Cell-selective modulation of the neuromuscular system in *Drosophila*

Kiel Graham Ormerod, Hon. B. Sc.

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Abstract:

The capacity for all living cells to sense and interact with their environment is a necessity for life. In highly evolved, eukaryotic species, like humans, signalling mechanisms are necessary to regulate the function and survival of all cells in the organism. Synchronizing systemic signalling systems at the cellular, organ and whole-organism level is a formidable task, and for most species requires a large number of signalling molecules and their receptors. One of the major types of signalling molecules used throughout the animal kingdom are modulatory substances (e.x. hormones and peptides). Modulators can act as chemical transmitters, facilitating communication at chemical synapses. There are hundreds of circulating modulators within the mammalian system, but the reason for so many remains a mystery. Recent work with the fruit fly, *Drosophila melanogaster* demonstrated the capacity for peptides to modulate synaptic transmission in a neuron-specific manner, suggesting that peptides are not simply redundant, but rather may have highly specific roles. Thus, the diversity of peptides may reflect cell-specific functions. The main objective of my doctoral thesis was to examine the extent to which neuromodulator substances and their receptors modulate synaptic transmission at a cell-specific level using *D. melanogaster*. Using three different modulatory substances, i) octopamine - a biogenic amine released from motor neuron terminals, ii) DPKQDFMRFa - a neuropeptide secreted into circulation, and iii) Proctolin - a pentapeptide released both from motor neuron terminals and into circulation, I was able to investigate not only the capacity of these various substances to work in a cell-selective manner, but also examine the different mechanisms of action and how modulatory substances work in concert to execute systemic functionality . The results

support the idea that modulatory substances act in a circuit-selective manner in the central nervous system and in the periphery in order to coordinate and synchronize physiologically and behaviourally relevant outputs. The findings contribute as to why the nervous system encodes so many modulatory substances.

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aspect of my life. You both inspire and motivate me to be a better person and I love you both.

Table of contents:

List of Figures:

List of tables:

List of Abbreviations:

5-HT: serotonin

BDSC: Bloomington *Drosophila* stock center

Ca^{2+}: calcium ion

cAMP: cyclic adenosine monophosphate

CNS: central nervous system

CS: Canton S.

DTKR: *Drosophila* tachykinin-like peptide receptor

EJP: excitatory junction potential

FR: FMRFa receptor

FR-IR: FMRFa receptor inverted repeat

GPCR: G-protein coupled receptor

gSAP: giant sarcomere associated protein

HL-3.1: Hemolymph-like saline

HL-6: Hemolymph-like saline

mepp: miniature end plate potential

Mg^{2+}: magnesium ion

K^{+}: potassium ion

mRNA: messenger Ribonucleic Acid.

NMJ: neuromuscular Junction

NT: neurotransmitter

OA: octopamine

PKA: protein kinase A

PLTX-II: ω-plectoxin-Pt1a

PNS: peripheral nervous system

Proct: proctolin gene

ProcR: proctolin receptor

TA: tyramine

TEM: Transmission Electron Microscopy

UAS: upstream activating sequence

VDRD: Vienna *Drosophila* RNAi Center

Chapter 1:

Introduction and Literature Review

1.01: General Introduction:

Communication between cells in a multicellular organism is arguably one of the most fundamental and critical processes in biology. Cell-to-cell communication and resource sharing enabled single-celled organisms to evolve into the multi-component, multi-faceted, complex, multicellular organisms which exist today. At a conceptual level, cellular communication may seem like a simple process, but it is one of the most diverse and complicated processes examined in science. Nearly all cells that comprise an organism need to be able to communicate and respond to signals from other cells in order to perform their very specialized tasks, often at critical times throughout that organism's life, to mediate critical physiological processes, to determine a cell's fate, and simply to survive and proliferate. Cellular communication is not restricted to cells within a single, multicellullar organism, or for communication within a single species. Intercellular communication is imperative between unicellular organisms to regulate population growth and to signal resource availability (e.g. bacteria and *Dictyostelium discoideum*). In complex multicellular organisms, being able to differentiate between self and non-self is imperative for survival, and requires an immune system to communicate with cells from different organisms and to generate appropriate defensive responses. One of the major forms of communication used throughout the animal kingdom is the release of small, soluble chemical factors, such as hormones and neurotransmitters, which can include both neuropeptides and biogenic amines. These molecules are used for communication on a local (cell-to-cell) level and also at a systemic level (by distribution via the circulatory system), and they regulate and/or mediate critical developmental, physiological and behavioural events. The importance of such signalling molecules in

regulating cell growth, proliferation and differentiation is demonstrated by the fact that breakdowns in cellular communication are associated with some of society's most severe and debilitating diseases and conditions (cancers, neurological disorders, obesity, diabetes, and many other conditions). However, before we can begin to understand and treat devastating diseases and conditions that affect our society, we must understand the basic components of cellular communication and what happens when these components malfunction or breakdown.

Chemical signalling plays a critically important role in the nervous system, where communication between nerve cells occurs by the release of chemical signals from one nerve cell to the next. There are billions of nerve cells (neurons) within the human brain, and they communicate by releasing chemicals called neurotransmitters (NTs). These transmitters are made, packaged and released at chemical synapses, specialized areas of association between two neurons. The postsynaptic cell often contains multiple receptors necessary for detecting and responding to NTs. Variability in the type of NTs as well as the type of receptor plays a major part in the ability of the nervous system to perform a wide range of functions. Further complexity and variability in cellular communication stems from the ability of other chemicals, called modulators, to alter communication between cells. A neuromodulatory substance is one that is released at or near a synapse or group of synapses and alters synaptic transmission by acting, with a variable time course, either pre-or postsynaptically, or both (Orchard et al, 1988).

Modulation

Biologically active modulatory substances mediate many types of signalling between cells such as autocrine, paracrine, endocrine and synaptic signalling. They play a

role during all stages of development and underlie a multitude of physiological and behavioural processes (Geary and Maule, 2010; Kastin, 2013; Yew et al, 1999). In the nervous system, biogenic amines (e.g. serotonin and dopamine) and neuropeptides can act as modulators. There are roughly 100 identified neuropeptides in the human CNS, and several hundred in invertebrates (Hummon et al., 2006; Hurlenius and Lagercrantz, 2001). Despite over half a century of investigation, it remains largely unknown why most vertebrate and invertebrate genomes encode such a large number of conserved peptides and their receptors. As molecular and genetic tools continue to develop, particularly in model murine and invertebrate systems, we are beginning to understand how neuropeptides modulate the activities of small populations of neurons and even individual neurons, and how modulation of these cells can alter physiological and behavioural outputs (Certel et a, 2010; Choi et al, 2011, Bargmann, 2012).

Cell-specific effects of modulation

Neuromodulators have been intensively studied for many decades, yet the reason why both vertebrate and invertebrate genomes encode often tens or hundreds of peptides, biogenic amines and other modulators still remains poorly understood. Typically, modulators are characterized by their effects at different synapses (e.g. neuron-neuron synapses or neuromuscular junctions) and by their effects on muscle contraction (Nichols et al, 2013). Often, multiple modulators have been demonstrated to have very similar or identical effects on target tissues, which has led some investigators to conclude that they are functionally redundant. However, scattered throughout the literature are examples of various modulators that have the capacity to preferentially affect some cells or cell-types, to affect some cells to a greater extent than others, or to evoke a response in some cells and no response in others (Evans, 1985; Anderson et al, 1988; Jorge-Rivera et al, 1996).

As electrophysiological, molecular, genetic and imaging techniques continue to develop, we are beginning to elucidate the functions of individual cells and small populations of cells within physiological systems (Certel et al, 2010; others). Consequently, we can now examine how modulators can alter the function of individual cells and how cell-selective modulation can influence physiological and behaviour events. There are numerous reports that various modulators can act on subsets of neurons in order to activate specific neural circuits to ultimately produce a specific behavioural outcome (Seleverston, 2010; Kravitz, 1988). This capacity of modulators to work in a cell-selective manner on individual cells or small neural circuits may be a reason why so many peptides are encoded within genomes. To date, most reports of cell-specific or cell-selective effects of modulators have described effects on neurons. The major aim or focus of this thesis is to examine a subset of modulatory substances within the model organism, *Drosophila melanogaster*, in order to determine whether these modulators possess the ability to work in a cell-selective manner on muscle cells.

1.02 Literature Review:

This thesis is primarily aimed at characterizing and differentiating between various neuromodulatory substances that influence cellular communication within a model organism. However, before reviewing the relevant literature it is important to establish the basic foundational information pertaining to cellular communication generationally.

Juxtacrine Signalling

Communication between two partnering cells can occur either by direct communication or by chemical-mediated signalling. Direct communication is also known as **juxtacrine signalling** and is a contact-dependent process. There are at least three major types of juxtacrine signalling. The first type involves a communication junction that directly links the cytoplasm of the two communicating cells. The two most prolific examples of these are: i) plasmodesmata in plants, which are channels that traverse the cell walls, and ii) gap junctions in animals (Lodish et al, 2013). Gap junctions (also known as a nexus or macula communicans) occur as a result of the formation of a channel, more typically many channels, which connect across the intercellular space between two cells. Once formed, these channels directly link the cytoplasm of the two cells, enabling the movement of small molecules and ions between the two cells. Gap junctions are found in all cells in the human body except skeletal muscle cells, and underlie many critical processes such as synchronization of the myocontractile cells of the heart, and electrical communication between nerve cells (Silverhorn et al, 2012).

The second type of juxtacrine signalling is **cell-cell signalling**, where typically the initiating cell, the inducer, exposes a protein ligand on the extracellular surface of the plasma membrane and the responding cell has a complimentary receptor for that ligand protein on the extracellular surface of its plasma membrane (Lodish et al, 2013). Binding of the ligand to its receptor induces a change in the responding cell. One of the best known examples of this is the Notch pathway (Gilbert, 2011). Cells expressing proteins known as Delta, Jagged or Serrate on the surface of their membranes activate neighbouring cells containing the receptor protein, Notch, in their membrane. Once these two molecules form a complex, Notch undergoes a conformational change and is cleaved by a protease (Gilbert, 2011). The cleaved component enters the nucleus and activates transcription factors to alter gene expression. It is important to note that other biomolecules such as saccharides and lipids can also act as inducing factors.

The third form of juxtacrine signalling **is cell-extracellular matrix signalling** (Lodish et al, 2013). The extracellular matrices are secreted molecules that make up the microenvironment of cells. This microenvironment produces the shape, size and strength of many tissues, like bone and cartilage. However, it provides more than just strength and support; it triggers signalling events by various surface growth factors and adhesion molecules (integrins). Proteins in the extracellular matrix directly influence the polarity, differentiation, proliferation, survival and behaviour of cells by communicating with the intracellular cytoskeleton and through the transmission of growth factors (Lodish et al, 2013). Integrins and proteoglycans are two major factors that detect and respond to physical and chemical changes in the environment, leading to a multitude of cellular changes (Kim et al., 2011).

Chemical Signalling

When examining communication between cells, it is imperative to be cognisant of all confounding possibilities, which is why this introduction began with an overview of all forms of communication. However, this thesis is primarily focused on the role of chemical communication between cells, and as such, will spend greater time and detail reviewing chemical signalling and the role of chemicals in communication not only locally, i.e. between two neighbouring cells, but also the role of chemical signalling at a distance.

Autocrine signalling occurs when chemical signals are produced by a cell, secreted, and then act directly upon receptors in the same cell that secreted them (Lodish et al, 2013). **Paracrine** signalling occurs between neighbouring cells and requires the synthesis and release of a chemical signalling molecule from a source cell that acts upon a neighbouring cell (Lodish et al, 2013). **Endocrine** signalling involves signalling molecules such as hormones, which act on target cells located at a distance from the site where the hormones are released (Lodish et al, 2013).

Signalling molecules

The types of signalling molecules that exist within an organism are highly numerous and vary in their structure and function. Not surprisingly, a large proportion of signalling molecules are proteins, but non-protein molecules such as steroid hormones, lipid-based molecules like eicosanoids, and even gaseous molecules like nitric oxide and carbon monoxide can serve as signalling molecules (Gomperts et al, 2009). To further complicate the situation, the types of receptors to which these molecules bind are just as

varied in their structure and function, possessing a wide variety of enzymatic activities; however, they are all proteins. The following section reviews basic information pertaining to the function and classification of modulators and their receptors, with a focus on modulatory substances in the nervous system.

A: Modulators in the nervous system

The research conducted in this thesis examines the role of modulators in an insect model species, *Drosophila melanogaster,* so relevant findings on modulatory substances in insects and other arthropods are highlighted; however, an overview of the general classification scheme for the major groups of modulatory substances is also presented.

A NT can be defined as a substance that is released at a chemical synapse and transiently alters the ionic permeability of the cell membrane of the postsynaptic cell (Orchard et al, 1988). In order for a putative substance to be classified as a NT, several criteria must be satisfied (Nichols et al., 2011). The substance must be produced inside the neuron, and precursor enzymes for the synthesis of the substance must also be present. Enough of the substance must be present within the neuron to elicit an effect on the postsynaptic cell. The substance must be released by the presynaptic neuron, and the postsynaptic neuron must contain the necessary receptors to respond to the substance. Finally, enzymes or a reuptake mechanism must be localized to the synapse to degrade or eliminate the continued action of the substance (Nichols et al, 2011). NTs can be classified broadly into two groups according to their function: excitatory substances are those which increase the probability that a neuron will fire an action potential, and inhibitory substances are those which decrease the probability that a neuron will fire an action potential. NTs have also been categorized according to their chemical structure and fall into six groups, which are: 1) acetylcholine, 2) amino acids, 3) neuropeptides, 4) monoamines, 5) purines, 6) lipids and gases (Thompson, 2009). It has also been suggested that single ions (such as synaptically released zinc) also meet the requirements for being considered NTs (Minami et al, 2002).

The classical NT is one that, according to Dale's principle, is released from all the synaptic terminals of a neuron, and these neurons release one and only one transmitter at their synapses. In this case, a neuron would perform the same chemical action at all of its synaptic connections, regardless of the identity of the target cell (Burnstock, 2004).

Mechanism of synthesis and release of neurotransmitters

Neurotransmitters are released from axon terminals, which are also referred to as nerve terminals, synaptic boutons or terminal boutons. Small molecule transmitters are usually synthesized within the bouton from chemical substrates or precursor molecules. The enzymes that catalyze the production of such transmitters are synthesized in the cell body and transported down the axon to the synaptic bouton. Neuropeptides, however, are encoded in the genome within genes for larger peptide molecules whose sequences are transcribed in the nucleus and translated within the cell body. These large "prepropeptide" molecules are cleaved by peptidase enzymes to produce biologically active oligopeptides that are typically 3-40 amino acids in length. These oligopeptides are packaged into vesicles that are transported down the axon to the synaptic terminals (Thompson, 2009).

Within the synaptic bouton, small-molecule NTs are stored in small synaptic vesicles that are typically 40 nm in diameter. These vesicles appear to be organized into two major pools: one is a readily releasable pool, consisting of vesicles that are docked and ready to release their contents into the synaptic cleft, and the other is a non-readily releasable pool, which constitute a reserve of vesicles that can become docked to the presynaptic membrane for subsequent transmitter release. Neuropeptides are stored in

larger vesicles that are referred to as dense core vesicles because their contents exhibit dense staining in transmission electron micrographs (van de Bospoort et al, 2012). Both types of vesicle release their contents by fusing with the plasma membrane in response to an increase in intracellular calcium.

The mechanisms underlying the docking and fusion of vesicles to the presynaptic membrane are best explained by the SNARE (soluble NSF attachment protein receptor) hypothesis, for which a Nobel prize in Medicine was awarded to Hansson and Rothman in 2013. For recent reviews see Rothman, (2014), Kaeser and Regehr, (2014.) Briefly, a set of proteins known as v(vesicle)-SNARES localized in the vesicle membrane interact with another set of proteins known as t(target)-SNARES localized in the presynaptic plasma membrane, and the protein-protein interaction directs some vesicles to an area of the presynaptic membrane known as the active zone. These vesicles form what is known as the readily releasable pool. When an action potential depolarizes the presynaptic bouton, it triggers the gating of voltage-gated calcium channels (VGCCs) resulting in the influx of calcium into the presynaptic terminal. Some proteins (e.g muc13, muc18) also tether VGCCs to the synaptic vesicles, enabling an efficient and rapid transition from the opening of VGCCs to calcium entering the bouton and binding to a calcium-sensing protein, synaptotagmin (Sudhof, 2013). The binding of calcium ultimately results in a conformational change in the SNARE-protein complex that promotes fusion of the docked vesicle to the presynaptic membrane and release of NT into the synaptic cleft. Once in the synaptic cleft, NT molecules diffuse rapidly and bind to proteinaceous receptors on the plasma membrane of the postsynaptic cell.

At the postsynaptic cell, NTs bind to protein-based receptors located in the postsynaptic plasma membrane. Under transmission electron microscopy (TEM) the postsynaptic plasma membrane often appears dark and thicker than a typical plasma membrane (Lauer et al, 2013). This is referred to as postsynaptic density and is a result of a vast accumulation of the cellular machinery, primarily G-protein coupled receptors (GPCRs) necessary for the detection and integration of chemical signals via NTs. The classical view of NT integration at the postsynaptic cell involves the NT binding to the receptor, leading to a transient change in the ionic permeability of the postsynaptic membrane. The effects are mediated through ligand-gated ion channels, also known as ionotropic receptors. These protein based structures are usually composed of at least two different domains, an extracellular domain which contains the ligand (NT) binding domain, and the transmembrane domain which contains the ion pore for the movement, or conductance of ions through the channel (Sato et al, 2008). Activation of an ionotropic receptor results in a direct flux of current into the postsynaptic cell. The composition of the receptor will alter a number of critical components which drastically affect its functionality. The ligand binding domain can be modulated by an allosteric binding site or by competitive binding of other ligands, blockers, agonists and antagonists. The ion pore itself can also be modulated in similar ways. The compositions of the receptor will also determine critical properties such as conductance, activation threshold, inactivation threshold, sensitivity, desensitization, and many other properties, all of which can have dramatic effects on efficient and effective communication between the two cells (Unwin, 1993).

In addition to the traditional, or classical view, chemical signal integration at the postsynaptic cell can involve a more complex and integrated signalling pathway. In many cases, activation of a postsynaptic receptor by a NT can initiate a second messenger system to evoke a response in the postsynaptic cell. The mechanism of action of such "non-classical" synaptic transmission and the second messenger cascade activated by receptor activation can vary enormously. Receptors that initiate such responses are known as metabotropic receptors (Unwin, 1993). The most common type of metabotropic receptors on the postsynaptic membrane are GPCRs. These are protein based molecules composed of 7 hydrophobic transmembrane spanning domains, with the N-terminus residing in the extracellular side of the membrane and the C-terminus on the intracellular side (Pin and Duvoisin, 1995). The functions of GPCRs are arguably as varied as those of ligand-gated ion channels.

Non-classical transmitters: Cotransmission

Just as there is a non-classical form of postsynaptic receptor activation, there is also a non-classical form of chemical communication from the presynaptic cleft. Generally speaking, classical transmitters are contained in small synaptic vesicles, whereas peptides are stored in large granular dense core vesicles (DCVs), but occasionally, transmitters are sometimes stored together with peptides in DCVs (Boarder, 1989). There is now a large body of literature which demonstrates the colocalization of multiple NTs and modulators at synapses in both vertebrate and invertebrate species. Additionally, literature also demonstrates colocalization of different types of postsynaptic receptors (e.g. nicotinic and ionotropic nucleotide receptors were identified in cholinergic terminals in the rat midbrain; Diaz-Hernandez et al, 2002). Evidence exists for co-release

of multiple excitatory substances, multiple inhibitory substances (GABA/glycine rat sacral dorsal commissural neurons) and even a combination of both excitatory and inhibitory substances (e.g. release of histamine, GABA, galanin, encephalin, and substance P in rat hypothalamus; Wu et al, 2002). GABA and a catecholamine (likely dopamine) are colocalized in neurons of the CPG controlling feeding-related behaviour in the mollusc *Aplysia* (Diaz-Rios et al, 2002). Colocalization has also been demonstrated in crayfish, cockroach and snail (Skiebe, 2003; Eckert et al, 2002; D'yakonova, 2002). Thus, it is now generally accepted that neurons can contain and release more than one chemical signalling molecule.

B: Cell-selective modulation.

Neuromodulators have been intensively studied for many decades, yet the reason why both vertebrate and invertebrate genomes often encode hundreds of modulators, hormones, peptides and their receptors still remains poorly understood. Modulators are typically characterized by their effects at various synapses (e.g. neuron-neuron, neuromuscular) and by their effects on muscle contraction. Often, multiple modulators have been shown to elicit very similar or identical effects on target tissues, which led many investigators to conclude that many modulators are functionally redundant (Hewes et al, 1998; Hooper and Marder, 1987; Nasbaum and Marder, 1988; Weimann et al, 1997; Evans 1984, 1994; Evans and Siegler, 1982; Brezina et al, 1996; Weiss et al, 1992; Worden et al, 1995). However, the literature does contain examples of various modulators that have the capacity to affect some cells or cell-types, while evoking no response in others (as described in succeeding sections below). As electrophysiological, molecular, genetic, imaging and other tools continue to develop, we are gaining a better understanding of the functions of individual cells and small populations of cells within physiological systems. Consequently, we can now examine how modulators can alter the physiological functions of individual cells and how cell-selective modulation can influence behaviour. On a more organismal level, there are also numerous reports that various modulators can act on subsets of neurons and their effector cells in order to activate specific neural circuitry to ultimately produce a specific behavioural outcome. This capacity of modulators to work in a cell-selective manner on individual cells or small neural circuits may be a reason why so many peptides are encoded within genomes. Here I review this idea of cell-selective modulation with an emphasis on the invertebrate literature.

This aspect of the literature review focuses on how modulators alter communication between neurons and between neurons and muscles. It also explores the capacity of neuromodulators to act systemically to activate and synchronize the 'biological circuitry' necessary for coordinating physiologically and behaviourally relevant processes. Behaviour is generated by the coordinated activities of sensory neurons, interneurons, motor neurons and muscle cells, and modulators have been shown to act on each of these cell types. Rhythmic behaviour is generated by circuits of neurons in the central nervous system, known as central pattern generators (CPGs), which produce rhythmic bursts of efferent impulses that activate muscles, and muscle contraction alters sensory feedback to the central pattern generators. Thus, muscles play important roles both as effector organs and in generating re-afferent signals that help shape the motor output to meet physiological or behavioural demands (Harris-Warrick and Johnson, 2010; Hooper and Dicaprio, 2004; Marder and Bucher, 2007; Marder et al, 2005; Marder and Calabrese, 1996, Selverston, 2010). An overview of the function of each of these (i) central output/ CPGs, ii) interneurons, iii) motor neurons, iv) muscle cells, and v) sensory feedback) is reviewed in the next few sections, and details are provided describing how modulators can alter their output, as well as the evidence for cell-selectivity in those systems.

Central pattern generators:

It is widely accepted that rhythmic behaviours are produced by CPGs within the central nervous system (CNS). CPGs are usually comprised entirely by interneurons, but in some invertebrates motor neurons are also part of the CPG (Hooper and Dicaprio, 2004; Selverston, 2010). The CPG produces rhythmic bursts of impulses that are

appropriately timed so that antagonistic muscles are activated out of phase (Marder and Calabrese, 1996). Studies of small neural networks such as those that generate the *Aplysia* gill withdrawl reflex, the escape response in *Tritonia,* and pond snail feeding/breathing provide insight into how circuits function to elicit rhythmic or reflexive behaviour (Mongeluzi et al, 1998; Ezzeddine and Glanzman, 2003; Straub et al, 2002; Spencer et al, 2002). Arguably the most well studied CPG system with regard to functional connectivity and modulation by biogenic amines and neuropeptides is the crab/lobster stomatogastric (STG) nervous system. The cells of the STG have been demonstrated to be modulated by over thirty neuromodulatory substances that are released by secretory organs or are secreted directly by axon terminals in the STG (Marder and Bucher, 2007). Many neurons within the STG have been recorded from directly using intracellular recordings and have been shown to respond to many endogenous modulatory substances. For example, exogenous application of eight different modulatory substances elicits characteristic but different forms of pyloric rhythm recorded simultaneously from the pyloric dilator, lateral pyloric and lateral ventricular nerve (Marder and Weimann, 1992). Hooper and Marder (1987) demonstrated that proctolin increases the amplitude and frequency of bursts produced by isolated pacemaker anterior burster neurons, isolated lateral pyloric and PY neurons respond to proctolin only when at or above threshold, and all other pyloric neurons are unaffected by proctolin. Thus, the STG represents a tremendous model system for examining cell-selective effects of chemical modulators on neurons (Marder and Bucher, 2007; Marder et al, 2005; Marder and Calabrese, 1996).

Sensory feedback

A historically debated topic in our understanding of the neuronal circuitry underlying behaviour is whether or not output from a CPG requires feedback from sensory neurons. While it is generally accepted that CPGs can produce rhythmic activity without external influences, in many isolated or reduced model systems it has been observed that the "fictive" motor pattern generated by the CPG is not identical to impulse patterns recorded from behaving animals with minimal disruption of sensory inputs (Ausborn, 2009; Beenhakker et al., 2005; Borgmann et al., 2009; Zill and Keller, 2009). This suggests that physiologically appropriate motor output requires afferent input from sensory systems (Blitz and Nausbaum, 2011, Fox et al, 2006, Song et al, 2007, Berni et al, 2012; Marder and Bucher, 2007). Sensory input has been demonstrated to help keep motor neuron bursts in phases that are appropriate for the behaviour, to terminate or activate motor patterns, and to modulate ongoing motor patterns (Brigmann and Kristan, 2008; Fetcho et al, 2008; Dubuc et al, 2008; Stein, 2009). Thus, in addition to the obvious role sensory neurons play by providing information about an animal's environment, inputs from re-afferent sensory neurons play a crucial role in modulating and fine-tuning the motor output generated by the CPG.

Given their importance in modulating the output from the CPG, it should seem likely that sensory neurons would be a key target for modulation by various chemical modulators. There is, however, a surprisingly limited number of investigations which examine the role of modulators on sensory neurons, and consequently even fewer which demonstrate the ability of these modulators to work in a cell-selective manner on sensory cells. Pasztor et al., (1988) isolated a proctolin-like substance from the peripheral sensory

endings of a lobster mechanoreceptor, the oval organ, and demonstrated stretch-dependent release of proctolin, suggesting that in this system proctolin may function to self-modulate sensory transduction. They also reported that parts of the lobster sensory neurons (afferent and branching dendrites) showed proctolin-like immunoreactivity (Pasztor et al., 1988). Other arthropod mechanoreceptors can be influenced by modulators as well (Pasztor and Bush, 1987, 1989; Pasztor and Macmillan, 1990; Cooper and Hartman, 1992; El Manira et al. 1991), and octopamine (OA) has been shown to modulate insect wing stretch receptors (Ramirez and Orchard, 1990). Pasztor and MacMillan, (1990) performed a comparative study using 7 different primary afferents from two arthropods, crayfish and lobster, and examined the responsiveness of the sensory cells to exogenous application of 3 different modulators (proctolin, octopamine and serotonin). Octopamine elicited excitatory effects on 6 of the 14 fibres and inhibitory effects on 2 of the fibres. Serotonin also had excitatory effects on 6 different fibers, but only 2 of those fibers were also responsive to octopamine. Serotonin also had inhibitory effects on the same two fibers as octopamine. Proctolin, however, enhanced receptor potential amplitudes and increased firing in 10 of the 14 fibres examined. Interestingly, Pasztor and MacMillan (1990) observed species-specific effects, where neurohormones were excitatory in one species and inhibitory or ineffective in the other. These results indicate cell-specificity with respect to effects of modulatory substances on sensory neurons, and they also indicate species specificity of neuromodulatory effects.

Biogenic amines and peptides in the CNS

I have already established that a large number of neuromodulatory substances are often encoded within genomes, and consequently the number of responsive neurons may

seem infinitely large. However, neuromodulatory neurons such as the aminergic and peptidergic ones are usually found in small numbers in most species of animals, representing less than 0.5% of neurons (Maeda, 2000; Maeda et al, 1991, Ungerstedt, 1971). The fields of innervation in these neurons are typically wide reaching with extensive arborizations facilitating broad-reaching impacts, and it is increasingly clear that at the level of small populations of neurons or individual neurons, considerable specificity exists in the fields of innervation and in their function (Alekeyenko et al, 2013; Andrews et al, 2014; Rezaval et al, 2012, Roy et al, 2007). While both anatomical and functional evidence exists to support this idea, the capacity to investigate the effects of neuromodulators on single cells, or small populations of cells within the central nervous system of an organism *in vivo*, has not been observed in many systems. In fact, outside of one or two model systems (*Drosophila* or *C.elegans*), attempts thus far are left with a fairly large, heterogeneous population of neurons (Bang et al, 2012; Brust et al, 2014; Dymecki et al, 2010).

Drosophila melanogaster is a good model system in which to manipulate and characterize the function of small populations of neurons within the CNS. There are approximately 100 octopaminergic neurons, 100 serotonergic neurons, and 80 proctolinergic neurons in the CNS of the fruit fly (Monastirioti et al, 1995; Alekseyenko et al, 2014; Anderson et al, 1988; Taylor et al, 2004). This relatively small number of neurons increases the feasibility of identifying the functions of octopamine, serotonin and proctolin within the CNS and the behavioural roles of these substances. *Drosophila* is also amenable to molecular and genetic techniques, with the potential to enable researchers to manipulate secretion and levels of expression of NTs and receptors in

single neurons or small populations of neurons *in vivo* (Alekseyenko et al, 2013). Mao and Davis (2009) examined eight different types of dopaminergic neurons that innervate the mushroom body neuropil of the *Drosophila* brain. The authors demonstrated that each type projects to a different region in the neuropil, and they postulated that these eight neuron types may differ in response properties and, thus, regulate different aspects of behaviour. Other studies in *Drosophila* have revealed that functionality can be associated with small subsets of neurons, such as the dopaminergic ones, influencing aversive reinforcement and appetitive memory output (Krashes et al 2009; Claridge-Chang et al, 2009). Recently studies have begun to demonstrate the function of 2-4 modulatory neurons in complex behavioural systems. Certel et al (2010) showed that three octopaminergic neurons in the suboesophageal ganglia altered male courtship behaviour and appeared to be necessary for guiding male behaviour in contact-dependent choice situations. Alekseyenko et al., (2010) narrowed down the possible roles of serotonin (5HT) and dopamine (DA) by genetically altering activity in subsets of DA and 5HT neurons. Altering expression of both DA and 5HT simultaneously abolished mid-and high-level aggression, selective disruption of 5HT signalling resulted in flies with a diminished ability to escalate fights, and elevating 5HT activity caused flies to escalate fights faster and fight at higher intensities. Acutely altering DA neurons made flies hyperactive and rarely social. Subsequently, Alekseyenko et al (2013) demonstrated that altering activity (by increasing or decreasing activity) in two sets of dopaminergic neurons in *Drosophila* (one from the T1 cluster and another from the PPM3 cluster) caused an increase in aggression. Bidaye et al (2013) screened 3400 fly lines for altered walking behaviour in flies, eventually narrowed expression down to two neurons (MAN

and MDN) and showed these two neurons caused flies to walk backwards, or to 'moonwalk'. As mentioned earlier, within the *Drosophila* CNS are relatively few peptidergic and aminergic positive neurons. While these cells are likely responding to peptides and biogenic amines released as transmitters, and cotransmitters; it does enable us to examine how and where cell-selective processes are employed, and the mechanisms underlying these processes.

Other research groups have also used these tools effectively to describe developmental processes in *Drosophila* (Chan and Kravtiz, 2007; Landgraf et al, 2003a, 2003b; Berni et al, 2012; Mozer and Sandstrom, 2012; Singh et al, 2010). Very little is known about whether the innervation patterns of peptidergic and aminergic neurons are conserved or completely overhauled during metamorphosis. Roy et al (2007) examined the morphology of serotonin-immunoreactive deutocerebral (CSD) interneurons during *Drosophila* development, and found dramatic changes in the number of dendritic branching points. Surprisingly, dendrites appear to almost completely retract after the larval stages and extend new projections (100's of branching points) during pupation and into adulthood. Singh et al (2010) showed that dendritic pruning and development are correlated with activity in single cells or small populations of neurons. More specifically, they demonstrated that a reduction in excitability in CSD neurons resulted in a lack of refinement of dendrites. They later demonstrated that wingless (Wg, *Drosophila* Wnt), levels increase with increased neuronal activity, and provide evidence that Wnt signalling stimulated by neural activity drives dendritic refinement. Thus, changes in activity in serotonergic neurons can dramatically change their field of innervation, and consequently the neural circuitry recruited by these cells. Together with the abovementioned roles of

these cells, and other aminergic and peptidergic cells in behaviour, altering the activity of single-cells can have broad-reaching effects. Zwart et al (2013) recently demonstrated that the dendritic arbors of motorneurons from *Drosophila* larvae are regulated on a cell-by-cell basis by the steroid hormone receptor ecdysone receptor-B2, which is followed by an enhancement of neuronal activity.These studies demonstrate that we have the tools necessary to manipulate and observe changes in small populations of neurons and even individual cells, and consequently we are beginning to understand the function of these cells within systems, and how modulation of these cells can alter physiological and behavioural output (Bargmann, 2012; Certel et al., 2010; Choi et al., 2011). These same tools can also be used to understand synaptic function and behavioural roles of transmitters and modulators. Molecular and genetic methods can be used to identify aminergic and peptidergic neurons in the CNS and to elucidate their physiological and behavioural roles. However, the neurons onto which the aminergic and peptidergic neurons project remain mostly unidentified in *Drosophila*.

Neuromuscular junctions

Much of the work on neuromodulation and chemical communication generally has focussed on synapses between neurons and muscle cells. A large number of neuromodulatory substances can affect the strength or efficacy of synaptic interactions and/or alter the intrinsic properties of neurons and muscles (Harris-Warrick et al, 1992; Marder and Calabrese, 1996; Jorge-Rivera et al, 1998). Studies in multiple species have demonstrated that many of the same substances can act on the same target tissue. In *Drosophila*, Hewes et al (1998) demonstrated that seven of the peptides encoded in the *Drosophila* FMRFa gene all increased the strength of nerve-evoked muscle contractions

in third instar larvae and concluded that these peptides appear to be functionally redundant. In the crustacean STG ganglion, the lateral pyloric neuron has been demonstrated to respond similarly to over 10 neuromodulators (Marder and Weimann, 1992; Hooper and Marder, 1987; Nasbaum and Marder, 1988; Weimann et al, 1997). Other invertebrate examples include the locust (Evans 1984, 1994; Evans and Siegler, 1982), *Aplysia* (Brezina et al, 1996; Weiss et al, 1992) and lobster (Worden et al, 1995). Nerve evoked contractions in a single stomach muscle from crabs are modulated by over 10 neuromodulatory substances, but the degree of modulation depends on the rate of nerve stimulation, and this dependence differs between modulators (Jorge-Rivera et al., 1998). Several other neuromodulators appear to be more effective at different stimulus frequencies (Brezina et al, 1996; Evans, 1984; Evans and Siegler, 1982; Weiss et al, 1993). The mode of action of these neuromodulators is less well studied, but act presynaptically to alter NT release and others act postsynaptically to enhance contractions downstream of muscle depolarization. Thus, even though neuromodulators appear to be functionally redundant, their mechanisms of action are not always identical. This could influence which subset of neurons and effector cells are recruited upon their release ultimately determining which cells influence specific physiological or behavioural paradigms, thereby enabling them to work in a cell-selective manner.

There are examples of modulators that have the capacity to affect some cells or cell-types while evoking no response or weaker responses in others. For example, in the crab gastric mill, the peptide allatostatin-3 decreases the initial amplitude of EJPs and enhances facilitation in one muscle (gm6) without altering EJP amplitude or facilitation in another (gm4), and proctolin increases EJP amplitude in muscle gm4 but not muscle

gm6 (Jorge-Rivera et al., 1998). Changes in synaptic facilitation indicate presynaptic mechanisms (Zucker, 1989), but it is unclear whether the changes in initial EJP amplitude in these studies reflect presynaptic or postsynaptic effects. In lobster stomach muscles GABA decreases the amplitude of EJPs in some muscles (gm6a and gm9) but not in others (the p1 muscle; Gutovitz et al., 2001). In crab opener muscle, DRNFLRFamide increases transmitter release from nerve endings of the fast excitatory axon but not the slow excitatory axon (Rathmayer et al., 2002), but postsynaptic effects were not examined. DRNFLRFamide induces contractions in superficial extensor muscles of crayfish but not in deep extensor or superficial flexor muscles (Quigley and Mercier, 1992). In the deep extensor muscles, DRNFLRFamide increases contractions evoked by muscle depolarization, indicating a postsynaptic effect (Mercier et al., 1993). In lobster, the pentapeptide proctolin increases the amplitude of EJPs in superficial extensor muscles and in both muscles associated with the muscle receptor organs (RM_1 and RM_2), but proctolin has no effect in the deep abdominal extensors (Pasztor and Golas, 1993). Serotonin and the lobster neuropeptide TNRNFLRFamide also increase EJPs and nerve-evoked contractions in these four lobster muscles, but serotonin is more effective on the tonic muscles and TNRNFLRFamide is more effective on the phasic muscles (Pasztor and Golas, 1993). In *Drosophila* larvae, the *Drosophila* neuropeptide DPKQDFMRFa increases the amplitude of EJPs elicited by stimulating motorneuron MN6/7-Ib but not those elicited by motor neuron MNSNb/d-Is (Dunn and Mercier, 2005). Taken-together, these studies provide evidence that although modulatory substances may appear to be functionally redundant, they can alter synaptic transmission at synapses between specific motor neurons and their target muscle cells. These observations raise the possibility that

modulation of selected synapses and muscles at the neuromuscular level might play a role in generating specific behaviours (Marder and Bucher, 2007; Marder et al, 2005; Marder and Calabrese, 1996).

Muscle

Studies examining behavioural effects of neuropeptides often focus on neural circuitry and overlook effects on effector cells (Hooper et al., 2007; Morris and Hooper, 2001). Arthropod muscles, however, integrate synaptic inputs from multiple axons that can be excitatory or inhibitory, and in many cases they contract in response to graded electrical signals or even to hormones (Atwood, 1976; Atwood and Cooper, 1995; Atwood et al., 1965; Meyrand and Marder, 1991; Peron et al., 2009). It has been postulated that modulation of centrally generated motor patterns in arthropods by NTs or hormones can be complemented by peripheral modulation at neuromuscular synapses and/or muscle fibres by the same or similar substances (Dickenson et al, 2015). In crab hearts, for example, FLRFamide peptides act centrally to increase the rate and amplitude of contractions by altering the rate of bursts generated by the cardiac ganglion, and they act peripherally to augment excitatory junctional potentials (EJPs) and muscle contractions (Fort et al., 2007). FLRFamides also act directly on crab stomatogastric ganglion to increase the pyloric rhythm and to evoke gastric mill activity, and they act peripherally to enhance EJPs and contractions in gastric mill muscles (Jorge-Rivera et al., 1998; Weimann et al., 1993). Thus, central and peripheral modulatory effects appear to be coordinated to produce physiologically appropriate changes in muscle performance. Few studies, however, have examined the possibility that peptidergic or aminergic modulators act directly on effector cells in a cell-specific manner. One such study was

with octopamine, which increases relaxation rate and cAMP levels more strongly in regions of the locust extensor-tibiae muscle that contain the highest proportions of slow and intermediate muscle fibers (Evans, 1985). Thus, although peripheral modulation by neuropeptides can involve cell-specific effects on neurons, there is a conspicuous lack of evidence that neuropeptides exhibit such specificity on muscle cells.

Drosophila melanogaster offers an opportunity to investigate cell-selective effects in muscles because all the fibers in body wall muscles of third-instar larvae are identifiable, and their innervation is known. Since a FMRFamide peptide elicits neuron specific effects at synapses on these muscle cells (Dunn and Mercier, 2005), it would be important to know whether this peptide also elicits cell-specific effects on muscle. Such information would indicate the extent to which cell-specific modulatory effects, which can occur in the CNS, extend to the peripheral level.

In *Drosophila*, the NT released from motorneuron terminals is generally glutamate (Jan and Jan, 1976), but some motorneurons in third instar larvae also contain co-transmitters (proctolin, octopamine, pituitary adenylate cyclase activating polypeptide and insulin-like peptide; Anderson et al, 1988; Monastirioti et al, 1995; Peron et al, 2009). Physiological effects of these co-transmitters on *Drosophila* larval muscles have not been well studied. Motor neurons containing proctolin and motor neurons containing octopamine innervate some but not all larval muscle cells (Anderson et al, 1988; Monastirioti et al, 1995), which suggests that modulation of these muscles could be cell specific. This possibility also provokes some fundamental questions about neuromodulation. Does the presence of a co-transmitter presynaptically coincide with

preferential or cell-selective effects on muscle cells? Does a neuromodulatory substance affect all muscle cells, or affect some cells more than others?

