The action of octopamine and tyramine on muscles of *Drosophila melanogaster* larvae.

Kiel G. Ormerod¹, Julia K. Hadden², Lylah D. Deady², A. Joffre Mercier¹, Jacob L. Krans²

¹Department of Biological Sciences, Brock University, Saint Catharine’s, Ontario, Canada
²Department of Neuroscience, Western New England University, Springfield, MA

Running Head: *Action of octopamine and tyramine on larval fruit fly muscle*

Contact Information: jkrans@wne.edu
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.

Keywords: neuromuscular junction, force, fight or flight, biogenic amine
Glossary / Abbreviations

5-HT: serotonin

Ca$^{2+}$: calcium

cAMP: cyclic adenosine monophosphate

CNS: central nervous system

EJP: excitatory junction potential

gSAP: giant sarcomere associated protein

HL-3.1: Hemolymph-like saline, revised by Feng et al., 2004 to reduce Mg$^{2+}$

HL-6: Hemolymph-like saline: Macleod et al., 2002

mepp: miniature end plate potential

Mg$^{2+}$: magnesium

K$^+$: potassium

NMJ: neuromuscular Junction

OA: octopamine

PLTX-II: ω-plectoxin-Pt1a

PNS: peripheral nervous system

TA: tyramine
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 suppress membrane excitability in many animals, including Drosophila (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 neurotransmitter 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 (reviews: 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 neurotransmitter (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 hornworm moth: 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.
Materials and Methods

1.0 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 H2O. 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.

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

2.0 Contraction

2.1 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: Sequim, WA, USA) 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 Δ animal length exceeded 5% (~200 μm of a 4 mm larvae; mean of 10 randomly selected videos was 3.62 ± 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-plectotoxin-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). 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.

2.2 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.

3.0 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. Some data have been reported previously in abstract form (Ormerod et al., 2012).
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-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 1). We examined several parameters of EJPs when recorded in saline containing OA, TA, and yohimbine and observed no significant differences in amine-driven changes 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 from this model 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 1). This ~+20% augmentation in HL-3.1 was not significantly different than that achieved in HL-6, containing 0.5 mM [Ca$^{2+}$] (Table 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 1, $P>0.1$; t-test). Application of 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 also examined saline containing 0.5 mM and 1.5mM calcium (Table 2; \( n_{\text{animal}} > 8 \), all metrics). We chose to examine the cell specific effects of amines in HL-6 because 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 upon 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 (\( 10^{-6} \) 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 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 $5.3 \times 10^{-6}$ 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] ($+6.04 \pm 0.88\%, P<0.01$). 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 ($5.5 \times 10^{-6}$ 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 upon contraction dynamics (Fig. 5D; $P>0.05$ using both analysis of variance 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 x 10^{-7} M, whereas 90% of maximal augmentation was attained in 1.4 x 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 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 ($F_{pk}$-syn = 3.21 mN vs. $F_{pk}$-direct = 3.26 mN; $t_{rise}$-syn = 1.066 s vs. $t_{rise}$-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 x $10^{-6}$ M [OA] in PLTX-containing saline vs. 5.3 x $10^{-6}$ 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 x $10^{-5}$ M [OA] in PLTX-containing saline vs. 3.8 x $10^{-5}$ M [OA] in control saline).
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 $5.5 \times 10^{-6}$ 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 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 ($\sim 25\%$) 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 $\sim 30\%$ 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\textsubscript{50} for force augmentation reported here (5.3 x 10\textsuperscript{-6} M) is similar to what Evans (1981) reported for the effect of OA on twitch amplitude in locust (3.3 x 10\textsuperscript{-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\textsuperscript{-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 \textit{decrease} in relaxation time (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 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), (b) developmental strategies (hemimetabolous vs. holometabolous; Konopova et al., 2011; Hoyle, 1983), (c) the evolution of different
intramuscular proteins (i.e. expression level of giant sarcomere associated proteins, discussed below), or (d) variations in recording methodology (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 $\approx +22\%$ 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 factor 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 neurotransmitter. 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 / neurotransmitters 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]. 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
The rate of tension (now synonymous with the \(\beta\)-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$_{2A}$ 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).
Acknowledgements and Funding

The authors thank Mr. Anthony Scibelli for technical support and the recordings he made for figure 2. This work was supported by a WNE Faculty Research Grant to JLK, a Discovery Grant from NSERC to AJM; NSERC PGS and a Queen Elizabeth II Scholarship in Science and Technology to KGO.


