contractions. Phentolamine also had no effect at either 10 or 100 µM concentrations.

Nerve-evoked contractions

Since the results presented above showed no effect of octopamine on muscle tonus or on glutamate-evoked contractions, octopamine's effects on nerve-evoked contractions were also re-assessed in this thesis. Initial trials were performed to develop a stimulus paradigm that would give consistent contraction amplitudes over time by minimizing effects of synaptic depression or facilitation that can accumulate over the course of each trial (Nicholls et al., 2012). In these trials, brief electrical stimuli (0.5 ms duration) were delivered in 250 ms bursts, and the burst frequency was varied (Figure 13). Four burst rates (0.5/min, 1/min, 2/min, and 4/min) were used, which corresponded to interburst intervals of 2 min, 1 min, 30 s and 15 s, respectively. Trials were performed in HL-6 saline containing 0.5 mM calcium or 1.5 mM calcium. Glutamate-evoked trials are typically performed in 0.5 mM calcium saline, which is below the average physiological concentration, but provides more stable and consistent contractions (J.Jung and A.J. Mercier, unpublished observations). Nerve-evoked contractions are typically elicted in 1.5 mM calcium containing saline (Ormerod, et al., 2013, 2016), and so in order to more closely examine the importance of extracellular calcium concentration on nerve-evoked contractions, and to allow a comparison between nerve-evoked and glutamate-evoked contractions, both levels of calcium where examined.

When larvae were perfused with 0.5 mM calcium saline, stimulus bursts delivered every two minutes, (0.5 / minute), elicited contractions with amplitudes that remained relatively stable, decreasing by only 0.067 mN (22 %) between the first and the tenth bursts (Figure 12). Delivering bursts 1 min apart also generated stable contractions, which dropped by only 0.006 mN (<1%) over ten bursts. When bursts were given every 30 seconds, (2 / minute) there was a

progressive increase in contraction amplitude, suggesting a build-up of facilitation leading to post-tetanic potentiation between bursts. Contractions increased from 0.67 mN to 1.18 mN, representing an increase of 57%. Delivering bursts 15 seconds apart also showed facilitation, with contraction amplitude increasing by an average of 49% between the first and tenth bursts. These results suggest that with low external calcium, bursts given in quick succession elicit synaptic facilitation from one burst to the next (Dickinson et al., 2015; Mahadevan et al., 2004; Mercier et al., 1984), but when the time between bursts is longer there is little to no net facilitation of depression.

When the same series of experiments was performed in saline that contained 1.5 mM calcium, the contraction amplitudes were more stable for all burst rates. With the higher level of external calcium there was no net facilitation or depression regardless of how much time ellapsed between bursts (Figure 12). All experiments examining effects of octopamine and its antagonist, phentolamine, on nerve-evoked contractions were conducted using saline containing 1.5 mM calcium because contractions were more stable in this saline.