Monastirioti et al, (1995) demonstrated that octopamine is found only in a subset of motorneurons innervating the larval bodywall muscles, specifically those neurons innervating fibers 12 and 13 but not 6 or 7. In Chapter 2, I examine the ability of this modulatory substance to work in a cell-selective manner on these muscle fibers. Dunn and Mercier, (2005) showed that DPKQDFMRFa increases EJP amplitude in muscle fibers 6 and 7, but only if they are elicited by one motor neuron (MN6/7-Ib) and not if they are elicited by another motor neuron (MNSNb/d-Is). In Chapter 3, I examine whether this peptide, which is thought to be released as a hormone (White et al, 1986), also works in a cell-selective manner on bodywall muscles of third-instar larvae. Anderson et al, (1988) demonstrated that motorneurons innervating muscle fibers 4, 12 and 13, but not 6 or 7 are immunoreactive for proctolin. Interestingly, Taylor et al, (2004) also demonstrated that proctolin is present in the ring-gland, the primary structure for the release of hormones. In Chapter 4, I examine the ability of proctolin to work in a cell-selective manner on the larval bodywall muscles, and I also attempt to examine how/why a neuromodulatory substance would be released both as a co-transmitter and as a peptide.

The three modulators examined in this thesis differ from each other with regard to the locations from which they are stored and released. OA is present in motorneuron terminals on numerous larval muscle cells in *Drosophila* (Monastirioti et al, 1995), and OA is thought to be contained within dense core vesicles (DCVs) and released as a co-transmitter (Peron et al, 2009). DPKQDFMRFa is only released as a hormone from neurosecretory cells found in the larval CNS and in those cells innervating the ring gland

(White et al, 1986). Proctolin is likely released both as a co-transmitter from DCVs at synapses predominantly on larval muscles 4, 12 and 13 (Anderson et al, 1988) and as a hormone from the ring gland (Anderson et al, 1988; Taylor et al, 2004). Thus, each of the three modulators will enable me to examine a unique question about the form of chemical communication utilized (i.e. paracrine vs. endocrine signalling, vs. both) and how such signalling might influence the physiological effects of each modulator.

1.03 Main objectives

The main objective of this thesis is to examine the capacity of modulatory substances to work in a cell-selective manner in *Drosophila melanogaster*. There are many lines of evidence for the ability of various neuromodulatory substances to alter the function or output in subsets of neurons, and that such cell-selection underlies activation of neural circuits that generate behaviour. I hypothesize that neuromodulatory substances can also work selectively on subsets of muscle cells to alter EJP amplitudes and/or muscle contractions. I propose that cell-specific modulation at the muscular and neuromuscular levels play roles in behaviour, and that peripheral modulation may be synchronized and integrated with central modulatory effects to produce physiologically appropriate changes in contraction of selected muscles or muscle cells. The research in this thesis utilizes a wide array of approaches, including electrophysiology, genetics, molecular biology, biochemistry, engineering and behavioural observations in order to characterize the physiological effects of several invertebrate modulators.

The first aim of this research is to investigate whether the cotransmittters/ biogenic amines, octopamine and tyramine, elicit cell-selective effects at the neuromuscular junction at the body-wall muscles of third-instar *Drosophila* larvae. Using a variety of electrophysiological and pharmacological approaches, this investigation demonstrates that both octopamine and tyramine alter communication at neuromuscular junctions. It further demonstrates that at higher concentrations octopamine selectively enhances synaptic communication to a greater extent in a subset of muscle fibers than others, and that this enhancement is conserved when examining force-generation in those cells.

The second aim of this research is to examine the ability of a neuropeptide hormone to work in a cell-selective manner at the neuromuscular junctions of *Drosophila* third-instar larvae. Using a variety of electrophysiological, genetic, molecular and force recordings, I differentiate and discriminate the mode of action of DPKQDFMRFa on pre- vs. postsynaptic cells as well as its direct action on muscle cells. I further demonstrate that the peptide selectively enhances synaptic communication from a subset of muscle fibers over others, and that selective enhancement is conserved on the enhancement of force generation. I also show that the ability of this peptide to work in a cell-selective manner is strongly correlated with the expression of the FMRFa receptor in muscle cells.

My third and final aim is to examine the ability of a putative cotransmitter and hormone to alter communication at the *Drosophila melanogaster* neuromuscular junction. Using a variety of electrophysiological, molecular, and behavioural approaches I am the first to demonstrate a variety of physiological and behavioural roles of proctolin in *Drosophila* larvae. I examine the physiological implications of releasing the same chemical modulator as a hormone and as a cotransmitter. My data show that proctolin is able to selectively activate subsets of muscle cells, and that it can enhance nerve-evoked contractions at lower concentrations when the motor neurons are stimulated at high impulse frequencies that are likely to release co-transmitters. The results also suggest that systemic release of proctolin can enhance contraction to a greater extent in muscle cells that receive proctolinergic innervation than in muscle cells that do not.

Overall, this thesis provides further evidence that various neuromodulatory substances can work in a cell-selective manner, and the findings may help to explain why the genome encodes so many peptides and their receptors.

Chapter 2:

Action of octopamine and tyramine on muscles of *Drosophila melanogaster* larvae.

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2.01 Abstract

Octopamine (OA) and tyramine (TA) play important roles in homeostatic mechanisms, behavior, and modulation of neuromuscular junctions in arthropods. However, direct actions of these amines on muscle force production that are distinct from effects at the neuromuscular synapse have not been well studied. We utilize the technical benefits of the *Drosophila* larval preparation to distinguish the effects of OA and TA on the neuromuscular synapse from their effects on contractility of muscle cells. In contrast to the slight and often insignificant effects of TA, the action of OA was profound across all metrics assessed. We demonstrate that exogenous OA application decreases the input resistance of larval muscle fibers, increases the amplitude of excitatory junction potentials (EJPs), augments contraction force and duration, and at higher concentrations (10^{-5} and 10^{-4} M) affects muscle cells 12 and 13 more than 6 and 7. Similarly, OA increases the force of synaptically driven contractions in a cell-specific manner. Moreover, such augmentation of contractile force persisted during direct muscle depolarization concurrent with synaptic block. OA elicited an even more profound effect on basal tonus. Application of 10^{-5} M OA increased synaptically driven contractions by ~1.1 mN but gave rise to a 28 mN increase in basal tonus in the absence of synaptic activation. Augmentation of basal tonus exceeded any physiological stimulation paradigm and can potentially be explained by changes in intramuscular protein mechanics. Thus, we provide evidence for independent but complimentary effects of OA on chemical synapses and muscle contractility.

2.02 Introduction

The biogenic amine octopamine (OA) is considered to be the invertebrate analog of norepinephrine, and investigations of OA's effects on various arthropod physiological systems have provided insight into fight or flight physiology (Adamo et al., 1995; Hoyle, 1975; Orchard et al 1982; Roeder, 2005). Effects of OA within the CNS have been studied in model arthropod preparations for several decades and have elucidated important physiological and homeostatic processes, such as energy liberation (Downer, 1979; Fields and Woodring, 1991; Mentel et al., 2003), modulation of metabolic rate, circulation, respiration and ion regulation (Battelle and Kravitz, 1978; Bellah et al., 1984; Blumenthal, 2003; Wierenga and Hollingworth, 1990) and establishment of social hierarchies (Kravitz, 1988). Modulatory actions of OA on synaptic potentials at the arthropod neuromuscular junction (NMJ) have been described in detail (Grundfest and Rueben, 1961; Kravitz et al., 1976; Wheal and Kerkut, 1976; Florey and Rathmayer, 1978; Keshishian et al., 1996; Nagaya et al., 2002). OA also elicits direct effects upon insect muscle fibers, altering contraction parameters such as basal tonus, peak force, and catch tension (Evans and O'Shea, 1978 and 1979; Evans and Siegler, 1982; Stevenson and Meuser, 1997). Nevertheless, the actions of OA on muscle force production and intramuscular targets have not been well investigated in behavioural contexts. Moreover, very few studies have attempted to distinguish intramuscular actions of OA from those at the NMJ (Fisher and Florey, 1983; Fox et al., 2006).

OA is synthesized *de novo* from the amino acid tyrosine via a two-step enzymatic conversion, first to tyramine (TA) then to OA. TA was once considered only an intermediary of OA biosynthesis, but has now been demonstrated to have its own

independent effects on synaptic transmission and to function through independent receptors (*Drosophila*: Nagaya et al., 2002; Bayliss et al., 2013. *C. elegans*: Alkema et al., 2005; Pirri et al., 2009. Acrididae: *Locusta etc*: Kononenko et al., 2009; Vierk et al., 2009; Homberg et al. 2013). The actions of OA and TA appear to vary considerably across arthropod preparations, in some cases even by sign (i.e. locust vs. fly: Evans and Siegler, 1982; Nagaya et al., 2002; Saraswati, et al., 2004; Walther and Zittlau, 1988). Although the action of OA is typically profound, there is some disagreement as to the action of amines within *Drosophila* preparations due in part to use of calcium-free, high magnesium saline (Kutsukake et al., 2000; calcium-free HL3 contains 20 mM Mg^{2+}), which is known to have anesthetic effects and supress membrane excitability in many animals, including *Drosophlia* (Chordata: Iseri and French, 1994; Arthropoda, Crustacea: Katz, 1936; Arthropoda, Insect – *Drosophila:* Feng et al., 2004). Indeed, whereas some report that TA greatly attenuates neuromuscular transduction (Roeder, 2005), others suggest that it has little or no action on the longitudinal muscle fibers of fly (Nagaya et al., 2002, Ormerod et al., 2012).

High levels of OA are found within insect central and peripheral nervous tissues where it functions as a NT and a neuromodulator (Roeder, 1999). Circulating levels of OA in the hemolymph of insects are also observed during stressful situations, where OA serves a neurohormonal role (Farooqui, 2007). OA has been shown to affect a number of behaviours (e.g. locomotion, flight, egg laying, aggressiveness, and ovulation) and is associated with major nervous system functions, such as desensitization and learning and memory (for reviews see, Roeder, 1999; Pflüger and Stevenson, 2005). These alterations in behaviour are accomplished through changes within the CNS as well as changes in the

periphery at muscles, all of which serve to alter muscle performance. Modulation at the peripheral level can be achieved by hormones, acting on both presynaptic and postsynaptic target cells, and by co-transmitters released from presynaptic terminals onto muscle cells at high impulse frequencies (Shakiryanova et al., 2005). OA acts as a neurohormone in insects (Roeder, 2005), and its presence in type II nerve endings in *Drosophila* larvae implicates OA as a NT (Monastirioti et al. 1995). OA has not been directly demonstrated to be co-transmitter, nor to be co-localized with other transmitters in *Drosophila* larval motoneurons; however, previous evidence suggests the presence of OA in dense core vesicles in other arthropods (Hoyle et al., 1980). It is necessary to distinguish effects upon neuromuscular synapses from direct effects on muscles (i.e. intramuscular or muscle membrane) that alter contractility in order to fully understand peripheral modulation.

Arthropod NMJs have long been used as models to study modulation of chemical synaptic transmission (Bradley et al., 1999) and provide several technical advantages. Arthropod muscles typically have a relatively small number of muscle cells, and in some cases the muscle cells are identifiable (e.g. Hoang and Chiba, 2001; Lnenicka and Melon, 1983; Velez and Wyman, 1978). In some of these model systems, the motoneurons have also been identified, and the patterns of innervation to specific muscle fibers have been well characterized [cockroach: Ahn and Full, 2002; Zill et al., 1981; fruit fly: Hoang and Chiba, 2001; crayfish: Lnenicka and Melon, 1983; Velez and Wymen, 1978; shrimp: Meyrand and Marder, 1991; locust: O'Shea et al., 1985; stick insects: Westmark et al 2009; tobacco hawkmoth: Weeks et al., 1997; mealworm: Hidoh and Fukami, 1987], making it possible to examine modulatory effects on chemical synapses between

identified synaptic partners. This also enables one to examine the inherent ability of modulators to act in a cell-specific manner. Modulatory substances like neurohormones, which interact systemically, need to do so in a coordinated manner, and thus cell-specificity would enable recruitment of selective circuitry.

Here we exploit the technical advantages offered by *Drosophila* larvae to distinguish between modulatory actions of OA on chemical synapses from direct effects on contractility of muscle cells. We utilized several strategies to make such a distinction in the location of OA action, including investigation of (1) passive membrane properties (i.e. membrane resistance), (2) principal components of EJPs, and (3) force production. Force augmentation by OA was characterized using the following three assays: (a) contractile force evoked via traditional electrical activation of the motor nerve (i.e. through the synapse), (b) basal muscle tonus in the absence of any synaptic activation, and (c) local depolarization concurrent with synaptic block. We provide evidence that in addition to its ability to augment muscle contractions and potentiate neuromuscular transduction, OA also augments evoked contractions downstream of chemical synapses. The OA-induced augmentation of basal tonus far exceeded augmentation of evoked contractions and is suggestive of a long-term, intramuscular change by an unidentified factor.

2.03 Materials and Methods

Animals and Basic Preparation

Drosophila melanogaster Canton S (CS) flies, obtained from the Bloomington *Drosophila* stock center, were used for all experiments. Flies were reared at 21°C on a 12:12 light-dark cycle and were provided with either a cornmeal-based medium (Boreal Laboratories Ltd., St. Catharines, Ontario, Canada) including dry yeast, or a Standard Diet (after David, 1962) consisting of 100 g yeast, 100 g glucose, 12 g agar and 10 mL propionic acid (mold inhibitor) combined in 1220 mL H_2O. Octopamine, tyramine, yohimbine, and cyproheptadine were acquired from Sigma Aldrich (St. Louis, MO).

Only early wandering stage third instar larvae were selected. Animals were collected from the sides of their culture vials and placed dorsal side up onto a dissecting dish containing either of two hemolymph-like *Drosophila* salines, HL-6 or HL-3.1, the compositions of which have been published (Macleod et al., 2002 and Feng et al., 2004, respectively). All of the experiments outlined herein were confirmed in both solutions except evoked contraction recordings (only HL-3.1). A semi-intact larval bodywall preparation (Paterson et al., 2010) was used for recording intracellular electrical signals and force (Fig. 1A.i). Briefly, larvae were incised along the longitudinal axis and pinned open. The segmental nerves could be severed near their exit from the ventral ganglion, and the CNS and all gut organs were removed. The bath was continuously perfused (0.7 mL/min, dish volume ~300 uL) with oxygenated physiological saline, except in the case of application of a toxin (§2.1, below), in which case saline containing the toxin was directly applied and not recirculated in an effort to avoid its residue / remnants confounding future experiments. Experiments followed the same basic application

routine: 10-15 minutes in control saline, application of amine, and wash-out for at least twice the duration of exposure to amine. In some experiments, muscle fibers 12 and 13, or fibers 6 and 7, were lesioned using fine dissection scissors. Unless noted, all bodywall muscles were intact.

Intracellular Recording

Intracellular recordings (Fig. 1A) were obtained using sharp micro-electrodes, produced from thin wall monofilament glass (WPI, Sarasota, FL, USA) using a Flaming-Brown micro-electrode puller (P-97, Sutter Instruments, Novato, CA, USA). Intracellular recordings were made from longitudinal muscle fibers 6, 7, 12 and 13 across abdominal segments 3, 4 and 5. The anatomy and position of longitudinal muscles (see Fig. 1A) in these centralized segments are highly conserved and function to shorten body length during rhythmic contractions of locomotion. Intracellular data from homologous muscle fibers (i.e. m. 6 and m. 7; m. 12 and 13) were combined and are reported as such. Synaptic potentials were elicited by stimulating all segmental motoneurons via a glass suction electrode, Grass S88 stimulator, and stimulus isolation unit (Grass Technologies, West Warwick, RI, USA). Single impulses were generated at 0.2 Hz, 0.5 ms pulse duration and ~115% of the voltage needed to attain maximal compound EJP amplitude. Stimulus frequency and voltage are described in text for contraction recordings as some experiments utilized direct stimulation of the muscle (§2.1, below).

EJPs were recorded using either an AxoClamp 2B (Molecular Devices, Sunnyvale, CA, USA) or Neurodata IR283A (Cygnus Technology, Delaware Water Gap, PA, USA) intracellular recording amplifier. Three principal components were measured from these recordings: (a) maximum amplitude (b) rise time constant (τ_{rise}: latency to

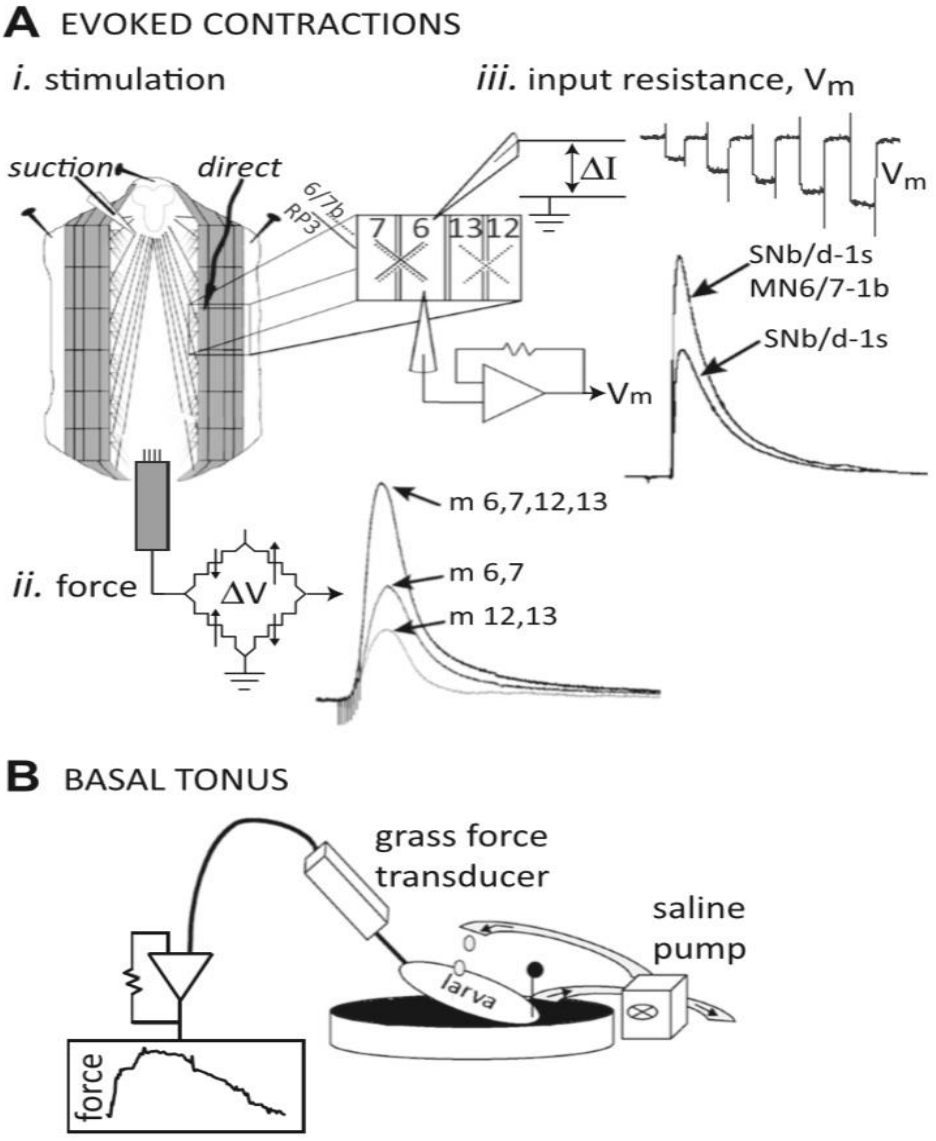

Figure 2.1: Semi-intact preparation used for recordings force and intracellular electrical signals. A: Larval body wall longitudinal muscles (m 6, 7, 12, and 13; gray) are shown in the schematic of a filleted larva and produced the gross majority of contractile force discussed here. Transverse muscles are indicated by fading light gray, outlined in the center of the schematic. *i*: segmental nerves are shown as black lines radiating from the ventral ganglion (*Top*). Both stimulating techniques, suction electrode and direct stimulating electrode, are indicated. *ii*: A hook placed upon the posterior portion of the preparation connects to the bam of the force tranducer, which utilizes a custom full Wheatstone bridge circuit made of silicon wafer. *iii*: An amplifier was used to inject current (*I*) and record voltage from a single intracellular electrode. Either muscle fibers were injected with a series of currents (4, 6, 8, 10, 12pA) and the voltage responses were recorded or membrane potential (V_M) was recorded concurrent with suction stimulation to presynaptic nerves. *B*: basal tonus was recorded with an established method (see text) and an FT03 Grass tension transducer and amplifier. The central nervous system (CNS) was eviscerated, and saline, with or without amines, was washed over the preparation.

reach ~63% of peak), and (c) decay time constant (τ_{decay}: latency to decay 63% from peak). Current injection was required for input resistance measurements and accomplished using the single-electrode voltage / current clamp technique.

Contractions

Synaptically Evoked Force

A force transducer was custom-designed and constructed using high gauge factor silicon wafer strain gauges (Micron Instruments, Simi Valley, CA, USA) and routed through an A-M-Systems DC amplifier (Model 3000: Sequuim, WA) at its lowest differential setting (50x). This transducer was utilized in all experiments recording evoked contractions (Fig. 1A.iii; after Paterson et al., 2010). Briefly, custom designed silicon wafers were placed in a full Wheatstone Bridge configuration around the weakest point of a 0.02" polycarbonate beam (1.5 cm *x* 5 cm), yielding a signal:noise limited resolution of ~600 nN. As with any force sensing device, the modulus of strain of the beam must be matched to the force generated. This newest generation of force beam in our laboratory was designed with whole body *Drosophila melanogaster* contractions in mind and provides favorable resolution (amount of silicon deformation) and stiffness (approach to isometric conditions).

Muscle fiber length was controlled using the following procedure (except during basal tonus recordings). Prior to acquiring experimental data, evoked contractions were monitored as muscle length was sequentially increased until reaching the peak of the length–tension curve. This peak was identified empirically as the muscle length just shorter than the length at which a decrease in force occurred. This method was utilized as one part of working toward the isometric condition. Additionally, video was acquired

through the microscope (TCA 5.0 MP, 8 fps, Ample Scientific, Norcross, GA, USA) while adjusting the muscle length. Length change during contraction was measured as the difference between the animal's length prior to and after contraction (analysis performed with Image J, NIH, Bethesda, MD, USA). Force data were rejected if animal length exceeded 5% (~200 μm of a 4 mm larvae; mean of 10 randomly selected videos was 3.62 $\pm$ 0.49 % or ~140 μm total animal length change). However, given that much of the bodywall tissue of these larvae can be modeled as a viscous material, small changes in total animal length cannot ensure that a particular segment's muscle fiber length does not change relative to the length of fibers in abutting segments.

Stimuli were delivered to segmental nerves through a glass suction electrode or directly to the muscles to evoke contraction. Stimulus duration was 700 μs during experiments measuring contractions, but reduced to 200 μs during V_m recordings. Duration was also reduced during direct muscle activation, and stimuli were delivered directly through the saline approximately two mm from the longitudinal muscles. Voltage was decreased an order of magnitude below that required for neuronal activation and then was increased progressively until contraction amplitude matched that of synaptically evoked contractions prior to changing to direct stimulation. In these latter experiments, a spider toxin (ω-plectoxin-Pt1a: PLTX-II, Alamone Labs, Jerusalem, Israel) was applied to block synaptic transmission. PLTX-II is a 44 amino acid peptidyl toxin produced by *Plectreurys tristis* and is known to effectively block voltage-gated, pre-synaptic calcium channels (Braton et al. 1987, Leung et al., 1989).

It has been well documented that the larval *D. melanogaster* preparation exhibits decay in several physiological properties (Macleod et al., 2002, Stewart et al., 1994).

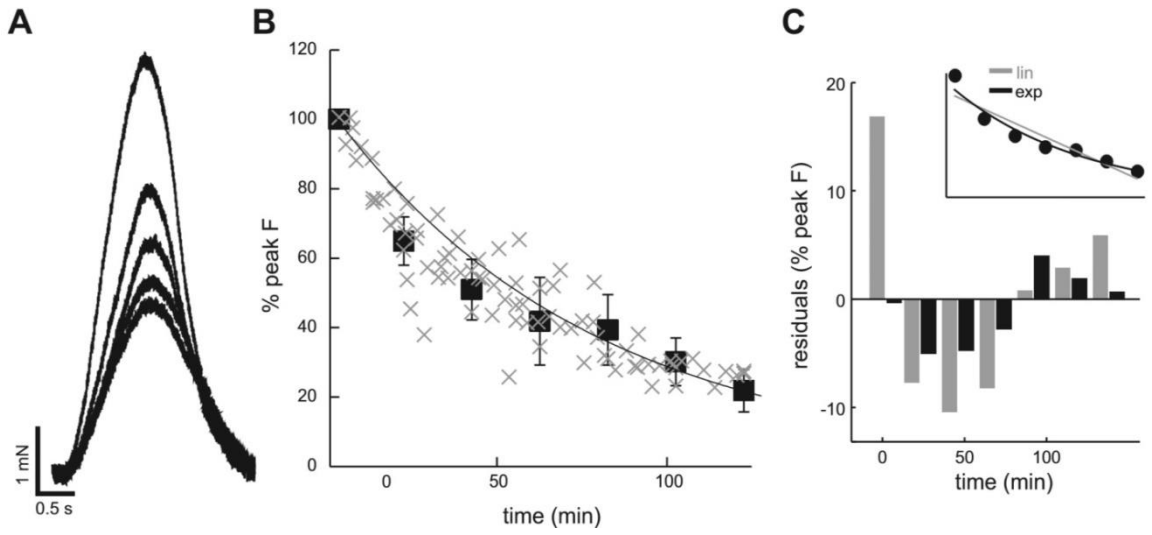

Figure 2.2: Time-dependent decrease in contraction amplitude. A: contraction force decreases with time, more notably in the first 30 min of recording than thereafter. B: therefore, an exponential function [peak force (F_{pk})= $e^{0.013 \times t}$] provides a statistically significant fit of the decay in contraction amplitude (R^2= 0.95, P<0.01, Pearson's correlation, n =10). Gray X are individual F_{pk} data from all 10 animals; black squares are mean F_{pk} values across all 10 animals. C: residuals from both linear (gray; F_{pk}= -0.0076t + 1; x = 7.48) and exponential (black; F_{pk}= $e^{-0.013 \times t}$; x = 2.74) functions were examined to identify the simplest function that reasonably fit the decay in contraction amplitude.

Extensive work has been done to maximize preparation longevity using hemolymph-like saline (Stewart, 1994, Krans et al., 2010). Moreover, scaling equations are routinely used to account for the progressively depolarized membrane potentials that often occur over time in larval bodywall muscle (Martin, 1976; Stevens, 1976; McLachlan and Martin, 1981). A descriptive model of decay in contraction force is necessary to quantify the change in force production at various times post-dissection. Although a given contraction may be lower than initial peak values obtained immediately post-dissection, it may actually correspond to an augmentation given the normal decay in contractile physiology. We quantified this physiologic 'run-down' in peak force over two hours of recording (Fig. 2). Decay in peak force evoked by equal trains of nerve stimulation - wherein no change in saline composition was administered – were better fit by an exponential decay function than a linear function (Fig. 2B and 2C; $R^2 = 0.95$ and 0.72, respectively; $P < 0.01$, both using *Pearson's Correlation*, n=10). In a minority of cases, a logarithmic fit (e.g. $F_{pk} = -0.155 \ln(t) + 1$; not shown) was also an acceptable model; i.e. two of these 10 experiments dedicated to quantifying decay were marginally better fit with a logarithmic function than an exponential function. Based on historical models of contraction run-down and a closer examination of residuals (Fig. 2C; the difference between observed data and fitting models), we chose to use an exponential fit. We evaluated 103 additional preparations for which some manipulation of the preparation saline was made but full reversal was attained, and in 87 of those 103 preparations (84%), $R^2 > 0.92$ using an exponential fit.

Basal Tonus

The posterior end of each dissected third-instar larva was pinned-down to a custom-made recording dish. The anterior of the larva was attached to a Grass FT03 tension transducer (Grass Instruments, Quincy, MA, USA) using a custom metal rod with a bent minuten pin at the distal end. The minuten pin was inserted into the larva in a manner which ensured muscle movements were parallel to the motion of the transducer spring. Care was taken to ensure that the preparation was not overstretched. The larva was raised slightly off the dish (~15°) to prevent friction and maximize contraction transduction. Contractions were amplified using a MOD CP122A amplifier (Grass Technologies, W. Warwick, RI, USA). The signal was digitized using a DATAQ DI-158U data acquisition device, then viewed and analyzed using DATAQ acquisition software. Solutions were applied directly to the larva using a peristaltic pump (0.7 mL/min, volume of dish ~300 uL). Excess solution was removed using continuous suction. Baseline recordings were taken for at least 5 minutes prior to exchanging saline for experimental solutions.

Data Analysis

EJPs were averaged into 30 second time intervals (six EJPs per interval) over each 15 minute trial, and each time point was then averaged over the replicate trials for each condition. Likewise, 8- 10 contractions were averaged every five minutes (using a 35 or 45 s inter-trial pause), and contraction trials typically lasted about two hours. Thus, hundreds of total repetitions for each experimental condition were used in computing averages. However, the number of replicates (n) reported indicates the number of

animals, not repetitions. Standard error of the mean is computed using the number of animals and is reported unless otherwise noted. Fit equations, correlation and Pearson's values, and t-test probabilities were generated using the statistics toolbox in MATLAB (Mathworks, Natick, MA, USA). Sigmaplot (Systat Software, San Jose, CA, USA) was used to generate logistic equations (three parameters plus intercept) and ANOVAs. Formulae are given in figure legends where possible, whereas statistical findings are reported in text.

2.04 Results:

We first characterized the [OA]- and [TA]-dependency of EJP peak amplitude when evoked via neural stimulation. At bath concentrations of greater than 10^{-7} M, OA augmented EJP amplitude significantly (Fig. 3; $P<0.01$, t-tests) in a dose-dependent manner ($P<0.01$; one way non-parametric analysis of variance), and the effect was reversible. In contrast, the action of TA on EJP amplitude was not significant at concentrations less than 10^{-6} M in HL-6 saline (Fig. 3D; $P>0.05$). TA did not significantly change rise or decay time constants at any concentration examined (data not shown). At concentrations at and above 10^{-6} M, TA significantly reduced the amplitude of EJPs (Fig. 3D), albeit much less so than OA's augmentation at a comparable concentration. Reversibility of amine modulation of EJP amplitude required approximately the same duration of washout as exposure at high doses (i.e. 10^{-4} M; Fig. 3C), but at all lower concentrations tested, EJP amplitude returned to control values in less than 5 min of washing in control saline (HL-6, perfusion rate = 0.7 mL/min). At 10^{-5} and 10^{-4} M [OA], the augmentation of EJP amplitude was significantly greater in muscles 12 and 13 than in muscles 6 and 7 (Fig. 3D; $P<0.01$, t-tests). Specifically, the mean augmentation of EJP amplitude in muscles 6 and 7 at the two highest concentrations of OA was +29.9% of control amplitude, whereas the mean augmentation of EJP amplitude in muscles 12 and 13 was +39.9%. In an attempt to ascertain if the OA-mediated effects on EJP amplitude were occurring in part through non-selective activation of tyramine receptors, we co-applied OA near its [EC_{50}] (10^{-6} M; Fig. 3D) and a tyramine receptor antagonist, yohimbine (10^{-5} M). There was no significant difference between values recorded during OA application alone and those recorded during co-

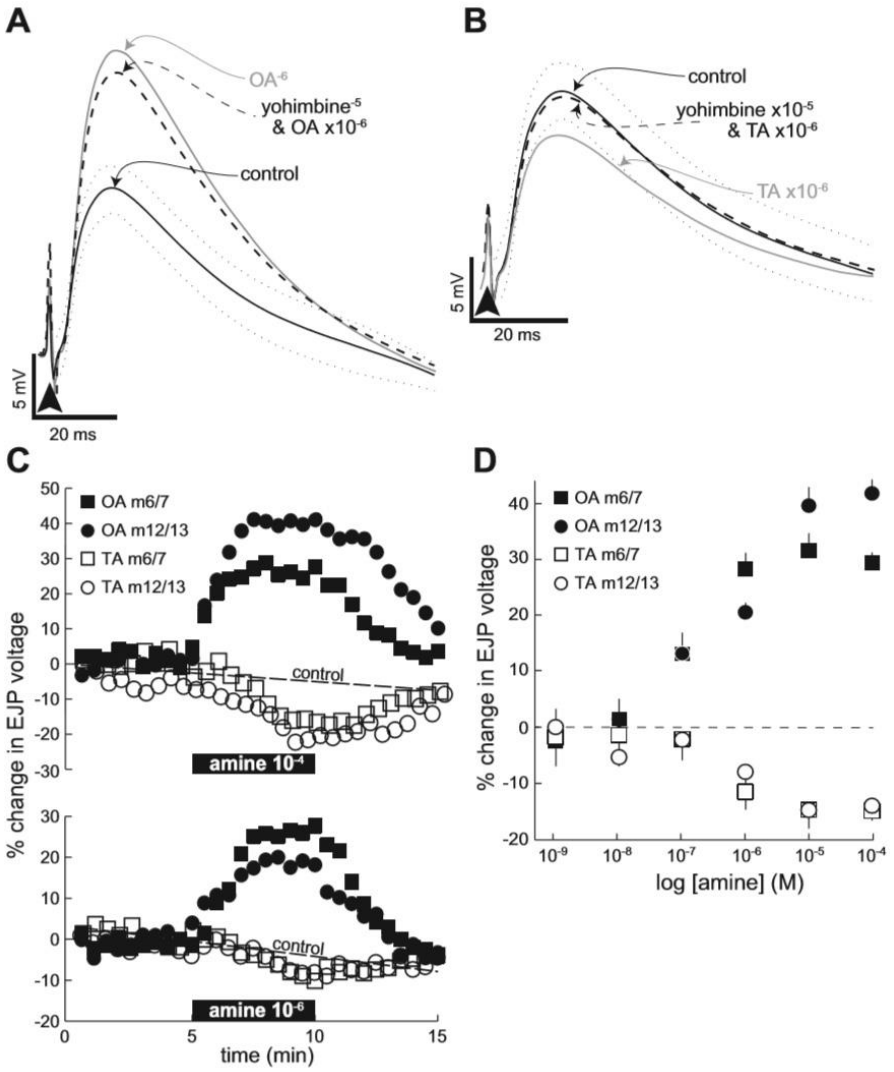

Figure 2.3: Amine modulation of excitatory junction potentials (EJPs) in HL-6 saline. A: an averaged EJP waveform (5 repetitions) recorded from muscle 6 (control; black) is plotted with 95% confidence intervals (dotted lines). Application of 10^{-6}M octopamine concentration ([OA]; gray) and coapplication of yohimbine (10^{-5}M) with 10^{-6}M [OA] (dashed line) augment EJP amplitude and duration. B: averaged EJP waveforms of 10^{-6}M tyramine concentration ([TA]; gray) and yohimbine 10^{-5}M coapplied with 10^{-6}M [TA] (dashed line) fall within the 95% confidence interval (dotted lines) of control saline (black). C: EJP amplitude is plotted over 15 min to demonstrate the rate of reversibility/washout. Horizontal black bars indicate when OA or TA was applied. Top: reversibility occurred with latency comparable to exposure duration when 10^{-4}M amine was applied, whereas reversibility of 10^{-6}M [OA] and 10^{-6}M [TA] (bottom) began immediately upon washout. D: EJP amplitude increased from control (dashed line) with OA application in a dose-dependent manner, whereas amplitude slightly decreased with TA application. The action on m 12/13 was greater than on m 6/7 at high doses of OA, whereas TA maintained a conservative effect on m 12/13 and m 6/7.

application of OA and yohimbine (e.g. 10^{-6} M [OA] + 10^{-5} M [yohimbine], Fig. 3A; P=0.1). However, yohimbine *did* block the TA-induced reduction in EJP amplitude (Fig 3B; 10^{-6} [TA] + 10^{-5} M [yohimbine]) as there was no statistical difference between control EJP values and those collected with co-applied yohimbine and tyramine (P>0.1, t-test).

We further examined these findings in HL-6 saline containing three times more calcium (i.e. 1.5 vs. 0.5mM) because external calcium concentration has been demonstrated to influence OA-mediated effects (Klassen and Kammer, 1985) (Table 2.1). We examined several parameters of EJPs when recorded in saline containing OA, TA, and yohimbine and observed no significant differences when using the different concentrations of [Ca^{2+}]. We next repeated these experiments with HL-3.1 saline since it is another commonly used saline for intracellular recording in this preparation. We first observed that EJPs were significantly larger in HL-3.1 than HL-6 saline both with 0.5 and 1.5 mM [Ca^{2+}] (P<0.01, t-test). Despite the larger initial EJP values, neither of the physiological salines nor the different calcium concentrations significantly altered the percent changes in EJP amplitude that we observed upon application of OA, TA or antagonist. For example, EJP amplitude was 32.3 + 0.8 mV in 10^{-6} M [OA] and 27.9 + 2.6 mV in control HL-3.1, yielding a +19% increase in amplitude (Table 2.1, P<0.05, t-test, n=5 animals, 19 muscles). This ~+20% augmentation in HL-3.1 was not significantly different than that achieved in HL-6, containing 0.5 mM [Ca^{2+}] (Table 2.1, P=0.26, t-test). Additionally, there was no significant change in EJP peak amplitude values recorded during bath application of OA when compared to EJP values obtained during co-application of OA and yohimbine (Table 2.1, P>0.1; t-test). Application of TA

Table 2.1: Summary of excitatory junctional potentials in two salines. Values are mean ± SE excitatory junction potential (EJP) amplitudes recorded from third-instar body wall muscle during application of octopamine (OA), tyramine (TA), and the antagonist yohimbine (YOH) in 2 different physiological salines (HL-3.1 and HL-6) and 2 different calcium concentrations ([Ca2]; 0.5 mM and 1.5 mM); $n_{animals} > 4$ for each metric. *Significant differences between salines.

	EJP Values in HL-6, mV		EJP Values in HL-3.1, mV	
	0.5 mM $[Ca^{2+}]$	1.5 mM $[Ca^{2+}]$	0.5 mM $[Ca^{2+}]$	1.5 mM $[Ca^{2+}]$
Control	16.47 ± 2.2*	21.34 ± 3.1	26.16 ± 2.99*	27.9 ± 2.6
10^{-6} M OA (~EC_{50})	20.18 ± 2.1 (22.50%)	26.85 ± 2.2 (25.80%)	31.89 ± 2.01 (22.27%)	32.3 ± 0.8 (19%)
10^{-6} M OA + 10^{-5} M YOH	20.77 ± 3.88 (26.1%)	26.63 ± 3.41 (24.8%)	33.28 ± 2.23 (27.60%)	34.4 ± 6.7 (25%)
10^{-6} M TA	14.83 ± 4.02 (−9.98%)	19.52 ± 5.07 (−8.54%)	23.57 ± 5.7 (−9.90%)	25.4 ± 4.1 (−9.0%)
10^{-6} M TA + 10^{-5} M YOH	15.81 ± 3.57 (−3.98%)	20.75 ± 4.10 (−2.77%)	25.55 ± 6.75 (2.33%)	27.7 ± 5.7 (−0.6%)

Table 2.2: Cell-specific effects of OA in HL-6 saline.