Figure 1. **Semi-intact preparation used for recording force and intracellular electrical signals.**

**A.** Larval bodywall 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 herein. 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 beam of the force transducer, which utilizes a custom full Wheatstone Bridge circuit made of silicon wafers. **iii.** An amplifier was used to inject current and record voltage from a single intracellular electrode. Muscle fibers were either injected with a series of currents (4, 6, 8, 10, 12 pA) and the voltage responses were recorded, or membrane potential was recorded concurrent with suction stimulation to presynaptic nerves. **B.** Basal tonus was recorded using an established method (see text) and an FTO3 Grass tension transducer and amplifier. The CNS was eviscerated and saline, with or without amines, was washed over the preparation.

Figure 2: **Time dependent decrease in contraction amplitude.** **A.** Contraction force decreases with time, more notably in the first 30 minutes of recording then thereafter (times after initial recording are given). **B.** Inasmuch, an exponential function \( F_{pk} = e^{-0.013 \times t} \) provides a statistically significant fit of the decay in contraction amplitude \( (R^2 = 0.95, P<0.01, \text{Pearson's} \) Correlation, \( n = 10 \)). Gray x’s are individual \( F_{pk} \) data from all 10 animals, solid squares are mean
Fpk values across all 10 animals. C. Residuals from both linear (gray: Fpk = -0.0076t + 1; \(\bar{x} = 7.48\)) and exponential functions (black: Fpk = e^{-0.013 \cdot t}; \(\bar{x} = 2.74\)) were examined to identify the simplest function that reasonably fit the decay in contraction amplitude.

Figure 3: Amine modulation of Excitatory Junction Potentials (EJPs) in HL-6 saline. A. An averaged EJP waveform (5 repetitions) recorded from muscle 6 (black) is plotted with 95% confidence intervals (dots). Application of 10^{-6} M [OA] (gray) and co-application 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 [TA] (gray) and yohimbine 10^{-5} M co-applied with 10^{-6} M [TA] (dashed) fall within the 95% confidence interval (dotted) of control saline (black) . C. EJP amplitude is plotted over 15 minutes 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 upon m 12/13 was greater than m 6/7 at high doses of [OA], whereas [TA] maintained a conservative effect upon m 12/13 and 6/7.

Figure 4: Total membrane resistance changes as a function of amine. 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 at even high concentrations of 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].

Figure 5: **Action of amines upon synaptically evoked force production.** A. Averaged contractions (n=8-10 repetitions, each) driven by 25 Hz stimulation of the motor nerve for one second: prior to application of OA (black: control HL-3.1 saline), during bath application of OA-containing saline (gray: [OA] $10^{-6}$ M), co-application of yohimbine ($10^{-5}$ M) with $10^{-6}$ M [OA] (dashed gray line), application of TA ($10^{-5}$ M) (light gray), and co-application of yohimbine ($10^{-5}$ M) and TA ($10^{-6}$ M) (dashed light gray). Crosshairs indicate the points used to compute time constants (t, latency from preceding force inflection: either onset of contraction or relaxation).

**B.** OA-dependent augmentation of contraction. The OA dependence of peak force ($F_{pk}$) from synaptically evoked contractions (n=33 total). The response of all four muscle fibers is shown in black squares (n=33). Open squares indicate that muscles 12 and 13 were ablated while m 6 and 7 were left intact (n=11). In contrast, filled circles indicate that m 12 and 13 were intact whilst 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). $\tau_{\text{rise}}$ [OA] decreases, reaching peak force in less time,
while $\tau_{\text{decay}}$ increases, maintaining force longer (see text). Standard deviation plotted in panel D.