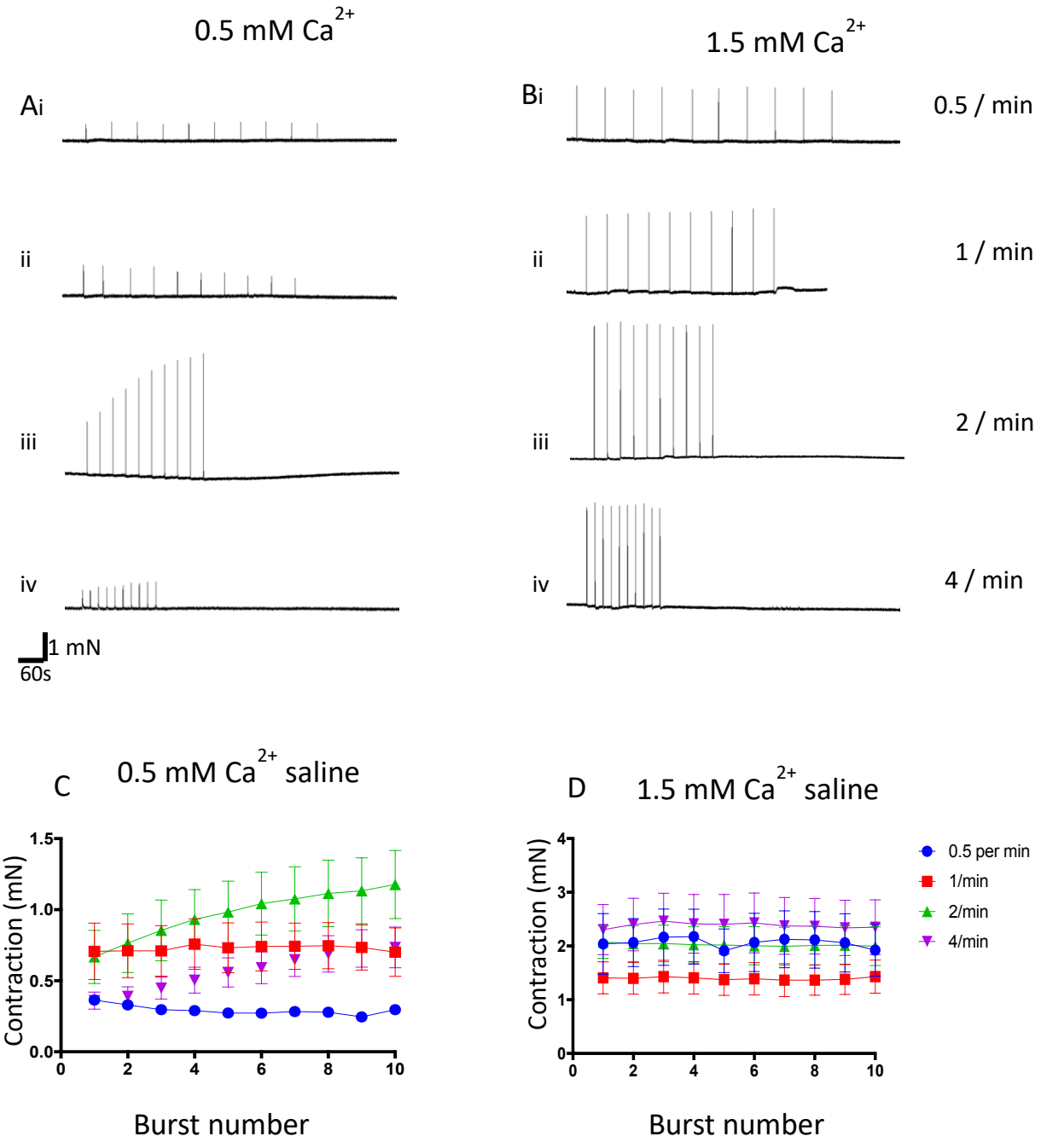


Figure 12: Effects of extracellular calcium and stimulus burst rate on nerve-evoked contractions of body wall muscles. In each trial, ten consecutive contractions were elicited by stimulating the segmental nerves using a suction electrode. Representative recordings show nerve-evoked contractions at different burst rates in HL-6 *Drosophila* saline containing 0.5 mM calcium (A) or 1.5 mM calcium (B). Averaged results (C) show interburst facilitation in 0.5 mM calcium at low burst rates (see key). (D) Shows the averaged results for interburst facilitation in 1.5 mM calcium saline. (Number of replicates: 0.5 mM Ca^{2+} 0.5/min n=6, 1/min n=9, 2/min n=7, 4/min n=7; 1.5 mM Ca^{2+} 0.5/min n=7, 1/min n=8, 2/min n=8, 4/min n=6)

In addition to re-examining effects of octopamine on nerve-evoked contractions, trials were performed using different intraburst stimulus frequencies, similar to the approach used by others to examine effects of proctolin (Ormerod et al., 2016). This approach was used because it seemed possible that, like proctolin, octopamine's ability to increase contraction amplitude might depend on neural activity, and, thus, effects of octopamine might not be apparent at some impulse frequencies but might become more pronounced at higher frequencies of stimulation. Direct nerve stimulation was performed using a suction electrode to suck up the nerves and stimulate directly. Three frequencies were chosen, a low frequency (5 Hz), a high frequency (50 Hz), and a mid-range frequency (32 Hz). When the nerves were stimulated at the low frequency (5 Hz) only the higher concentration of 1 µM octopamine was able to enhance the amplitude of the contractions (Figure 13). The lower concentration of octopamine (0.01 µM) did not alter the amplitude of the contractions as compared to saline ($p=0.733$).

It was predicted that if octopamine was being released from the presynaptic terminal during nerve stimulation and acting on the post-synaptic cell to enhance contractions, then adding an octopamine antagonist to the bath solution should block the effects of endogenous octopamine and decrease contraction amplitude. To test this prediction 10 µM phentolamine was added to the bath solution during the nerve-evoked stimulation. At this concentration, phentolamine did not alter contraction amplitude during 5 Hz nerve stimulation. Interestingly, when 1 µM octopamine was applied with the antagonist phentolamine at 10 µM, there was no enhancement of nerve-evoked contractions. This suggests that the bath applied phentolamine was able to block the effect of the bath applied octopamine.