	0.5 mM $[Ca^{2+}]$	1.5 mM $[Ca^{2+}]$
MF 6/7 EJP control	15.8 ± 3.2	20.3 ± 3.6
MF 6/7 10^{-6} M OA	20.2 ± 2.8 (27.7%)	26.3 ± 3.8 (29.9%)
MF 12/13 EJP control	16.6 ± 5.8	22.7 ± 4.9
MF 12/13 10^{-6} M OA	20.0 ± 4.5 (20.0%)	28.1 ± 5.7 (23.7%)

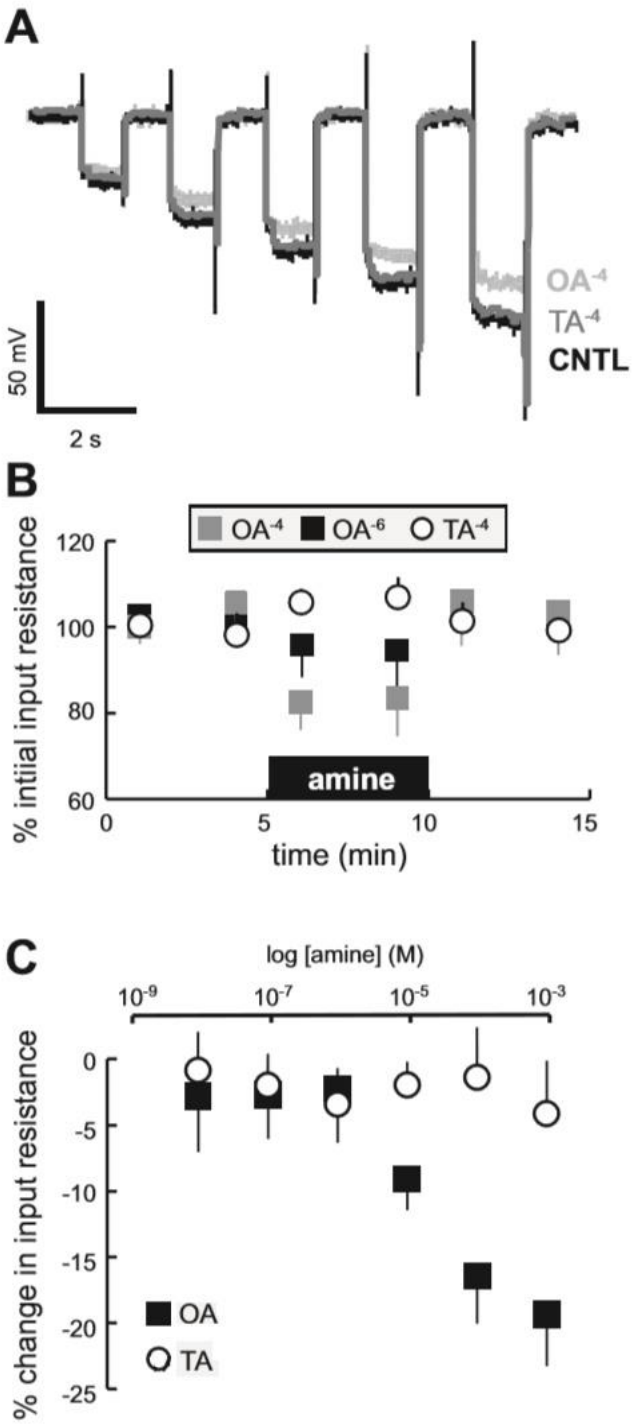

Fig. 2.4. Total membrane resistance changes as a function of amine concentration. A: a series of hyperpolarizing current pulses was passed (4, 6, 8, 10, and 12 pA), and the resultant voltages from muscles 6, 7, 12, or 13 were recorded. B: time series data are given to demonstrate the rapid reversibility of changes in total membrane resistance even at high [OA](i.e.,10^{-4}M)(filled markers) and the insignificant change after exposure to high [TA] (open markers). C: total membrane resistance changed in a dose-dependent manner with OA but not TA.

once again attenuated EJP amplitude in HL-3.1 saline as it did in HL-6 (i.e. 10^{-6} M [TA], $P<0.01$, t-test).

Cell-specific differences were examined in HL-6 saline containing 0.5 mM and 1.5mM calcium (Table 2.2) since in general, the action of amines on EJP amplitude was not different between the two salines (i.e. percent change was comparable and not statistically different between HL-3.1 and HL-6). We measured voltage deflections to brief (~1s) pulses of hyperpolarizing current steps (Fig. 4A) to estimate input resistance of the muscle cells and evaluate the action of the amines on muscle membrane properties. At concentrations greater than 10^{-5} M, OA significantly decreased input resistance (Fig. 4B; $P<0.01$ for 10^{-5} through 10^{-3} M [OA]) and did so in a dose-dependent manner (Fig. 4C, $P<0.05$; one way non-parametric analysis of variance). TA did not have a significant dose-dependent action upon input resistance ($P<0.05$; one way non-parametric analysis of variance), though at the highest dose examined, there was a statistical difference between input resistance estimated in TA and control saline ($P<0.05$; t-test).

Octopamine modulated several components of contraction force. Most notably, at even low doses, octopamine increased the peak amplitude of contractions elicited by stimulating the motor nerve at 25 Hz (Fig. 5A). Modulation of contraction force was significantly dependent upon changes in [OA] (Fig. 5B, one way non-parametric analysis of variance, $P<<0.01$). The OA-dependent augmentation of force saturated above 10^{-4} M [OA] and yielded $+32.3 \pm 6.7\%$ greater force than observed in controls (black squares Fig. 5B). When combined with the tyramine receptor antagonist, yohimbine (10^{-5} M), [OA] at 10^{-4} M augmented force $+29.34 \pm 2.26\%$, which was not statistically different from OA alone ($P>0.05$; t-test). There was minimal augmentation observed at

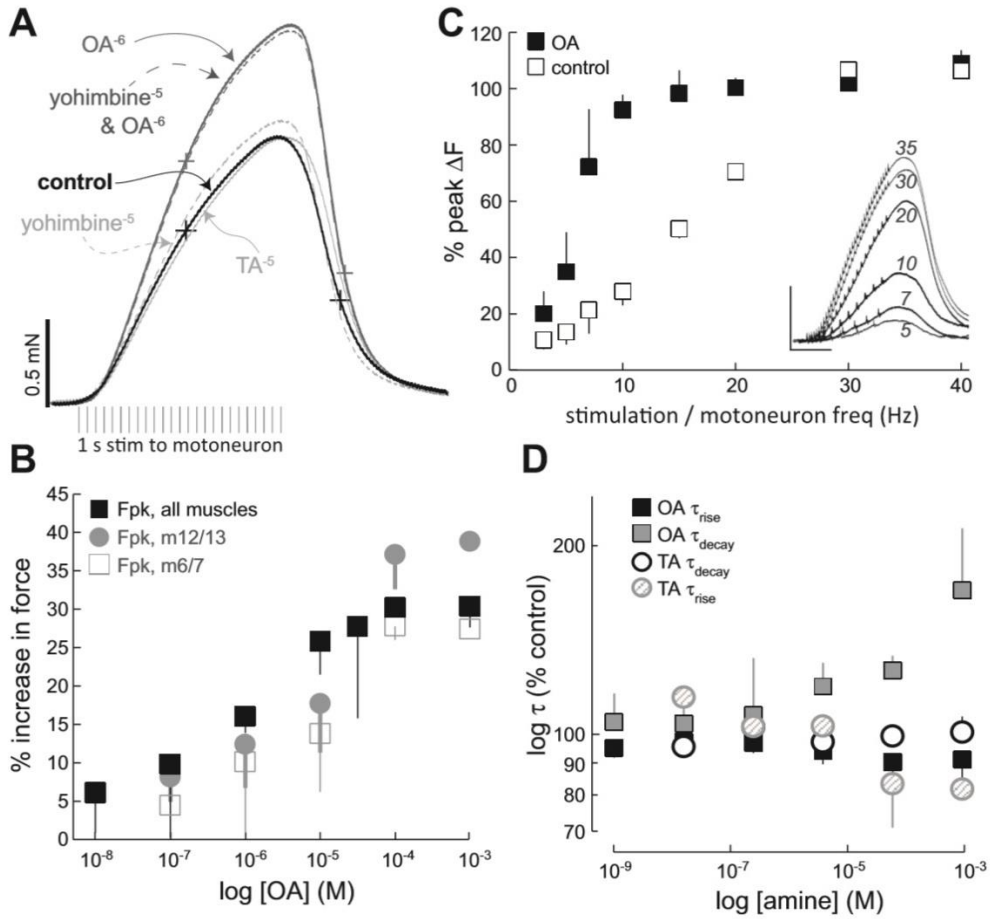

Figure 2.5: Action of amines on synaptically evoked force production. A: averaged contractions (n=8–10 repetitions each) driven by 25-Hz stimulation of the motor nerve for 1 s: prior to application of OA (black: control HL-3.1 saline) and during bath application of OA-containing saline (gray: [OA] 10^{-6}M), coapplication of yohimbine (10^{-5}M) with 10^{-6}M [OA] (dashed gray line), application of TA (10^{-5}M) (light gray), and coapplication of yohimbine (10^{-5}M) with TA (10^{-6}M) (dashed light gray). Cross hairs indicate the points used to compute time constants (τ, latency from preceding force inflection: either onset of contraction or relaxation). B: OA-dependent augmentation of contraction: OA dependence of F_{pk} from synaptically evoked contractions (n= 33 total). The response of all 4 muscle fibers is shown with black squares (n=33). Open squares indicate that m 12 and 13 were ablated while m 6 and 7 were left intact (n=11). In contrast, gray circles indicate that m 12 and 13 were intact while m 6 and 7 were ablated (n=12). C: force-frequency curve for OA and control groups. The force-frequency (motoneuron) relationship shifts left in OA-containing saline (5.5^{-6}M [OA] selected from the dose-response curve above; EC_{50} of contraction) Inset: muscle contraction (raw recordings) at varying frequencies. D: time constants of contraction with TA application (circles) are not significantly different from control values. In contrast, time constants with OA application (squares) change in a dose-dependent manner consistent with increased contraction duration (n=31 preparations). τ_{rise} [OA] decreases, reaching peak force in less time, while τ_{decay} [OA] increases, maintaining force longer (see text). Standard deviation is plotted in D.

concentrations of 10^{-8} - 10^{-7} M [OA], about +8 $\pm$ 6%, that may be attributable to the modest scaling routine utilized to counteract physiologic run-down (Methods). The dose at which 50% of OA augmentation was achieved was estimated using a standard logistic equation and was 5.3 x 10^{-6} M [OA].

Since high doses of octopamine induced a greater change in EJP amplitude among muscles 12 and 13 than muscles 6 and 7, we next evaluated the cell-specificity of its action upon contraction force by ablating either muscles 6 and 7 or muscles 12 and 13. Greater augmentation was observed when muscles 12 and 13 were left intact than when muscles 6 and 7 were left intact (Fig. 5B). Across all doses tested, augmentation of force in muscles 12 and 13 was +28.59 $\pm$ 1.88% greater than in muscles 6 and 7. The augmentation of force in muscles 12 and 13 was significantly greater than the value obtained with all fibers intact at 10^{-4} and 10^{-3} M [OA] (+6.04 0.88%, $P<0.01$). Likewise, the augmentation of force observed at these concentrations in muscles 6 and 7 was significantly lower than that observed when all fibers were intact (-4.5 $\pm$0.64%, $P=0.0094$). The greater modulation of contraction in muscles 12 and 13 compared to 6 and 7 corresponds well with the cell-specific effects of OA on EJP amplitude (Fig. 3D).

We hypothesized that the OA-induced augmentation of contraction force would shift the motoneuron frequency – force relationship to the left, yielding greater forces in octopamine than control saline from otherwise equivalent motoneuron trains. We tested this hypothesis by selecting a single concentration of octopamine (5.5 x 10^{-6} M, approximating the EC_{50} indicated above) and measuring force of contraction prior to, during, and after OA application. The three-parameter logistics equations utilized to describe these frequency-force curves suggest that OA decreased the motoneuron

frequency required to generate 90% of the maximal force, nearly in half, from 23.01 Hz in control saline to 12.75 Hz in OA-containing saline (Fig. 5C). Likewise, there was a shift in the frequency required of the motoneuron to yield 50% of tetanic force from 10 Hz in control saline, to 6.9 Hz in OA-containing saline (Fig. 5C).

Octopamine also increased the duration of contractions, both by decreasing rise time and increasing decay time. At the highest concentration of OA examined, 10^{-3} M, the force associated with evoked contractions occasionally required more than 2 s to decay after synaptic activation, yielding a very large and variable increase in mean decay time (101.7 $\pm$ 62.68 ms; +76.45 $\pm$ 47.12% increase; Fig. 5D). The OA-dependent increase in decay times persisted in a dose-dependent manner across all doses examined, and was statistically significant at concentrations greater than 10^{-5} M [OA] ($P=0.049$ at 10^{-5} M, $P \leq 0.011$ at higher [OA]). Likewise, though of opposite sign, rise time of contraction progressively decreased with [OA] (Fig. 5D; $P =0.052$ at 10^{-5} M, $P<0.01$ at higher [OA]). Although TA decreased contraction amplitude and duration slightly at higher doses, there was no statistically significant dose-dependent action on contraction dynamics (Fig. 5D; $P>0.05$ with both ANOVA and Pearson's test). Likewise, the application of yohimbine had no statistical effect on contraction amplitude (Fig. 5A: $P>0.05$, t-tests) when applied alone or co-applied with amine.

We next evaluated changes in basal tonus – in the absence of synaptic activity – as an indication of extrajunctional octopaminergic modulation. In the great majority of experiments OA was bath applied to preparations used exclusively for basal tonus (Fig. 1B) that eliminated synaptic discharge via evisceration of the CNS. However, in a few experiments, stimulation of severed segmental nerves was utilized whilst measuring basal

tonus (Fig. 1A) to provide simultaneous comparison of augmentation to basal tonus with augmentation of synaptically driven contractions. For example, application of 10^{-5} M [OA] induced a large, slow contraction that reached a stable level after about three minutes (Fig. 6A). This same concentration of OA yielded a +21.4% increase in contraction force evoked by stimulation of the segmental nerve at 25 Hz for 1 s (Fig. 6A, insets at far left and right). This latter effect corresponded to ~1.1 mN and was *many* times smaller than the slow, progressive increase in basal tonus, which was 28 mN (Fig. 6A; $P<0.05$, one way analysis of variance). Basal tonus increased with [OA] in a dose-dependent manner (Fig. 6B). To evaluate the magnitude of augmentation and compare it to that observed upon synaptic activation, we fit the change in basal tonus with a simple logistic equation (Fig. 6C; total force, sufficiently consistent across animals). The concentration of [OA] at which 50% of the total augmentation in basal tonus was observed, was determined to be 8.8×10^{-7} M, whereas 90% of maximal augmentation was attained in 1.4×10^{-5} M [OA].

There was no statistical effect of TA on basal tonus at concentrations less than 10^{-5} M (Fig. 6C). However, at 10^{-4} M, TA did have a statistically significant effect upon basal tonus, but the effect was very short lived (less than 30 seconds) and was not observed in all preparations investigated.

Given this indication that a profound post-synaptic action of OA may exist independently of synaptic activation, we next examined forces driven by direct depolarization. These experiments utilized the spider toxin PLTX-II, which is a known pre-synaptic voltage-gated calcium channel blocker (Branton et al., 1987). Early work characterizing the action of the PLTX toxin did not use multiple stimuli as we are

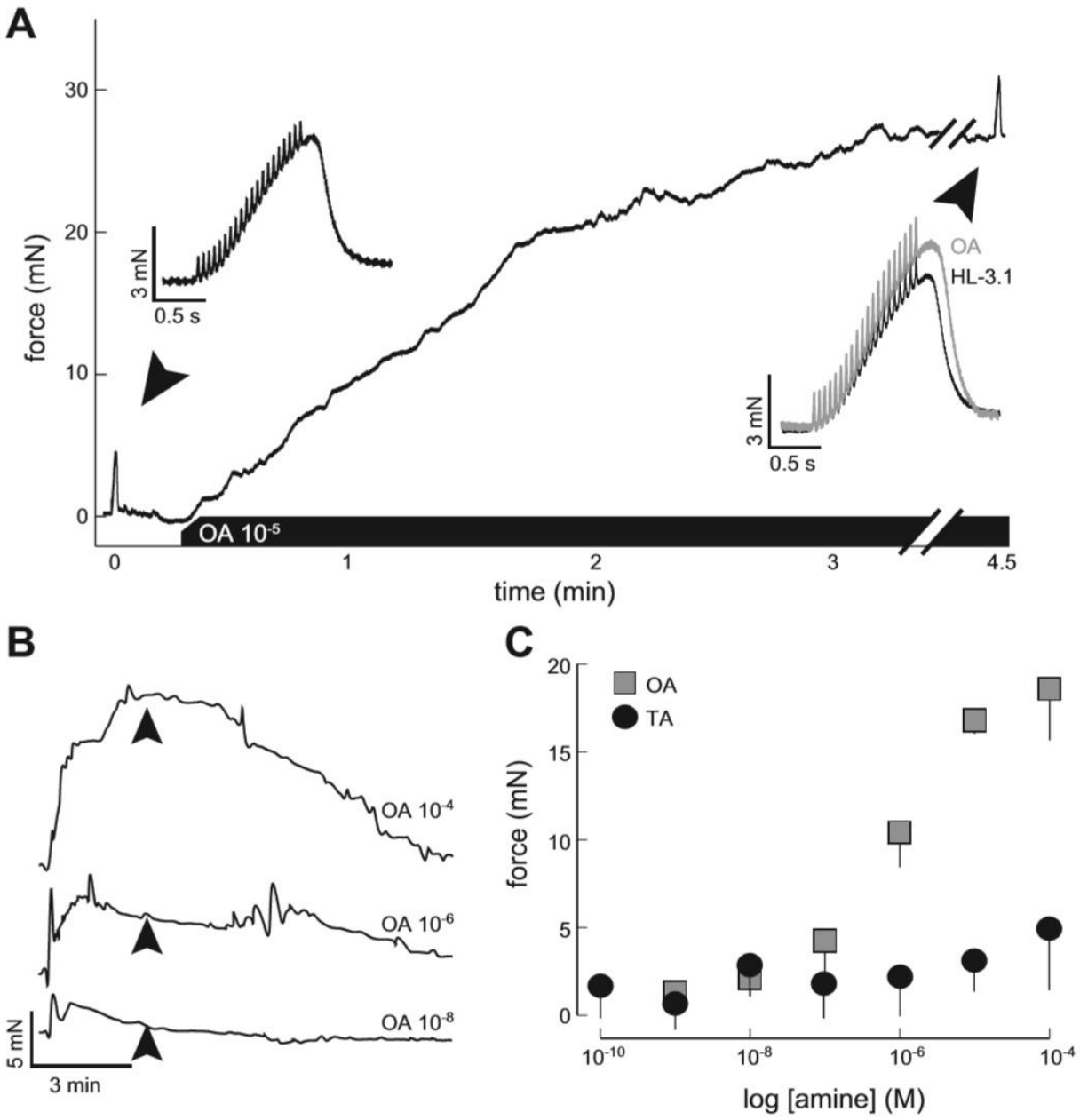

Fig. 2.6. Action of amines on basal tonus. A: force was recorded while stimuli were delivered to the segmental nerves at 25 Hz (duration= 1 s) immediately prior to application of OA (left inset: black) and ~4 min later (right inset: gray; again 25 Hz for 1 s), at the peak of OA's action on basal tonus. B: with the segmental nerves severed and CNS removed, 5 min of OA application augmented basal tonus. The amplitude of augmentation was measured 3 min (arrowheads) after application. C: OA increased basal tonus in a dose-dependent manner in preparations lacking electrical stimulation. TA's action on basal tonus was not consistent or significant at any concentration other than 10^{-4}M. Contraction data represent mean force gain with each amine concentration.

accustomed to in driving contractions. We therefore recorded the persistent failure of the synapse in two series of experiments (Fig. 7). Axonal stimulation of the motoneuron in the presence of 10^{-8} M [PLTX-II] yielded persistent decay in the amplitude of contraction force and a complete abolition of force within 30 minutes (Fig. 7A). The persistent loss of contraction during axonal stimulation corresponded to a loss of synaptic depolarization of the muscle (Fig. 7B). However, contractions were recovered with direct depolarization by ejecting the axons from the glass stimulating electrode and moving the electrode 2 mm further from the NMJ (Fig.7C). Contraction amplitude and rise slopes were not statistically different between synaptic and direct stimulation of the muscle ($F_{pk\text{-}syn}$ = 3.21 mN vs. $F_{pk\text{-}direct}$ = 3.26 mN; $t_{rise\text{-}syn}$ = 1.066 s vs. $t_{rise\text{-}direct}$ = 1.073 s; $P>0.05$, n=8-10 each pair). Contraction decay times were substantially longer and more variable in saline containing PLTX and OA than in controls (data not shown). Octopaminergic augmentation of contractions was maintained in the absence of synaptic depolarization, though at a consistently lower magnitude (Fig. 7D). Indeed, there was no significant difference in the rise slope of logistic functions used to fit these two data sets – either with or without synaptic activation (Fig. 7D; peak rise in force per log M [OA] = 9.5%, peak rise in force per log M [OA]+PLTX = 8.7%, $P>0.05$). Specifically, the concentration of OA at which 50% of the maximal effect was observed was between 10^{-5} and 10^{-6} M in both cases (9.3×10^{-6} M [OA] in PLTX-containing saline vs. 5.3×10^{-6} M [OA] in control saline), and 90% of the effect was obtained between 10^{-4} and 10^{-5} M [OA] in both conditions (8.0×10^{-5} M [OA] in PLTX-containing saline vs. 3.8×10^{-5} M [OA] in control saline).

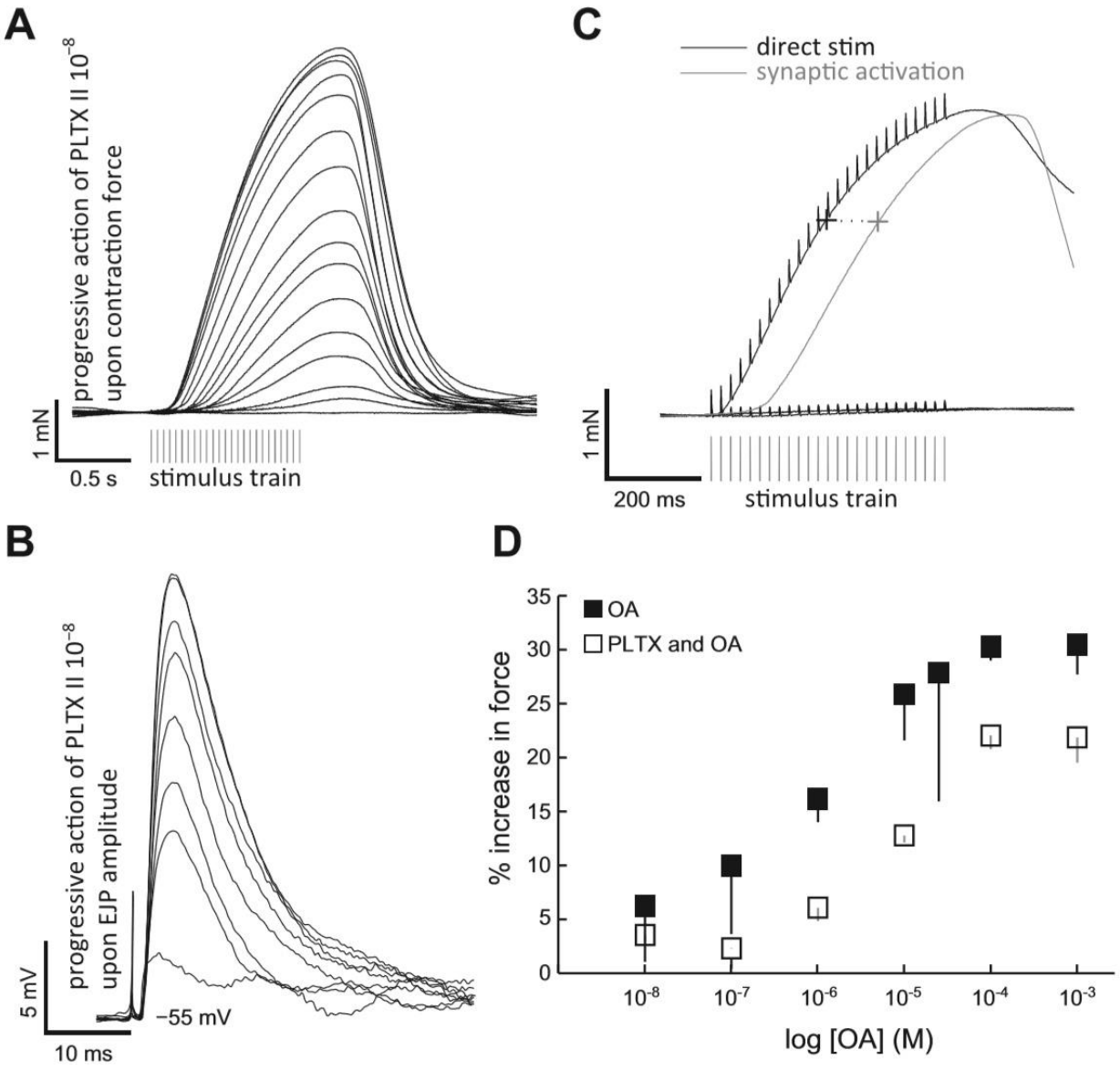

Fig. 2.7. Directly activated contractions are also augmented by OA. A: with application of ω-plectoxin-Pt1a (PLTX II), contraction amplitude (not averaged) decreases with time; each trace reflects a 2-min interval. Stimuli are delivered to the segmental nerve/motoneurons at 25 Hz for 1 s. B: EJPs (not averaged) recorded from the longitudinal muscles demonstrate the progressive failure of the synapse; each trace reflects a 40-s interval. C: contraction traces (not averaged) before and after 30-min exposure to PLTX. The synaptically evoked contraction immediately preceding application of PLTX is shown in gray (no artifacts). Lines with stimulus artifact were collected on direct stimulation/ depolarization (black). Points at which rise time constants were measured are marked on each contraction trace. The stimulus trains (identical) used to evoke each contraction are shown in gray beneath the contractions. D: OA augmentation of contraction amplitude when evoked synaptically (filled squares; replotted from Fig. 5) or via direct stimulation of the muscle in PLTX (open squares, n=22).

2.05 Discussion

We have demonstrated that octopamine elicits distinct but complimentary actions on muscle cells and on neuromuscular synapses. EJP amplitudes increased by ~35% (all muscles, 10^{-4} and 10^{-5} M [OA]), and the force associated with synaptically driven contractions increased similarly, ~32%. OA also augmented force significantly in directly stimulated muscles after blocking neuromuscular synapses. The significant OA-induced reduction in input resistance and dramatic increases in muscle tonus, far exceeding synaptically driven changes in force-production, provide additional evidence for an independent postsynaptic action of OA. Additionally, OA was found to consistently potentiate EJPs in some fibers to a greater extent than others, thereby providing evidence for cell-specificity. OA also significantly shifted the motoneuron frequency – force relationship to the left; 90% of maximum force was obtained in 5.5×10^{-6} M [OA] at only 55% of the stimulus frequency required in control saline. The greatly increased relaxation / decay time of contractions, taken together with augmented force, suggests a robust action upon muscle contractile properties and work potential. The co-application of the selective tyramine receptor antagonist yohimbine (Donini and Lange, 2004) indicates that even at high concentrations of OA application, the effects are unlikely to be confounded by tyramine receptor activation. We further tested this by using cyproheptadine, a blocker of amine receptors shown to only weakly antagonize octopamine receptors in locust, but block tyramine receptors slightly better (Orchard and Lange, 1986). Once more, the action of cyproheptadine, when co-applied with the biogenic amine, was insignificant (data not shown).

Octopamine elicited a dose-dependent decrease in muscle fiber input resistance, suggesting that OA opens ion channels and/or greatly activates exchanger rates at the muscle membrane (Fritz et al., 1979; Walther and Zittlau, 1998). Given the significant reduction in input resistance, Ohm's law predicts a concurrent reduction in EJP amplitude. However, application of OA demonstrated a substantial dose-dependent *increase* in EJP amplitude relative to control preparations, suggesting that the drop in input resistance is more than compensated by an increase in synaptic current. It has been shown previously that octopamine increases mepp frequency (Evans, 1981; O'Gara and Dewes, 1990), but previous reports do *not* indicate an effect of OA on mepp amplitude (Evans, 1981). Nonetheless, we observed a significant decrease in muscle membrane resistance ($\leq 20\%$) *and* an increase in post-synaptic potential amplitude. A plausible explanation is that OA increases EJP amplitude by increasing the amount of transmitter released per nerve impulse. Hidoh and Fukami (1987) reported that OA increased EJP amplitude in mealworm larvae (*Tenebrio molitor*) by roughly 40% at concentrations at or above 10^{-6} M. They also observed a significant increase in mepp frequency and no change in mepp amplitude following OA application (Hidoh and Fukami, 1987). These observations, coupled with a significant increase in quantal content, led them to speculate an increase in intracellular Ca^{2+} was responsible for the increased EJP amplitude. More recently, OA has been shown to enhance transmitter release in *Aplysia* neurons via an increase in calcium entry at synaptic boutons (Jin et al., 2012). Our demonstration of unwavering EJP augmentation (~25% at 10^{-6} [OA]) by OA in saline with either 0.5 (HL-6) or 1.5 mM [Ca^{2+}] (HL-3.1) supports a pre-synaptic effect consistent with those previously reported.

Of the principle parameters of contraction force altered by OA, the most noticeable *initially* was a significant increase in synaptically evoked force of contractions. High concentrations (i.e. 10^{-4} M [OA]) generated ~30% greater force than observed in control saline, which was comparable to the observed increases in EJP amplitude (~35%) at the same OA concentration. OA has previously been demonstrated to have an effect on twitch amplitude in a variety of arthropod species (O'Gara and Dewes, 1990), though interestingly, it is sometimes opposite in sign (Evans, 1981; Evans and O'Shea, 1978, 1979; Evans and Siegler, 1982). OA potentiates striated muscle contractions in crayfish (Fisher and Florey, 1983), lobster (Kravitz et al., 1980) and crab (Rane et al., 1984). The EC_{50} for force augmentation reported here (5.3×10^{-6}M) is similar to what Evans (1981) reported for the effect of OA on twitch amplitude in locust (3.3×10^{-6}M). OA application also significantly increased the relaxation time (decreased rate of decay) of contractions in the present study. Here again, the effects observed on EJPs translated well to force recordings; the decay time constant for EJP was +22% greater than control (10^{-5} M [OA]) and the decay time constant for synaptically driven contraction force at the same concentration was +30% greater than control. A number of previous arthropod investigations report a significant *increase* in the relaxation rate of twitch amplitudes and muscle contractions which is opposite in sign to our results (O'Gara and Dewes, 1990, O'Shea and Evans, 1979, Whim and Evans, 1988, 1989). In most cases, however, OA application increases EJP amplitudes which correlate well with increases in the force of twitch amplitude. The apparent differences in the rates of relaxation could be attributable to several factors: (a) muscle type is quite variable within a species (e.g. slow-twitch vs. fast-twitch; Wiersma et al., 1938; Atwood et al, 1965) and across life stages (i.e. insect

flight vs. larval muscles; Dudley, 2000; Patterson et al., 2010), developmental strategies (hemimetabolous vs. holometabolous; Konopova etal, 2011; Hoyle, 1983) or (b) the evolution of different muscle mechanisms (i.e. expression level of giant sarcomere associated proteins, discussed below).

Although the OA-dependent changes in contraction force during synaptically driven recordings were significant, they were small in comparison to the effects of OA on basal tonus. Application of 10^{-5} M [OA]-containing saline resulted in a 1.1 mN increase in the force generated by synaptic activation, compared to a 28 mN change in basal tonus without synaptic activation. Not only do these results highlight a profound postsynaptic effect, but the 28 mN of basal tonus augmentation is drastically larger than what has been observed under normal physiological stimulation paradigms (Paterson et al., 2010). This provides additional evidence that OA may be working on extrajunctional receptors or influencing other intramuscular properties (discussed below). To verify that the effects on basal tonus were independent of the synapse, we pharmacologically blocked the presynaptic contribution and directly depolarized muscles. Force was augmented ~+22% under these conditions (at 10^{-4} and 10^{-3} M), suggesting that only about one-third of the +32% augmentation of force via synaptic activation is attributable to larger EJPs. Previous reports on the effects of OA consistently demonstrate a reduction in basal tonus of skeletal muscle in locust and cricket (O'Gara and Dewes, 1990, O'Shea and Evans, 1979, Whim and Evans, 1988, 1989). Similar to the effects on relaxation rate, the unexpected effects we report on basal tonus may be attributable to several factors (some biological factors are indicated above). However, a second explanation for these findings involves the recording apparatus utilized in most studies. Commercial force transducers

(i.e. Grass FT03) often rely on a large spring constant that maintains a particular muscle preparation length. Our force beams are matched specifically to the tissue of larval *D. melanogaster* and thus utilize a far lesser modulus of elasticity of the force of the beam itself (and associated smaller spring constant). If internal muscle resistance is decreased by fight-or-flight hormones – hypothetically to empower greater contractions – then, a large transducer spring constant could effectively mask increased force capabilities by simply resisting length change more effectively. Nevertheless, our basal tonus observations provide further evidence for an independent postsynaptic target of OA. Moreover, these results demonstrate that in the presence of a modulatory substance, the EJP is not necessarily the sole indicator of force production, which suggests that caution should be taken in drawing conclusions about muscular force from electrophysiological data alone.

In addition to the dose-dependent increases in EJP amplitude following OA application, OA potentiated EJPs more strongly in some muscle fibers (12 and 13) than in others (6 and 7). Monastirioti et al. (1995) demonstrated differential OA expression within motoneuron subtypes innervating *Drosophila* larval body wall muscles using immunoreactivity. They concluded that OA-immunoreactive boutons innervated muscles 12 and 13, but not 6 and 7. If the presence of OA-immunoreactivity is tightly correlated with the capacity to be modulated by exogenous OA, we would have predicted little or no increase in EJP amplitude for fibers 6 and 7. Thus, the presence or absence of OA within synaptic boutons does not correlate well with the ability of the innervated muscle fibers to respond to exogenous OA application. This conclusion is consistent with the accepted view that OA acts as a neurohormone (e.g. during fight or flight) in addition to its

function as a NT. Our data indicate that the absence of OA-immunoreactivity in muscles 6 and 7 does not exclude them from modulation by OA, but may indicate the presence of additional cellular machinery (i.e. receptors) in muscles 12 and 13 since that pair exhibited greater augmentation of EJP amplitude and decay time.

Our data support that OA can and does act in a cell-specific manner in muscles. We thus sought to determine whether this cell-specific difference in EJP potentiation extended to force generation. Using an ablation technique, we eliminated muscles 6 and 7 from our recordings and examined OA-dependent force changes associated with synaptically driven contractions. With only muscles 12 and 13 intact, at 10^{-4} and 10^{-3} M [OA] we observed a +38 $\pm$ 5% increase in force, a +6.0 $\pm$ 0.9% increase over the augmentation observed with all muscles intact. Next, we eliminated muscles 12 and 13, leaving 6 and 7 intact, and observed a +28 $\pm$ 2% augmentation in force at 10^{-4} and 10^{-3} M [OA], a -4.5 $\pm$ 0.6% decrease compared to all fibers intact. These results demonstrate that the cell-specific effects upon EJPs correspond to complementary cell-specificity in force augmentation.

We demonstrated here that OA's metabolic precursor TA, decreased the amplitude of EJPs, although modestly relative to the actions of OA, as previously shown (Nagaya, 2002). Interestingly, TA's inhibitory effect at the synapse did not translate to a reduction in force production (Fig. 5) or basal tonus (Fig. 6). Additionally, co-application of OA and the TA selective antagonist yohimbine did not affect the amplitude or time constants of evoked muscle contractions. Thus, both the synaptic and muscle specific effects of OA appear to be independently modulated *and* independent of any non-specific interaction with TA receptors.

It has been shown for a multitude of neuromodulators / NTs within the *Drosophila* CNS that signaling molecules often recruit specific subsets of neurons in order to produce / alter a specific behaviour. Examples include effects of OA on male social behaviour, (Certel et al., 2010) and the roles of dopamine in stress (Neckameyer and Weinstein, 2005) and of 5-HT in sleep (Yuan et al., 2008). While behaviours are controlled and coordinated centrally, the effector cells should be modulated in a manner that complements changes in motor output generated within the CNS. OA has provided evidence for a role in coordinating behaviour, from the CNS (Certel et al, 2010) to the periphery (Saraswati et al, 2004; Fox et al, 2006). Here we demonstrate independent but complementary actions of OA at the peripheral level.

Putative mechanism and model of octopamine neuromuscular modulation.

Given that co-application of the well-established tyramine receptor antagonist, yohimbine (Orchard and Lange, 1985; Saraswati et al., 2004) with OA yielded no significant deviation from octopaminergic augmentation, we offer the following explanation for the independent, complimentary pre- and post-synaptic effects. Several decades of research support the hypothesis that different isoforms of OA receptors are localized pre-and post-synaptically. The original classification scheme for octopamine receptors, as suggested by Evans (1981), made a clear distinction between two main classes of OA receptors ($OCTOPAMINE_1$ and $OCTOPAMINE_2$) based mainly upon pharmacological characterizations. $OCTOPAMINE_1$ receptors typically yield increases in intracellular calcium, whereas $OCTOPAMINE_2$ is thought to mediate the activation of adenylate cyclase and subsequently modified $[cAMP]_i$. It is likely then, that a variant of

the $OCTOPAMINE_1$ receptor (potentially an isoform of the OAMB receptor) is present at the presynaptic bouton. In the context of current results, it seems plausible that a member of the $OCTOPAMINE_2$ receptor is expressed postsynaptically. A subgroup of $OCTOPAMINE_{2(B)}$ was stated to be located postsynaptically - on the muscle - and mediate an increase in the relaxation rate of tension (now synonymous with the β-adrenergic-like octopamine receptors; Evans and Maqueira, 2005). Therefore, the OA-induced changes postsynaptically are potentially attributable to the activation of a second messenger system. *Drosophila* possesses many cyclic nucleotide gated channels, such as cAMP-dependent K^+-channel, which could account for the drop in input resistance (Delgado et al, 1991; Wicher et al., 2001). Interestingly, adenylate cyclase activation typically results in cAMP-dependent phosphorylation of protein kinase A (PKA). PKA has been demonstrated to activate L-type calcium channels, which are speculated to be localized postsynaptically to larval bodywall muscle in *Drosophila* (Basavappa et al., 1999). This activation of L-type calcium channels could also be responsible for the changes in input resistance and account for an enhancement in intracellular calcium concentrations, likely yielding increased force-production.

However, our data also show a drastic increase in basal tonus, over 25 times the augmentation observed during synaptically driven contractions. An effect as prolonged as OA's effect on basal tonus could reflect changes in vital intramuscular proteins contributing to force production. Actin, myosin, and troponin / tropomyosin interactions cannot account for the prolonged, augmented state of contractility often observed in muscle physiology (e.g. Blaschko effect or catch tension; Krans, 2010). Recent evidence provides support that elastic proteins of the muscle (giant sarcomere associated proteins

or gSAPs) that interact with actin / myosin may be responsible for such phenomena (arthropods: Hooper and Thuma, 2005, Hooper et al., 2008; chordates: Nishikawa et al., 2012). If gSAP function is indeed similar across phyla, then any number of the gSAPs may form a cross-bridge facilitating an indirect, long-lasting bond between actin and myosin resulting in the persistent effect on basal tonus observed here (Nishikawa et al., 2012). It is noteworthy that PKA has previously been demonstrated to phosphorylate titin (Kruger and Linke, 2006), and that in chordate fibers, calcium influx – here putatively augmented by PKA –increases titin stiffness (Labeit *et al.*, 2003). Thus, the downstream action of OA on OCTOPAMINE$_{2B}$ receptors being a change in [cAMP] provides a pathway for physiologic modulation of the gSAPs, which is consistent with the exceptional change in basal tonus reported here and changes in work capacity reported elsewhere (Evans and Siegler, 1982).

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Chapter 3:

Cell-selective modulation of the *Drosophila* neuromuscular system by a neuropeptide

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3.01 Abstract:

Neuropeptides can modulate physiological properties of neurons in a cell-specific manner. The present work examines whether a neuropeptide can also modulate muscle tissue in a cell-specific manner, using identified muscle cells in third instar larvae of fruit flies. DPKQDFMRFa, a modulatory peptide in the fruit fly *Drosophila melanogaster*, has been shown to enhance transmitter release from motor neurons and to elicit contractions by a direct effect on muscle cells. We report that DPKQDFMRFa causes a nifedipine-sensitive drop in input resistance in some muscle cells (6 and 7) but not others (12 and 13). The peptide also increased the amplitude of nerve-evoked contractions and compound excitatory junctional potentials (EJPs) to a greater degree in muscle cells 6 and 7 than 12 and 13. Knocking down FMRFa receptor (FR) expression separately in nerve and muscle indicate that both presynaptic and postsynaptic FR expression contributed to the enhanced contractions, but EJP enhancement was due mainly to presynaptic expression. Muscle-ablation showed that DPKQDFMRFa induced contractions and enhanced nerve-evoked contractions more strongly in muscle cells 6 and 7 than cells 12 and 13. *In situ* hybridization indicated that FR expression was significantly greater in muscle cells 6 and 7 than 12 and 13. Taken together, these results indicate that DPKQDFMRFa can elicit cell-selective effects on muscle fibres. The ability of neuropeptides to work in a cell-selective manner on neurons and muscle cells may help explain why so many peptides are encoded in invertebrate and vertebrate genomes.

