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

Authors: Kiel G. Ormerod¹, Jacob L. Krans² and A. Joffre Mercier¹.

Affiliations: ¹Brock University, St. Catharines, Ontario, Canada, L2S 3A1, ²Western New England University, Springfield, MA, USA 01119

Corresponding Author: Kiel G. Ormerod, Brock University, St. Catharines, Ontario, Canada, L2S 3A1, Department of Biological Sciences. kielormerod@gmail.com

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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.
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 (Yew et al., 1999; Geary and Maule, 2010; Kastin, 2013). There are roughly 50 identified neuropeptides in the human CNS, and several hundreds in invertebrates (Hurlenius and Lagercrantz, 2001; Hummon et al., 2006). 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 (Certel et al., 2010; Choi et al., 2011; Bargmann, 2012). 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 and Kravitz, 1984; Marder and Calabrese, 1996; Selverston 2010; Harris-Warrick, 2011). 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 neurotransmitters 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 DPKQDFRFa 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.

**Materials and Methods**

**Fly Stocks**

*Drosophila melanogaster* Canton S. (CS) flies, obtained from Bloomington *Drosophila* stock center (BDSC), 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 cerevisiae*), 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* (BDSC), 24B-GAL4 (BDSC) and *tubP-GAL4* (BDSC). *elav-Gal4* was used for pan-neuronal expression of the UAS-FR-IR transgene (Luo et al., 1994; Sink et al., 2001). 24B-GAL4 (Luo ed fet al., 1994; Brand and Perrimon, 1993) 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 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 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, 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), 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 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 CaCl₂. The force recordings depicted in Figure 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) NaHCO₃: 10; Sucrose: 115; Trehalose: 5; NaCl: 70; KCl: 5; MgCl: 4; HEPES: 5; CaCl₂: 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 5 min 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
30 min 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-fluoresce (4
hrs in 1:100 anti-DIG-fluoresce: blocking solution). Prior to microscopy, tissues were washed
(3 x 5 min in TBS, 3 x 1 min in dH20). Tissue was imaged using 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 2C-F, 3A-D). For Figures 4B-E,
5A-B, 6A, 7A-D, 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 6B and 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 2B, 4A, and 9B.

Results

Input resistance

Cell-specific effects of DPKQDFMRFa on muscle cells were first assessed by estimating input resistance (Figure 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 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 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 of 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 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 2E) or muscle cell 13 (Two-way RM ANOVA,
F= 0.870, P=.503, Figure 2F). Control recordings with no peptide application demonstrated
stable input resistance values over 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 3A) and 7 (Two-way RM ANOVA, F =
1.598, P= 0.165, Figure 3B) and resulted in no change in input resistance in cells 12 (Two-way
RM ANOVA, F=0.649, P=0.663, Figure 3C) and 13 (Two-way RM ANOVA, F = 0.620,
P=0.685, Figure 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 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 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 them
(One-way ANOVA, F= 2.939, P>0.05, Figure 4B-E black diamonds). A dose-response was
constructed from recordings made from muscle cell 6. The EC\textsubscript{50} for the effect of DPKQDFMF\textsubscript{a}
on EJPs was 4.1x10\textsuperscript{-8} M (Figure 4A, right). At 1x10\textsuperscript{-6} M, DPKQDFMF\textsubscript{a} increased EJP
amplitude in all four muscle cells (muscle 6: One-way RM ANOVA, F=9.578, P=0.008, Figure
4B; muscle 7: One-way RM ANOVA, F=9.427, P=0.005, Figure 4C; muscle 12: One-way RM
ANOVA, F=13.703, P=0.003, Figure 4D; muscle 13: One way RM ANOVA, F= 9.621,
P=0.007, Figure 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 DPKQDFMF\textsubscript{a}
application resulted in a return to baseline values in all cases. Application of DPKQDFMF\textsubscript{a}
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 DPKQDFMF\textsubscript{a} 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\textsuperscript{-7} M DPKQDFMF\textsubscript{a}, 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 4B, C), and EJP enhancement was similar between muscles 12 and 13 (Figure 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 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 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 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 6B) was not significantly different from CS larvae or from 24B larvae at the same time point (69.1 ± 14.0%, Figure 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 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 6B), 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 the 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 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 \textit{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 7A-C, black diamonds). This effect, described previously and termed “rundown,” has been reported on several occasions (Stewart et al., 1994; Macleod et al, 2002; Krans et al., 2010; Ormerod et al., 2013). In sham-operated preparations with no muscle cells ablated (Figure 7A), application of $1 \times 10^{-6}$ M DPKQDFMRFa after five minutes of stimulation increased nerve-evoked contractions to $126 \pm 8\%$ 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 7A). In preparations with muscle cells 6 and 7 intact and 12 and 13 ablated (Figure 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 7B). When muscle cells 12 and 13 were left intact and 6 and 7 were ablated (Figure 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 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 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 8A, no-peptide application). Figure 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 ± 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
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 8B). 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 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 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 7A) and after rundown (Figure 8A-Canton S larvae). The ability of
DPKQDFMRFa to counter-act 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 (Hewes and Taghert, 2001; Clark et al., 2008, 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 9A). The EC$_{50}$ for peptide-induced contractions was 6.6x10$^{-8}$ M, as estimated from the dose-response curve (Figure 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 9B). This peptide concentration induced contractions in preparations with and without muscle ablation (Figure 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 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 10A, and areas of the muscle we used for analysis are shown in Figure 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 10C).

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 -10 mV 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 dihydropyridine-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.
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 (MNSNbd-Ib). The difference suggests that other motor neurons may be responsive to this peptide. Muscle cells 6 and 7 are innervated by MNSNbd-I and occasionally by MNSNbd-II, in addition to MN6/7-Ib (Hoang and Chiba, 2001). Since DPKQDFMRFa does not enhance EJPs elicited by stimulating MNSNbd-I (Dunn and Mercier, 2005), it is possible that the peptide may modulate MNSNbd-I.

RNAi 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 RNAi 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 EC₅₀ value for peptide-induced contractions (6.6
x $10^{-8}$ M) was only slightly higher than that reported previously for nerve-evoked contractions (2.5 x $10^{-8}$ M, Hewes et al., 1998), and threshold for both effects was between 1 x $10^{-8}$ and 1 x $10^{-9}$ M (Figure 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 10) correlated with larger contractions in 6 and 7 in the presence of DPKQDFMRFa (Figures 7-9). However, cells 12 and 13 did contain mRNA for FR even though they showed no change in input resistance in response to DPKQDFMRFa (Figure 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.
References:


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Figure Legends:

Figure 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. 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.

Figure 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.

Figure 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 4: DPKQDFMRFa enhances excitatory junctional potentials greater in some cells. A: LEFT- Representative EJP traces from fiber 6 before (Control) and after peptide application (10-6 M 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%).

Figure 5: Co-application of DPKQDFMRFa and nifedipine does not alter the amplitude of EJPs. Insect 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. 5A: 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). 5B: 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 EC₅₀ concentration of DPKQDFMRFa also showed a greater enhancement of EJPs in cells 6 and 7 compared to 12 and 13.

Figure 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 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.

Figure 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.

Figure 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.

Figure 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 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.