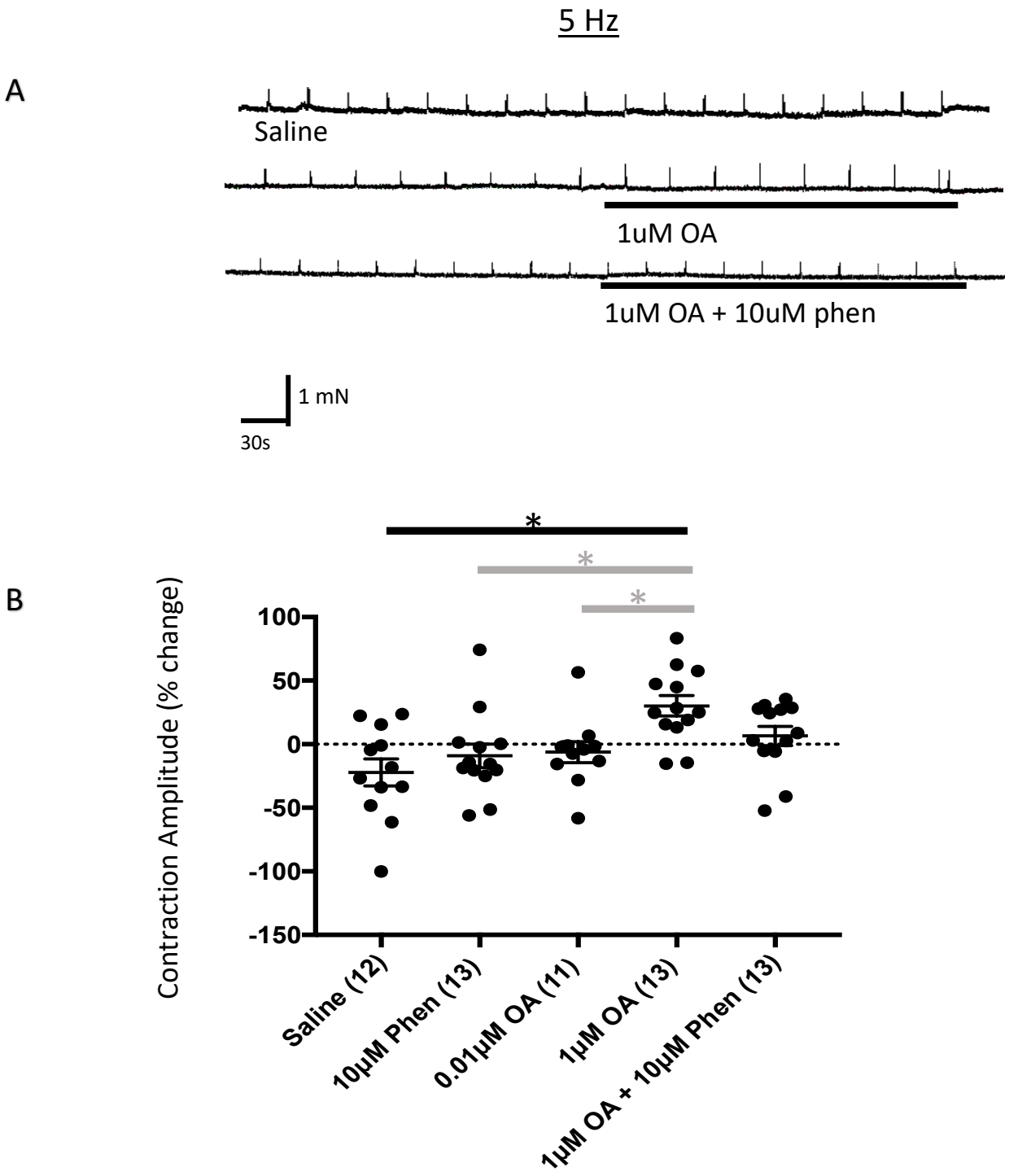
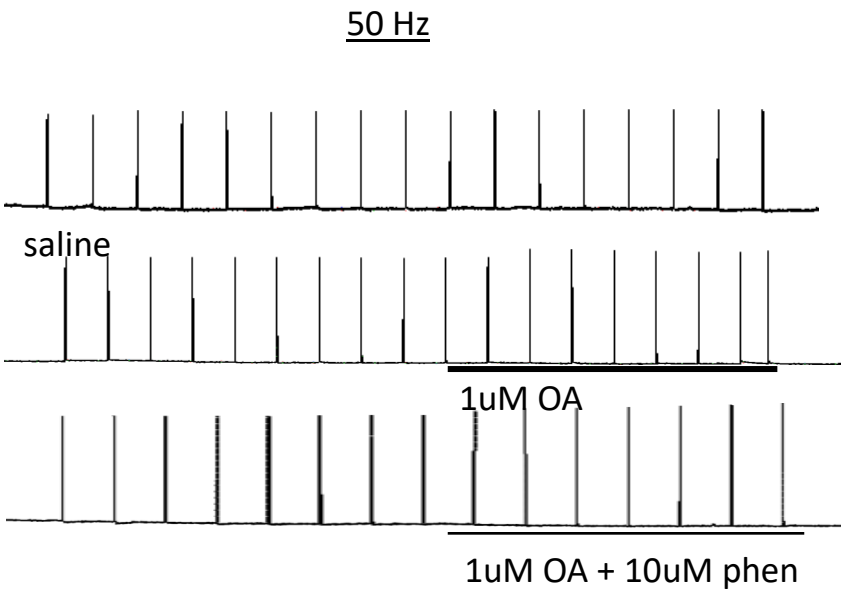


Figure 13: The effects of octopamine on low frequency (5 Hz) nerve-evoked contractions of *Drosophila* body wall muscles. (A) sample recordings of nerve-evoked contractions at 5 Hz frequency in saline (control) and octopamine (1µM). (B) 1 µM octopamine significantly enhanced contraction amplitude over saline (p=0.001). ([10µM phen vs 1µM OA, p=0.0167]; [0.01µM OA vs 1µM OA, p=0.443])

With low frequency stimulation, (5 Hz), only a relatively high concentration of 1 µM octopamine was able to significantly enhance nerve-evoked muscle contractions (Figure 13). If the ability to enhance contractions increases with activity, it can be predicted that with a high frequency of stimulation a lower concentration of octopamine in the bath should increase the amplitude of the contractions. At the highest frequency stimulation tested here, 50 Hz, the same trend that was seen in the low, 5 Hz, frequency was seen again. That is, 1 µM octopamine was able to significantly enhance the nerve-evoked contractions (one-way ANOVA, Tukey's multiple comparisons test, $p=0.046$, $F(4, 41)$), but, the 0.01 µM octopamine did not alter the contractions. Thus, the predicted drop in concentration of octopamine to enhance contraction was not seen. However, only two concentrations were tested here, more concentrations at smaller intervals could be tested to further examine at what concentration octopamine fails to enhance nerve-evoked contractions. The octopamine antagonist, phentolamine (10 µM), alone did not alter the nerve-evoked contractions as was predicted but it did decrease the enhancement of bath applied 1 µM octopamine (Figure 14).