3.02 Introduction

Biologically active peptides mediate many types of signalling between cells, such as autocrine, paracrine, endocrine and synaptic signalling. Peptides play vital roles during all stages of development and underlie a multitude of physiological and behavioural processes (Geary and Maule, 2010; Kastin, 2013; Yew et al., 1999). There are roughly 50 identified neuropeptides in the human CNS, and several hundreds in invertebrates (Hummon et al., 2006; Hurlenius and Lagercrantz, 2001). Despite over half a century of investigation, it remains largely unknown why most vertebrate and invertebrate genomes encode such a large number of conserved peptides and their receptors. As molecular and genetic tools continue to develop, particularly in model murine and invertebrate systems, we are beginning to understand the function of small populations of cells and even individual cells within systems, and how modulation of these cells can alter physiological and behavioural output (Bargmann, 2012; Certel et al., 2010; Choi et al., 2011). A growing body of literature exists to support the view that different modulators can act on different subsets of neurons in order to activate specific neural circuits and/or inhibit others and ultimately produce a specific behavioural outcome (Harris-Warrick, 2011; Harris-Warrick and Kravitz, 1984; Marder and Calabrese, 1996; Selverston 2010). This concept of "neuron-specific" or "circuit-specific" modulation may help explain why the CNS contains so many neuropeptides.

Investigations of the mechanisms through which neuropeptides modulate and regulate behaviour often focus on neural circuitry and sometimes overlook effects on muscle cells, despite the fact that muscle performance is the final objective of the motor output pattern (Hooper et al., 2007; Morris and Hooper, 2001). This is understandable in

studies of chordate twitch fibres, where current dogma indicates that muscle impulses follow motor neuron impulses one-to-one, so that the strength, duration and speed of contraction are more easily predicted from the impulse pattern in the motor axons. Invertebrate muscles, however, integrate information from synaptic inputs differently because they are often innervated by multiple excitatory axons, sometimes receive inhibitory inputs and, in many cases, contract in response to graded electrical signals or even in response to hormones (Atwood, 1976; Atwood and Cooper, 1995; Atwood et al., 1965; Peron et al., 2009). Among invertebrates, modulation of centrally generated motor patterns by NTs or hormones can be complemented by peripheral modulation at neuromuscular synapses and/or muscle fibres by the same or similar substances (Ormerod et al., 2013). In crab hearts, for example, FLRFamide peptides act centrally to increase the rate and amplitude of contractions by altering the rate of bursts generated by the cardiac ganglion, and they act peripherally to augment excitatory junctional potentials (EJPs) and muscle contractions (Fort et al., 2007). FLRFamides also act directly on crab stomatogastric ganglion to increase pyloric rhythm frequency and to evoke gastric mill activity, and they act peripherally to enhance EJPs and contractions in gastric mill muscles (Jorge-Rivera et al., 1998; Weimann et al., 1993). Thus, central and peripheral modulatory effects appear to be coordinated to produce physiologically appropriate changes in muscle performance.

Although there is a growing body of evidence to indicate that peptides and other modulators can act in a cell-specific manner on neurons, few studies have examined the possibility that peptidergic or aminergic modulators may also work in a cell-specific or tissue-specific manner on effector cells. Perhaps the best example is for octopamine,

which increases relaxation rate and cAMP levels more strongly in regions of the locust extensor-tibiae muscle that contain the highest proportions of slow and intermediate muscle fibers (Evans, 1985). Likewise, in *Drosophila* larvae, octopamine increases EJP amplitude and nerve-evoked contractions more strongly in some muscle fibres than others (Ormerod et al., 2013). In the crab gastric mill, allatostatin-3 decreases the initial EJP amplitude and enhances facilitation in one muscle (gm6) without altering EJP amplitude or facilitation in another (gm4), and proctolin increases EJP amplitude in muscle gm4 but not muscle gm6 (Jorge-Rivera et al., 1998). It was not clear, however, whether the changes in initial EJP amplitude in these studies were caused by presynaptic or postsynaptic effects; changes in synaptic facilitation reflect presynaptic rather than postsynaptic mechanisms (Zucker, 1989). In lobster stomach muscles, GABA was found to decrease the amplitude of EJPs in some muscles (gm6a and gm9) but not in others (the p1 muscle; Gutovitz et al., 2001). In crab opener muscle, DRNFLRFamide increased transmitter release from nerve endings of the fast excitatory axon but not the slow excitatory axon (Rathmayer et al., 2002), but postsynaptic effects were not examined. This same peptide induced contractions in superficial extensor muscles of crayfish but not in deep extensor or superficial flexor muscles (Quigley and Mercier, 1992), but the possibility that DRNFLRFamide might augment contractions evoked by muscle depolarization was not examined. Thus, although peripheral modulation by neuropeptides can involve cell-specific effects on neurons, there is a conspicuous lack of evidence that neuropeptides exhibit such specificity on muscle cells.

Here we examine the question of whether or not a neuropeptide can elicit cell-selective effects post-synaptically on individual muscle cells, using *Drosophila*

melanogaster as a model system. The muscle cells of third instar larvae are uniquely identifiable, and details of synaptic innervation of these cells have been well characterized (Hoang and Chiba, 2001). We investigated the most abundant peptide encoded in the *Drosophila dFMRF* gene, DPKQDFMRFa, which has been isolated and purified from *Drosophila* tissue and is thought to be released as a neurohormone (Nambu et al., 1998; Nichols, 1992; White et al., 1986). Previous work showed that this peptide can increase transmitter release from motor neurons in a cell-specific manner (Dunn and Mercier, 2005; Klose et al., 2010), and that it acts directly on muscle cells to elicit slow contractions (Clark et al., 2008; Milakovic et al., 2014). We now present evidence that DPKQDFMRFa alters input resistance preferentially in some muscle cells and elicits stronger contractions in these cells. We also show that the peptide increases the amplitude of nerve-evoked contractions, that postsynaptic mechanisms contribute to this effect, and that the effect is stronger in some muscle cells than in others. These findings support the view that peripheral modulatory effects can be selective for individual muscle cells.

3.03 Materials and Methods

Fly Stocks

Drosophila melanogaster Canton S. (CS) flies, obtained from Bloomington *Drosophila* stock center, were used for all control trials unless otherwise indicated. All flies were provided with commercial fly media (Formula 4-24 Instant *Drosophila* medium, Plain, 173200), including dry yeast (*Saccharomyces cereviciae*), and were reared at 21°C, constant humidity and on a 12:12 light-dark cycle. To investigate effects of knocking down expression of the mRNA encoding the FMRFamide receptor (FR), a transgenic line containing a FR inverted repeat (FR-IR) downstream of an upstream activating sequence (UAS) was obtained from Vienna *Drosophila* RNAi Center (VDRD #9594). Three tissue-specific drivers were used to examine reduced FR expression: *elav-GAL4*, *24B-GAL4* and *tubP-GAL4*. *elav-Gal4* was used for pan-neuronal expression of the UAS-FR-IR transgene (Luo et al., 1994; Sink et al., 2001). *24B-GAL4* (Brand and Perrimon, 1993; Luo ed fet al., 1994) was used to express *UAS-FR-IR* in all larval somatic muscles (Schuster et al., 1996). *tubP-GAL4* is an insert on the third chromosome that is balanced over TM3, *Sb* and allows for ubiquitous expression of Gal4 (Lee and Luo, 1999).

Dissection

Wandering, third-instar larvae were utilized for all experiments. Larvae were collected from the sides of their culture vials and then placed immediately onto a dissecting dish containing a modified hemolymph-like (HL6) *Drosophila* saline (Macleod et al, 2002) with the following composition (in mM): 23.7 NaCl, 24.8 KCl, 0.5

$CaCl_2$, 15.0 $MgCl_2$, 10.0 $NaHCO_3$, 80.0 Trehalose, 20.0 Isethionic acid, 5.0 BES, 5.7 L-alanine, 2.0 L-arginine, 14.5 glycine, 11.0 L-histidine, 1.7 L-methionine, 13.0 L-proline, 2.3 L-serine, 2.5 L-threonine, 1.4 L-tyrosine, 1.0 valine (pH = 7.2). DPKQDFMRFa was custom synthesized by Cell Essentials (Boston, MA, U.S.A.). With the exception of the force recordings made in Figure 3.7, in all experiments requiring physiological saline, HL6 was used (please see 'force recordings' below). Where noted, 10μM Nifedipine was applied (Sigma-Aldrich, Oakville, Ontario, Canada).

Larvae were pinned dorsal-side up at the anterior and posterior most parts of the larvae. A small incision was made along the dorsal midline, and the larvae were eviscerated. All nerves emerging from the central nervous system (CNS) were severed, and the CNS, including ventral nerve cord and the right and left lobes, was removed, leaving long nerve bundles innervating the body wall muscles. The body wall was pinned out, exposing the body-wall muscles. This preparation allowed recording excitatory junctional potentials (EJPs), input resistance and muscle contractions (Figure 3.1).

Electrophysiological Recordings

Compound EJPs were elicited by stimulating all severed abdominal nerves using a suction electrode connected to a Grass S88 stimulator via a Grass stimulus isolation unit (Grass Technologies, Warwick, RI, USA). Impulses were generated at 0.2 Hz. EJPs were recorded using sharp, glass micro-electrodes containing a 2:1 mixture of 3M potassium chloride : 3M potassium acetate. Signals were detected with an intracellular electrometer (Warner Instrument Corporation, model IE:210), viewed on a HAMEG oscilloscope and sent to a personal computer via an analog-to-digital converter (Brock University,

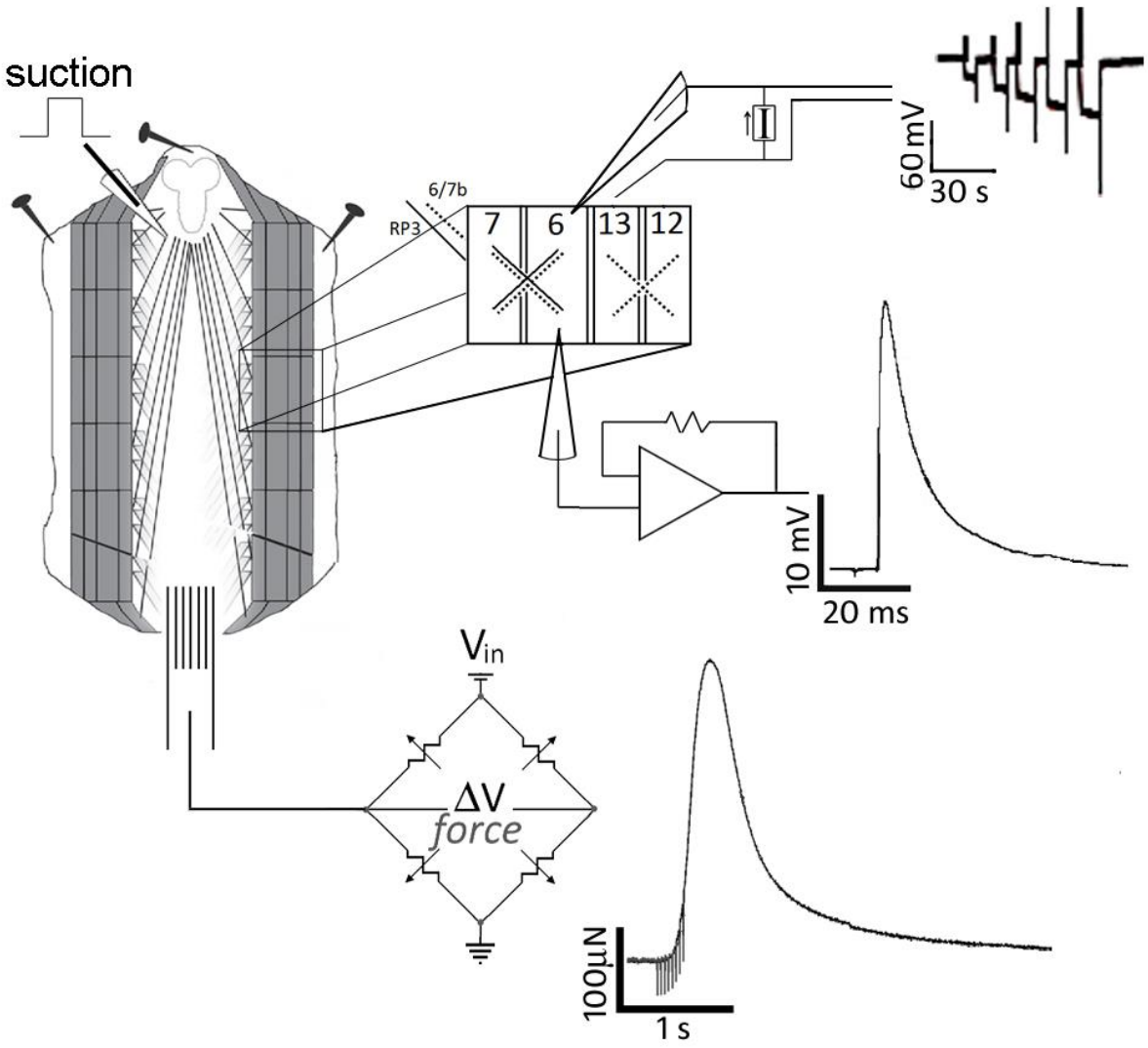

Figure 3.1: Schematic representation of the *Drosophila* third-instar larval semi-intact preparation used for intracellular and force recordings. Emphasis is placed on the subset of longitudinal muscle cells examined in this study, larval body wall muscles (m 6, 7, 12, and 13; in gray). Each abdominal segment is innervating by a segmental nerve, shown as black lines originating from the ventral ganglion. In all experiments the ventral ganglion was removed, and physiological saline was washed over the preparation. Right top: A bridge circuit enabled the injection of a known series of currents (4, 6, 8, 10, 12 nA) across the membrane and recording of the voltage response using a single intracellular electrode. Right middle: Compound excitatory junctional potentials were recorded by stimulating all segmental nerve branches and intracellularly recording from one of the four cells of interest. Bottom right: For some force recordings, a hook was place on the posterior end of the preparation, and connected to the beam of a custom force transduce (full Wheatstone bridge circuit made of silicon wafers, see Ormerod et al. 2013). Other force recordings and basal tonus were recorded using a Grass FT03 tension transducer and amplifier.

Electronics division). Signals were acquired and processed in digital format using custom made software (Brock University, Electronics division). Microsoft Excel™ was used for further analysis. The acquisition software detected the maximum amplitude of each EJP. For each trial, EJP amplitudes were averaged over 30 s time intervals (6 responses), and each 30 s average was plotted over the 15 min trial, generating 30 data points.

Solutions and dissection used during input resistance measurements were identical to those described above, except that 10μM Nifedipine was used where noted. A high-impedance bridge amplifier (Neurodata IR283A, Cygnus technology, Inc. Intracellular Recording Amplifier) was used to inject current and record voltage responses from single muscle cells using single, sharp intracellular electrodes containing 3 M potassium sulfate. Each muscle cell was injected with a series of currents (4, 6, 8, 10, 12 nA), and voltage responses were recorded. The current injection series was performed 6 times throughout a 15 minute recording period at time points 1, 4, 6, 9, 11, and 15 min. To calculate the input resistance, current and voltage values were used to generate V vs. I curves, and the slope of each curve was calculated for each of the 6 time points per muscle cell. The values were divided by the initial slope-value (time point 1) and expressed as a percentage of the initial value.

Force recordings

In some experiments, where contractions were compared with and without ablating specific muscle cells (Figure 3.7), force was detected using a custom force transducer composed of four silicon wafer strain gauges (Micron Instruments, Simi Valley, CA, USA) in full Wheatstone bridge configuration and mounted about the narrowest part of a polycarbonate beam (Ormerod et al., 2013; Patterson et al, 2010). The

transducer operates linearly between 1μN and 2N and exhibited no temperature sensitivity between 10 and 30°C. Signals were detected and amplified using a differential amplifier (model 3000, A-M Systems, Carlsborg, WA, USA) with no online filtering. All other force recordings were made using a Grass FT03 Force-Displacement Transducer connected to a Grass MOD CP122A amplifier. Contractions were elicited using electrical stimuli from a Grass S48 stimulator, which delivered bursts of eight impulses at 32 Hz every 15 s.

All force recordings were made using 1.5mM $CaCl_2$. The force recordings depicted in Figure 3.7 were conducted using the modified hemolymph-like saline HL3.1 (Stewart et al., 1994), the standard physiological saline used in the laboratory where these trials were conducted. HL3.1 contained (in mM) $NaHCO_3$: 10; Sucrose: 115; Trehalose: 5; NaCl: 70; KCl: 5; MgCl: 4; HEPES: 5; $CaCl_2$: 1.5 (pH = 7.2). There were no qualitative differences between the two salines with regard to the peptide's ability to enhance contractions. We have also previously demonstrated that these two salines do not alter octopamine-induced enhancements of EJPs (Ormerod et al, 2013). Larvae were dissected as described above for EJP recordings. To attach the larvae to the force transducer, a hook was made from fine dissection pins and placed onto the posterior end of the larvae, after which all remaining pins except the anterior pin were removed. In select trials, a fine angled tip dissecting knife was used to selective ablate a subset of muscles in each of the hemi-segments. Care was taken to avoid any damage to any other tissue in the larvae.

Passive changes in muscle force

Following dissection, the anterior dissection pin was replaced with the Grass FT03 tension transducer (Grass Instruments, Quincy, MA, USA) as described previously (Clark et al., 2008; Milakovic et al., 2014). Contractions were amplified using a MOD CP 122A amplifier (Grass Telefactor, West Warwick, RI, USA), digitized using DATAQ data acquisition (Model DI-145, Akron, OH, USA), and viewed using WinDaq software (DATAQ instruments). The recording dish had a volume of ~0.2–0.4 ml and was perfused continuously at a rate of 0.7 ml per min. Excess fluid was removed by continuous suction.

RT-qPCR

Specific details for RT-qPCR are reported elsewhere (Milakovic et al., 2014). Briefly, total RNA was isolated using Norgen's Total RNA Purification Kit (St Catharines, ON, Canada), 500 ng of total RNA were reverse transcribed with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), and SYBR Green qPCR Supermix (Invitrogen) was added to cDNA and primers. Samples were amplified for 40 cycles in a thermocycler (Bio-Rad) for 5 min at 95°C, 15 s at 95°C, 90 s at 58°C and 30 s at 72°C. Primers sequences have been reported previously (Milakovic et al., 2014).

***in situ* hybridization**

Whole dissected (see above) third-instar larvae were fixed in a 4% paraformaldehyde solution overnight. Pre-hybridization washes (5 x 5 min in PBS, 1 x 5min in SSC) were followed by hybridization of the tissues samples with DIG-labelled sense and antisense probes overnight in a hybridization chamber at 60°C. Post-hybridization washes (2 x 5 min in SSC at 60°C, 1 x 30min in SSC + 50% formamide 60°C, 1 x 5 min in SSC). Subsequently, tissue was washed (4 x 5 min in TBS, 1 x 30 min

in blocking solution) prior to incubation with anti-DIG-fluorescene (4 hrs in 1:100 anti-DIG-fluorescene: blocking solution). Prior to microscopy, tissues were washed (3 x 5 min in TBS, 3 x 1min in in dH20). Tissue was imaged on confocal microscopy (Nikon series 1000). Intensity of fluorescence was quantified using image J software (NIH). For each sample the perimeter of each of the four cells was outlined in Image J, and a region of interest within the perimeter was defined in each cell to compare fluorescent staining between the fibres. Care was taken to ensure that each region of interest represented more than 50% of fibre area in each optical section and that no superficial or deep layers interfered with the outlined area in any of the optical sections. To account for cell volume, we took a 50 image Z-stack for each sample. The average pixel-intensity for each cell over the 50-image stack was compared across the four cells. By setting the muscle cell with the greatest relative amount of transcript expression to 100, we obtained a quantitative measure of transcript expression between the four cells (muscle cells 6, 7, 12, 13) of interest.

Statistical analyses

Statistical significance was assessed using SigmaPlot™ software. For comparisons within conditions a one-way ANOVA was used if the data were normally distributed and the variance was homogenous. If these two conditions were not met, a comparable non-parametric test was used. For comparisons both within and between conditions a two-way repeated measures ANOVA, or comparable non-parametric test was used (Figures 3.2C-F, 3.3A-D). For Figures 3.4B-E, 3.5A-B, 3.6A, 3.7A-D, 3.8A, to determine between group differences (if peptide application altered the parameter of interest), we averaged all time points for each trial into three bins; before peptide

application, during application and during the washout, and performed a one-way repeated-measures ANOVA. For Figures 3.6B and 3.8B, we isolated averaged data points at the 8 minute time point (3 minutes into peptide application) and performed a one-way ANOVA across all conditions. In all cases if a significant difference was obtained a Tukey (for ANOVA) or Dunn's (for ANOVA on ranks) post-hoc test was performed to establish specific differences. GraphPad™ software was used for generating dose-response curves in Figures 3.2B, 3.4A, and 3.9B.

3.04 Results

Input resistance

Cell-specific effects of DPKQDFMRFa on muscle cells were first assessed by estimating input resistance (Figure 3.2). Input resistance was determined by measuring slope resistance six times during each 15 minute recording session (at 1, 4, 6, 9, 11 and 14 min time points). Resting membrane potential values are typically ~-42 to -44mV, and there is no statistical difference across the four fibers of interest (fiber 6: 44.5 ± 9.2mV, fiber 7: 42.5 ± 9.5mV, fiber 12: 42.3 ± 8.9mV, fiber 13: 44.1 ± 9.4mV, Kruskal-Wallis one-way analysis of variance on ranks, H=2.12, P=0.548). Input resistance values were typically in the range of 3-5MΩ (Figure 3.2A). A dose-response curve was constructed using muscle cell 6. We avoided the possibility of desensitization completely by using a naïve preparation for each concentration. The EC_{50} for the effect of DPKQDFMRFa on input resistance was $1.3x10^{-7}$ M (Figure 3.2B). Application of $1x10^{-6}$ M DPKQDFMRFa elicited a significant reduction in the input resistance of muscle cells 6 after one minute and four minutes of peptide application (24±8% and 26±7%, respectively, Two-way repeated measures (RM) ANOVA, F = 13.281, P<0.001, Tukey post-hoc, P<0.05, Figure 2C,), and in muscle cell 7 after one minute and 4 minutes of peptide application (14±5%and 18±6% , Two-way RM ANOVA, F= 19.284, P<0.001, Tukey post-hoc, P<0.05; Figure 3.2 D). The input resistance returned to control values within one minute of saline wash. Interestingly, DPKQDFMRFa did not elicit a significant change in input resistance in muscle cell 12 (Two-way RM ANOVA, F= 0.716, P = 0.612, Figure 3.2E) or muscle cell 13 (Two-way RM ANOVA, F= 0.870, P=.503, Figure 3.2F). Control recordings with no peptide application demonstrated stable input resistance values over

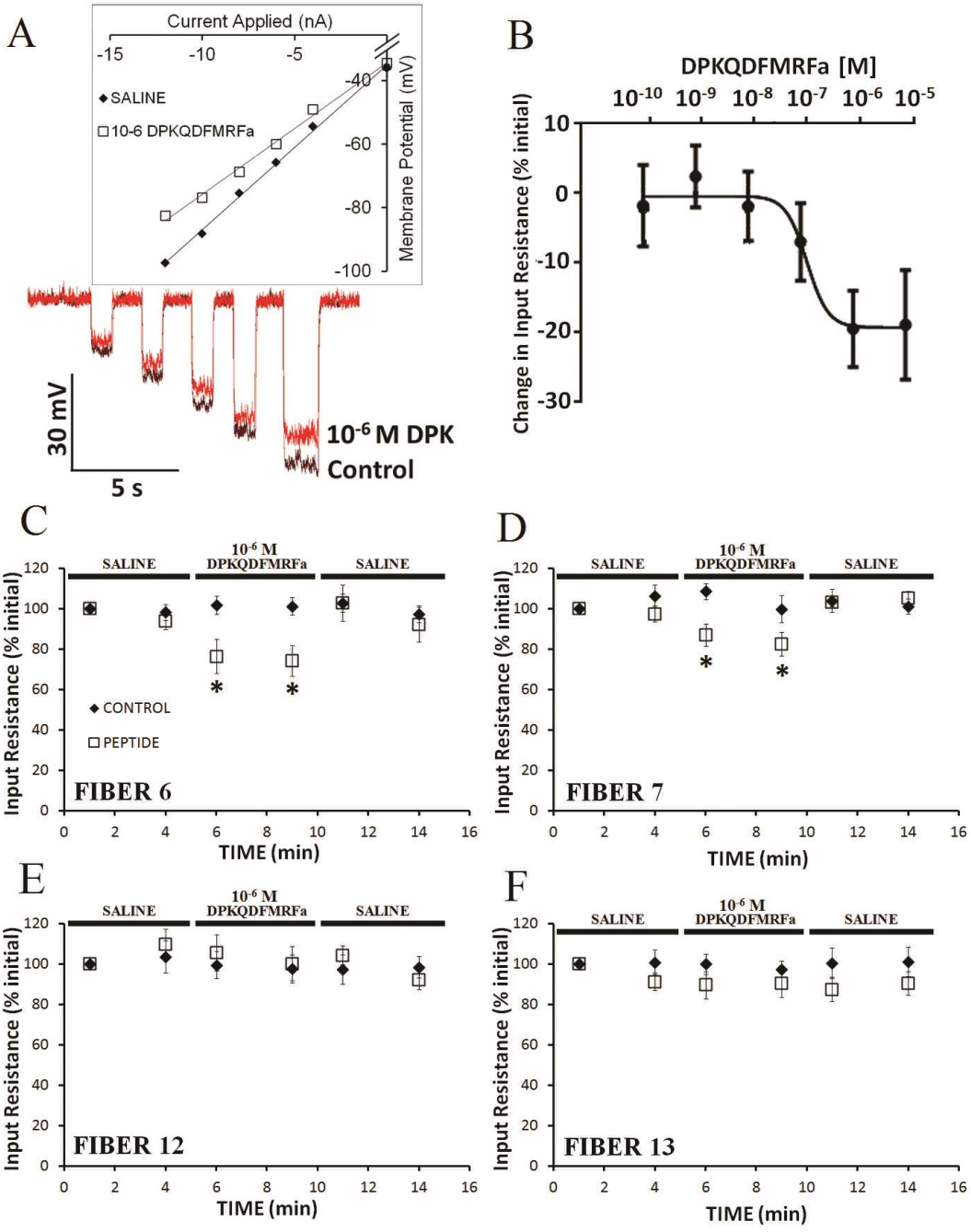

Figure 3.2: DPKQDFMRFa significantly reduced input resistance in cells 6 and 7, but not in 12 and 13. A: Top: Current-voltage curve from muscle cell 6 before (SALINE) and after peptide application (10^{-6} DPKQDFMRFa).Bottom: Representative voltage traces from muscle cell 6 in the presence of saline (Control) and in the presence of 10^{-6} M DPKQDFMRFa (10^{-6} M DPK) in response to a series of square, hyper-polarizing current pulses (4, 6, 8, 10, 12nA).B: Dose-response curve taken from input resistance recordings in muscle cells 6.C-D: DPKQDFMRFa significantly reduced the input resistance in cells 6 and 7, both acutely after one minute of application, and after four minutes of application of DPKQDFMRFA. E-F: DPKQDFMRFa does not alter the input resistance in cells 12 and 13. In both cells the effect was reversible following a saline washout. * denotes $P<0.05$.

the 15 minute recording period. Thus, DPKQDFMRFa modulated input resistance of muscle cells in a cell-specific manner.

Clark et al, (2008) demonstrated that DPKQDFMRFa-induced contractions require extracellular calcium and are blocked by nifedipine and nicardipine, suggesting the involvement of calcium influx through L-type calcium channels. We, therefore, sought to determine if the cell-specific reduction in input resistance showed a similar relationship to L-type channels. Co-application of nifedipine with DPKQDFMRFa prevented the reduction in input resistance in cells 6 (Two-way RM ANOVA, $F = 0.909$, $P=0.478$, Figure 3.3A) and 7 (Two-way RM ANOVA, $F = 1.598$, $P= 0.165$, Figure 3.3B) and resulted in no change in input resistance in cells 12 (Two-way RM ANOVA, $F=0.649$, $P=0.663$, Figure 3.3C) and 13 (Two-way RM ANOVA, $F = 0.620$, $P=0.685$, Figure 3.3D). Thus, it appears that DPKQDFMRFa-dependent reduction in input resistance in cells 6 and 7 requires L-type calcium channels.

EJPs

We next examined the implications of the cell-specific reduction in input resistance on compound excitatory junctional potentials (EJPs) in the larval body-wall muscles. Figure 3.4A (left) depicts representative EJP traces before and after application of 10^{-6}M DPKQDFMRFa. At the stimulus frequency utilized (0.2 Hz) there was a gradual decrease in EJP amplitude over the recording period due to low-frequency synaptic depression (Figure 3.4B-E, black diamonds), as reported previously in this preparation (Dunn and Mercier, 2005) and at other arthropod synapses (Bruner and Kennedy, 1970; Bryan and Atwood, 1981). Low-frequency depression occurred in all four muscle cells, and the degree of depression was not significantly different between

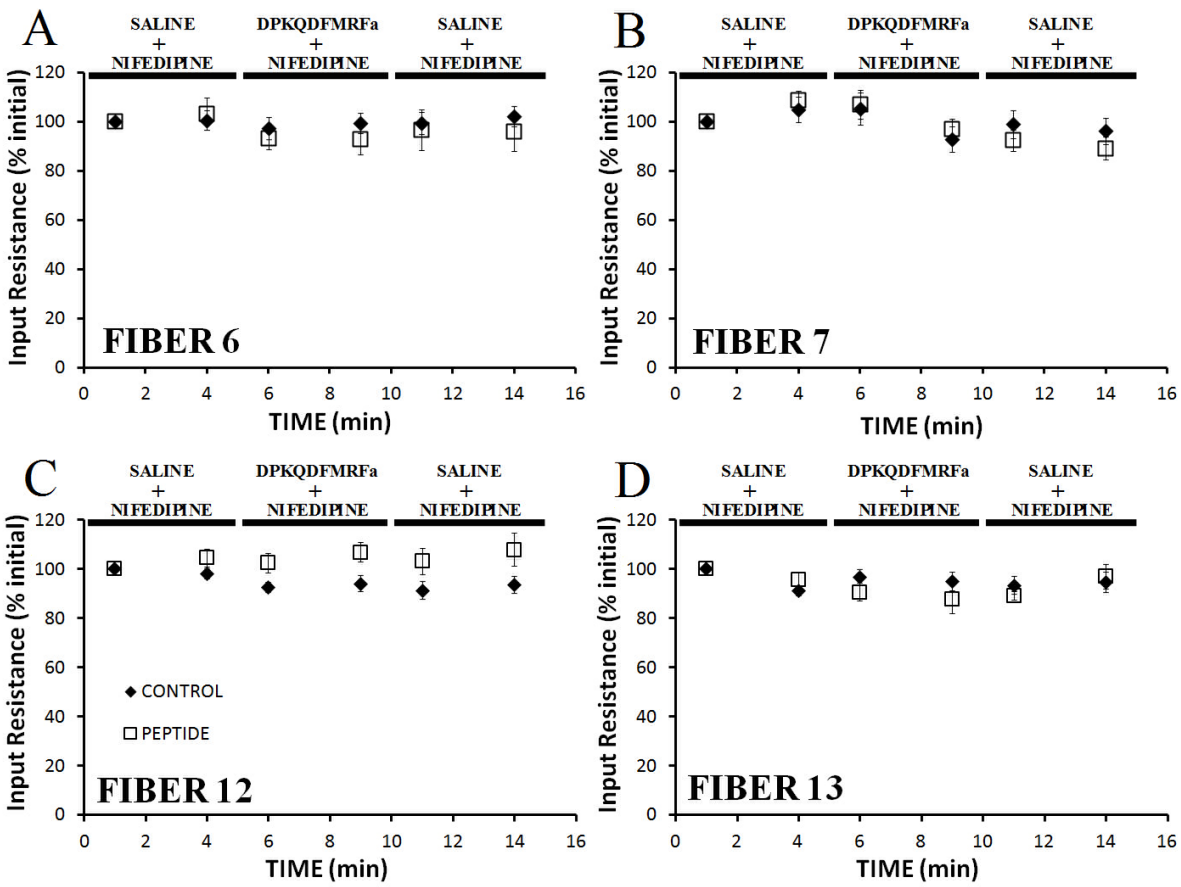

Figure 3.3: Nifedipine blocks DPKQDFMRFa-induced reduction in input resistance. A-B: Co-application of 10μM Nifedipine with DPKQDFMRFa blocked the reduction in input resistance. C-D: Cells 12 and 13 are not affected by application of DPKQDFMRFa or by co-application of DPKQDFMRFa and the L-type selective calcium channel blocker Nifedipine.

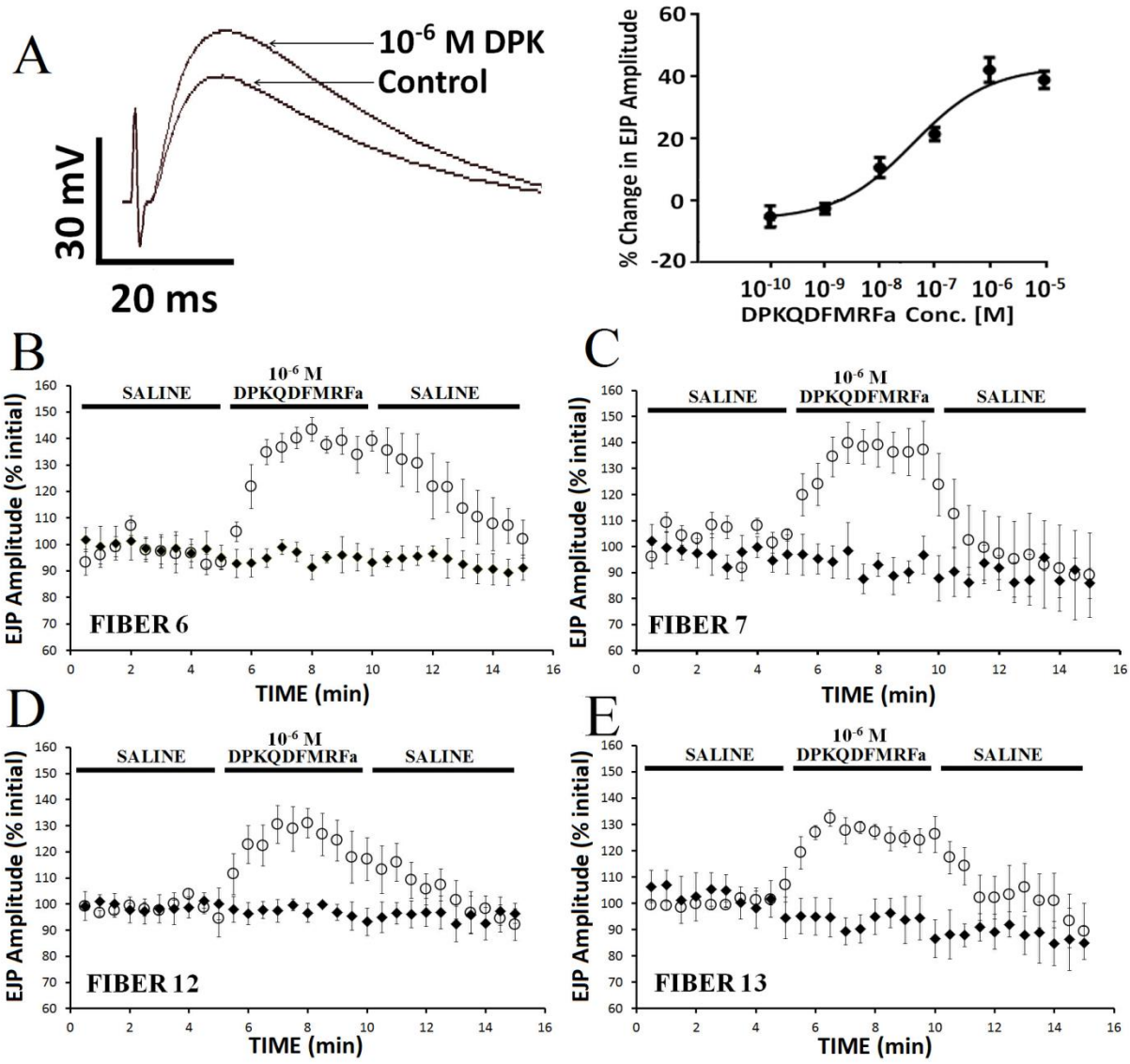

Figure 3.4: DPKQDFMRFa enhances excitatory junctional potentials greater in some cells. A: LEFT- Representative EJP traces from fiber 6 before (Control) and after peptide application (10-6M DPK); RIGHT- Dose response curve for the effect of DPKQDFMRFa on compound EJPs in muscle cell 6.. B-E: shows that application of 10^{-6} M DPKQDFMRFa elicits a significant enhancement in EJP amplitude in all four cells investigated. Closer examination reveals that EJPs are potentiated to a greater extent in cells 6 and 7 (~40%) that in cells 12 and 13 (~30%).

them (One-way ANOVA, F= 2.939, P>0.05, Figure 3.4B-E black diamonds). A dose-response was constructed from recordings made from muscle cell 6. The EC_{50} for the effect of DPKQDFMRFa on EJPs was $4.1x10^{-8}$ M (Figure 3.4A, right). At $1x10^{-6}$ M, DPKQDFMRFa increased EJP amplitude in all four muscle cells (muscle 6: One-way RM ANOVA, F=9.578, P=0.008, Figure 3.4B; muscle 7: One-way RM ANOVA, F=9.427, P=0.005, Figure 3.4C; muscle 12: One-way RM ANOVA, F=13.703, P=0.003, Figure 3.4D; muscle 13: One way RM ANOVA, F= 9.621, P=0.007, Figure 3.4E). The increase was approximately 40% in cells 6 and 7 and approximately 30% in cells 12 and 13 (3 minutes into peptide application; fiber 6: 43.5 ± 3.4%, fiber 7: 38.2 ± 6.5%, fiber 12: 31.0 ± 3.7%, fiber 13: 27.2 ± 2.7%). The increase in EJP amplitude peaked after about three minutes in all cells investigated, and saline washout following DPKQDFMRFa application resulted in a return to baseline values in all cases. Application of DPKQDFMRFa also decreased the time-to-peak of the EJP by 28±9% (paired-t-test, t=-10.710, P<0.001) and decreased the decay time by 24±19% (paired-t-test, t=-11.229, P<0.001) in cells 6 and 7. Such changes in EJP time course are fairly consistent with the drop in input resistance, which would shorten the time constant of the postsynaptic membrane.