Figure 6: **Action of amines on basal tonus.** **A.** Force was recorded whilst stimuli were delivered to the segmental nerves at 25 Hz (duration = 1 s) immediately prior to application of OA (inset left: black), and about four minutes later (inset right: 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, five minutes of octopamine application augmented basal tonus. The amplitude of augmentation was measured three minutes (black arrows) post-application. **C.** OA (squares) increased basal tonus in a dose-dependent manner in preparations lacking electrical stimulation. TA’s action (circles) on basal tonus was not consistent, nor significant at any concentration other than $10^{-4}$ M. Contraction data represent mean force gain with each [amine] concentration.

Figure 7: **Directly activated contractions are also augmented by octopamine.** **A.** Upon application of PLTX, contraction amplitude (not averaged) decreases with time; each trace reflects a two minute 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 minutes exposure to PLTX. The synaptically-evoked contraction immediately preceding application of PLTX is shown in gray (no artifacts). Lines with stimulus artifact were collected upon 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; re-plotted
from Figure 5) or via direct stimulation of the muscle in PLTX (open squares, n=22).
Table 1: **Summary of excitatory junctional potentials in two salines.** EJPs recorded from third-instar body wall muscle during the application of OA, TA, and the antagonist yohimbine (YOH) in two different physiological salines (HL-3.1 and HL-6) and two different calcium concentrations (0.5 mM and 1.5 mM; \( n_{\text{animals}} > 4 \) for each metric). † denotes significant differences between salines.

Table 2: **Cell-specific effects of OA in HL-6 saline.**
A  EVOKED CONTRACTIONS

\[ \text{input resistance, } V_m \]

\[ \Delta V \]

\[ m_{6,7,12,13} \]

\[ m_{6,7} \]

\[ m_{12,13} \]

\[ \text{snb/d-1s} \]

\[ \text{mn6/7-1b} \]

B  BASAL TONUS

\[ \text{grass force transducer} \]

\[ \text{saline pump} \]
A

B

C

1 mN 0.5 s

% peak F

time (min)

residuals (% peak F)

time (min)
A

OA m6/7
OA m12/13
control
yohimbine-5 & OA x10^-6

B

control
yohimbine x10^-5 & TA x10^-6
TA x10^-6

C

% change in EJP voltage

0 5 10 15
0 10 20 30 40

D

% change in EJP voltage

log [amine] (M)

OA m6/7
OA m12/13
TA m6/7
TA m12/13

amine 10^-4
amine 10^-6

5 mV
20 ms
A

![Graph with data points for OA-4, OA-6, and TA-4 showing changes in input resistance over time.]

B

![Bar graph showing % initial input resistance over time for OA-4, OA-6, and TA-4.]

C

![Scatter plot showing % change in input resistance against log [amine] (M) for OA and TA.]

Legend:
- OA-4
- OA-6
- TA-4

The figure shows the effect of amine on the input resistance of neurons over time and across different concentrations.
A progressive action of PLTX II 10^{-8} upon contraction force

0.5 s stimulus train

1 mN

B progressive action of PLTX II 10^{-8} upon EJP amplitude

-55 mV

10 ms

C

direct stim
synaptic activation

1 mN

200 ms stimulus train

D

% increase in force

log [OA] (M)

OA
PLTX and OA
### Table 1

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<th>EJP values (mV) in HL-3.1</th>
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<td>0.5 mM [Ca^{2+}]</td>
<td>1.5 mM [Ca^{2+}]</td>
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<td><strong>Control</strong></td>
<td>16.47 ± 2.2† (22.50%)</td>
<td>21.34 ± 3.1</td>
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<td>10^{-6} M OA (~EC_{50})</td>
<td>20.18 ± 2.1 (26.1%)</td>
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<tr>
<td>+ 10^{-5} M YOH</td>
<td>20.77 ± 3.88 (26.1%)</td>
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<td>10^{-6} M TA</td>
<td>14.83 ± 4.02 (-9.98%)</td>
<td>19.52 ± 5.07 (-8.54%)</td>
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<tr>
<td>+ 10^{-5} M YOH</td>
<td>15.81 ± 3.57 (-3.98%)</td>
<td>20.75 ± 4.10 (-2.77%)</td>
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### Table 2

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