A



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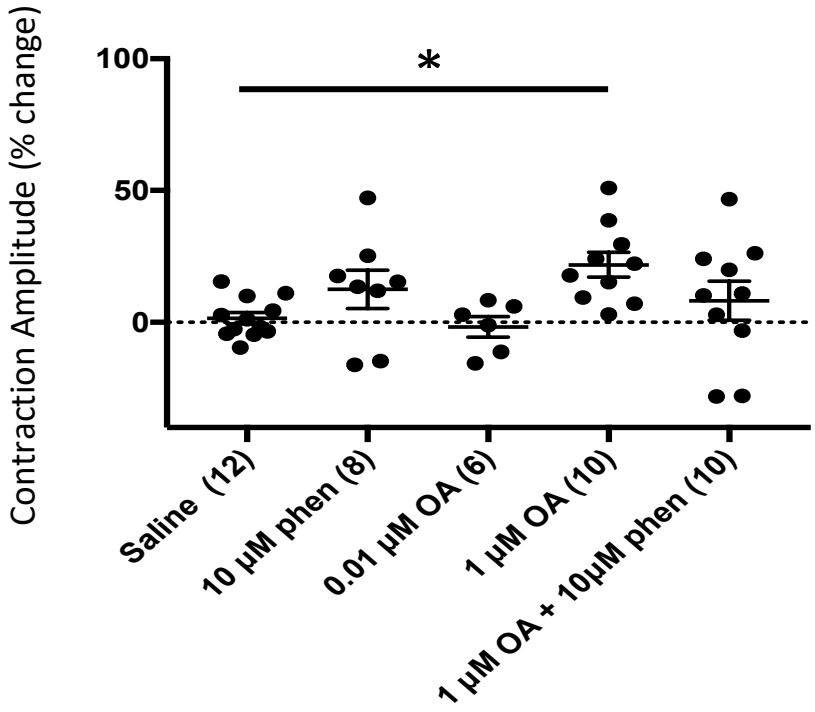

Figure 14: The effect of octopamine on high frequency (50 Hz) nerve-evoked contractions of *Drosophila* body wall muscles. (A) Sample recordings of nerve-evoked contractions elicited at 50 Hz. (B) 1 µM octopamine significantly enhanced contraction amplitude over saline control (p=0.046)

There was no shift in threshold for octopamine to enhance nerve-evoked contractions seen in this thesis with increasing the frequency of stimulation from 5 Hz to 50Hz. These frequencies may be out of the physiological range and not truly representing an *in vivo* environment. Using fictive locomotion techniques motor neurons in the Drosophila have been shown to have stereotypical firing rates between 21 and 42 Hz (Chouhan et al. 2012). Based on those frequencies a mid-range frequency of 32 Hz was chosen to examine the effects of octopamine on nerve-evoked contractions. Proctolin's ability to enhance nerve-evoked contractions in *Drosophila* dropped by two orders of magnitude between 2 Hz and 32 Hz stimulation frequencies (Ormerod et al., 2016).

When the nerve was stimulated at 32 Hz, nearly every solution applied during nerve-stimulation enhanced the amplitude of the nerve-evoked contractions (Figure 15). As was seen with both high (50 Hz) and low (5 Hz) stimulus frequencies, phentolamine applied alone during 32 Hz stimulation did not alter the amplitude of contractions at either concentration tested, (10 µM and 100 µM). Interestingly, at this frequency of stimulation both octopamine concentrations tested (0.01 µM and 1 µM) elicited a significant increase in contraction amplitude ([saline vs 0.01 µM OA, $p=0.001$], [saline vs 1 µM OA, $p=0.0001$]). This suggests that 32 Hz may represent an activity level that is optimal for octopamine to enhance contractions. The effect of 0.01 µM octopamine was blocked by 10 µM phentoloamine (one-way ANOVA; saline vs (10 µM phen + 1 µM OA), $df=88$, $p=0.002$), but the effect of 1 µM octopamine was not blocked by phenolamine at either 10 µM or 100 µM (one-way ANOVA; saline vs (10 µM phen + 1 µM OA), $df=88$, $p=0.0005$; saline vs (100 µM phen + 1 µM OA), $df=88$, $p=0.0009$).