Since nifedipine prevented DPKQDFMRFa from decreasing input resistance in muscle cells 6 and 7, we next sought to determine whether or not L-type calcium channels might contribute to the potentiation of EJP amplitude. We used $1x10^{-7}$M DPKQDFMRFa, which was very close to the EC_{50} concentration for the reduction in input resistance. Since enhancement of EJPs by the peptide was similar between muscles 6 and 7 (Figure 3.4B, C), and EJP enhancement was similar between muscles 12 and 13

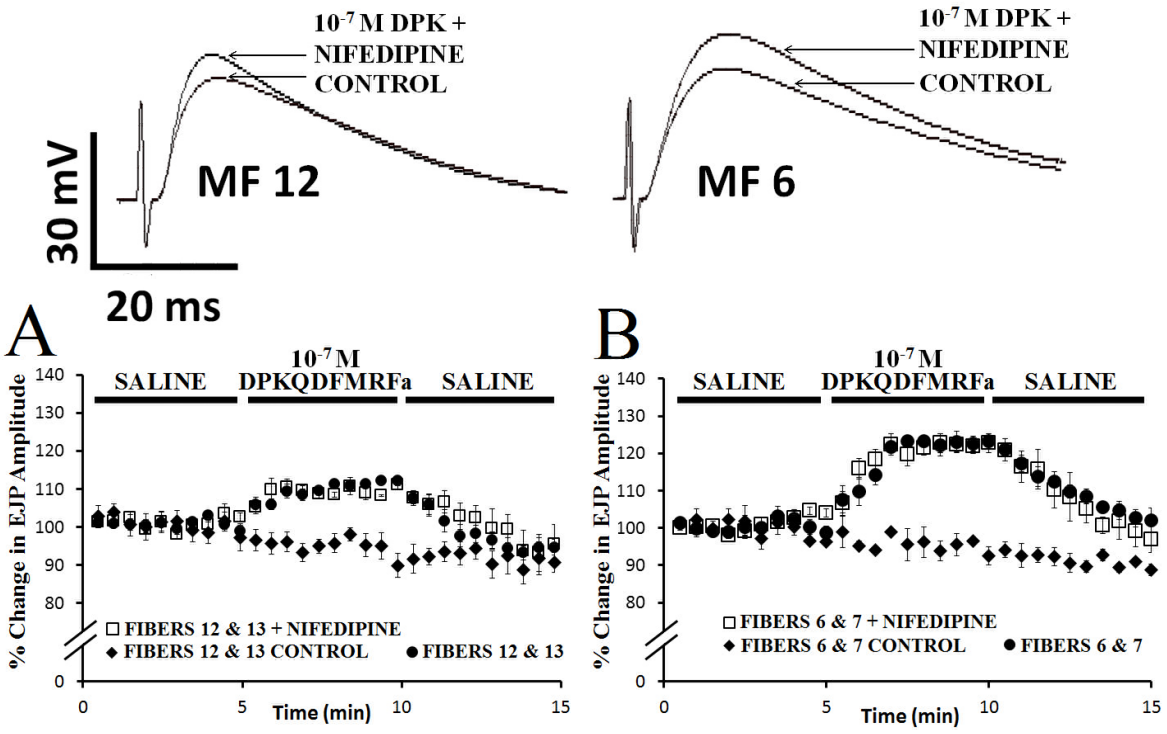

Figure 3.5: Co-application of DPKQDFMRFa and nifedipine does not alter the amplitude of EJPs. Inset above: (left fiber 12, right fiber 6) representative EJP traces from a control trial (no peptide) and an EJP trace following co-application of 10^{-7}M DPKQDFMRFa and 10μM nifedipine. A: pooled data from muscle cells 12 and 13 with no peptide added (closed diamonds), pooled data from muscle cells 12 and 13 with peptide added (closed circles) and pooled data from cells 12 and 13 with peptide and nifedipine added (open squares). B: pooled data from muscle cells 6 and 7 with no peptide added (closed diamonds), pooled data from muscle cells 6 and 7 with peptide added (closed circles) and pooled data from cells 6 and 7 with peptide and nifedipine added (open squares). Combining recordings taken in cells (A) 12 and 13 or (B) cells 6 and 7 demonstrates that co-application of 10μM nifedipine with 10^{-7} M DPKQDFMRFa does not alter the amplitude of EJPs compared to the effect of 10^{-7} M DPKQDFMRFa alone. Additionally, comparing A vs. B also demonstrates that a closer approximation of the EC_{50} concentration of DPKQDFMRFa also showed a greater enhancement of EJPs in cells 6 and 7 compared to 12 and 13.

(Figure 3.4D, E), data were combined for these two cell pairs. Co-application of nifedipine did not alter the enhancement of EJPs by the peptide in any of the muscle cells (fibers 12 and 13- Figure 3.5A: One-way ANOVA, F=0.183, P= 0.682; fibers 6 and 7 - 5B: One-way ANOVA, F=0.028, P=0.871). The concentration of nifedipine utilized ($1x10^{-5}$ M) was slightly higher than the IC_{50} ($3x10^{-6}$ M) previously reported to inhibit L-type channels in *Drosophila* muscle cells (Morales *et al.*, 1999). At $1x10^{-7}$ M, DPKQDFMRFa elicited a significantly larger increase in EJP amplitude in cells 6 and 7 than in 12 and 13 (increases at eight minutes were 23.3 ± 2.1% for 6 & 7 pooled and 11.3 ± 1.9% for 12 & 13 pooled; One-way ANOVA, F=35.723, P<0.001, Figure 3.5A-B).

Knock-down of FMRF-R pre- and postsynaptically

To examine the contribution of the FMRFamide receptor (FR) to the potentiation of EJPs, the UAS-RNAi / Gal4 system was used to knock down receptor expression presynaptically (in nerves), postsynaptically (in muscles) and ubiquitously (Figure 3.6A, B). In control trials with CS larvae, $1x10^{-6}$ M DPKQDFMRFa increased EJP amplitude by 66 ± 12%. Knocking down FR expression in muscle cells (*24B-Gal4 / UAS-FR-IR*) appeared to cause a small reduction in the potentiation induced by DPKQDFMRFa, but the potentiation after 3 minutes of peptide application (53 ± 9%, Figure 3.6B) was not significantly different from CS larvae or from 24B larvae at the same time point (69.1 ± 14.0%, Figure 3.6B). Knocking down FR expression in nerves (*Elav-Gal4 / UAS-FR-IR*) significantly reduced the DPKQDFMRFa-induced increase in EJP amplitude after 3 minutes of peptide application (23 ± 7%, Figure 3.6B) compared to CS larvae and Elav controls at the same time point (59.4 ± 15.5%; Kruskal-Wallis one-way analysis of variance on ranks, H=37.723, P<0.001, Dunn's post-hoc analysis, P<0.05, Figure 3.6B),

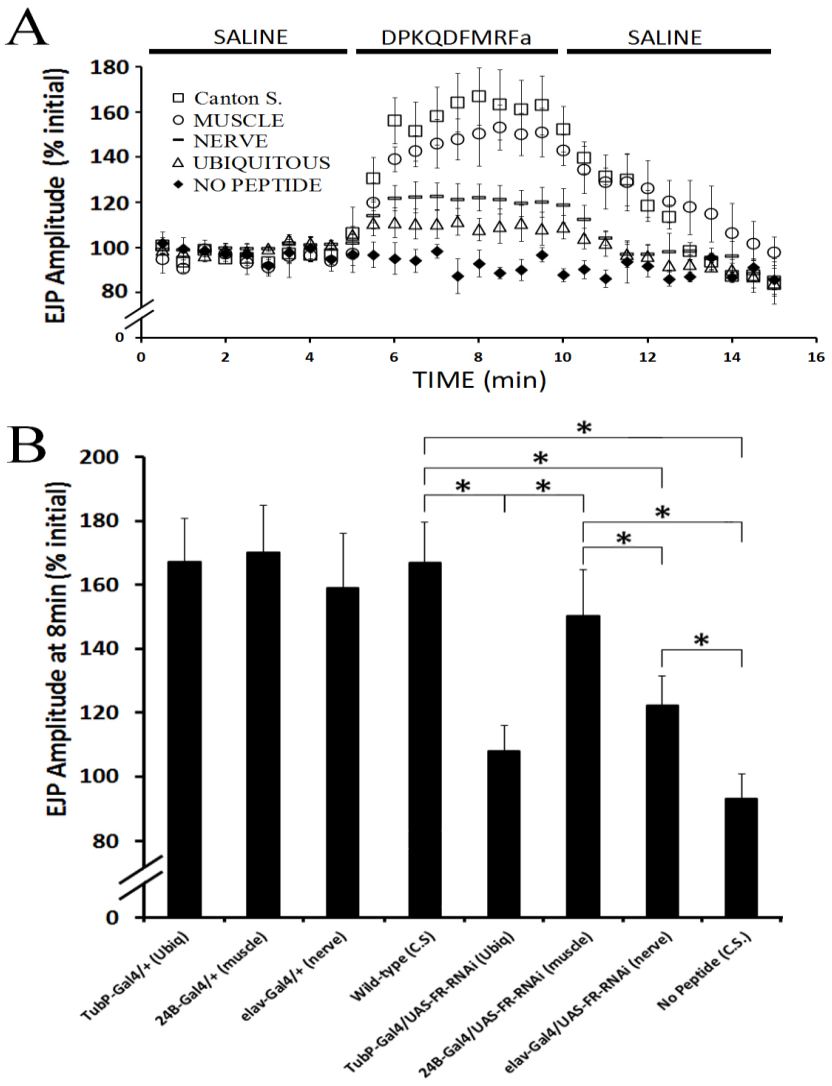

Figure 3.6: DPKQDFMRFa-induced enhancement of EJPs is largely dependent upon presynaptic FMRFa receptor (FR) expression. A: Using the Gal4/UAS system to knock-down expression of FR separately in muscle, nerve and ubiquitously. Knocking down FR expression postsynaptically (MUSCLE) did not alter the ability of the peptide to enhance EJPs compared to wild-type (Canton S.) controls. Knocking down FR expression presynaptically (NERVE) significantly reduced the peptide-induced enhancement of EJPs compared to controls. Lastly, knocking down FR expression ubiquitously (UBIQUITOUS) also significantly reduced the peptide-induced (10^{-6}M DPKQDFMRFa) enhancement of EJPs compared to controls. B: EJP amplitude at 8 minutes for all control and knock-down lines illustrates the predominant role presynaptic FR expression has on DPKQDFMRFa-mediated increases in EJP. EJPs in both the nerve and ubiquitous knock-downs are significantly reduced compared to CS controls, but the reduction is greater in the ubiquitous knock-down highlighted by a lack of statistical difference from no-peptide controls. DPKQDFMRFa-induced increases in EJP amplitude in all Gal4 driver lines were not statistically different from CS controls. * denotes $P<0.05$.

but the peptide still elicited a significant increase in EJP amplitude when compared to control trials with no DPKQDFMRFa application ($P<0.05$). Knocking down FR expression ubiquitously (*tubP-Gal4 / UAS-FR-IR)* reduced the peptide-dependent increase in EJP amplitude to only 11 ± 7%, which was significantly different from both CS and tubP control (*tubP-Gal4/+*) larvae after 3 minutes of peptide application (66.5 ± 13.6%, $P<0.05$). None of the outcross control lines was significantly different from CS controls (% increases in EJP amplitude were as follows: tubP-Gal4/+: 66.5 ± 13.6, 24B-Gal4/+: 69.1 ± 14.0, Elav-Gal4/+: 59.4 ± 15.5).We previously confirmed knock-down of the FR using qPCR to quantify expression in each of our lines (Milakovic et al., 2014). Ubiquitous (*tubP-Gal4 / UAS-FR-IR*) knockdown lines had the largest reduction in transcript levels, relative to wildtype controls, with ~90% reduction. Expression was reduced in muscle (*24B-Gal4 / UAS-FR-IR*) and nerve (*Elav-Gal4 / UAS-FR-IR*) knockdown lines by 77% and 60%, respectively.

Nerve-evoked contractions

To determine whether the peptide might enhance contractions to a greater degree in some muscle cells than others, an isometric force transducer was used to quantify changes in the amplitude of muscle contractions that were evoked using bursts of electrical stimuli applied every 15 s (eight stimuli at 32 Hz within each burst) to all the segmental nerves. This stimulus protocol is within the range of motor output patterns underlying contractions recorded from tethered larvae (Paterson et al., 2010). Muscle cells 6 and 7 contributed roughly 50% of the ventral longitudinal force generated by semi-intact preparations, and muscle cells 12 and 13 contributed roughly 30% (see representative traces top of Figure 3.7), consistent with cellular volume / sarcomeric

potential. To determine whether or not DPKQDFMRFa affected individual muscle cells to the same degree, we used cell ablation to eliminate selected pairs of muscle cells (either 6 and 7, or 12 and 13) that contribute to longitudinal force production, and then compared the peptide's effects on nerve-evoked contractions (Ormerod et al, 2013). It is important to note that a large number of the longitudinal muscles (e.g. dorsal muscle cells 1-3, 9-11) which would typically contribute to larval peristalsis are also ablated during dissection, but all other cells were left intact for recording contractions unless we deliberately ablated them to assess their contribution to the force generated. There are 30 muscle cells per abdominal hemisegment, and cells other than 6, 7, 12, and 13 could contribute to longitudinal contractions and might even be modulated by the peptide. To distinguish the contributed of cells 6 and 7 (not 12 and 13), these fibers were ablated after the initial dissection, and contractions of these preparations were compared with control preparations that were identical in every respect except that no cells were ablated following the initial dissection. The difference between contractions of preparations with and without selected cell ablation indicates the contribution of the selected muscle fiber pair (6 & 7, or 12 & 13) to the contraction. Thus, the longitudinal force production examined here does not provide a comprehensive depiction of forces involved in *in vivo* locomotion, but rather, highlights muscles of the ventral bodywall, which contacts the animal's substrate.

In the absence of peptide, nerve-evoked contractions decreased to approximately 40-60% of their initial amplitude during the first five minutes of stimulation and were relatively stable thereafter (Figure 3.7A-C, black diamonds). This effect, described previously and termed "rundown," has been reported on several occasions (Krans et al.,

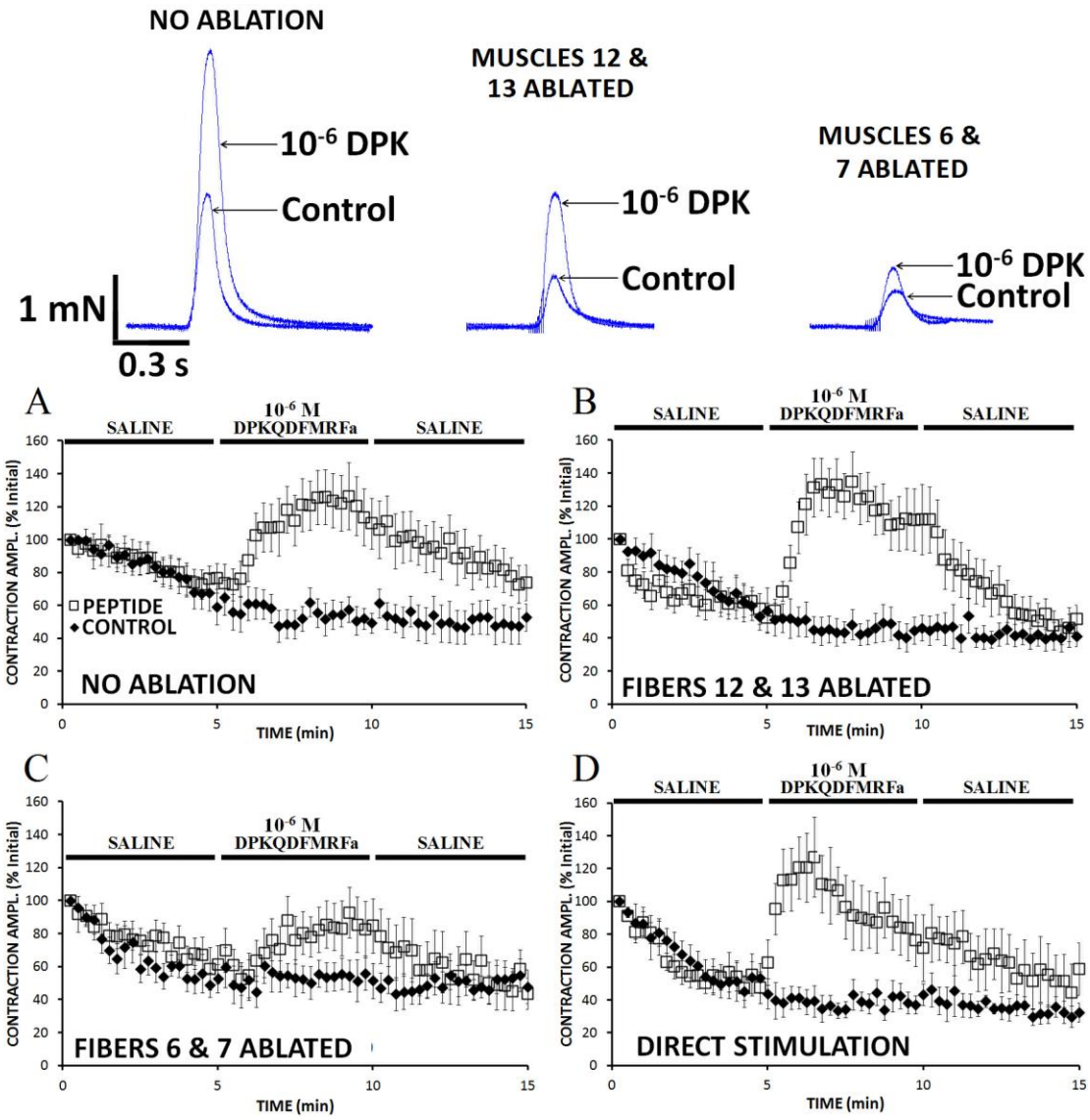

Figure 3.7: DPKQDFMRFa application enhanced evoked contractions in muscle cells 6 and 7 more than in muscle cells 12 and 13. A-D: evoked isometric contractions in third-instar larvae exhibit physiologic rundown during the recording period, as previously described (Ormerod et al. 2013). A: Recordings from semi-intact preparations with no muscle ablation reveal that exogenous application of 10^{-6}M DPKQDFMRFa induced a significant increase in the amplitude of evoked contractions. B: The amplitude of evoked contractions in preparations with cells 12 and 13 ablated (leaving 6 and 7 intact) were also significantly enhanced following the application of 10^{-6}M DPKQDFMRFa. C: The peptide-mediated enhancement of evoked contraction in preparations with muscle cells 6 and 7 ablated (leaving 12 and 13 intact) were greatly attenuated compared to preparations with no ablation or preparations with cells 12 and 13 ablated. D: Attempts to bypass nervous stimulation using direct stimulation of muscle cells also demonstrated a significant enhancement of contraction amplitudes. * denotes $P<0.05$.

2010; Macleod et al, 2002; Ormerod et al., 2013; Stewart et al., 1994). In sham-operated preparations with no muscle cells ablated (Figure 3.7A), application of $1x10^{-6}$ M DPKQDFMRFa after five minutes of stimulation increased nerve-evoked contractions to 126 ± 8% of their initial amplitude, which was more than double the force generated in control trials at the same time point but with no peptide applied (57 ± 8% of initial amplitude; One-way RM ANOVA, $F=11.210$, $P<0.001$, Tukey post-hoc, $P<0.05$, Figure 3.7A). In preparations with muscle cells 6 and 7 intact and 12 and 13 ablated (Figure 3.7B), the effect of the peptide was nearly identical to that observed in preparations with no ablation, increasing contractions to a level (132 ± 16% of initial amplitude) that was more than double the value observed in control trials with no peptide (49 ± 11%; One-way RM ANOVA, $F=14.759$, $P<0.001$, Tukey post-hoc, $P<0.05$, Figure 3.7B). When muscle cells 12 and 13 were left intact and 6 and 7 were ablated (Figure 3.7C), the effect of DPKQDFMRFa was reduced compared to intact preparations and to preparations with cells 12 and 13 ablated, but peptide application did cause a significant increase in force compared to controls with no peptide (92 ± 15% of initial value, compared to 54 ± 10% for control trials, One way RM ANOVA, $F=4.751$, $P=0.030$, Tukey post-hoc, $P<0.05$,Figure 3.7C). Together, these results indicate that in addition to contributing more to total longitudinal force, muscle cells 6 and 7 also contribute more to the enhancement of contractile force induced by DPKQDFMRFa.

In an attempt to bypass nerve stimulation and examine direct effects of the peptide on the muscle cells, we applied the same impulse bursts to the muscle cells using extracellular wire electrodes (Figure 3.7D), as described elsewhere (Ormerod et al., 2013). (No cell ablations were performed in these trials, and the stimulus intensity was

decreased an order or magnitude from that used for nerve stimulation.) These preparations also showed "run-down" of contraction amplitude over the first five minutes, and subsequent application of $1x10^{-6}$ M, DPKQDFMRFa enhanced contraction amplitude to 127 ± 24% of initial value, which was not significantly different from the increase observed in non-ablated preparations subjected to nerve stimulation.

We also assessed DPKQDFMRFa-induced changes in nerve-evoked contractions in the muscle, nerve and ubiquitous FR knock-down lines to distinguish postsynaptic and presynaptic contributions to the peptide's effect. To minimize the impact of rundown in these trials, we waited a sufficient amount of time (5-10 min) for force recordings to stabilize before starting the experimental procedures. This reduced rundown to less than 15% over the 15 minute recording period (Figure 3.8A, no-peptide application). Figure 3.8B shows the peptide-induced increase in force at three minutes of peptide application, which was at or near the maximal effect (Figure 8A). In CS flies, $1x10^{-6}$ M DPKQDFMRFa elicited a 59.3 $\pm$ 10.9% increase in force compared to its no peptide control). Knocking down expression of the FR in the nerve resulted in a significant reduction in the peptide-induced increase in force production compared to the control trials (35.8 ± 7.3%, One-way ANOVA, $F=113.220$, $P<0.001$, Tukey post-hoc, $P<0.05$, Figure 3.8B). Reducing FR expression in muscle also caused a significant reduction in the peptide-induced increase in contractions compared to CS trials (29.0 ± 9.7%, $P<0.05$, Figure 3.8B).

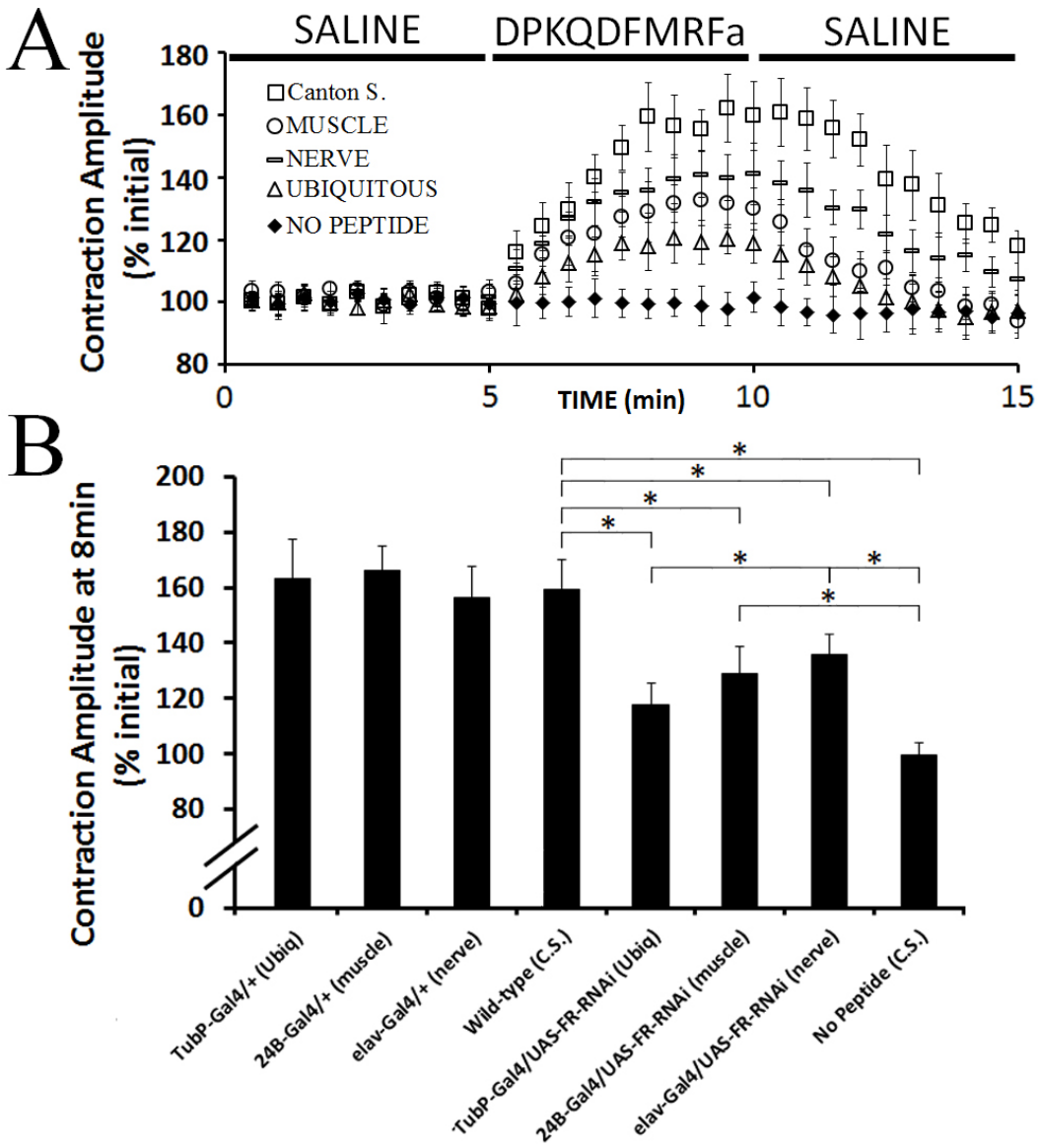

Figure 3.8: Pre and postsynaptic FR expression is required for DPKQDFMRFa-induced increases in evoked contraction amplitude. A: Using the Gal4/UAS system to knock-down expression of FR separately in muscle, nerve, and ubiquitously. Knocking down FR expression presynaptically (NERVE) significantly reduced the 10^{-6} M DPKQDFMRFa-induced enhancement of evoked contractions compared to controls. Knocking down FR expression postsynaptically (MUSCLE) also significantly reduced the peptide-induced enhancement of evoked contractions compared to wild-type (Canton S) controls. Knocking down FR expression ubiquitously (UBIQUITOUS) also significantly reduced the peptide-induced enhancement of evoked contractions compared to controls. B: Evoked contraction amplitudes at 8 minutes for all control and knock-down lines. Peptide-induced increases in the amplitude of evoked contractions were significantly reduced in all three knock-down lines. Both the nerve and muscle knock-down lines were significantly different from no-peptides controls, the ubiquitous knock-down was not significantly different from no-peptide controls. DPKQDFMRFa-induced increases in evoked contractions amplitude in all Gal4 driver lines were not statistically different from CS controls. * denotes $P<0.05$.

These results suggest that both presynaptic and postsynaptic receptors contribute to the peptide's ability to enhance muscle contraction. Reducing FR expression ubiquitously also resulted in a significant reduction in the response to DPKQDFMRFa compared to CS (18.0 ± 7.7%, $P<0.05$, Figure 3.8B). The effects of the peptide on nerve-evoked contractions in CS larvae were not statistically different from any of the uncrossed driver lines (*24B-Gal4, Elav-Gal4 and tubP-Gal4*; $P>0.05$, Figure 3.8B).

It is also noteworthy that the ability of the peptide to increase nerve-evoked contractions in preparations with no muscle cells ablated was qualitatively and quantitatively similar during rundown (Figure 3.7A) and after rundown (Figure 3.8A-Canton S larvae). The ability of DPKQDFMRFa to counteract the effects of rundown on contraction amplitude suggests that this peptide may play a role in sustaining contraction size.

Changes in Tonus

Previously it has been demonstrated that DPKQDFMRFa elicits small, sustained muscle contractions in third instar larvae through a direct action on muscle cells (Clark et al., 2008; Hewes and Taghert, 2001; Milakovic et al., 2014) . To examine whether these peptide-induced contractions exhibit cell-specificity, we assessed the effects of ablating pairs of muscle fibres (representative traces in Figure 3.9A). The EC_{50} for peptide-induced contractions was $6.6x10^{-8}$ M, as estimated from the dose-response curve (Figure 3.9B). To compare effects of DPKQDFMRFa on different muscle fibres, a concentration of $1x10^{-7}$ M was selected, since this was slightly above the EC_{50} value but below the maximal (saturating) effect (Figure 3.9B). This peptide concentration induced

contractions in preparations with and without muscle ablation (Figure 3.9C). Contractions were reduced significantly by ablation of cells 6 and 7 or 12 and 13, and contractions were significantly smaller when 6 and 7 were ablated than when 12 and 13 were ablated (One-way ANOVA, $F=39.194$, $P<0.001$, Tukey post-hoc, $P<0.01$, Figure 3.9C).

Receptor distribution

Finally, we wanted to determine whether cell-specific differences in peptide responsiveness could be attributable to differences in FR expression. Initial attempts to design an antibody against the FR protein were unsuccessful, so we examined changes in transcript expression (representative image in Figure 3.10A: areas of the muscle used for analysis are shown in Figure 3.10B). Muscle fiber 7 had the highest FR expression compared to the other 3 muscle fibers, so it was arbitrarily set to 100% (Figure 10C). Muscle fiber 6 had, on average, 90.5 ± 6.8% expression compared to muscle 7. Muscle 12 showed 71.9 ± 5.9% expression, and muscle 13 exhibited 52.2 ± 6.0 % expression relative to muscle 7. Expression levels in fibers 6 and 7 were not statistically different from one another ($P>0.05$). Expression levels in fibers 12 and 13 were also not statistically different from one another ($P>0.05$), but expression in fibers 6 and 7 was statistically different from expression in fibers 12 and 13 (Kruskal-Wallis one-way analysis of variance on ranks, $H=39.487$, $P<0.001$, Tukey post-hoc, $P<0.05$, Figure 3.10C).

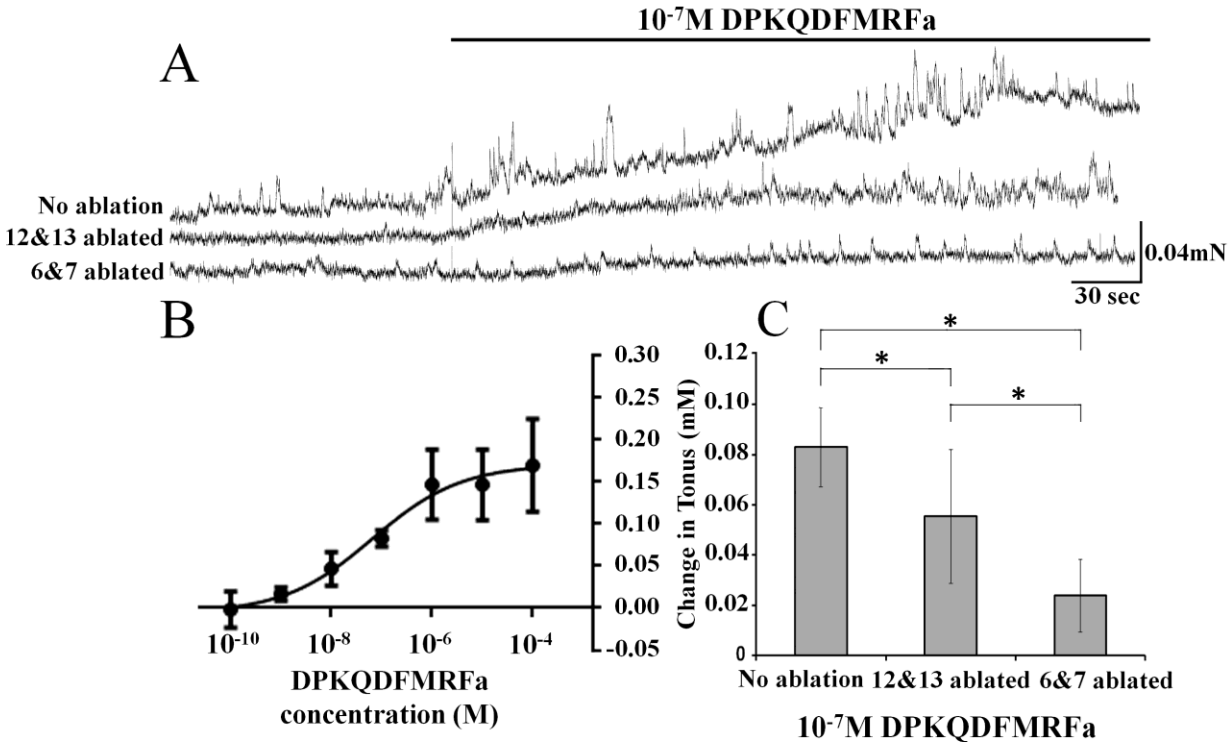

Figure 3.9: DPKQDFMRFa-induced sustained contractions are larger in cells 6 and 7 than in 12 and 13. A.no ablation: Representative trace of 10^{-7} M DPKQDFMRFa-induced contraction in semi-intact preparation with no cells ablated. A.12&13 ablated and A.6&7 ablated depict representative traces of peptide-mediated contractions in preparations with muscle cells 12 and 13 ablated, and 6 and 7 ablated, respectively. B: Dose-response curve for the effect of DPKQDFMRFa on sustained contractions in intact preparations (no ablation). Note: the frequency and amplitude of the asynchronous, phasic contractions were not examined. C: The average change in tonus induced by DPKQDFMRFa is compared between preparations with no ablation, with cells 12 and 13 ablated and with cells 6 and 7 ablated. Ablating both sets of cells (12 and 13, 6 and 7) significantly reduced the amplitude of peptide-induced sustained contractions compared to no ablation controls ($P<0.05$). Peptide-induced contractions in preparations with cells 6 and 7 ablated were significantly lesser than those preparations with 12 and 13 ablated ($P<0.05$).

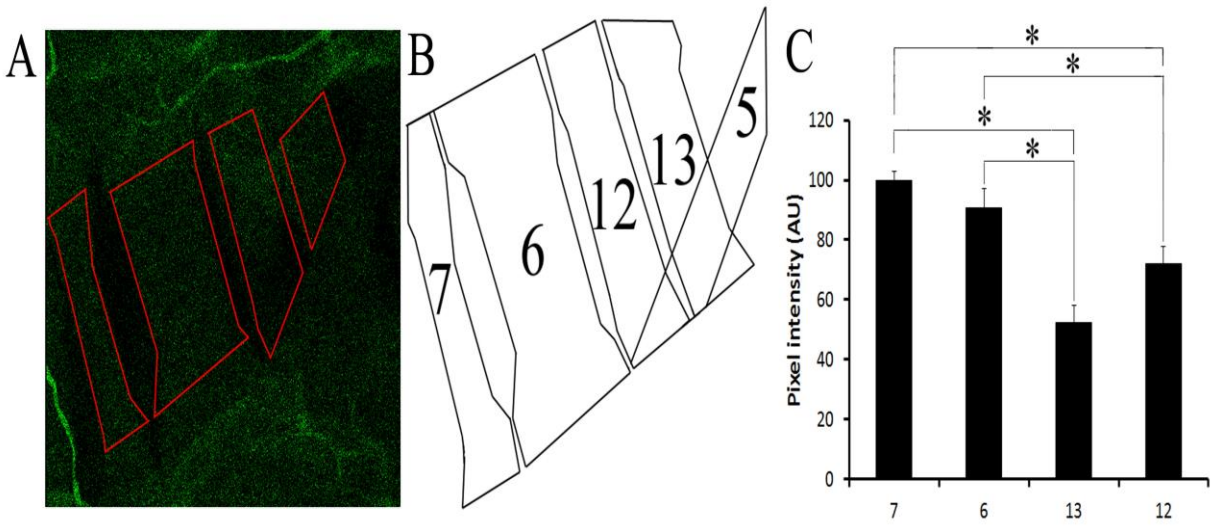

Figure 3.10: Muscle cells 6 and 7 have significantly greater FR expression compared to cells 12 and 13. A: Representative confocal microscope image from a single focal plane showing the four muscle cells. The red outline represents the area of each fiber used for pixel intensity analysis. B: Schematic outline of the four muscle cells of interest and muscle fiber 5, which was avoided during analysis of fibers 12 and 13. C:*In situ hybridization* analysis for the expression of FR revealed that cell 7 had the highest relative amount of expression compared to the other three cells. There was no significant difference between cells 6 and 7. Both muscle cells 12 and 13 were statistically different from muscle cells 6 and 7. * denotes $P<0.05$.

3.05 Discussion

We provide evidence that a *Drosophila* neuropeptide, DPKQDFMRFa, elicits cell-selective effects on muscle fibres of third-instar larvae. DPKQDFMRFa induced a significant reduction in input resistance in muscle cells 6 and 7 but not in cells 12 and 13. EJP amplitude increased in all four muscle cells investigated, but the increase elicited by $1x10^{-7}$ M DPKQDFMRFa was significantly higher in fibres 6 and 7 than in 12 and 13. Knocking down FMRFa receptor (FR) expression separately in nervous and muscle tissue demonstrated that enhancement of EJP amplitude was largely dependent upon presynaptic FR expression. Muscle-ablation experiments demonstrated that DPKQDFMRFa enhanced nerve-evoked contractions more strongly in muscle cells 6 and 7 than in cells 12 and 13. Contractions induced directly by the peptide were also larger in cells 6 and 7 than in 12 and 13. Finally, FR expression was significantly greater in cells 6 and 7 than in 12 and 13. Taken together, these results indicate that DPKQDFMRFa can elicit greater modulatory effects on some muscle cells than others. This preferential modulation, which we refer to as "cell-selective", appears to involve differential expression of the peptide's receptor.

A reduction in input resistance indicates increased cellular conductance and suggests the activation of ion channels in the plasma membrane, although enhanced activation of exchangers in the muscle membrane can have a comparable effect (Fritz et al, 1979; Walther and Zittlau, 1998). The ability of nifedipine to abolish the drop in input resistance suggests that DPKQDFMRFa might activate dihydropyridine-sensitive, L-type calcium currents known to be present in the plasma membrane of these muscle cells (Gielow et al., 1995). However, such L-type currents are activated by voltages (-40 to -

10mV cf. Geilow et al., 1995) slightly above the range of resting membrane potential values in the present work (-42 to -44 mV). Moreover, input resistance measurements reported here were elicited by hyperpolarizing rather than depolarizing pulses. Thus, it seems unlikely that DPKQDFMRFa activates such L-type currents. These *Drosophila* muscles also contain amiloride-sensitive, T-like currents (Gielow et al., 1995). However, DPKQDFMRFa-induced contractions are reduced by nifedipine but are not sensitive to the T-type blockers, amiloride and flunarizine (Clark et al., 2008). Thus, although the postsynaptic effect of the peptide appears to be mediated by dihydrypyridine-sensitive currents, the channels underlying such effects require further characterization. Other putative hormones, such as crustacean cardioactive peptide, proctolin and DRNFLRFamide (Donini and Lange, 2002; Nykamp et al., 1994; Quigley and Mercier, 1997) also require extracellular calcium to induce contractions in arthropod muscles. In addition, YIRFa elicits contractions and activates inward current in muscles of the flatworm *Schistosoma mansoni*, and both effects are antagonized by inhibitors of L-type channels (Novozhilova et al., 2010). These findings suggest that several peptide modulators may induce contractions in invertebrate muscles by activating calcium channels in the plasma membrane.

A 20-25% decrease in input resistance, as observed in cells 6 and 7 during peptide exposure, would be expected to cause a proportional decrease in EJP amplitude if the synaptic current remained constant. Previous studies, however, demonstrated that DPKQDFMRFa increases synaptic current (Hewes et al., 1998) via an increase in the number of quanta of transmitter released per nerve impulse (Klose et al., 2010). The overall increase in EJP amplitude in cells 6 and 7 would suggest that the magnitude of the

increase in synaptic current exceeds the magnitude of the drop in input resistance. Indeed, 0.5-1x10^{-6} M DPKQDFMRFa was reported to increase synaptic current by 51-55% (Hewes et al., 1998; Klose et al., 2010), which exceeds the magnitude of the drop in input resistance reported here. A 40% increase in the amplitude of compound EJPs is reported here for cells 6 and 7 in response to 1x10^{-6} M DPKQDFMRFa. This value is higher than that reported previously for comparable peptide concentrations (20% for 0.5-1x10^{-6} M; Dunn and Mercier, 2005; Klose et al., 2010) when simple EJPs were recorded in muscle cell 6 while stimulating only one motor axon (MNSNb/d-Ib). The difference suggests that other motor neurons may be responsive to this peptide. Muscle cells 6 and 7 are innervated by MNSNb/d-Is and occasionally by MNSNb/d-II, in addition to MN6/7-Ib (Hoang and Chiba, 2001). Since DPKQDFMRFa does not enhance EJPs elicited by stimulating MNSNb/d-Is (Dunn and Mercier, 2005), it is possible that the peptide may modulate MNSNb/d-II.

RNA_i experiments previously showed that the ability of DPKQDFMRFa to increase synaptic current requires expression of FR and another peptide receptor, *Drosophila* myosuppressin receptor 2 (DmsR2) in *Drosophila* neurons (Klose et al., 2010). Our results corroborate these findings by showing that the peptide's ability to increase the size of compound EJPs requires FR expression in neurons. Reducing FR expression in muscle cells, however, had no significant effect on the peptide's ability to increase EJP amplitude. These observations indicate that enhancement of EJPs by DPKQDFMRFa results primarily from presynaptic effects, and that postsynaptic effects of the peptide contribute little (if anything) to the increase in EJPs. The small (23%) increase in EJP amplitude that persists following FR knockdown in neurons probably

results from residual expression of FR and/or expression of DmsR2. FR expression was reduced by 60% in these larvae, but these measurements were made using whole larvae rather than isolated nervous systems. Thus, although RNA_i successfully reduced FR expression, we have not estimated the degree of knockdown precisely in each tissue.