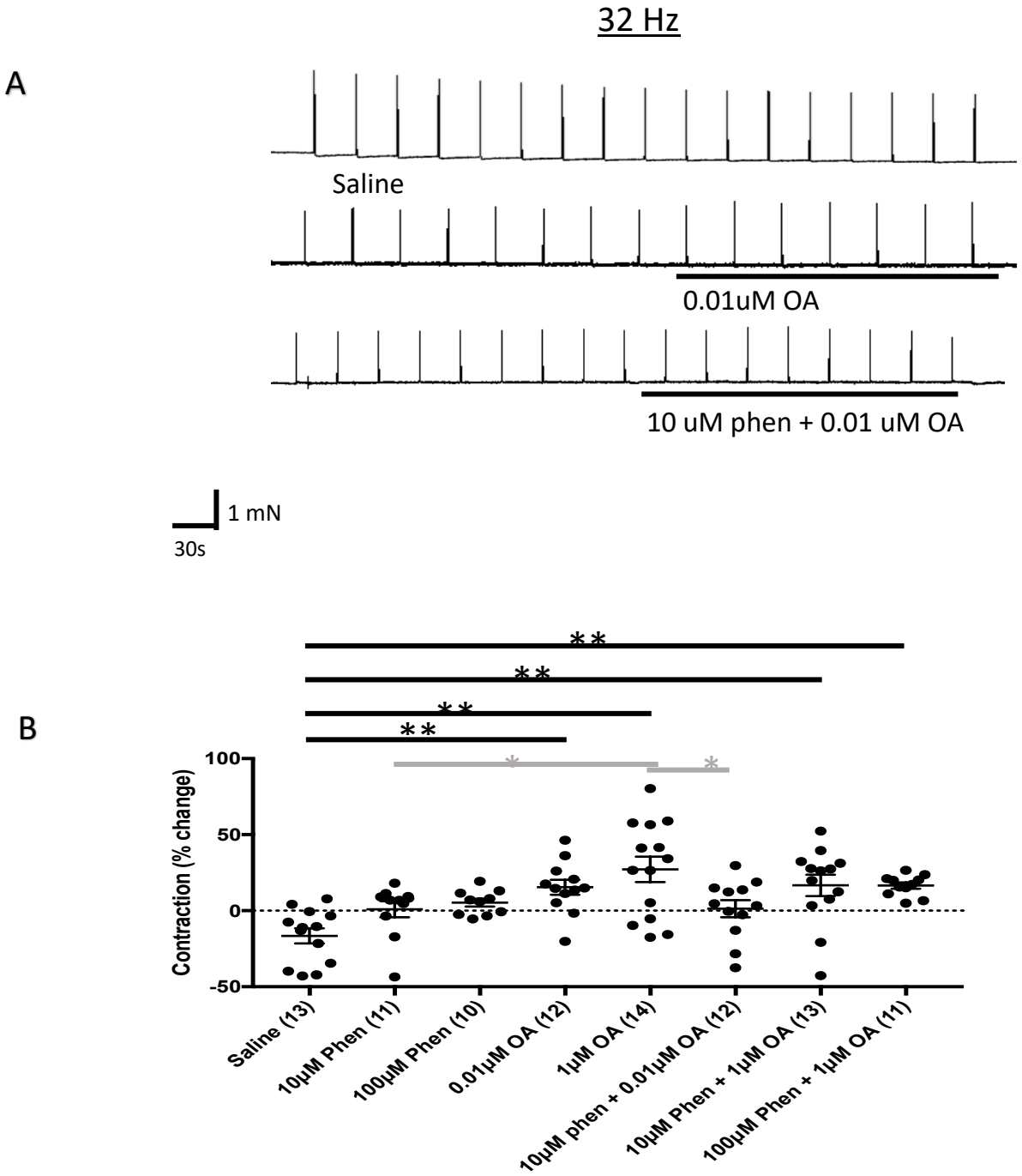

Figure 15: The effect of octopamine on *Drosophila* body wall muscle contractions evoked by nerve stimulation at 32 Hz. (A) Representative recordings of nerve-evoked contractions at 32 Hz frequency. (B) Octopamine significantly enhanced contraction amplitude at 0.01 µM and 1 µM as compared to saline. 1 µM octopamine enhanced contractions even in the presence of phentolamine. (*=p<0.05, **=p<0.005, and ***=p<0.0005)

Discussion

This thesis examined co-transmission at neuromuscular junctions of *Drosophila* larvae, focusing on two modulators that are thought to act as co-transmitters in this system, based on their presence in the synaptic terminals and on their reported ability to alter postsynaptic potentials and/or nerve-evoked contractions. The aim was to present further evidence that one or both substances function as co-transmitters in this system. One approach was an attempt to mimic release of the excitatory transmitter, L-glutamate, by directly applying it to the muscles, and to determine whether or not octopamine and proctolin alter the amplitude of glutamate-evoked contractions. This approach avoided simultaneous release of co-transmitters with glutamate, as would be expected to occur during nerve stimulation. In these experiments proctolin increased the amplitude of glutamate-evoked contractions, which is consistent with proctolin acting as a co-transmitter. The present data, however, are also consistent with proctolin acting as a neurohormone (rather than as a co-transmitter). A more convincing demonstration of co-transmission would require evidence that proctolin is released at physiologically effective concentrations by physiologically relevant levels of impulse activity in the motor neurons. Although proctolin release from motor neurons has been demonstrated in crayfish (Bishop et al., 1987), it has not been possible to detect or quantify proctolin release from *Drosohphila* neurons, due mainly to the small size of fruit fly larvae (Ormerod et al., 2018).