Although FR expression in muscle does not appear to contribute substantially to the enhancement of EJPs, it does contribute to the enhancement of muscle contraction. Knock-down of the FR in muscle cells caused a significant decrease in enhancement of nerve-evoked contractions by DPKQDFMRFa, and this reduction was similar to the effect of knocking down FR in nerve cells. Thus, the peptide's ability to increase the amplitude of nerve-evoked contractions involves presynaptic and postsynaptic mechanisms. The latter mechanisms are most likely reflected in the ability of DPKQDFMRFa to induce contractions, which are reduced by knocking down FR expression in muscle cells (Milakovic et al., 2014). If the same postsynaptic mechanisms that induce contractions also contribute to the enhancement of nerve-evoked contractions, both modulatory effects should exhibit the same pattern of muscle cell specificity, at least to some extent (i.e. barring any overriding influence of presynaptic modulatory effects on transmitter output that could influence contractions of all four muscle cells). Indeed, cell ablation showed that muscle cells 6 and 7 contributed more than 12 and 13 to both the peptide's ability to induce contractions and to enhance nerve-evoked contractions. A similarity between the ability of DPKQDFMRFa to induce contractions and its enhancement of evoked contractions is also reflected in the peptide's dose-dependence. The EC_{50} value for peptide-induced contractions (6.6×10^{-8} M) was only slightly higher than that reported previously for nerve-evoked contractions (2.5×10^{-8} M, Hewes et al.,

1998), and threshold for both effects was between 1 x 10^{-8} and 1 x 10^{-9} M (Figure 3.9B; Clark et al., 2008; Hewes et al., 1998).

Higher FR expression in muscle cells 6 and 7 than in cells 12 and 13 (Figure 3.10) correlated with larger contractions in 6 and 7 in the presence of DPKQDFMRFa (Figures 3.7-3.9). However, cells 12 and 13 did contain mRNA for FR even though they showed no change in input resistance in response to DPKQDFMRFa (Figure 3.2). Thus, our data indicate that the simple presence or absence of a receptor does not necessarily ensure that a particular modulatory effect will be observed. There could be several reasons for this, such as cell-specific differences in post-translational modification of the nascent receptor protein, turnover rates in the membrane or rates of inserting the receptor into the plasma membrane. Although our data indicate that the DmsR1 and DmsR2 receptors do not contribute to the ability of DPKQDFMRFa to induce contractions, we have not ruled out the possibility that these receptors might contribute to other effects of this peptide, such as reduction in input resistance.

We do not know which biochemical signalling pathways in the muscle cells give rise to peptide-induced contractions and/or peptide-enhancement of evoked muscle contractions. Peptide-induced contractions require extracellular calcium and are antagonized by dihydropyridines (Clark et al., 2008) but do not appear to involve calcium/calmodulin-dependent protein kinase (CaMKII), cAMP, cGMP, arachidonic acid, or linoleic acid, and the involvement of IP_3 and phospholipase C also seems unlikely (Milakovic et al., 2014). They do, however, require FR expression in muscle cells and are sensitive to pertussis toxin, which confirms the involvement of this G-protein coupled receptor (Milakovic et al., 2014). Presynaptic mechanisms through

which DPKQDFMRFa enhances transmitter output and augments EJP amplitude include activation of at least two receptors (FR and DmsR2), release of calcium from internal stores and activation of CaMKII (Dunn and Mercier, 2005; Klose et al., 2010). Thus, presynaptic and postsynaptic modulatory effects of this neuropeptide appear to involve distinct intracellular signalling pathways. Octopamine has also been shown to elicit presynaptic and postsynaptic effects at neuromuscular junctions of locust (Evans, 1981) and *Drosophila* (Ormerod et al., 2013) via distinct signalling systems.

The present results confirm that a neuropeptide can act directly on muscle fibres in a cell-selective manner, eliciting greater modulatory effects in some than in others. Although each muscle fibre in the *Drosophila* larval body wall is a single cell, each fibre acts as a separate muscle and is typically referred to as a muscle (e.g. Huang and Chiba, 2001). This poses the question of whether our observations with *Drosophila* larvae represent cell-specificity *per se*, or whether they reflect selective modulation of different muscles. Previous work with the crab gastric mill (Jorge-Rivera et al., 1998) showed that aminergic and peptidergic modulators elicited differential effects on EJPs in two different muscles, gm4 and gm6, which might support the notion of muscle-specific modulation. That study reported differential effects on synaptic facilitation, which is modulated presynaptically (Zucker, 1989), and no attempt was made to examine postsynaptic effects directly. Thus, differential effects on gastric mill muscles gm4 and gm6 (Jorge-Rivera et al., 1998) are likely to result from differential effects on the motor nerve terminals. GABA, however, can also act as a selective modulator on gastric mill muscles of the lobster, acting presynaptically via $GABA_A$-like receptors to enhance excitatory transmission onto three muscles (GM6a, gm9, and p1), and acting postsynaptically via

$GABA_B$-like receptors to increase conductance in muscles gm6a and gm9 but not in muscle p1 (Gutovitz et al, 2001). Thus, muscles can be modulated selectively by postsynaptic mechanisms even when they share common presynaptic modulatory effects. Cell-selective modulation within one muscle has been reported for octopamine, which increases cAMP levels to a greater extent in tonic and intermediate fibers of locust extensor tibiae muscle than in phasic fibres of the same muscle (Evans, 1985). These observations support the notion that cell-selective modulation within a given muscle may be related to tonic vs. phasic fibre types. Octopamine also increases both EJPs and evoked contractions more strongly in *Drosophila* larval muscles 12 and 13 than 6 and 7, and it can induce contractions directly (Ormerod et al., 2013). Thus, octopamine appears to be capable of modulating individual muscle cells selectively via a direct action in addition to whatever presynaptic effects it may elicit.

Functional implications of fibre-selective and muscle-selective modulation by peptidergic and aminergic neurohormones are not yet known. Selective enhancement of contractions of tonic or phasic muscle fibre types could play an important role during activation of slow or fast movements in arthropods, which exhibit great diversity of contractile properties, both within and between muscles (Atwood, 1976; Atwood et al., 1965; Gunzel et al., 1993). Indeed, inhibition of tonic fibres in a given muscle is thought to reduce "drag" during movements generated by faster fibres (Ballantyne and Rathmayer, 1981; Wiens, 1989). It is interesting that DPKQDFMRFa modulates *Drosophila* muscle cells 6 and 7 to a greater extent than 12 and 13, while octopamine has the opposite effect (Ormerod et al., 2013). This suggests that different modulators may have complementary functions in the peripheral nervous system, potentiating synaptic

transmission and contraction more at different subsets of muscles or muscle cells. Such differential modulation might play a role in locomotion in *Drosophila* larvae, such as enhancing the contraction of medial muscle cells during forward movement and enhancing contraction of lateral muscle cells during turning. Interestingly, octopaminergic nerve terminals are found on muscle 4 (which is located laterally) and muscles 12 and 13 (which are lateral to 6 and 7), but not on the most medial muscles, 6 and 7 (Keshishian et al., 1988). Our findings also open the question of whether modulation within the central nervous system, to elicit selected motor output patterns, is matched by peripheral modulation of selected muscle cells and the motor nerve terminals on them. Cell-selective modulation in the peripheral and central nervous systems may help to account for the presence of so many peptidergic signalling molecules.

3.06 Acknowledgments

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Chapter 4:

Characterizing the physiological and behavioural roles of proctolin in *Drosophila melanogaster*.

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Abstract

The neuropeptide proctolin (RYLPT) plays important roles as both a neurohormone and a co-transmitter in arthropod neuromuscular systems. We used third-instar *Drosophila* larvae as a model system to differentiate synaptic effects of this peptide from its direct effects on muscle contractility and to determine whether proctolin can work in a cell-selective manner on muscle fibers. Proctolin did not appear to alter the amplitude of excitatory junctional potentials, but did induce sustained muscle contractions in preparations where the CNS had been removed and no stimuli were applied to the remaining nerves. Proctolin-induced contractions were dose-dependent, were reduced by knocking down expression of the *Drosophila* proctolin receptor in muscle tissue, and were larger in some muscle cells than others (i.e. larger in fibers 4, 12 and 13 than in 6 and 7). Proctolin also increased the amplitude of nerve-evoked contractions in a dose-dependent manner, and the magnitude of this effect was also larger in some muscle cells than others (again, larger in fibers 4, 12 and 13 than in 6 and 7). Increasing the intra-burst impulse frequency and number of impulses per burst increased the magnitude of proctolin's enhancement of nerve-evoked contractions and decreased the threshold and EC_{50} concentrations for proctolin to enhance nerve-evoked contractions. Reducing proctolin receptor expression decreased the velocity of larval crawling at higher temperatures, and thermal preference in these larvae. Our results suggest that proctolin acts directly on body-wall muscles to elicit slow, sustained contractions and to enhance nerve-evoked contractions, and that proctolin affects muscle fibers in a cell-selective manner.

Introduction:

Signalling molecules such as neuropeptides and biogenic amines are found extensively throughout the animal kingdom, and the genomes of most organisms encode many such molecules and their receptors. There are roughly 100 neuropeptides in the human CNS and several hundred in invertebrates, and they can exert a wide range of biological actions during all stages of development (Kastin et al, 2013, Geary and Maule, 2010; Yew et al., 1999; Hummon et al., 2006; Hurlenius and Lagercrantz, 2001, Ma et al; 2008; Christie, 2014). Despite intensive investigations over the past half-century we are only beginning to understand why most vertebrate and invertebrate genomes encode such a large number of signalling molecules and their receptors. One hypothesis to rationalize this complexity of signalling molecules is that different modulatory substances may act on different subsets of neurons to activate specific neural circuits and/or inhibit others, in order to elicit specific physiological or behavioural outcomes (Harris-Warrick and Kravitz, 1984; Marder and Calabrese, 1996; Selverston, 2010; Dickinson et al., 2015; Ormerod et al., 2015). This concept of modulators working in a 'circuit-specific' manner may help to explain why so many peptides and their receptors are conserved within genomes. Intercellular signalling molecules include "classical NTs," which are released at chemical synapses and alter ionic conductance and voltage in postsynaptic target cells, and "neuromodulators," which elicit physiological effects without directly altering trans-membrane voltage (Orchard et al., 1989). Neuromodulators can be secreted as hormones into the circulation, allowing them to act on distant target cells, or they can be released as co-transmitters at chemical synapses (Kastin, 2013). Modulation by signalling molecules occurs at various levels within nervous and neuromuscular systems, including sensory

neurons, interneurons, motor neurons and even muscle cells. For example, neuromodulators can act in a cell-selective way to recruit specific sets of synaptically connected interneurons into activity patterns that generate rhythmic movements. Together, these sets of interneurons comprise CPGs, whose activities are regulated by neuromodulators (Harris-Warrick, 2010; Selverston, 2010; Hooper and DiCaprio, 2004; Marder and Calabrese, 1996; Marder and Bucher, 2007). Indeed, the participation of a given interneuron within a specific CPG depends on the presence of specific modulator substances outside the cell and the expression of receptors to those modulators in the cell membrane. The effectiveness of motor output patterns is also affected by neuromodulators that, in arthropods, can alter both the release of NTS at neuromuscular synapses and the responsiveness of muscle cells to NTs (Orchard et al., 1989; Marder and Calabrese, 1996; Dickinson et al., 2015). Modulatory substances also act on sensory neurons, changing their sensitivity to sensory stimuli (Pasztor and Bush, 1987, 1989; Pasztor and Macmillan, 1990; El Manira et al., 1991, Ramirez and Orchard, 1990). Thus, all the key components responsible for generating behaviour (sensory neurons, interneurons, motor neurons and muscle cells) can be modulated by neuropeptides and biogenic amines. Our current thinking is that modulatory actions at all these levels work in concert to generate physiologically and/or behaviourally appropriate responses in the context of constantly changing internal and external environments.

In addition to cell-specific effects on neurons, our work and that of others has shown that aminergic and peptidergic modulators can enhance nerve-evoked contractions and EJPs more strongly in some muscle cells than others (Gutovitz et al., 2001; Jorge-Rivera et al., 1998; Ormerod et al., 2013; 2015; Pasztor and Golas, 1993). In some cases, the

difference in effectiveness is due to preferential effects on presynaptic cells, and in others, the difference is postsynaptic in origin. Our recent work with *Drosophila* 3rd instar larvae showed that the *Drosophila* peptide DPKQDFMRFamide elicits stronger contractions and enhances nerve-evoked contractions more strongly in some muscle fibers than others, and these effects appeared to correlate with differences in expression of the FMRFamide receptor in the muscle fibers (Ormerod et al., 2015). The physiological implications for such cell-selective effects in muscle and the roles they may play in behaviour are unknown. Nonetheless, the occurrence of cell-selective modulation among muscle cells may help to explain the apparent need for so many peptide and aminergic modulators in any given species.

The modulatory actions of DPKQDFMRFamide in *Drosophila* larvae appear to reflect neurohormonal effects, since this peptide is contained in a neurosecretory structure, the ring gland, and the motor axons innervating the larval body wall muscles do not contain FMRFamide-like immunoreactivity (White et al., 1986). Other modulators, such as proctolin (RYLTP), can be released both as hormones and as co-transmitters. In 3rd instar *Drosophila* larvae, proctolin is contained in the ring gland and in motor axons and terminals innervating some, but not all, body wall muscles (Anderson et al., 1988; Taylor et al., 2004). This arrangement suggests one possible explanation for cell-selective effects in muscle, namely that effects of a particular hormonal modulator might be restricted to muscle fibers that also receive that substance as a co-transmitter. Alternatively, fibers which do not receive that co-transmitter might respond to its release as a hormone, if the purpose of the hormone is to recruit or modulate all muscle fibers. In

the latter case, the role of co-transmission might be to preferentially modulate subsets of muscle fibers.

The primary purpose of this paper was to determine whether proctolin can elicit cell-selective effects on muscle fibers in *Drosophila* 3rd instar larvae. Proctolin is known to elicit contractions and to enhance nerve-evoked contractions in muscles of many arthropod species (Baines et al, 1990; Bensen et al., 1981; Erxleben et al., 1995; Lange et al., 1990; Kimura et al., 1989; Mercier and Lee, 2002; Jorge- River et al., 1998). In *Drosophila* larvae, proctolin is present in the ring gland, indicating a hormonal function, and it is also present in motor axons and terminals innervating muscle cells 4, 12 and 13 but not those axons innervating muscle cells 6 and 7 (Anderson et al, 1988; Taylor et al, 2004). We used cell ablation techniques to determine whether or not proctolin induces and/or enhances contractions to the same extent in all these fibers. The results indicate that proctolin can elicit modulatory effects in all these muscle cells, but the modulatory effects are more pronounced in muscle cells 4, 12 and 13. We also show that the effects of proctolin are reduced by knocking down a G-protein coupled receptor (GPCR) previously identified as a proctolin receptor in *Drosophila*, and that modulation of nerve-evoked contractions by proctolin depends on stimulus frequency.

Drosophila has also proven to be an effective and high-throughput system in which to assess the behavioural role for modulators, or to assess the behavioural ramifications of altered modulator expression (Sokolowski, 2001). Numerous assays have been designed to study larval behaviour in *Drosophila*, such as olfaction (Shaver et al., 1998), foraging (Pereira et al., 1995), locomotion (Heiman et al., 1996; Suster et al., 2003), thermotaxis (Kwon et al., 2008) and phototaxis (Xiang et al., 2010), and there are

also well defined assays to examine adult behaviours, such as thermotaxis (Sayeed and Benzer, 1966), geotaxis (Skandalis et al., 2011), learning and memory (Tully and Quinn, 1985). Since such tools have been used previously to elucidate putative functions of other modulators (octopamine: Certel et al., 2010; serotonin: Alekseyenko et al., 2014; dopamine: Alekseyenko et al., 2013), we also attempted to make use of these tools to uncover potential behavioural roles of proctolin.

Materials and methods:

Fly lines:

Drosophila melanogaster Canton S. (CS) flies, obtained from Bloomington *Drosophila* stock center, were used for all control trials unless otherwise indicated. All flies were provided with commercial fly media (Formula 4-24 Instant *Drosophila* medium, Plain, 173200), including dry yeast (*Saccharomyces cereviciae*), and were reared at 21°C, constant humidity and on a 12:12 light-dark cycle. To examine the effects of knocking down expression of the mRNA encoding the proctolin receptor (ProcR), a transgenic line containing dsRNA for RNAi against the ProcR downstream of an upstream activating sequence (UAS) was obtained from BDSC (29414). Two tissue-specific drivers were utilized to examine reduced ProcR expression: *elav-GAL4*, and *24B-GAL4*. *elav-Gal4* was used for pan-neuronal expression of the UAS-ProcR-IR transgene (Luo et al., 1994; Sink et al., 2001), and *24B-GAL4* (Luo et al., 1994; Brand and Perrimon, 1993) was used to express *UAS-FR-IR* in all larval somatic muscles (Schuster et al., 1996).

Dissection

In all physiological experiments, wandering, third-instar larvae were used. Larvae were isolated from the sides of their culture vials and were placed immediately onto a dissecting dish containing a modified hemolymph-like (HL6) *Drosophila* saline (Macleod et al, 2002) with the following composition (in mM): 23.7 NaCl, 24.8 KCl, 0.5 $CaCl_2$, 15.0 $MgCl_2$, 10.0 $NaHCO_3$, 80.0 trehalose, 20.0 isethionic acid, 5.0 BES, 5.7 L-alanine, 2.0 L-arginine, 14.5 glycine, 11.0 L-histidine, 1.7 L-methionine, 13.0 L-proline, 2.3 L-serine, 2.5 L-threonine, 1.4 L-tyrosine, 1.0 valine (pH = 7.2). Larvae were pinned

dorsal-side up at the anterior and posterior ends, a small incision was made along the entire dorsal midline, and the visceral organs were removed. All nerves emerging from the central nervous system (CNS) were severed at the ventral nerve cord, and the CNS, including ventral nerve cord and the right and left lobes, was removed, leaving long nerve bundles innervating the body wall muscles. This preparation was sufficient for muscle contraction recordings. In order to record intracellularly from muscle cells, the body wall was pinned out, and the body-wall muscles were viewed under a dissecting microscope.

Electrophysiological Recordings

Compound EJPs were elicited by stimulating all severed abdominal nerves using a suction electrode connected to a Grass S88 stimulator via a Grass stimulus isolation unit (Grass Technologies, Warwick, RI, USA). Impulses were generated via the stimulator at 0.2 Hz. EJPs were recorded using sharp, glass micro-electrodes containing a 2:1 mixture of 3M potassium chloride : 3M potassium acetate. Signals were detected with an intracellular electrometer (Warner Instrument Corporation, model IE:210), viewed on a HAMEG oscilloscope and sent to a personal computer via an analog-to-digital converter (Brock University, Electronics division). Signals were acquired and processed in digital format using custom made software ("Evoke", Brock University, Electronics division). Microsoft Excel™ was used for further analysis. The acquisition software detected the maximum amplitude of each EJP. For each trial, EJP amplitudes were averaged over 30 s time intervals (6 responses). Proctolin was obtained from Abbiotec (350353, San Diego, CA, USA).

Nerve-evoked Contractions

All force recordings were obtained using a Grass FT03 Force-Displacement Transducer connected to a Grass MOD CP122A amplifier. Nerve-evoked contractions were elicited using bursts of electrical stimuli from a Grass S48 stimulator. Impulse bursts 250 ms in duration were applied every 15 seconds. All force recordings were made using HL6 physiological saline (described above) except that the $CaCl_2$ concentration was 1.5 mM. Larvae were dissected as outlined above. To attach the larvae to the force transducer, a hook was made from a fine minuten pin and placed onto the posterior end of the larvae. In selected trials, a fine angled tip dissecting knife was used to selectively ablate a subset of muscles in each hemi-segment, as described elsewhere (Ormerod et al, 2013). Care was taken to avoid damage to any other tissue in the larvae.

Muscle cells 6 and 7 contributed approximately 50% of the ventral longitudinal force generated by these semi-intact preparations, and muscle cells 12 and 13 contributed roughly 30%, and muscle cells 4, 12 and 13 contributed approximately 70%, which was consistent with cellular volume / sarcomeric potential (Ormerod et al, 2013). To determine whether or not proctolin enhanced muscle cells in a cell-selective manner, we used cell ablation to eliminate sets of muscle cells that contribute to longitudinal force production, and then compared proctolin's effects on nerve-evoked contractions. All other muscle cells were left intact for recording contractions unless deliberately ablated, or ablated during dissection. To determine the relative contribution made by these muscle cells, contractions were compared to those of control preparations that were identical in every respect except that no proctolin was applied.

Proctolin-induced contractions

To determine whether proctolin would induce contractions in the absence of nerve stimulation, larvae were dissected as described above, and the anterior pin was replaced with a custom minuten pin attached to a Grass FT03 tension transducer (Grass Instruments, Quincy, MA, USA) as described previously (Clark et al., 2008; Milakovic et al, 2014, Ormerod et al , 2013; 15). Contractions were amplified using a MOD CP 122A amplifier (Grass Telefactor, West Warwick, RI, USA), digitized using DATAQ data acquisition (Model DI-145, Akron, OH, USA), and viewed using WinDaq software (DATAQ instruments). Data were subsequently exported to Microsoft Excel™ for further analysis and graphing. The recording dish had a volume of ~0.2–0.4 ml and was perfused continuously at a rate of 0.7 ml per min. Excess fluid was removed by continuous suction.

RT-qPCR

To quantify changes in ProcR expression in knock-down lines, total RNA was isolated from whole larvae (5 larvae per replicate) using Norgen's Total RNA Purification Kit (St. Catharines, ON, Canada), 500 ng of total RNA were reverse transcribed with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) and PCR was performed to validate primers (see Table 4.1). For real-time qPCR, SYBR Green qPCR Supermix (Invitrogen) was added to the cDNA and the primers. Each sample was amplified for 40 cycles in a thermocycler (Bio-Rad) as follows: 5 min at 95°C, 15 s at 95°C, 90 s at 56°C and 30 s at 72°C. The delta delta Ct (2–ΔΔCt) method was used for

data analysis, and ribosomal protein 49, a house-keeping gene, was used for data normalization.

Table 4.1: Primers used for PCR.

Primers	Sequence
rp49 forward	5'GATCGTGAAGAAGCGCCA 3'
rp49 reverse	5' CGCTCGACAATCTCCTTG 3'
ProcR forward	5' TGTGCCGCATACAACGACTA 3'
ProcR reverse	5' GCTATCAGGCGACCCGTATT 3'

Larval thermal preference

To assess thermal preference, a linear thermal gradient of 15°C to 30°C was generated using an aluminum dish machined into a rectangular lane (48mm x 20mm x 9 mm), which was placed on top of two Peltier units, each positioned at either end of the lane. Each Peltier unit had an independent digitally controlled power supply box (Brock University Electronics Division), ensuring accurate and constant temperature extremes. The temperature range and linear nature of the gradient were confirmed using a thermal camera (FLIR SC660, FLIR Systems, Inc.), connected to a computerized data acquisition system (Examine-R, FLIR Systems, Inc.). During experiments, the thermal gradient was monitored using two thermocouples, one at each end of the lane. Third-instar larvae were removed from stock vials without anaesthesia, and were washed in distilled water (to avoid contaminating the testing surface with food) and placed in the center of the lane. Testing was performed in the dark, and images were captured under infrared light using a Vario-Sonnar Super Steady Shot© camcorder (Sony Carl Zeiss); the video stream was captured to still images every second using HandyAvi software (AZcendant Software, Tempe, AZ). ImageJ (NIH) software was used to assess the final positional co-ordinates and the corresponding temperature for each larva after 10 minutes, and mean positioning

was calculated as a measure of thermal preference. Prior to experimentation any potential side or wall bias was assessed by examining larval distribution in the lane set ubiquitously at 22°C. We arbitrarily divided the lane into four equal quadrants and observed no statistically significant differences between them, nor were there any side or wall biases.

Velocity of locomotion

We made use of our gradient testing apparatus from the thermotaxis experiments and knowledge that *Drosophila* larvae show a negative phototaxis to assess larval locomotory velocity (Xiang et al, 2010). Larvae were placed at one end of the lane, with the entire gradient chamber set to a constant temperature 21°C. Then using fiber-optic cables, light was cast directly onto the larvae, which caused them to crawl away. We recorded their behaviour on a video camcorder (see above) and calculated their velocity over a distance of 4 mm using ImageJ software.

Statistics

SigmaPlot™ software was used to examine statistical significances. To look for comparisons within conditions a one-way ANOVA was used if the data were normally distributed and the variance was homogenous. In circumstances where these two conditions were not met, a comparable non-parametric test was used. Specifically, in Figures 4.6, 4.7, 4.8B, a Kruskal-Wallis one-way analysis of variance by ranks was used with a Dunn's post hoc test. For comparisons both within and between conditions a two-way repeated measures ANOVA was used. In all cases if a significant difference was obtained a Tukey (for ANOVA) or Dunn's (for ANOVA on ranks) post-hoc test was

performed to establish specific differences. GraphPad™ software was used for generating dose-response curves, and for calculating EC_{50} concentrations.

Results:

Excitatory Junctional Potentials

We first assessed the effect of proctolin on 5 different muscle fibers (Fibers 4, 6, 7, 12 and 13) to determine if this peptide also elicits differential effects on muscle cells. Electrical stimuli were delivered to severed nerve branches continuously at 0.2 Hz, and EJPs were recorded for 15 min (5 min in saline, 5 min in proctolin and 5 min of washout with saline). At the stimulus frequency used there was a gradual decrease in EJP amplitude over the 15 min recording period in control recordings with no peptide (Figure 4.1A). Such a reduction in EJP amplitude has been referred to as low-frequency depression and has been reported previously for this preparation (Dunn and Mercier, 2005) and in other arthropod muscles (Bruner and Kennedy, 1970; Bryan and Atwood, 1981). The degree of low-frequency depression was not significantly different between the five fibers in control trials with no peptide after 10 minutes (Two-way ANOVA, DF=58, between: F=0.448, P=0.642; within: F=1.48, P=0.237). Figure 4.1B (top) depicts representative EJPs from muscle cell 6 (Fiber 6) at the start of a recording (0 min) and after 5 minutes of saline application followed by 5 minutes of saline with 10^{-8} M proctolin (10 min). Application of 10^{-10} to 10^{-8}M proctolin did not consistently alter the amplitude of EJPs in any of the identified muscle fibers investigated (Figure 4.1B, bottom). The slight reduction in amplitude in Figure 4.1 (raw traces, 1B top) is due to low-frequency depression and not an inhibitory effect of proctolin.

Proctolin-induced Changes in Tonus

To determine whether proctolin could elicit contractions in third-instar larval body-wall muscles, changes in muscle force were recorded in dissected larvae with no

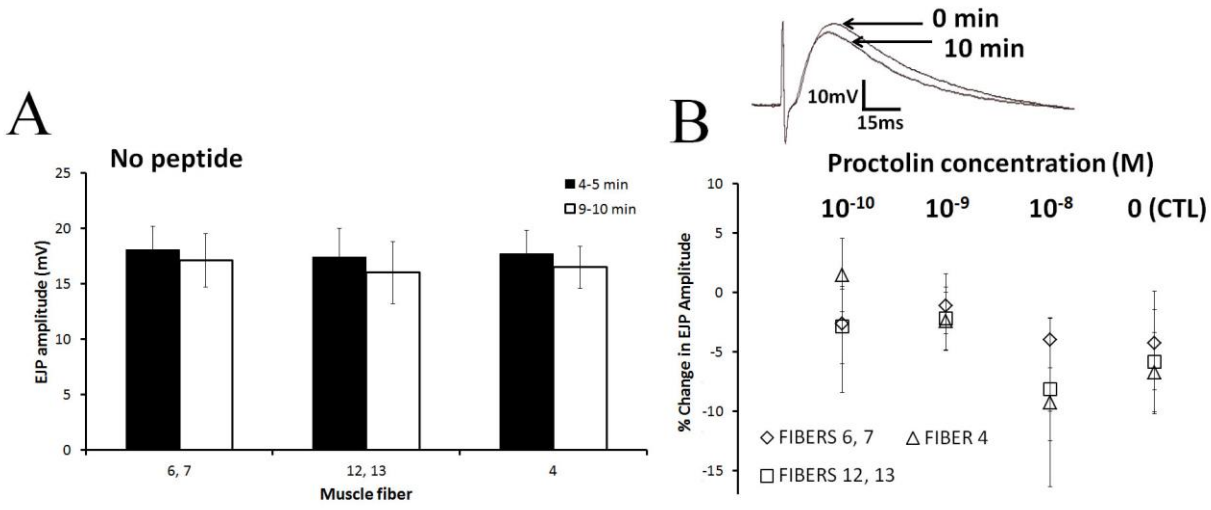


Figure 4.1: Proctolin application does not alter the amplitude of excitatory junctional potentials (EJPs). A: Average EJP amplitudes across the 5 muscle fibers of interest at 4-5 min and 9-10 min in physiological saline to emphasize change in EJP amplitude as a function of time is conserved across these fibers. Error bars: SEM, N=6-9. B: EJPs were recorded intracellularly in *Drosophila* muscle fibers prior to and during exogenous application of 10^{-10} to 10^{-8}M proctolin. Each trial consisted of 5 min of saline application followed by 5 min of proctolin application and subsequently a 5 min washout period. EJPs were elicited using continuous 0.2Hz stimulation, and in each trial the average amplitude of 6 EJPs recorded between 9.5 and 10min are shown (corresponding to 4.5-5min of proctolin application). None of the muscle fibers showed a significant change in EJP amplitude at any of the proctolin concentrations shown. Error bars: SEM, N=6-9.

CNS and no nerve stimulation. Figure 4.2A depicts representative recordings of muscle contractions from larvae following the application of 10^{-10}, 10^{-7} and 10^{-5}M proctolin. Exogenous application of proctolin elicited small, sustained muscle contractions (increases in muscle tonus) that were dose-dependent (Figure 4.2). The threshold for eliciting sustained contractions was between 10^{-9}M and 10^{-8}M, and the effect saturated at or above 10^{-5}M. The EC_{50} for the effect of proctolin on sustained contractions was 8.5 x 10^{-7}M (Figure 4.2B).

Proctolin and other neuropeptides have previously been demonstrated to require external calcium to elicit their effects on muscle fibers (Lange et al, 1987; Nwoga and Bittar, 1985; Schwarz et al, 1980; Wilcox and Lange, 1995). To determine if proctolin-induced contractions in *Drosophila* require external calcium, the concentration of calcium in the physiological saline was altered. Figure 4.2C demonstrates the effects of altering the external calcium concentration (0mM, 0.5mM, and 1.5mM) on the ability of proctolin (10^{-8}M, 10^{-7}M, and 10^{-6}M) to induce sustained contractions in body-wall muscle. Reducing the external calcium from 1.5mM to 0.5mM significantly reduced the amplitude of contractions in 10^{-8}M and 10^{-6}M proctolin, and reducing calcium from 1.5mM to 0mM significantly reduced the amplitude of contractions at all three proctolin concentrations examined (Figure 4.2C: Two-way ANOVA, $F=66.415$, $DF=68$, $P<0.001$, Tukey post hoc, $P<0.05$). To examine the possibility that calcium influx through L-type channels might be necessary for the contractions, we co-applied 10^{-6}M proctolin with the L-type selective blocker, Nifedipine at a concentration ($1x10^{-6}$ M) slightly lower than the IC_{50} ($3x10^{-6}$ M) reported to inhibit L-type channels in *Drosophila* muscle cells was used (Morales *et al.*, 1999). Co-application of 10^{-6}M nifedipine and 10^{-6}M proctolin

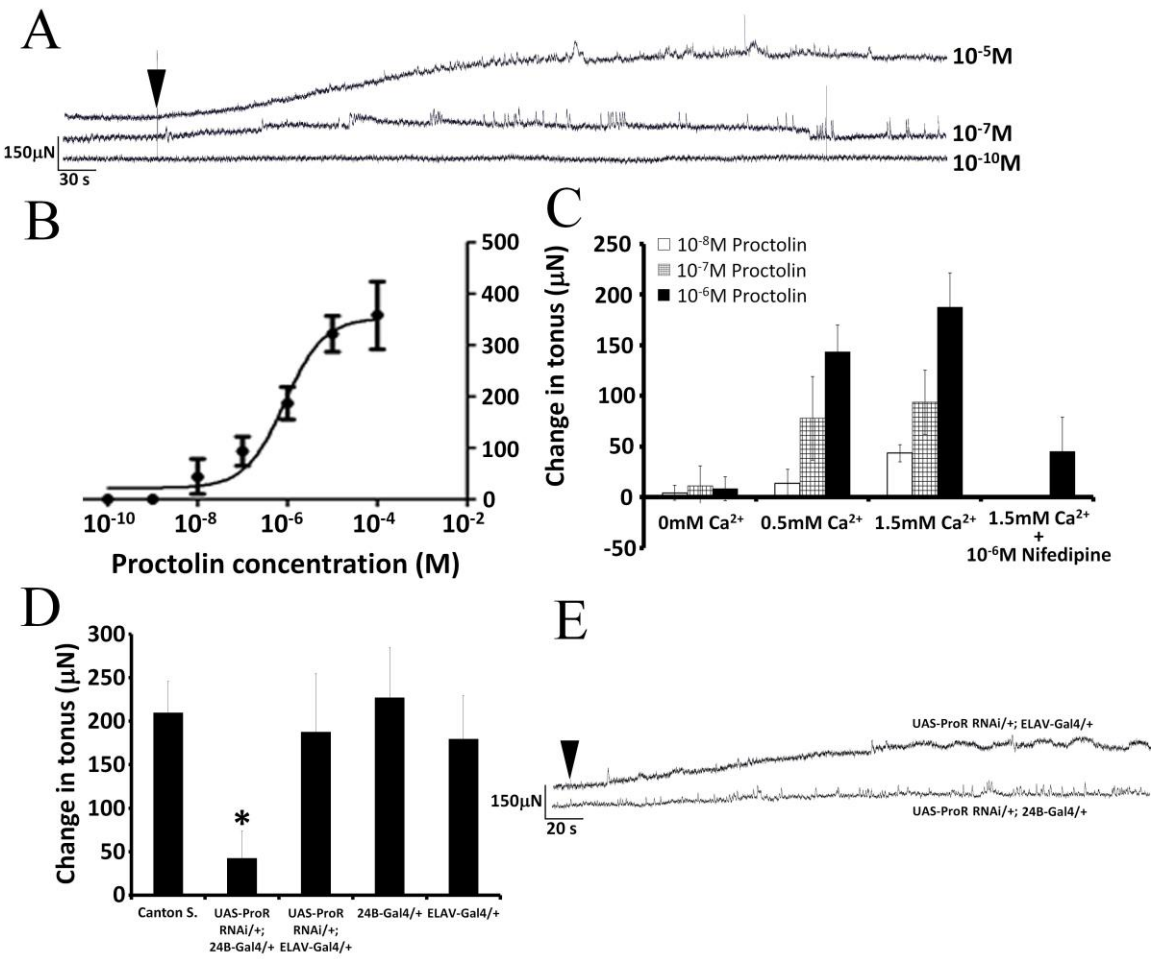


Figure 4.2: Proctolin induces dose-dependent sustained contractions in third-instar larvae. A: representative force traces of proctolin-induced (10^{-5}M, 10^{-7}M, and 10^{-10}M) contractions from semi-intact preparations. Arrow indicates when proctolin was applied. B: Dose-response curve for the effect of proctolin on sustained contractions. Independent replicates for each concentration, Error bars: SD, N=8-18. C: Effects of altering the external calcium concentration (0, 0.5, and 1.5mM Ca^{2+}) in saline on the ability of exogenous proctolin (at 10^{-8}M to 10^{-6}M) to evoke sustained contractions in third-instar larval body-wall muscles. Co-application of 10^{-6}M Nifedipine with 1.5mM Ca^{2+} saline significantly reduced the amplitude of 10^{-6}M proctolin-induced sustained contractions. Error bars: SD, N=5-7. D: Using the Gal4/UAS system to knockdown the expression of the proctolin receptor separately in muscle (UAS-ProcR RNAi/+; 24B-Gal4/+) and in nervous tissue (UAS-ProcR RNAi/+; Elav-Gal4/+) shows that 10^{-6}M proctolin-induced changes in tonus require proctolin receptor expression in muscle tissue, but not in nervous tissue. Error bars: SD, N=7-8. *-indicated $P<0.05$, compared to Canton S. controls. E. Representative traces from muscle and nerve knock-down lines. Arrow indicates when proctolin was applied.

significantly reduced the amplitude of sustained contractions compared to application of 10^{-6}M proctolin alone (Figure 4.2C: Mann Whitney Rank Sum, T=29, P<0.01).

To determine whether proctolin-induced changes in tonus are elicited via this G-protein coupled receptor (GPCR, the proctolin receptor (ProcR)) and are dependent upon post-synaptic receptor localization, we used the Gal4/UAS system to drive the expression of RNAi against the proctolin receptor in a tissue-selective manner to knock-down receptor expression in nervous and muscle tissue. Figure 4.2D depicts changes in tonus elicited by 10^{-6}M proctolin for control lines (Canton S (wild-type) and Gal4 driver lines (24B-Gal4/+, and ELAV-Gal4/+) that were not crossed with RNAi- knockdown line) and to lines where the ProcR was knocked-down postsynaptically in muscle cells (UAS-ProcR RNAi/+; 24b-Gal4/+) and in nervous tissue (UAS-ProcR RNAi/+; ELAV-Gal4/+). Knocking down ProcR expression selectively in muscle fibers significantly reduced the proctolin-induced changes in tonus compared to control lines, but knocking down ProcR in nervous tissue did not (One-way ANOVA, F=17.452, P<0.001, Tukey post-hoc P<0.05). There were no statistically significant differences between the responses to proctolin between control lines (One-way ANOVA, F=0.975, P=.419). Figure 4.2E depicts representative traces from nerve (top) and muscle (bottom) knock-down lines. qPCR was carried out to confirm knock-down of proctolin receptor expression across the various lines. Expression was reduced in muscle (24B-Gal4/UAS-ProcR) and nerve (elav-Gal4/UAS-ProcR) by 67% and 53% respectively (Milakovic et al., 2014).

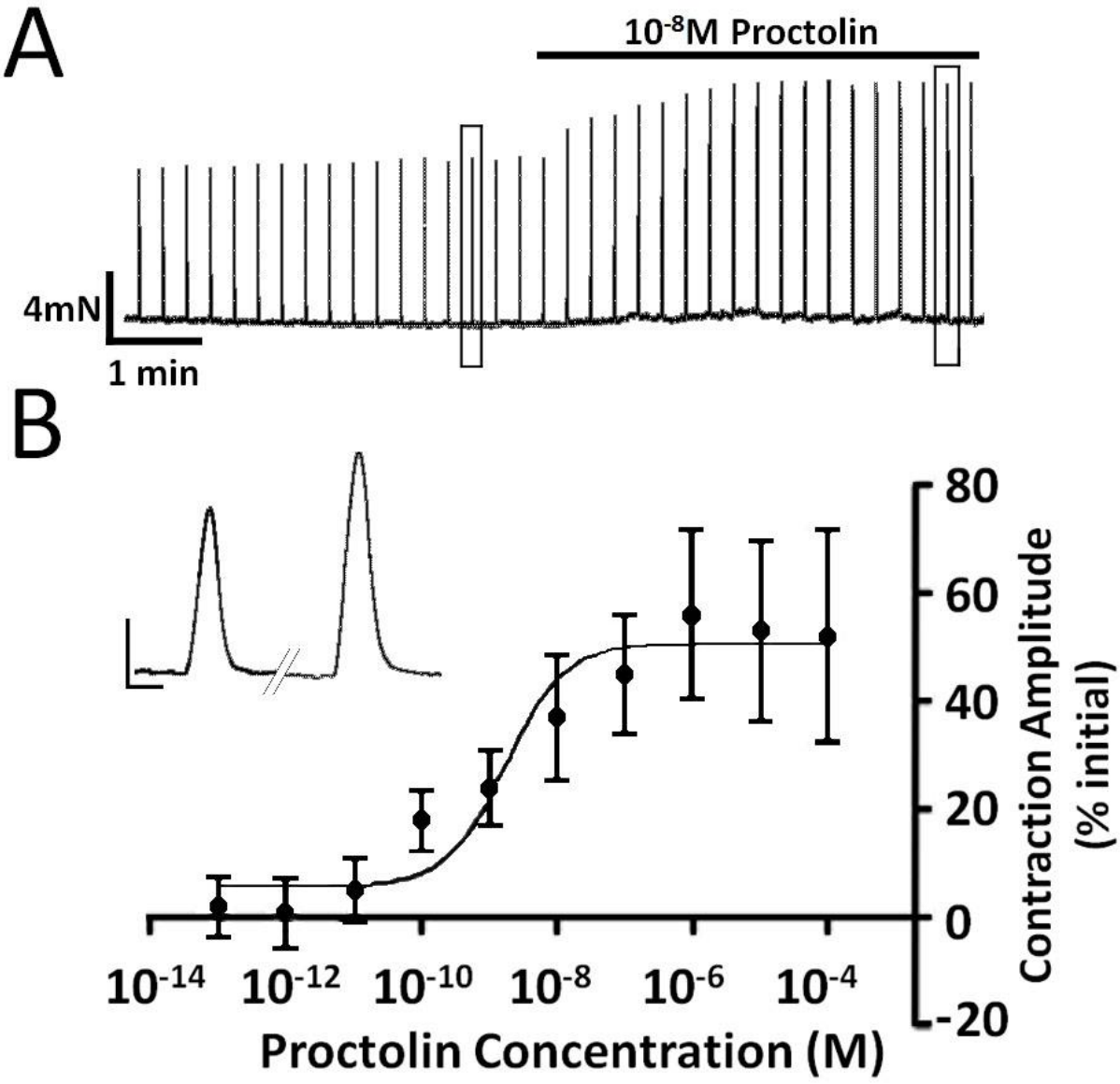


Figure 4.3: Proctolin application increased the amplitude of evoked contractions. **A**: representative trace of the temporal effects of proctolin application on contractions that were evoked using a 250 ms duration burst at 32Hz, every 15 seconds. The representative trace is about 9 minutes in duration. The black application bar depicts when 10^{-8}M proctolin was applied. The two boxes (one prior to proctolin application and one roughly 4.5min after 10^{-8}M proctolin) indicate which individual contractions were taken to create the inset shown in the middle-left. **INSET**: single evoked contraction before (left) and after (right) 10^{-8}M proctolin. The scale bars for the inset: 0.4mN, 0.2s. **B**: Dose-response curve for the effect of proctolin on nerve-evoked contractions. Independent replicates for each concentration, error bars: SD, N=7-10.

Nerve-evoked Contractions

We next examined whether exogenous application of proctolin would alter nerve-evoked contractions in this larval preparation. Initially, we used a previously established stimulus protocol to evoke and record contractions (32 Hz intra-burst impulse frequency, 250ms burst duration, 1 burst every 15s; Ormerod et al, 2013). This stimulus protocol is within the range of motor output patterns underlying contractions recorded from tethered larvae (Paterson et al., 2010). Figure 4.3A depicts a 10min recording showing that 10^{-8}M proctolin increased nerve-evoked contractions and shows the dose-dependence for this enhancement of nerve-evoked contractions. Interestingly, the threshold for proctolin to alter nerve-evoked contractions (approximately 10^{-11}M) was 2-3 orders of magnitude lower than its threshold for eliciting contractions. At this intra-burst impulse frequency (32 Hz), the dose-response relationship was quite broad, with a saturating effect at 10^{-6} M, and the EC_{50} value was 1.7×10^{-9}M.

We next examined whether proctolin-induced enhancement of nerve-evoked contractions was sensitive to changes in the intra-burst impulse frequency. Figure 4.4 illustrates the effects of 10^{-9}M proctolin on nerve-evoked contractions for several intra-burst frequencies. In these experiments peak force was expressed as a percentage of the maximal contraction elicited with 50 Hz stimulation, which elicits maximal contractions in these preparations (Ormerod et al, 2013). The concentration of proctolin used, which was very close to the EC_{50} value, caused significant increases in nerve-evoked contractions at intra-burst frequencies of 10-40Hz but not at intra-burst frequencies of 2-5 Hz (Figure 4.4B). Thus, proctolin was more effective at increasing nerve-evoked contractions at higher intra-burst frequencies.

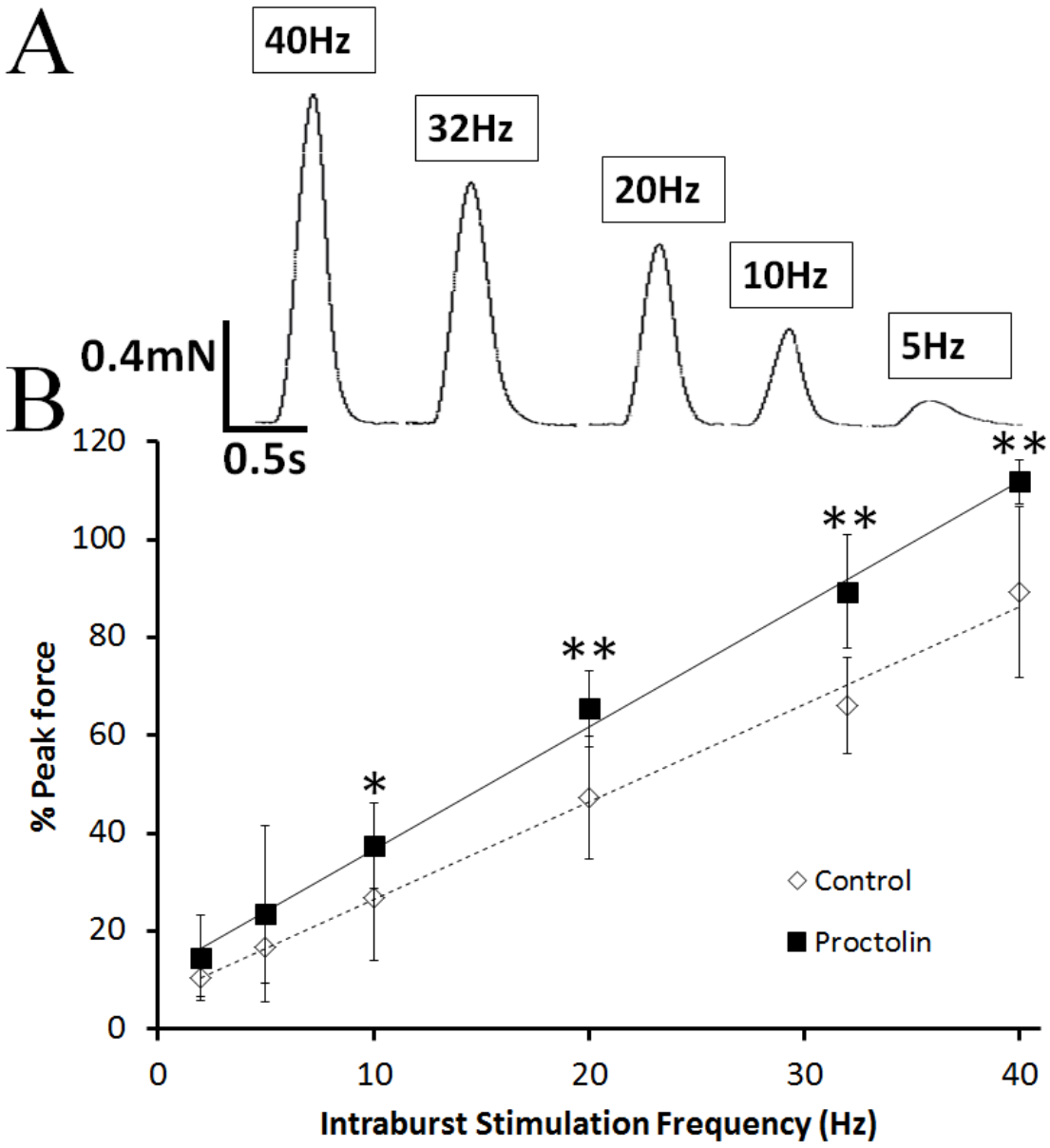


Figure 4.4: Force-frequency curve for control and proctolin groups. **A**: Representative traces shown above depict the effect of varying the intra-burst frequency on evoked-contractions from control animals. **B**: The effect 10^{-9}M proctolin on nerve-evoked contractions was determined for various intra-burst frequencies (motor neuron stimulation) in third-instar larvae. Proctolin enhanced nerve-evoked contractions at higher impulse frequencies but not at low intraburst frequencies. Maximal force was estimated using a single 50Hz burst (250ms duration) at the end of each trial. Statistical comparisons between contractions with and without proctolin were made using a Mann-Whitney test (*P<0.05; **P<0.01). Error Bars: SD, n=7-10.

Table 4.2: Increasing impulse frequency within bursts increases the effectiveness of proctolin at enhancing nerve-evoked contractions. A dose-response curve was generated for the 5 listed stimulus-rates. Both the threshold for enhancement of contractions and the EC_{50}-values for proctolin decreased with increases in intra-burst stimulus rate.

Intraburst Impulse Rate (Hz)	Number of impulses	Threshold	EC_{50}
2	1	10^{-10}-10^{-9}M	8.4 x 10^{-8}M
5	2	10^{-10}-10^{-9}M	4.0 x 10^{-8}M
10	3	10^{-11}-10^{-10}M	1.8 x 10^{-8}M
20	6	10^{-11}-10^{-10}M	4.6 x 10^{-9}M
32	9	10^{-12}-10^{-11}M	1.7 x 10^{-9}M

To further explore the frequency-dependent effects of proctolin, dose-response curves were generated for 5 of the intraburst stimulus frequencies used in Figure 4.3 and were used to estimate threshold and EC_{50}-values (Table 4.2). The threshold for proctolin to enhance nerve-evoked contractions decreased as the intraburst stimulus frequency was raised (Table 4.2). Thresholds were between 10^{-10} and 10^{-9}M for 2 and 5Hz stimulation, between 10^{-11} and 10^{-10}M for 10 and 20 Hz, and between 10^{-12} and 10^{-11}M for 32 and 40 Hz stimulation (Table 4.2). There was also a concomitant reduction in the EC_{50} concentration as the intra-burst impulse frequency increased (Table 4.2).

Muscle-cell ablation

Anderson et al, (1988) demonstrated that the motor neurons innervating muscle fibers 3, 4, 12, and 13 contain the highest relative abundance of proctolin immunoreactivity (14-34%), with very little apparent expression (0-4%) in the other 26 fibers. Thus, to determine if these fibers are preferentially modulated by proctolin, we used an established cell-ablation technique, where muscle cells of interest are serially ablated (Ormerod et al, 2013; 2015), to assess the relative contribution of selected muscle fibers to proctolin-induced enhancement of nerve-evoked contractions (Figure 4.5). Contractions were expressed as % of initial value and were plotted against time. At 10^{-8}M, proctolin-enhanced nerve evoked contractions in all cases. The effect of proctolin was reduced only slightly by ablating muscles cells 6 and 7 or by ablating muscle cells 12 and 13. Ablating cells 4, 12 and 13, however, appeared to reduce proctolin's effectiveness more. To quantify the effect of proctolin, nerve-evoked contractions at 8-10 min in each proctolin trial (corresponding to 4-5 min of proctolin exposure) were averaged, and the averaged value at 8-10 min control trials (with no proctolin) was

subtracted. This difference was then expressed as a percentage of the maximal contraction elicited by a 50 Hz impulse burst in that trial. This procedure corrected for differences in force between the different preparations and provided a measure of proctolin's ability to enhance nerve-evoked contractions in the subset of fibers remaining after ablation (Figure 4.6). With no cells ablated, 10^{-8}M proctolin enhanced contractions by up to 78% of maximal contraction. Ablating cells 6 and 7 significantly decreased the effectiveness of proctolin to 62% of maximal force (a 16% drop in effectiveness compared to no ablation). Ablating cells 12 and 13 significantly decreased proctolin's effect to 52% of maximal contraction (a 26% drop compared to no ablation), and ablating cells 4, 12 and 13 significantly decreased proctolin's ability to enhance contractions to 47% of maximal contraction (a 31% decrease in the modulatory effect of proctolin). Thus, modulation of muscle cells 4, 12 and 13 appeared to contribute proportionately more to proctolin's effectiveness than did modulation of cells 6 and 7. (Figure 4.6: Kruskal-Wallis, $H=123.455$, $P<0.001$, Dunn's post hoc, $P<0.05$).

We next sought to determine if proctolin-induced contractions in the absence of neuron stimulation (Figure 4.2) might also reflect cell-selective effects. To determine this, contractions were elicited using 10^{-6}M proctolin, as in Figure 4.2, but selective cell ablation was performed. Ablating muscle fibers 6 and 7 significantly reduced the force of proctolin-induced contractions compared to control preparations with no ablation (Figure 4.7: Kruskal-Wallis ANOVA on ranks, $H=42.803$, $P<0.001$, Dunn's Post hoc, $P<0.05$). Ablating muscle fibers 12 and 13 also significantly reduced the force of sustained contractions compared to control preparations ($P<0.05$), and ablating fibers 4,

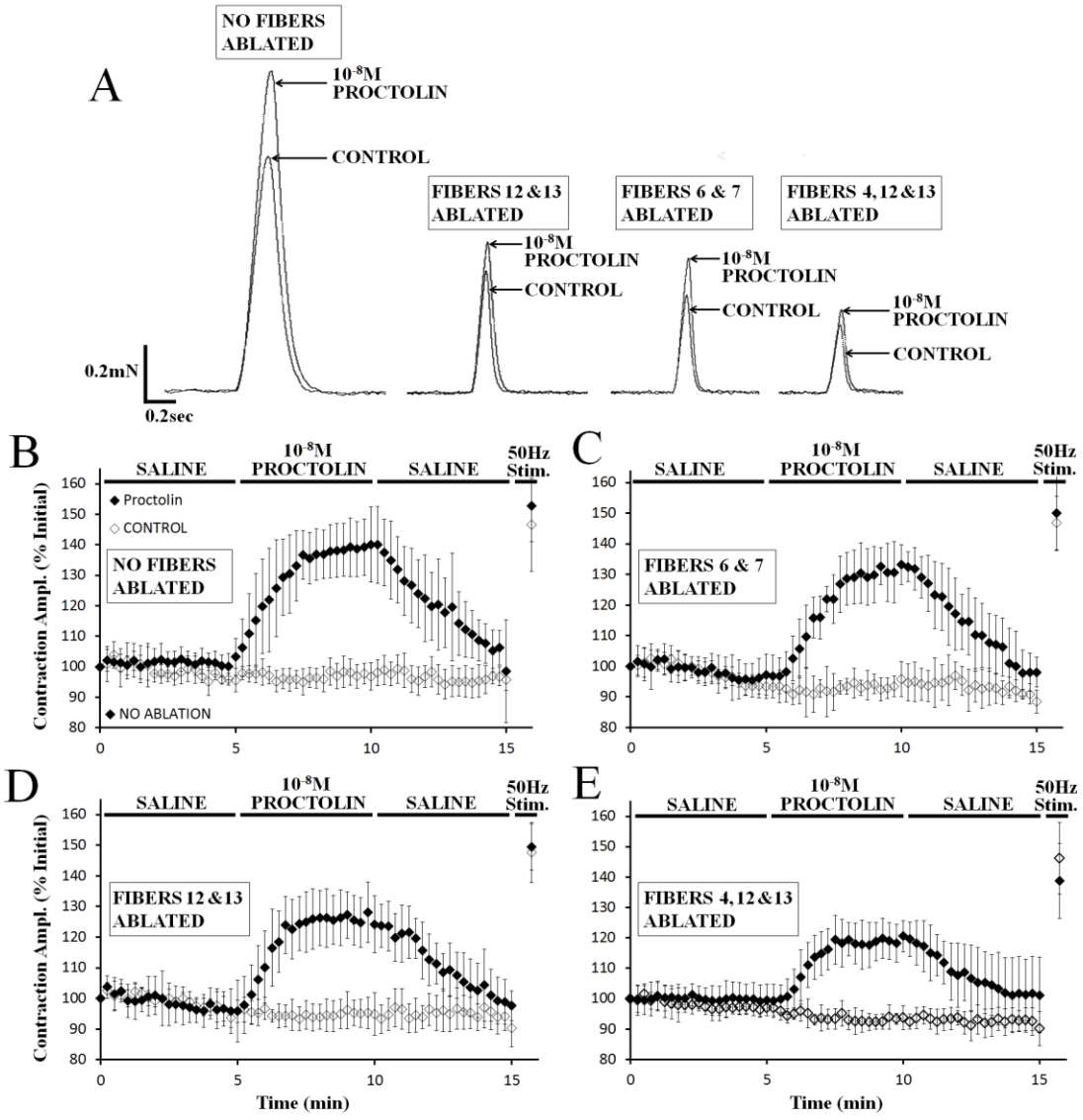


Figure 4.5: Proctolin application increased the amplitude of nerve-evoked contraction in muscle cells 4, 12, and 13 more than in muscle cells 6 and 7. **A**: Representative traces from intact preparations and those preparations with muscle cells ablated, before and after 10^{-8}M proctolin application. **B**: Nerve evoked contractions from semi-intact preparations with no muscle cell ablation show that 10^{-8}M proctolin application induced a significant increase in contraction amplitude ($P<0.001$). **C**: 10^{-8}M proctolin significantly increased the amplitude of nerve-evoked contractions in preparations with muscle cells 6 and 7 ablated ($P<0.001$). **D**: 10^{-8}M proctolin significantly increased the amplitude of nerve-evoked contractions in preparations with muscle cells 12 and 13 ablated ($P<0.001$). **E**: 10^{-8}M proctolin significantly increased the amplitude of nerve-evoked contractions in preparations with muscle cells 4, 12, and 13 ablated ($P<0.001$). **B-E** error bars: SD, N=6-9.

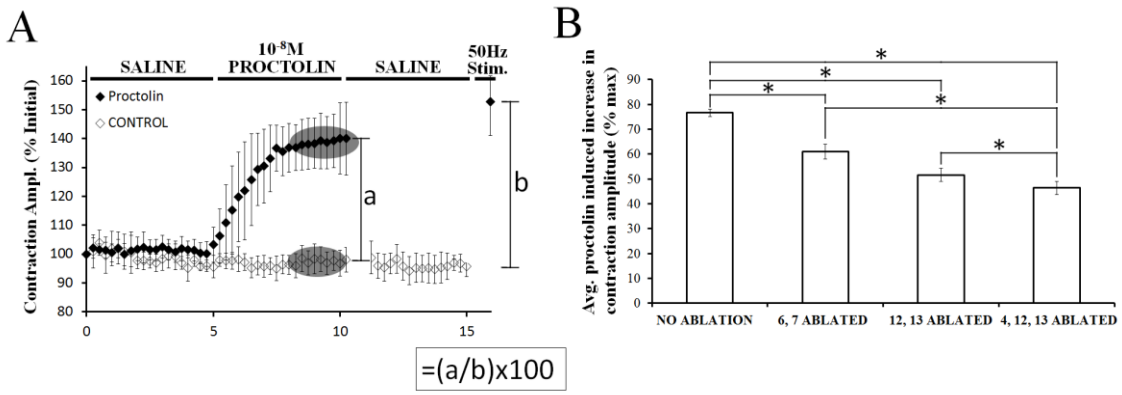


Figure 4.6: Proctolin increases the amplitude of evoked contractions to a greater extent in muscle fibers 4, 12 and 13 than muscle fibers 6 and 7. A: Data from Figure 4.5B are used to demonstrate how proctolin-induced increases in contraction amplitude were expressed as a function of maximal contraction. Within each trial, contraction amplitudes (expressed as % of initial contraction) were averaged during the 8-10 min time period for both proctolin and "no-proctolin" application trials. The averaged amplitude for the trials with no proctolin was subtracted from amplitude obtained in each of the proctolin application trials to estimate the increase in nerve-evoked contraction attributed to proctolin (a). The maximal contraction value for that preparation (b) was estimated by subtracting the averaged contraction at 15 min in control trials from the contraction elicited by a single burst of impulses at 50 Hz in the presence of the peptide. The ratio a/b was then multiplied by 100 to estimate the relative effectiveness of proctolin in each trial. This procedure estimates the modulatory effect on only the muscle fibres present and allowed a comparison between preparations with different levels of ablation by compensating for differences in total muscle mass. B: Effects of cell ablation on modulation by 10^{-8}M proctolin were assessed by estimating the relative effectiveness as described in A. Error bars: SD. (Kruskal-Wallis, $H=123.455$, $P<0.001$, Dunn's post hoc, *$P<0.05$).

12 and 13 significantly reduced the force of sustained contractions compared to controls ($P<0.05$). A statistically significant difference was also observed between preparation with fibers 6 and 7 ablated and those preparations with fibers 4, 12 and 13 ablated. Thus, as with nerve-evoked contractions, fibers 4, 12 and13 appeared to contribute more to the proctolin-induced contractions.

Behavioural Experiments

In addition to examining physiological effects of proctolin in third-instar D*rosophila* larvae, we examined its possible roles in behaviour. Given proctolin's ability to enhance muscle contractions, we first examined whether knocking down the proctolin receptor would alter the ability of larval to crawl. A light avoidance assay was employed to estimate maximal crawling speed of larvae in Canton S. larvae, muscle and nerve-selective ProcR knock-down lines, as well as the corresponding genetic controls (Figure 4.8A). At room temperature, Canton S. flies had an average velocity of 0.08mm/s, which was not significantly different from either of the knock-down lines (Elav-Gal4/UAS-RNAi ProcR or 24B-Gal4/UAS-RNAi ProcR; Two-way ANOVA with Tukey for pairwise comparisons, $P>0.05$). Given that larval crawling in *Drosophila* and neuromodulation by neuropeptides have both been demonstrated to be temperature-dependent, along with many other biological processes, we examined larval crawling velocity at 5 different temperatures, ranging from 16-36°C (Dunn and Mercier, 2005; Ohyama et al, 2013). In response to step-wise increments of 5°C, Canton S. larvae crawled at a significantly faster velocity with each temperature increase up to 36°C, at which larval velocity appeared to saturate (Two-way ANOVA, Tukey $P<0.05$). At 16°C, there were no significant differences between crawling velocities of the Canton S. and

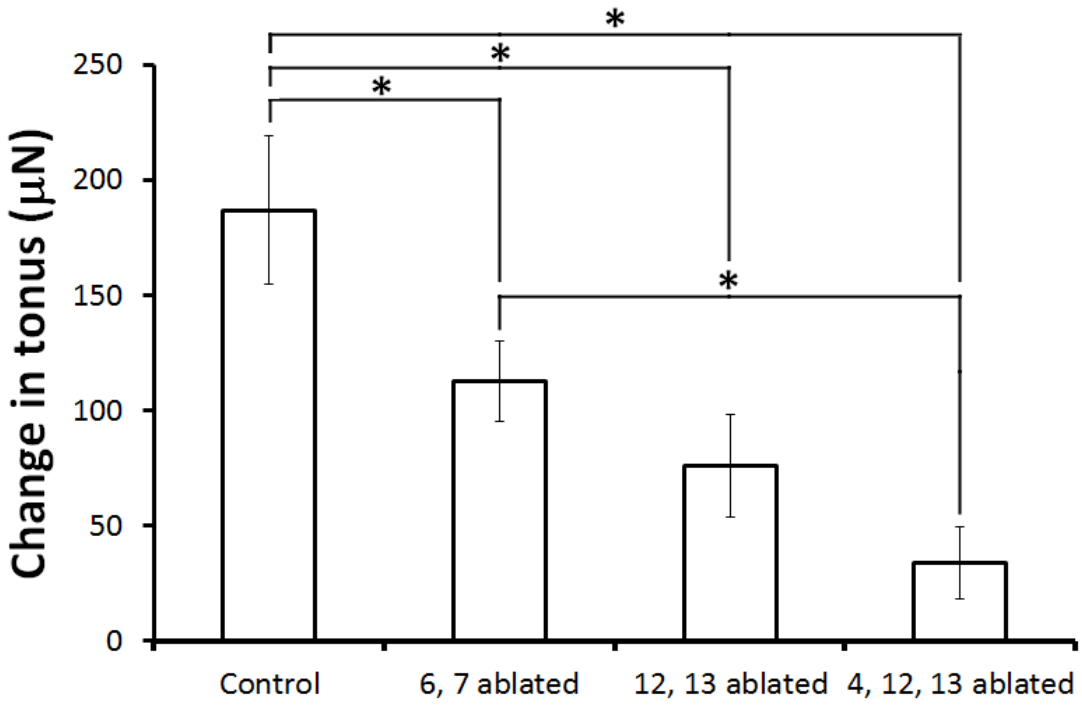


Figure 4.7: Proctolin induced sustained contractions are larger in cells 4, 12 and 13 than in cells 6 and 7. Average change in tonus induced by 10^{-6}M proctolin application compared across preparations with: no ablation (Control); with cells 6 and 7 ablated; 12 and 13 ablated; and cells 4, 12, and 13 ablated. Significances were observed between the controls and all other groups, and between those preparations with cells 6 and 7 ablated and those preparations with cells 4, 12, and 13 ablated (* $P < 0.05$). Error bars: SD, N=10-19.

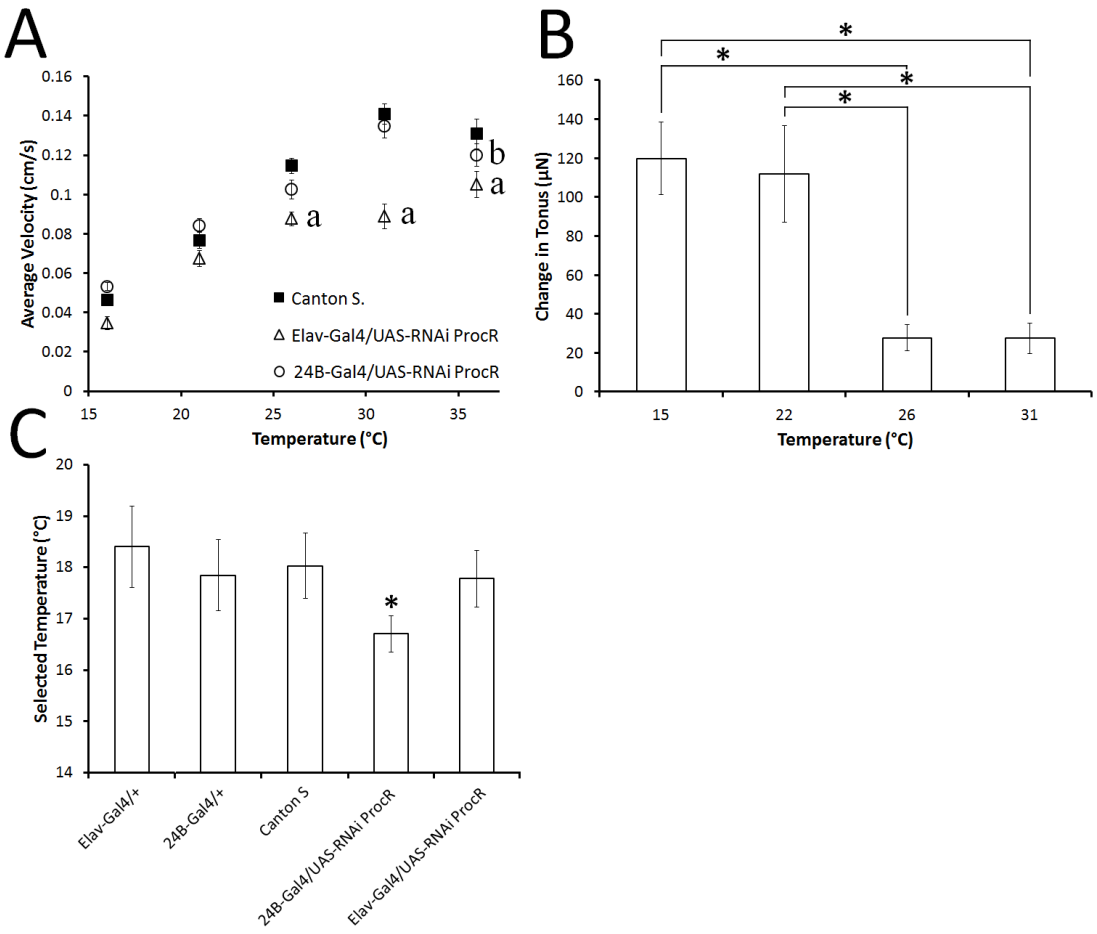


Figure 4.8: Reducing the expression of the proctolin receptor alters larval speed and thermal preference. **A**: Using the Gal4/UAS system to knock down the expression of the proctolin receptor in muscle (24B-Gal4/UAS-RNAi ProcR) and in nervous tissue (Elav-Gal4/UAS – RNAi ProcR), to determine if reducing proctolin receptor expression alters the velocity of larval crawling at different temperatures. b- indicates significant differences between control and muscle (24B) trials. Error bars: SEM, N=14-19. **B**: The ability of proctolin to increase changes in tonus is temperature-dependent. Increasing temperature decreases the force of sustained contractions elicited by 10^{-6}M proctolin application. Error bars: SD, N=10, * P<0.05. **C**: Using the Gal4/UAS system to knock-down proctolin receptor expression revealed that altering proctolin receptor expression in muscle tissue significantly alters the preferred temperature of larvae. Error bars: SD, N=18-20, *P<0.05.

proctolin receptor knock-down groups. Increasing temperature above 21°C increased crawling velocity in larvae with reduced proctolin receptor expression in nervous tissue and muscle tissue, but velocity was significantly lower ($P<0.05$) at 26-36°C in larvae with reduced expression in nervous tissue than in Canton S. larvae. At 36°C, larvae with reduced proctolin receptor expression in muscle tissue were also significantly slower compared to Canton S. controls ($P<0.05$). Muscle and nerve control lines (Elav-Gal4/+; 24B-Gal4/+) were also tested and none showed a significant difference in larval crawling velocity when compared to Canton S. larvae. Taken together, the results indicated that altering proctolin receptor expression affects the temperature-dependence of crawling velocity.

To further explore the temperature-dependence of proctolin's effects in third-instar larvae, we next examined proctolin-induced contractions at different temperatures.. Contractions induced by 10^{-6}M proctolin at 16°C and 21°C were not significantly different (Figure 4.8B: Kruskal-Wallis one way ANOVA, $H=23.674$, $P<0.001$, Tukey test for pairwise comparisons, $P>0.05$). Increasing the temperature to 31 or 36°C, however, significantly decreased proctolin-induced contractions to approximately 24% of the amplitude observed at 16 and 21°C ($P<0.05$). One possible confounding effect was the role of pH during these experiments. We monitored the saline pH during the experiment, and found a strongly negative correlation ($R^2=0.999$) with increasing temperature. At 16°C pH=7.30, at 36°C pH= 7.06, thus for every degree Celsius change a 0.012pH shift occurred.

We next examined whether altering proctolin receptor expression altered the thermal preference of third-instar larvae (Figure 4.8C). Canton S. flies had a preferred

temperature of 18.0 ± 0.6 °C, and the nerve-selective proctolin receptor knock-down line had a preferred temperature of 17.8 ± 0.5°C. The muscle-selective proctolin receptor knock-down line had a significantly decreased preferred temperature of 16.7 ± 0.4°C (One Way ANOVA, $F=3.529$, $P=0.037$, Tukey test for pairwise comparisons, $P<0.05$). No significant differences were observed between muscle and nerve genetic control lines ($P<0.05$).

Discussion:

To date, few studies have examined the physiological role of proctolin in *Drosophila* (Taylor et al., 2004). Here we characterize several roles for proctolin in body-wall muscles in third-instar *Drosophila* larvae. Proctolin induced sustained contractions in these muscles after removing the CNS and in the absence of stimulation to the remaining nerves. These contractions were dose-dependent, were reduced by knocking down expression of the *Drosophila* proctolin receptor in muscle tissue, and were larger in some muscle cells than others (i.e. larger in fibers 4, 12 and 13 than in 6 and 7) as indicated by fiber ablation experiments. Proctolin also increased the amplitude of nerve-evoked contractions in a dose-dependent manner, and the magnitude of this effect was also larger in some muscle cells than others (again, larger in fibers 4, 12 and 13 than in 6 and 7). The magnitude of proctolin's enhancement of nerve-evoked contractions also increased with increasing intra-burst stimulus frequency (from 2 to 40 Hz). Taken together, these observations indicate that proctolin acts directly on body-wall muscles to elicit slow, sustained contractions and to enhance nerve-evoked contractions, and proctolin appears to do so in a cell-selective manner.

Initial attempts at examining a behavioural role for proctolin revealed that altering expression of the proctolin receptor in the nervous system reduced larval crawling velocity, compared to control lines, at temperatures of 26°C or higher, and that altering proctolin receptor expression in muscle tissue only negatively influenced larval crawling velocity at 36°C. Knocking down proctolin receptor expression in muscle tissue also decreased the preferred temperature of larvae from 18.0°C to 16.7°C. Interestingly, increasing the temperature to 26°C and 31°C greatly diminished the ability of proctolin to

induce sustained contractures in body-wall muscle. These observations indicate that proctolin's modulatory effects on muscle contraction are temperature-dependent and suggest that proctolin may play a role in mediating behaviours associated with temperature preference, and especially in avoidance behaviours of elevated temperatures (McKemy, 2007).

Johnson et al. (2003) identified and characterized the *Drosophila* proctolin receptor by expressing genes for "orphaned" *Drosophila* GPCRs in HEK cells and examining responses to numerous modulators. Only one receptor (the one studied here) was highly selective for proctolin compared to 14 other modulators investigated. The only other agent reported to activate this receptor was substance P, which was effective at a concentration that was 10,000 times greater than that of proctolin. Substance P has previously been identified in the *Drosophila* CNS, and in cell lines derived from the *Drosophila* CNS, but we were unable to identify a receptor for substance P within the *Drosophila* genomic database (Nassel et al., 1990; Ui-Tei et al., 1995). Our results demonstrate that proctolin-induced contractions were significantly reduced by knock-down of this receptor in *Drosophila* muscle tissue and, thus, help to confirm its identity as a bona fide *Drosophila* proctolin receptor. The inability of receptor knock-down in neurons to reduce proctolin-induced contractions confirms that this effect of proctolin is mediated postsynaptically and not presynaptically.

The proctolin-induced contractions of *Drosophila* body-wall muscles required extracellular calcium and were blocked by 10^{-6}M nifedipine, which has been shown by others to block L-type calcium channels in these muscle fibers (Gielow et al., 1995). Observations similar to ours have been reported for cockroach hyperneural muscle

(Wegener and Nassel, 2000), where the actions of proctolin requires calcium entry through L-type channels in the plasma membrane to trigger release of calcium from intracellular stores. A similar dependence on extracellular calcium has been reported for proctolin-induced contractions in numerous arthropod muscles, including locust oviduct muscles (Lange et al, 1987; Wilcox and Lange, 1995), lobster opener muscle (Schwarz et al, 1980), barnacle muscle (Nwoga and Bittar, 1985) and *Lucilia* body wall muscle (Irving and Miller, 1980), and proctolin is reported to increase calcium channel activity in the plasma membrane of crayfish abdominal flexor muscles (Bishop et al., 1991). The prolonged time course of proctolin-induced contractures and the requirement of a GPCR imply the involvement of second messengers. Proctolin has been shown by others to activate adenylate cyclase (Hiripi et al., 1979; Wright et al., 1986), and both cyclic adenosine monophosphate (cAMP) and inositol tris-phosphate (IP_3) have been implicated as mediators of proctolin-induced contractions (Erxleben et al., 1995; Lange et al., 1989). The intracellular signalling pathways underlying effects of proctolin in *Drosophila* remain to be elucidated.

Although proctolin increased the amplitude of nerve-evoked contractions, it had no significant effect on EJP amplitude at concentrations of $1x10^{-10}$ to $1x10^{-8}$M in any of the five *Drosophila* muscle fibers examined. This suggests that proctolin increases force generation by acting down-stream of postsynaptic depolarization. Similar effects of proctolin have been reported in crayfish abdominal flexor muscles (Bishop et al., 1987), locust oviduct muscles (Lange and Orchard, 1984; Orchard and Lange, 1986) and crab ventilatory muscles (Mercier and Wilkens, 1985). Direct evidence that proctolin enhances excitation-contration coupling has been reported for isolated muscle fibers of the isopod

crustacean, *Idotea baltica*, where proctolin decreases membrane conductance and potassium channel activity and, in some fibers, increases excitability (Erxleben et al., 1995). In other arthropod muscles, however, proctolin does increase EJP amplitude and appears to do presynaptically (Beilin and Pasztor, 1989; Belanger and Orchard, 1993; Jorge-Rivera, 1998; Rathmayer et al., 2002).

Proctolin increased the amplitude of nerve-evoked contractions in *Drosophila* larvae to a greater degree when the frequency and number of impulses delivered to the motor axons was increased. Other reports also indicate that modulation of evoked contractions is sensitive to changes in nerve impulse pattern. Serotonin and dopamine increased nerve-evoked contractions in crab gastric mill muscle (gm4) more substantially at 20-40 Hz than at lower frequencies (with a constant burst duration of 0.5 s; Jorge-Rivera et al., 1998). In the same muscle, however, proctolin and the crustacean peptide TNRNFLRFamide increased nerve-evoked contractions less substantially at 30-40 Hz than at lower impulse frequencies (Jorge-Rivera et al., 1998). In *Drosophila* larval body wall muscles, octopamine increased nerve-evoked contractions more effectively at 5-20 Hz (with 1 s burst duration) than at higher intra-burst frequencies (30-40 Hz; Ormerod et al., 2013). Thus, different modulatory substances can be either more effective or less effective at increasing nerve-evoked contractions when the frequency and number of stimuli increase. The mechanisms underlying such frequency-dependent effects of aminergic and peptidergic modulators are not known.

One possible rationale for bath application of proctolin being more effective at higher frequencies in our experiments is that these stimuli may release proctolin and other peptides that are present as co-transmitters in synaptic terminals of the

motorneurons. Belanger and Orchard (1993) showed that proctolin was released from motor neurons innervating locust ovipostior opener muscles by 30Hz stimulation but not by10 Hz stimulation. In *Drosphila* larvae high frequency stimulation of motor axons supplying the body wall muscles increases both the mobilization of dense core vesicles in the synaptic terminals and the release of neuropeptides (Shakiryanova et al., 2005). Thus, increased release of proctolin or other co-transmitters at the higher stimulus frequencies in our experiments might contribute to the increased amplitude of nerve-evoked contractions. We were unable to address this question directly because the amount of proctolin released from *Drosophila* larvae is below the detection level of current analytical methods. Using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry, we found that extracts from ten larvae contained a peak at 649 Daltons that was just above detection level and was identical in mass to a proctolin standard (data not shown). Dircksen et al. (2011) also identified a proctolin-specific peak at 649 using MS in another arthropod, *Daphnia pulex*. Since electrical stimulation typically releases 6-8% of endogenous stores or proctolin in locust muscle (Belanger and Orchard, 1993; Lange, 2002), other approaches are needed to assess release of proctolin and other co-transmitters in *Drosophila*.

Our data show that increasing the frequency and number of stimuli applied to the motor axons decrease the threshold concentration and EC_{50} for proctolin to increase nerve-evoked contractions. To our knowledge, this is the first report of any modulatory substance having such an effect. This finding suggests that caution is needed in interpreting threshold and EC_{50} values for modulation of nerve-evoked processes, and this might be particularly important for modulatory substances that are released as co-

transmitters. Increasing the frequency and number of nerve impulses increases synaptic release of peptide co-transmitters, including proctolin (Belanger and Orchard, 1993; Shakiryanova et al., 2005). We propose that such an effect may effectively lower the concentration needed for bath application of the same substance to elicit a noticeable effect on the postsynaptic cell. We tested this hypothesis by comparing the effectiveness of proctolin to increase nerve-evoked contractions between muscle cells that receive proctolin as a co-transmitter with muscle cells that do not. We reasoned that bath applying a sub-maximal concentration of proctolin (that would not "overpower" the effects of co-transmission) would increase nerve-evoked contractions to a greater degree in muscle cells innervated by motor neurons that contain proctolin (muscles 4, 12 and 13) than in muscle cells that do not (muscles 6 and 7; Anderson et al., 1988). Ablating muscle cells 4, 12 and 13 reduced the effect of proctolin to a greater extent than did ablating cells 6 and 7, supporting the hypothesis that co-transmission reduces threshold and EC_{50} concentations. Since the effectiveness of proctolin was assessed on all muscle cells that remained after ablation and were quantified as a percentage of maximal contraction induced by tetanic stimulation of those fibers, the differences cannot be attributed to differences in muscle fiber size. Instead, our data indicate differential modulation of muscle fibers, with proctolin increasing nerve-evoked contractions to a greater degree in cells 4, 12 and 13 than in cells 6 and 7.

There are alternative explanations for the reduction in threshold and EC_{50} that occurred in response to increasing the number and frequency of nerve impulses. Such changes in the stimulus regimen could increase the release of co-transmitters other than proctolin, and these may alter intracellular signaling pathways and "prime" the

postsynaptic cell to increase its responsiveness to proctolin (Meyrand and Marder, 1991). On the other hand, proctolin's abilitity to increase evoked contractions depends on postsynaptic depolarization and concommitant opening of calcium channels in the plasma membrane (Bishop et al., 1987; 1991), both of which should increase with increases in the frequency and number of impulses. Thus, the differential modulation of nerve-evoked contraction could be explained if changes in the stimulus regimen generated larger depolarization in muscle cells 4, 12 and 13 than in 6 and 7, or if the proctolin receptor is more highly expressed in cells 4, 12 and 13. Since proctolin also elicited larger contractions in cells 4, 12 and 13, the latter explanation seems plausible.