Octopamine, on the other hand, did not increase the amplitude of glutamate-evoked contractions, which does not support a role for octopamine as a co-transmitter, or at least not one that acts directly on muscle. In fact, the data suggest that glutamate-evoked contractions were smaller in the presence of octopamine, but this trend was not statistically significant (7

mM Glu vs. 7 mM Glu + 1 µM octopamine, p=0.121). The inability of octopamine to alter glutamate-evoked contractions is surprising for at least two reasons. First, the octopamine receptor, Octβ2R, shows strong expression in the body wall muscles of third instar larvae (El-Kholy, et al., 2015). Second, octopamine has been reported previously to induce contractions of body wall muscles in third instar *Drosophila* larvae and to enhance nerve-evoked contractions of these muscles (Ormerod et al., 2013). The increase in nerve-evoked contractions can be attributed, at least in part, to octopamine's ability to increase EJP size (Ormerod et al., 2013), which appears to be caused by increased neurotransmitter release, since octopamine increases evoked EJPs without altering the amplitude of spontaneous miniature EJPs (Koon et al., 2011). Such a presynaptic effect of octopamine would not be observed with glutamate-evoked contractions because the nerve terminals are not activated. Octopamine's reported ability to induce contractions, however, probably reflects a postsynaptic effect because it occurs in the absence of nerve stimulation and because octopamine reduces input resistance in body wall muscle fibers (Ormerod et al., 2013). In the present work octopamine failed to induce contractions at concentrations of 0.01 µM and 1 µM. Although 0.01 µM is below the threshold reported for octopamine to induce contractions, 1 µM is above the reported threshold and has been shown to generate contractions that were half the maximum amplitude elicited by octopamine (Ormerod et al., 2013). In the present work, it was considered possible that the octopamine used was old and oxidized, but the same results were obtained with a newly purchased batch of octopamine.

The discrepancy between the present results (i.e. failure of octopamine to induce contractions) and those reported by Ormerod et al. (2013) might result from differences in

calcium concentration in the physiological salines. In the present work, octopamine's ability to induce contractions and to enhance glutamate-evoked contractions was examined using saline containing 0.5 mM calcium. Ormerod et al. (2013) demonstrated that octopamine induces contractions and enhances nerve-evoked contractions using saline containing 1.5 mM calcium. Effects of octopamine on locust skeletal muscle require extracellular calcium (Evans, 1984; Evans & O'Shea, 1978). Proctolin's effects are also calcium-dependent; decreasing extracellular calcium concentration from 1.5 mM to 0.5 mM reduces the threshold for proctolin to elicit contractions of *Drosophila* larval muscles (Ormerod et al., 2016). This would suggest that the failure of octopamine to induce contractions in the present experiments may be due to the lower extracellular calcium levels, and that octopamine might induce contractions in saline containing a higher concentration of calcium. On the other hand, this thesis examined effects of octopamine on nerve-evoked contractions in saline containing 1.5 mM calcium, and no increases in tonus were apparent (e.g. Figures 13-15). The reason for the discrepancy between the present results and those of Ormerod et al. (2013) is currently unresolved and warrants further investigation. It would probably be worthwhile to re-examine octopamine's ability to alter tonus and to modulate glutamate-evoked contractions in salines of varying calcium levels. The low calcium concentration used here (0.5 mM) is below the average physiological concentration (1.5 mM ± 0.7, Stewart et al., 1994) but was selected because glutamate elicits contractions of variable time course and amplitude in 1.5 mM calcium (J. Jung and A.J. Mercier, unpublished observations).

Although the present work did not confirm a previous report that octopamine induces contraction of larval body wall muscles (Ormerod et al., 2013), it did confirm that octopamine

increases the amplitude of nerve-evoked contractions. Data presented here indicate that this effect of octopamine depends on neural activity, since 0.01 µM octopamine increased the amplitude of contractions elicited by bursts of 32 Hz stimuli but did not change the amplitude of contractions elicited by bursts of stimuli at 5 Hz or 50 Hz (Figures 13-15). Thus, the 32 Hz stimulus pattern appears to represent an optimal activity level for modulation by a low concentration of octopamine, compared to the other stimulus patterns used in the present work. This level of activity (9 impulses at 32 Hz within each burst) was "intermediate" compared to the other two paradigms (2 impulses at 5 Hz, and 13 impulses at 50 Hz). The mechanisms underlying activity dependence for each of these modulators are still unknown.

Octopamine's ability to increase the amplitude of nerve-evoked contractions may involve presynaptic mechanisms. Octopamine has been shown to increase EJP amplitudes without altering the size of spontaneous miniature EJPs (Koon et al., 2011; Ormerod et al., 2013). Thus, quantal size does not change, which would indicate that octopamine does not alter responsiveness of the muscle cell to glutamate. The absence of a change in quantal size suggests that the increase in evoked EJP amplitude may involve an increase in the number of quanta of transmitter released per nerve impulse (Nicholls et al., 2012). The effects of octopamine may also involve postsynaptic mechanisms. Octopamine's reported ability to induce contractions is thought to represent a direct effect on body wall muscle cells (Ormerod et al., 2013), and at concentrations of 10 µM and greater, octopamine decreases input resistance in these muscle fibers (Ormerod et al., 2013). One or both of these effects, however, might involve presynaptic mechanisms, since 100 µM octopamine evokes release of neuropeptides from the motor neurons by activating PKA and triggering release of calcium from

internal stores in the synaptic terminals (Shakiryanova et al., 2011). Thus, it is possible that the drop in input resistance and contractions attributed to octopamine could actually be mediated by release of neuropeptides, particularly at high octopamine concentrations. Further work is thus needed to identify either presynaptic and/or postsynaptic effects of octopamine, and attention should be paid to the concentrations used to elicit such effects.

Several trials were conducted to determine whether an antagonist of octopamine would reduce nerve-evoked contractions and, thus, provide evidence for nerve-evoked release of octopamine as a co-transmitter. Phentolamine was selected because it is an α-adrenergic receptor blocker that reversibly blocks the Octβ class of octopamine receptor (Hana & Lange, 2017). This receptor class, specifically Octß2R, is expressed in body wall muscles of 3rd instar *Drosophila* larvae (El-Kholy et al., 2015) and is responsible for mediating presynaptic effects of octopamine on motor nerve terminals (Koon et al., 2011). Phentolamine blocks the ability of octopamine to inhibit contractions of oviduct muscles in the bug *Rhodnius prolixus,* at concentrations as low as 0.1-10 µM (Hana & Lange, 2017). Thus, if octopamine is released and acting as a co-transmitter at neuromuscular junctions in *Drosophila* larvae, 10 µM phentolamine would be expected to reduce the amplitude of nerve-evoked contractions of the body wall muscles. In the present work, however, treatment with phentolamine alone did not alter contraction amplitude at concentrations of 10 µM and 100 µM. The lower concentration of phentolamine, however, appeared to antagonize effects of bath applied octopamine in some cases. When compared to saline controls, 1 µM octopamine caused a significant increase in contractions elicited by 5 Hz and 50 Hz stimulation, but the increase was not statistically significant when 1 µM octopamine was combined with 10 µM phentolamine (Figures 13 and

14). This same concentration of phentolamine, (10 µM), prevented 0.01 µM octopamine from increasing contraction amplitudes significantly during 32 Hz stimulation (Figure 15). These results suggest that phentolamine was an effective antagonist in at least some cases. The failure of phentolamine to block effects of 1 µM octopamine during 32 Hz stimulation (Figure 15) might suggest that high amounts of octopamine were released during this stimulus regime, so that the combined effects of bath applied octopamine and locally released octopamine were able to overcome the effects of a competitive antagonist. Such a scheme might account for the fact that phentolamine blocked octopamine's effect during 5 Hz stimulation and not at 32 Hz. It is more difficult, however, to explain why octopamine's effect during 50 Hz stimulation was blocked by phentolamine, since this higher stimulus frequency would be expected to release more transmitter. Perhaps stimulation at 50 Hz during the five minute "baseline" period before octopamine application depleted releasable stores of octopamine, so that the combined effects of bath-applied and locally released octopamine were actually lower during 50 Hz stimulation. There is some evidence for both short-term and long-term synaptic depression in the body wall muscles of the *Drosophila* third in-star larva (Guo & zhong, 2006) , but in the absence of corroborative data, such an explanation is purely speculative. In any case, phentolamine was able to antagonize at least some of the effects of bath-applied octopamine. Thus, the failure of phentolamine to reduce the amplitude of nerve-evoked contractions in the absence of bath-applied octopamine does not support the hypothesis that octopamine is released as a co-transmitter in this system.

The findings discussed above are not congruent with previous work (Koon et al., 2011), which provided convincing evidence that octopamine release from motor neurons of

Drosophila larvae acts on Octß2 receptors on the motor neurons, and that these receptors act as autoreceptors to increase transmitter output and to promote axonal outgrowth. That work involved methods to selectively alter expression of Octß2R. Pharmacological agents are often less precise. Although phentolamine is known to act as an antagonist on the octopamine receptors in *Drosophila,* it isn't the only antagonist that is available. Mianserin is also a known octopamine antagonist in this system and may be more potent than phentoloamine as it has a high affinity for the neuronal octopamine receptor in locust (Roeder 1990). Because the results obtained using phentolamine as an antagonist did not meet the initial prediction of a reduction in contraction amplitude, the experiment should be repeated with other antagonists, such as mianserin, gramine and epinastine (Hana & Lange, 2017; Rosenberg et al., 2007), to verify the present results. It is possible that phentolamine may not be the most effective antagonist of octopamine receptors in *Drosophila* body wall muscles.

The experiments with proctolin were designed to determine whether or not higher concentrations of L-glutamate would decrease the threshold necessary for proctolin to enhance evoked contractions. The rationale for these experiments was that increasing glutamate concentration would mimic the increased release of glutamate that occurs when the motor neurons are stimulated electrically with an increasing number of impulses and with increasing stimulus frequency. Since such changes in motor pattern are reported to decrease the threshold concentration for proctolin to enhance contractions (Ormerod, et al., 2015), it was predicted that raising the glutamate concentration would lower the threshold for proctolin to enhance contractions. The results show that proctolin was able to enhance glutamate-evoked contractions in a dose-dependent manner, as would be expected for a cotransmitter that acts

directly on muscle cells. The threshold for proctolin to enhance these contractions was 100 nM at low glutamate concentrations (3-5 mM) but decreased to 10 nM proctolin when the glutamate concentration increased to 7 mM. The threshold for proctolin increased back to 100 nM at higher glutamate concentrations (10-20 mM). These results indicate that the relationship between proctolin threshold to enhance contractions and glutamate concentration has a "U-shape"; the threshold decreased with increased glutamate concentration peaking at 7 mM glutamate, and then increased again with increased glutamate concentrations.