To at least some extent, the cell-selective modulation of muscle contraction reported here mirrors the effects of the *Drosophila* peptide DPKQDFMRFamide, which increases nerve-evoked contractions to a greater degree in muscle cells 6 and 7 than in 12 and 13 (Ormerod et al., 2015). The cell-selective modulation by the latter peptide appears to result from differences in expression level of the FMRFamide receptor between these muscle fibers (Ormerod et al., 2015), but it remains to be determined whether differences in expression of the proctolin receptor also occur. The apparent complementarity of modulation of these muscle fibers by proctolin and DPKQDFMRFamide suggests that these two peptides may play some complementary role in either recruiting or maximizing the contraction of specific subsets of muscle cells during specific behaviours. Based on these findings and what has been observed in the CNS, we speculate that different peptides may elicit similar effects at the cellular level but serve to recruit specific groups of neurons and muscle cells to generate specific behaviours (Harris-Warrick and Kravitz,

1984; Marder and Calabrese, 1996; Selverston, 2010; Certel, 2010; Alekseyenko et al., 2014).

The behavioural experiments reported here indicate that a pathway involving the proctolin receptor may mediate the ability of larvae to detect and respond to an altered thermal environment. Larvae with reduced proctolin receptor expression in muscle tissue exhibited a preference for lower temperatures and a reduced crawling velocity at 36°C. Although reducing proctolin receptor expression in the nervous system had no effect on temperature preference, it reduced larval crawling velocity at 25-36°C and was more effective in altering crawling velocity than was altering receptor expression in muscle. Over-expression of the *Proct* gene in *Drosophila* leads to an increase in heart rate (Taylor et al, 2004), but to our knowledge no other physiological or behavioural effects of altering expression of proctolin or its receptor have been reported. Proctolin is known to play important roles in insects by regulating contractions of leg muscles (Adams and O'Shea, 1983) and of muscles associated with digestion and reproduction (Belanger and Orchard, 1993; Brown and Starratt, 1975; Lange, 2002; Orchard et al., 2011), but possible links between proctolin signaling and temperature-dependent behaviours have not been suggested previously. Our physiological contraction data indicated that proctolin is less effective at inducing contractions at higher temperatures. This observation could suggest that proctolin is less effective at inducing muscle contractions at higher temperatures and therefore might lend support to the lower contraction velocity results we obtained with the ProcR transgenic lines. However, we also noticed that pH decreased (pH=7.06) when temperature was increased to 36°C. While seemingly minor, a 0.1-0.2 shift in pH is physiologically relevant and could affect both pre- and post-synaptic

cellular function (Sinning and Huber, 2013). It is unlikely that such a shift in pH occurs within the animal during fluctuations in ambient temperature, so these results may not reflect conditions existing within the animal.

The present results suggest that proctolin receptors in muscle play a role in temperature preference, and that proctolin receptors in neurons play an important role in increasing crawling speed at high ambient temperatures. Since bath application of proctolin did not significantly alter EJP amplitude, it seems unlikely that synaptic terminals of motor neurons are targets of proctolin's action. Thus, the temperature-dependent increase in crawling speed is probably mediated by effects either within the central nervous system or on sensory afferent inputs. In *Drosophila* larvae, proctolin immunoreactivity occurs in about 50 neuronal cell bodies distributed across the brain, subesophageal, thoracic and abdominal regions of the central nervous system (Anderson et al., 1988), but distribution of proctolin receptors is not known. The chordotonal organs and peripheral mechanoreceptors, play a key role in temperature synchronization of behavioural activity in *Drosophila* (Sehadova et al., 2009), and mutating the Pyrexia transient receptor potential channel, expressed in the chordotonal organs, alters temperature entrainment of the circadian clock (Wolfgang et al, 2013). Proctolin alters the sensitivity of sensory neurons associated with several arthropod mechanoreceptors (Pasztor and Bush, 1989; Pasztor and Golas, 1993; Pasztor et al., 1988; Pasztor and MacMillan, 1990). Thus, it is possible that proctolin might act on sensory neurons to help mediate temperature detection or to modulate neural circuits that mediate temperature-dependent changes in larval locomotion and temperature selection.

Chapter 5:

Conclusions and perspectives

General Discussion

This thesis has focused on the ability of modulatory substances in the model organism, *Drosophila melanogaster*, to work in a cell-selective manner. Vertebrate and invertebrate genomes encode hundreds-to-thousands of neuropeptides and their receptors, yet the reason why there are so many is not clear. Published literature contains examples of various modulators that have the capacity to affect some cells or cell-types, while evoking no response in others. There are also numerous reports that various modulators can act on subsets of neurons and their effector cells in order to activate specific neural circuitry to ultimately produce a specific behavioural outcome. This capacity of modulators to work in a selective manner on individual cells or small neural circuits may be a reason why so many peptides are encoded within genomes.

The major findings in this thesis are summarized in Table 5.1 and Figure 5.1. Briefly, I observed cell-selective effects for the three different modulators based on their ability to alter membrane properties, to enhance EJP amplitude, to alter nerve-evoked contractions and to induce sustained contractions independently of nerve stimulation. Octopamine and proctolin affected muscle fibers 12 and 13 to a greater extent than fibers 6 and 7. Octopamine was found to enhance the amplitude of EJPs to a greater extent in fibers 12 and 13, and a similar cell-selectivity was observed for the ability of octopamine to enhance nerve-evoked contractions. Proctolin did not alter the amplitude of EJPs; however, it did increase the amplitude of nerve-evoked contractions, and did so more strongly in muscle fibers 4, 12 and 13 than in fibers 6 and 7. This cell-selective effect was also observed when looking at proctolin's ability to induce sustained contractions in the

Table 5.1: Summary figure of effects of the primarily modulatory substances investigated through this thesis.

	Fiber selectivity	EJPs	Nerve-Evoked Contractions	Direct Contractions
Octopamine	12, 13 > 6, 7	↑	↑	↑↑
DPKQDFMRFa	12, 13 < 6, 7	↑	↑↑	↑
Proctolin	12, 13 > 6, 7	-	↑↑	↑

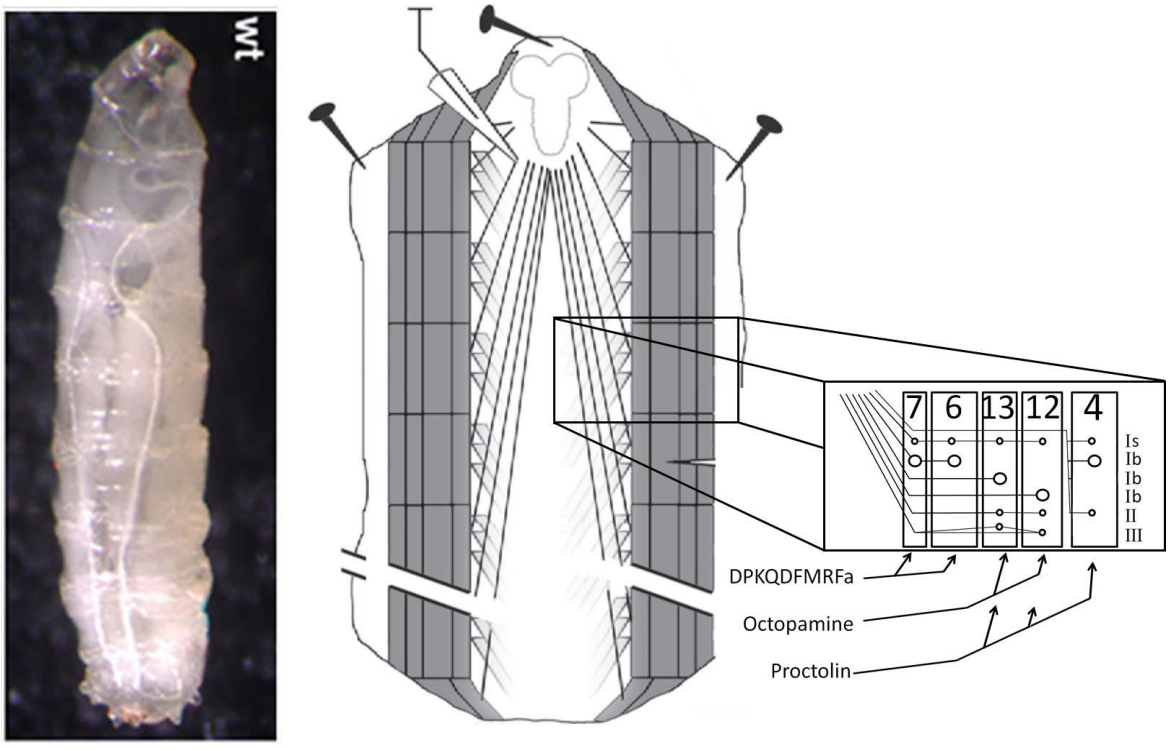


Figure 1: Summary of cell-selective effects of the modulatory substances studied in this thesis. Left: bright-field image of third instar larvae. Middle: cartoon depiction of dissected third-instar larval preparation with an emphasis on the abdominal segments and innervation patterns for fibers 6, 7, 12 and 13. Right: Cartoon depiction summarizing the three neuromodulatory substances used in this thesis, and the muscle fibers they are selective for.

body-wall muscle of these larvae. The neuropeptide DPKQDFMRFamide increased the amplitude of EJPs to a greater extent in cells 6 and 7 than fibers 12 and 13, and it also increased the amplitude of nerve-evoked contractions to a greater extent in fibers 6 and 7 than fibers 12 and 13. DPKQDFMRFamide exhibited the same cell-selectivity for its ability to induce sustained contractions. I demonstrated that the ability of DPKQDFMRFamide to enhance EJPs is mainly derived from a presynaptic mechanism, but the ability to enhance nerve-evoked contractions required the presence of the FMRFamide receptor in pre- and postsynaptic cells. Lastly, I provided evidence that differences in the effectiveness of DPKQDFMRFamide on cells 6, 7, 12 and 13 correspond with differences in FMRFamide receptor expression.

In addition to demonstrating that three different modulatory substances found in *Drosophila* have the capacity to elicit cell-selective modulation at neuromuscular junctions and muscle fibers, the present results suggest that these peripheral sites play critical roles in behaviour by integrating hormonal signals with the motor output patterns that are generated in the CNS. It is known that the motor output selectively activates particular muscles by recruiting specific motor axons, and the contractions of these muscles are determined by the structure of the motor pattern (i.e. number of impulses per burst, intraburst impulse frequencies and number of bursts per minute). Since some muscle fibres are innervated by synaptic terminals that contain co-transmitters (e.g. octopamine and proctolin), co-transmitter release (which is presumably sensitive to the impulse frequency) increases contractions in these specific cells. The present findings suggest that hormones can also enhance contraction in a cell-selective manner, partly by potentiating transmitter release from specific synaptic terminals, and partly via direct

effects on muscle cells, which are greater in some fibers than in others. Perhaps the most surprising finding is that the threshold for proctolin's ability to increase nerve-evoked contractions is reduced by about two orders of magnitude when the motor neurons are stimulated at higher impulse frequencies (Chapter 4). This result demonstrates an interaction between hormonal effects and the motor output pattern. The ability of octopamine to increase EJP amplitude has shown previously to depend on motor neuron impulse frequencies in crayfish opener muscle (Breen and Atwood, 1983), but no studies, to my knowledge, have shown that threshold and EC_{50} values for modulation of chemical synapses or of muscle contraction depend on impulse frequency. This new finding suggests that low hormonal concentrations can act preferentially to enhance synaptic transmission and contraction in muscle cells that are activated at high impulse frequencies, and that higher hormonal concentrations might enhance contractions on a greater number of muscle fibers.

The findings in this thesis demonstrate how complex information processing in arthropod muscle cells can be, and they reaffirm the notion that effector cells need to be considered as a key regulatory site in the generation of behaviour and not simply as output cells. Cell selective modulation of neurons and muscle cells suggests that although neuropeptides can elicit identical physiological effects at the cellular level, different peptides may be needed to recruit specific neural circuits, potentiate specific neuromuscular synapses and enhance contractions preferentially in specific muscle cells. This may help to explain why there are so many neuropeptides and why they have been thought to be functionally redundant.

Investigations of the mechanisms through which neuropeptides modulate and regulate behaviour often focus on neural circuitry and overlook effects on muscle cells (Hooper et al., 2007; Morris and Hooper, 2001). Although there is a growing body of evidence to indicate that peptides and other modulators can act in a cell-specific manner on neurons, few studies have examined the possibility that peptidergic or aminergic modulators may also work in a cell-specific or tissue-specific manner on effector cells. The work conducted in this thesis, demonstrate that cells outside the CNS have the capacity to respond to and integrate information from chemical signals. Thus, we are continuing to gain traction for the idea that central and peripheral modulatory effects appear to be coordinated to produce physiologically appropriate changes in muscle performance.

I will next speculate upon a few putative mechanisms of action, with a focus on how these modulatory substances might elicit their cellular effects, and how these relate to the ability of the substances to work in a cell-selective manner.

Involvement of GPCRs

The three modulatory substances investigated in this dissertation appear to elicit their cellular effects via distinct, bona fide GPCRs. I have not yet ruled out the possibility that DPKQDFMRa may elicit its effects via a ligand coupled ion channel, as FMRFamide does in molluscs (Cottrell, 1997). However, DPKQDFMRFa-induced contractions are reduced dramatically by knocking down the FR gene, which encodes a GPCR, and by treatment with pertussis toxin, which suggests the involvement of a G_0-GPCR (Milakovic et al, 2014). I also provide evidence that this peptide elicits cell-selective effects on

muscle fibers that correspond to differences in expression of the peptide receptor. The mechanisms underlying modulation by OA are more difficult to define, given the number of unique OA/TA receptors. Using a genetic approach to identify the receptor and/or intracellular pathway(s) underlying the effects of OA reported in this thesis would be more challenging given the possible number and iterations of receptors. Nonetheless, there is evidence to suggest that OA exerts some of its effects by altering muscle-specific proteins encoded within the *D. melanogaster sls* (sallimus) gene (discussed in chapter 2). It is not beyond reason to consider that these proteins may also be expressed in a cell-selective manner, enabling a subset of muscle cells to be activated by the second messenger system (e.g. a phosphorylation-based system) initiated by this modulator. I demonstrated that the physiological effects of proctolin appear to be mediated, at least partially, through the proctolin receptor. While I did not perform experiments to show that the proctolin receptor is differentially localized or expressed in some cells, I have shown this to be the case for another peptide receptor (Ormerod et al, 2015).

Overall, I have shown that each of the modulators investigated in this dissertation is able to work in a cell-selective manner and appears to do so by activating a distinct GPCR. Consequently, selective release of these modulators would trigger the selective activation or enhancement of a subset of muscle fibers in the larval bodywall. These findings, together with the fact that other labs have demonstrated that modulators also elicit cell-selective effects on neurons within the CNS, further support the idea that modulators can act on subsets of neurons and on subsets of their effector cells in order to generate a specific behavioural outcome.

Location and release mechanisms are important to modulator functionality

DPKQDFMRFa and OA elicit effects at similar concentrations, typically showing thresholds at or above 10^{-9}M. They both alter resting membrane properties, EJPs, and nerve-evoked contractions, and they both induced sustained muscle contractions, thereby increasing muscle tonus. Thus, at the cellular level, the physiological effects of these two modulators are similar in many respects. The primary differences between these two modulatory substances are: i) OA preferentially affects muscle fibers 12 and 13, but DPKQDFMRFa preferentially affects fibers 6 and 7, and ii) OA is a much more effective at increasing muscle tonus than is DPKQDFMRFa (20mN vs 0.08mN, indicating a 250x greater effect for OA). However, the concentration of OA used to investigate effects on sustained contractions (Figure 2.6, Chapter 2) is very high, and such a high concentration might only occur during a unique physiological stimulation circumstance (discussed below). Perhaps it is not where the substance is stored, but how that substance is released that is critical to its function.

Release of cotransmitters from dense core vesicles at neuromuscular junctions

Generally speaking, classical transmitters are contained in small synaptic vesicles (SSVs), whereas neuropeptides and monoamines are stored in large granular dense core vesicles (DCVs), but occasionally, transmitters are sometimes stored together with peptides and monoamines in DCVs (Kuffler et al, 1987; Friend, 1976). There is now a large body of literature which demonstrates the colocalization of multiple NTs and modulators at synapses in both vertebrates and invertebrates (Burnstock, 2003; Burnstock 2009). In *Drosophila* third instar-larvae, a number of neuromodulatory substances have been shown to colocalize with glutamate at neuromuscular junctions, including OA and

proctolin which are both contained within DCVs (Anderson et al, 1988; Monastirioti et al, 1995; Peron et al, 2009; Taylor et al, 2004). There is substantial imaging-based evidence to demonstrate where various neuromodulatory substances are contained at synapses; however, little functional evidence exists to demonstrate how they are released, and even less evidence exists to demonstrate the physiological circumstances underlying their release.

Chapter 2 (Ormerod et al, 2013) describes how altering stimulation rate affects the ability of OA to enhance nerve evoked contractions. I found that OA was most effective at enhancing the amplitude of contractions evoked by stimulus frequencies between 5 and 20Hz. High (20+ Hz) motorneuron stimulation frequencies liberate greater amplitude contractions and consequently the force generated is closer to saturation, and therefore there is less capacity for potentiation by modulators. OA was found to profoundly affect membrane contractility (250x greater than DPKQDFMRFa), and thus these effects might reflect hyper-responsiveness of the muscle to the release of modulators. This idea of modulators making muscle more responsive to stimulation was previously demonstrated by Meyrand and Marder, (1991), who showed that preconditioning muscle fibers from shrimp with YGGFMRFa 'primed' the muscle to be much more responsive to communication from the motorneuron. Their findings indicated that neuropeptides alter the physiological state of muscles, making them contract more strongly when the motor neurons are activated at the same impulse frequency. The present findings, however, show that responsiveness to modulation depends on motor neuron impulse frequency, and the effects of OA suggest that there is an upper limit to the enhancement of muscle contraction.

Chapter 4 describes similar experiments with proctolin and reports that the peptide's ability to enhance nerve-evoked contractions increased with increasing the stimulation rate until maximal contraction amplitude was observed, indicating a "saturation point" for the modulatory effect. Shakiryanova et al. (2005) demonstrated that mobilization and exocytosis of DCVs increases with stimulus frequency, the interaction between stimulus frequency and proctolin's ability to increase nerve-evoked contractions may involve the release of co-transmitters from the synaptic terminals of the motor neurons at high frequencies. Since proctolin can be released both as a hormone and as a cotransmitter, I predicted that applying proctolin in the bathing solution at a modest concentration ($1x10^{-8}$ M), and stimulating at 32Hz would selectively enhance contractions in muscle cells that receive proctolinergic innervation, specifically muscle fibers 4, 12 and 13. The results supported my prediction. This result has implications for hormonal effects of proctolin. If proctolin is released at low concentrations (e.g. $1x10^{-8}$ M or lower; see Table 4.2), high frequency activity in the motor neurons that contain proctolin would trigger exocytosis of DCVs and increase the local concentration of proctolin in the synaptic clefts for a subset of muscle cells (4, 12 and 13). This would enhance nerve-evoked contractions in this "muscle group" preferentially over other fibers. During other circumstances, however, secretion of higher concentrations of proctolin into the circulation might increase the contraction of more muscle cells. It is not clear what physiological conditions or behaviours might require cell-selective differences in muscle contraction. The lateral placement of cells 4, 12 and 13 compared to cells 6 and 7, however, suggests that turning might involve greater contractions in the former fibers

(see below), whereas movement on a straight path, particularly at high speed, might require enhancement of contractions in all 5 muscle cells studied in this thesis.

Taken together, the results presented here indicate that not only do these modulatory substances have the capacity to work in a cell-selective manner, but two of the modulatory substances investigated also appear to work in a frequency-dependent manner. Frequency-dependent modulatory effects were also reported by Jorge-Rivera et al. (1998) for NMJs in crab stomach muscles, where modulation varied with the rate of presynaptic stimulation and was different for several aminergic or peptidergic modulators. Several other neuromodulators appear to be more effective at different stimulus frequencies (Brezina et al, 1996; Evans, 1984; Evans and Siegler, 1982; Weiss et al, 1993).

Physiological and behavioural implications of cell-selective modulation.

The overarching theme of this dissertation is to examine the capacity of modulatory substances to work in a cell-selective manner in order to help to explain why so many neuropeptides and their receptors are encoded in the genomes and transcriptomes of vertebrates and invertebrates. The concept that modulators can act on subsets of neurons and their effector cells in order to activate specific neural circuitry to ultimately produce a specific behavioural outcome was postulated decades ago and continues to be supported by leaders in the fields of modulators and neural circuitry (Harris-Warrick, 2011; Harris-Warrick and Kravitz, 1984; Marder and Calabrese, 1996; Selverston 2010).

In order for neuromodulatory substances to activate a specific subset of neural circuitry and effector cells, each modulator would need to affect each of the major

components that comprise a 'biological circuit'. Behaviour is generated by the coordinated activities of sensory neurons, interneurons, motor neurons and muscle cells, and modulators have been shown to act on each of these cell types. Unfortunately, very few studies have examined the ability of modulators to coordinate activity across these different major components. Here I investigate the ability of several modulators to affect communication and coordinate actions at neuromuscular synapses and on muscle fibers. To show that these areas are vital for regulating circuitry underlying behaviour, deficits in stereotyped behaviours would need to be observed. I performed a series of behavioural experiments on both the larval and adult forms of *Drosophila* with altered FR and ProcR receptor expression. (I omitted OA due to the large number of receptor isoforms.) Reducing FR expression in neurons has been previously shown to negatively affect larval locomotion (Klose et al, 2010). I also generated evidence to show locomotory defects in adults as reducing FR expression negatively affected adult geotaxis. Appendixes 1-4 demonstrate the effects of 4 behavioural assays I conducted on the larval and adult forms of Drosophila with reduced FMRFa receptor expression in muscle and nervous tissue. I observed a significant reduction in the acute geotactic response following knocking-down FMRFa receptor expression separately in muscle and nervous tissue. However, altering FMRFa receptor expression had no significant effects on larval or adult thermal preference (Appendixes 2-3) or on their capacity for learning in memory when subjected to a learning and memory assay (Appendix 4). Klose et al, (2010) demonstrated that the escape responses of larvae to bright light are impaired in mutants of both receptors (FMRFa receptor and DmsR-2) that respond to DPKQDFMRFamide. Knock-down of both receptors in neurons, but not in muscle, also impaired larval escape responses. This

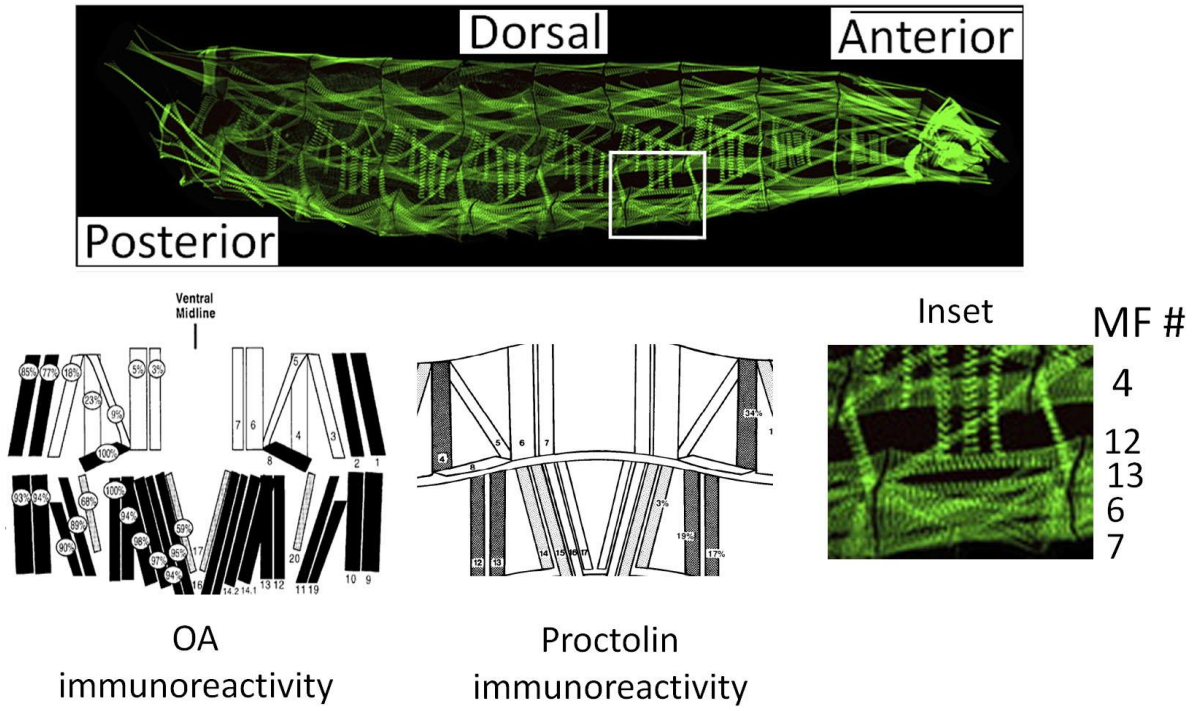


Figure 5.2: Top: scratch-GFP transgenic *Drosophila* line which drives the expression of GFP in muscle fibers in third-instar larvae (Hughes and Thomas, 2004). Bottom: percentage of OA and proctolin immunoreactivity in the motorneuron innervating the bodywall muscles of third-instar larvae (from Monastrioti et al, 1995 and Anderson et al, 1988). Bottom right: inset from top image to magnify muscle fibers 4, 6, 7, 12, and 13 and their anatomical location in the larvae. Fibers 6 and 7 are located directly next to the ventral midline, fibers 12 and 13 are lateral to fibers 6 and 7, and muscle fiber 4 is the lateral most fiber.

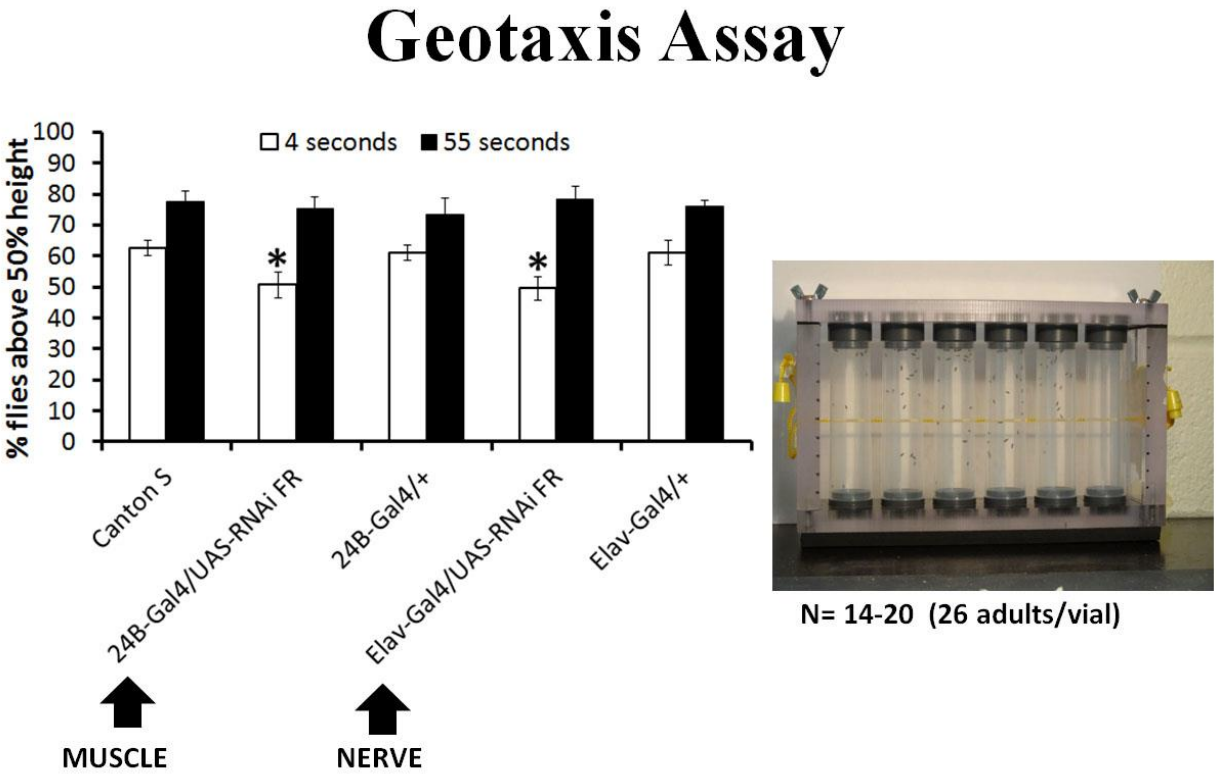
suggests that DPKQDFMRFamide acts mainly on neurons, either in the CNS or at motor nerve terminals, to help mediate escape behaviour, but on both neurons on muscles to mediate their geotactic response. In Chapter 4 I demonstrated that altering ProcR expression appears to alter the thermal preference and the velocity of larval crawling. Saraswati et al (2003), genetically reduced OA expression in larvae and observed gross locomotory deficits. Thus, altering expression of the receptors for any of the modulators explored in this thesis appears to negatively affect larval crawling.

These deficits in larval crawling observed for all three modulators are not surprising given the fact that all three have been shown to affect force generation in larval bodywall muscle. However, we have yet to examine any cell-selective ramifications of altered expression. With respect to OA, Saraswati et al. (2003) demonstrated reduced locomotion and an increased propensity to turn when OA concentrations were reduced. Monastirioti et al (1995) showed greater OA immunoreactivity in bodywall muscle fibers located laterally, which suggests that modulation of lateral fibers by OA may help to mediate turning behaviour (Figure 5.2). Similar expression patterns have been shown for proctolin, with the highest expression found in fibers located laterally (Figure 5.2). This may seem functionally redundant at first; however, I demonstrated that these two substances appear to operate optimally at different motorneuron frequencies, which would suggest that proctolin and OA might exert their respective modulatory effects under different behavioural or physiological circumstances. DPKQDFMRFa enhanced contractions most strongly in muscle fibers anatomically located closest to the ventral midline (fibers 6 and 7), previously shown to generate the most force during crawling (Paterson et al, 2010). Klose et al, (2010), also demonstrated that bath application of

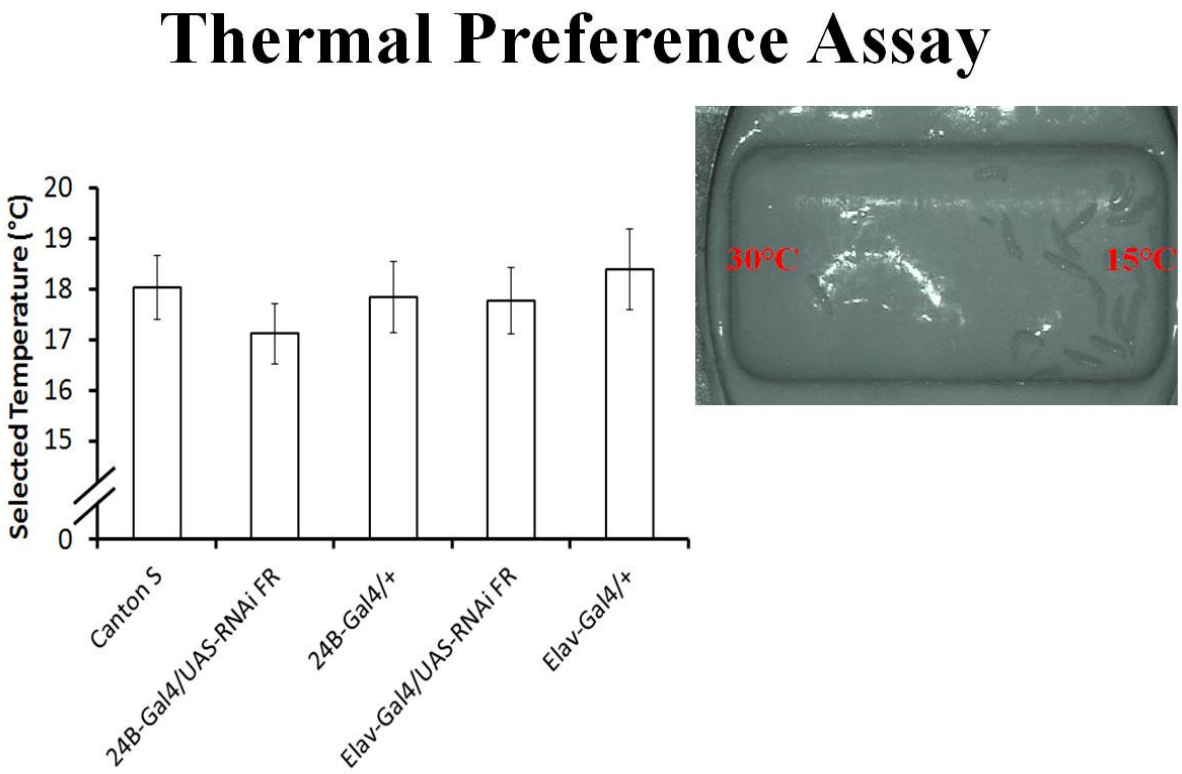
DPKQDFMRFamide decreased ‘run-down’ of the centrally generated locomotory rhythm. A justification is also provided in Chapter 3 for the idea that this peptide might be constantly expressed to prevent/reduce muscle run-down. Thus, each of these modulators could serve a unique function in regulating behaviour.

This thesis has focused on the ability of modulatory substances in the model organism, *Drosophila melanogaster*, to work in a cell-selective manner. I characterized the physiological effects of OA, DPKQDFMRFa, and proctolin at larval NMJs and their direct effects on muscle fibers. In doing so I provided evidence to demonstrate that each modulator can operate in a cell-selective manner, and that neuromuscular synapses and muscle fibers can serve as key targets for cell-selective modulation. In the context of work published elsewhere, the present findings suggest a critical role for cell-selective modulation in the CNS and at the periphery in behaviour. Each modulator could regulate or activate a specific set of neurons and muscle cells in order to generate a specific behaviour, such as larval turning or forward peristalsis. These studies demonstrate that cells outside the CNS have the capacity to respond to and integrate information from chemical signals, and may do so in a cell-selective manner. This capacity of modulators to work in a cell-selective manner on individual cells or small neural circuits may be a reason why so many peptides are encoded within genomes.

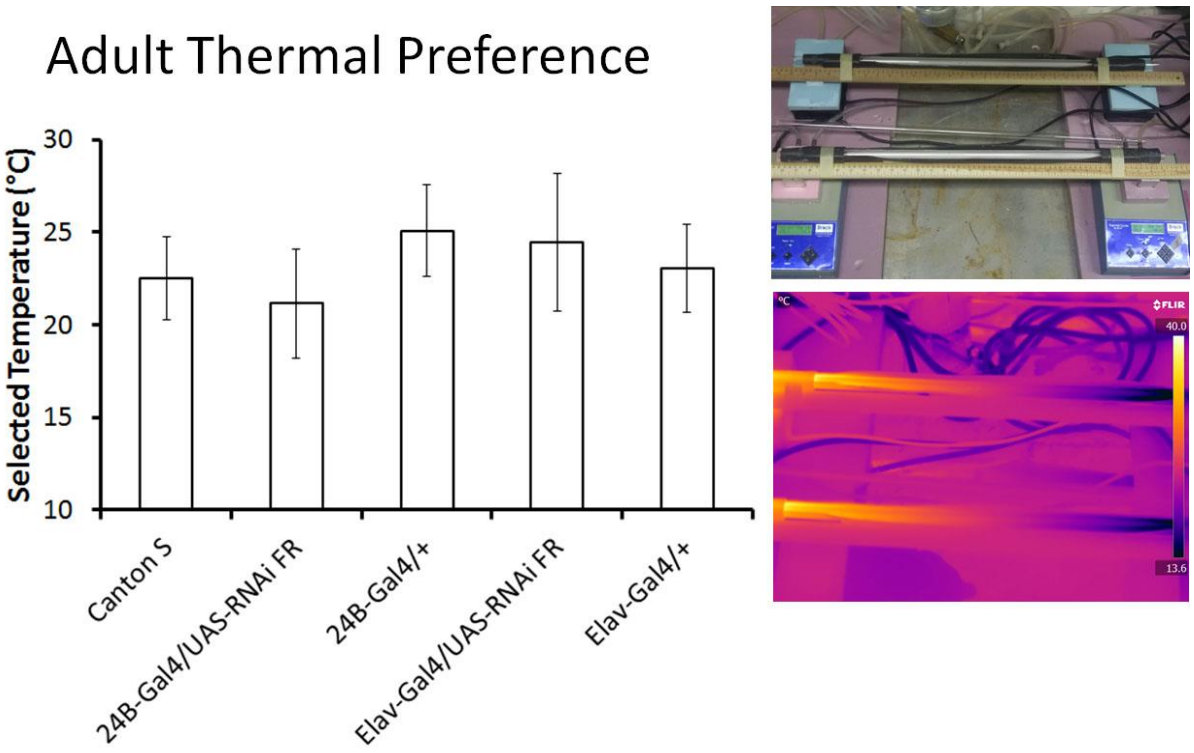
Appendix



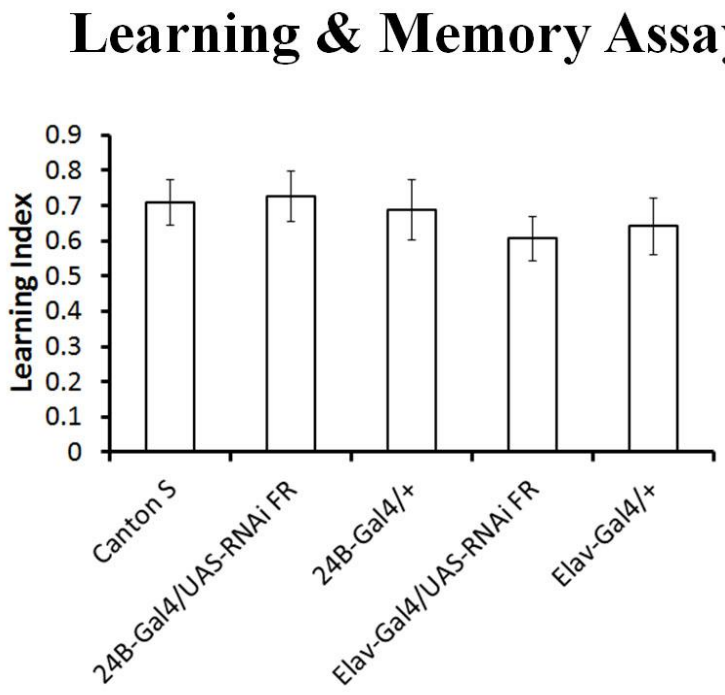
App. 1: Reducing expression of FMRFamide receptor in nervous or muscle tissue significantly reduces the acute (4s) geotactic response in adult flies. Left: Number of flies which climbed above the 50% height of the vial (see right for image) in control, transgenic and driver control lines after 4 and 55 seconds of an abrupt drop (see Skandalis et al, 2010 for methods). One-way ANOVA, * $P<0.05$.



App. 2: Reducing expression of FMRFamide receptor in nervous or muscle tissue does not alter third-instar larval preference.



App. 3: Reducing expression of FMRFamide receptor in nervous or muscle tissue does not alter adult larval preference.



App. 4: Reducing expression of FMRFamide receptor in nervous or muscle tissue does not alter adult learning and memory . Adult flies were subjected to a modified version of the Tully and Quinn (1985) T-Maze, which examines olfactory-based associative learning and memory. Flies (75-100 at a time), between one and eight days post-eclosion, were placed within a copper-grid wire lined acrylic chamber through which air and odours could flow. Flies were classically conditioned to associate one of two odours, 4-Methylcyclohexanol (MCH; Aldrich – 101123210) or 3-Octanol (OCT; Sigma-Aldrich – W358126), with a 90V shock as the negative stimulus. The odours were produced by dissolving these chemicals in mineral oil at a 10^{-6} dilution. Compressed air was forced through the mineral oil at a constant flow rate (500 mL/min), and the odour stream was diverted to appropriate test chambers. A no odour condition was produced simply by bubbling air through mineral oil. Half of the flies were trained to associate the shock with MCH, and the other half were trained to associate the shock with OCT. Each training session consisted of 90 s with air, 60 s with shock and odour #1, 30 s with air, 60 s with odour #2, and 30 s with air. After training, the flies were transferred down an elevator to a point where they were able to choose between two collection tubes, one containing odour #1 and the other with odour #2. The performance of the flies was calculated as in Tully and Quinn (1985). A learning index was determined as the fraction of flies that avoided the shock-associated odour minus the fraction of flies that preferred the shocked-associated odour. Since we trained flies to associate shock with either odour, we averaged the two odour-shock groups for each group. If all avoided the shock-associated odour (perfect learning), the index value would be 1; if all preferred the shock-associated odour, the index value would be -1. A learning index value was computed for each group 2, 5 and 10 minutes after training. Ten independent groups of 75-100 flies were assessed for each group.

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