Ormerod et al. (2015) stimulated the motor axons that innervate *Drosophila* body wall muscles, and reported that changing the stimulus pattern from one stimulus applied every 15 s to bursts of up to 9 impulses at 32 Hz within each burst decreased the threshold for proctolin to enhance contractions by two orders of magnitude (from < 1 nM to < 0.01 nM). They did not observe any increase in proctolin threshold as nerve activity increased within this range. Thus, it was expected that the threshold for proctolin to enhance glutamate-evoked contractions would continue to decrease with increasing glutamate concentrations or at least stabilize at high glutamate concentrations. This expectation, however, is based on the assumption that the range of glutamate concentrations used here elicts comparable contractions to those generated over the range of nerve stimuli used by Ormerod et al. (2016). No attempt was made in this thesis to compare nerve stimulation with glutamate-evoked contractions. It has been reported, (Jung et al., 2018), however, that contractions elicited by 3-20 mM glutamate are approximately 8-70% of maximal contractions elicited by 300 mM KCl, and this range corresponds well to contraction amplitudes elicited by electrical stimuli with intraburst frequencies of 5-32 Hz in saline containing 1.5 mM calcium (Ormerod et al., 2016). In the

present work, however, contractions elicited by 3-20 mM glutamate ranged from 2% to 29% of KCl-induced contractions (Figure 4). This range should correspond to roughly the lower half of the range of nerve-evoked contractions reported by Ormerod et al. (2016). Over this contraction range, increasing glutamate concentration would be expected to decrease the threshold for proctolin by one order of magnitude (Ormerod et al., 2016), but the threshold would not be expected to increase within this range. Although the reduction in threshold for proctolin between 3 mM and 7 mM glutamate could contribute to the activity-dependence reported earlier for proctolin, the increase in threshold between 7 mM glutamate and 10 mM glutamate, cannot explain such activity-dependence. Thus, postsynaptic mechanisms resulting from increased glutamate release with increasing stimulus frequency probably contribute to some of the activity-dependence of proctolin's effectiveness, but such postsynaptic mechanisms do not account for all of the activity-depencence.

The goal of this part of the thesis was not to mimic the entire effect of increasing the frequency and number of impulses applied to motor neurons, but only to mimic an increase in glutamate release that would occur under such circumstances. The treatment regime used here circumvented the potential release of proctolin and/or other cotransmitters from synaptic terminals, which would probably increase with such increases in impulse activity. Since the results do not entirely match the predictions, it seems possible that cotransmitter release may contribute to the reduction in proctolin threshold at high stumulus frequencies and with increased number of stimuli per burst (Ormerod et al., 2016).

Since glutamate depolarizes the body wall muscles of Drosohphila 3rd instar larvae (Jan & Jan, 1976), the reduction in threshold for proctolin to enhance glutamate-evoked

contractions reported here might involve depolarization of the muscles or the consequent increase in intracellular calcium concentration that results from opening L-type calcuim channels (Gu & Singh, 1997) and calcium-dependent release of calcium from the sarcoplasmic reticulum (Peron et al., 2009; Sullivan et al., 2000). Calcium is necessary for many pathways and second messenger systems (Nicholls et al., 2012). It is also able to act as a second messenger itself. When a muscle cell is depolarized the membrane bound voltage-gated calcium channels open and allow calcium to enter the cell. This calcium binds to ryanodine and /or IP3 receptors on the endoplasmic reticulum (ER) which causes the release of calcium from internal stores (Hoyle, 1983). Since calcium is a ubiquitous second messenger (Nicholls et al, 2012), a variety of intracellular signaling pathways could potentially mediate activity-dependent changes in the threshold for proctolin to enhance contractions. This area requires further study.

One interesting finding was the time-dependence of proctolin's ability to enhance glutamate-evoked contractions (Figure 8). It was predicted that applying proctolin for four minutes before glutamate would increase the effectiveness of proctolin compared to simultaneous application of proctolin and glutamate for one minute, due to the extra time provided for activating second messanger pathways. Applying proctolin before glutamate, however, decreased proctolin's effectiveness completely, since proctolin did not increase contraction amplitude with this paradigm (Figure 8). This result suggests that the intracellular signals mediating proctolin's ability to enhance glutamate-evoked contractions are transient, lasting less than four minutes. Many intracellular signaling pathways modulate synaptic properties over periods of several minutes, including pathways involving cAMP, cGMP, calmodulin and protein kinase C (e.g. Badhwar et al., 2005; da Silva & Lange, 2008; Friedrich et

al., 1998; Noronha & Mercier, 1995). Nitric oxide, however, is labile and has a half-life of only 3-5 s in physiological solutions (Ignarro, 1990), and has been shown to mediate effects of cholinergic receptor activation in the insect, *Maduca sexta* (Zayas et al., 2000, 2002). These effects involve activation of nicotinic receptors that trigger an increase in intracellular calcium levels in some neurons (Zayas et al., 2000). Since proctolin's ability to enhance glutamate-evoked contractions is transient, further work should consider nitic oxide as a possible candidate for mediating proctolin's effects on *Drosophila* muscle. It would also be worthwhile to determine whether proctolin enhances glutamate's ability to increase intracellular calcium.

In conclusion, although many of the original predictions were not met this thesis does provide some insight into co-transmission and synaptic modulation. Evidence is provided that postsynaptic mechanisms may contribute to activity-dependent modulation by proctolin, but that such mechanisms do not appear to account fully for activity-dependent modulation. Proctolin enhances glutamate-evoked contractions, but its effectiveness is time-sensitive, lasting only briefly. The experiments performed with octopamine do not corroborate previous reports that this substance induces contractions of larval body wall muscles. Experiments with phentolamine do not fully support a role for release of octopamine as a co-transmitter, but this work requires further study.

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