

Peptide-Induced Modulation of Synaptic Transmission and Escape Response in *Drosophila* Requires Two G-Protein-Coupled Receptors

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Neuropeptides are found in both mammals and invertebrates and can modulate neural function through activation of G-protein-coupled receptors (GPCRs). The precise mechanisms by which many of these GPCRs modulate specific signaling cascades to regulate neural function are not well defined. We used *Drosophila melanogaster* as a model to examine both the cellular and behavioral effects of DPKQDFMRFamide, the most abundant peptide encoded by the dFMRF gene. We show that DPKQDFMRFamide enhanced synaptic transmission through activation of two G-protein-coupled receptors, *Fmrf* Receptor (*FR*) and *Dromyosuppressin* Receptor-2 (*DmsR-2*). The peptide increased both the presynaptic Ca^{2+} response and the quantal content of released transmitter. Peptide-induced modulation of synaptic function could be abrogated by depleting intracellular Ca^{2+} stores or by interfering with Ca^{2+} release from the endoplasmic reticulum through disruption of either the ryanodine receptor or the inositol 1,4,5-trisphosphate receptor. The peptide also altered behavior. Exogenous DPKQDFMRFamide enhanced fictive locomotion; this required both the *FR* and *DmsR-2*. Likewise, both receptors were required for an escape response to intense light exposure. Thus, coincident detection of a peptide by two GPCRs modulates synaptic function through effects of Ca^{2+} -induced Ca^{2+} release, and we hypothesize that these mechanisms are involved in behavioral responses to environmental stress.

Introduction

Neuropeptides modulate numerous physiological processes and behaviors by acting as neurotransmitters, neuromodulators, and hormones. Despite their prevalence and broad functions, the precise mechanisms by which specific peptides influence individual behaviors remain unclear. These mechanisms can be elucidated by determining their actions on specific neurons, receptors, and intracellular signaling pathways.

FMRFamides are oligopeptides that end in the sequence Phe-Met-Arg-Phe-NH₂ and act as both hormones and neuromodulators; they act on G-protein-coupled receptors (GPCRs) in the central and peripheral nervous systems (Greenberg and Price, 1992; Espinoza et al., 2000; Vilim et al., 2010). In mammals, GPCRs sensitive to FMRFamide peptides regulate muscle contraction, antinociception, feeding behavior, and learning and

memory (Panula et al., 1996). In *Caenorhabditis elegans*, hypoxia-enhanced sensory perception is regulated by a circuit that involves the FMRFamide-like neuropeptide FLP-21 and its GPCR, the neuropeptide Y receptor-related protein NPR-1 (Pocock and Hobert, 2010). The *npr-1* gene has been associated with a number of different behaviors, including foraging, aggregation, and aerotaxis (de Bono and Bargmann, 1998; Chang et al., 2006; Gloria-Soria and Azevedo, 2008). While it is clear that GPCRs play essential roles in regulating a variety of behaviors, the signaling proteins and pathways activated by these receptors remain largely unresolved.

Drosophila has many features that make it an ideal model to study the function of neuropeptides and their cognate receptors. A wide array of molecular genetic tools are available in *Drosophila*, and its genome has been fully sequenced (Adams et al., 2000). Larval neuromuscular junctions are readily accessible glutamatergic synapses that allow both electrophysiological and optophysiological recordings. *Drosophila* is also an ideal model for combining behavioral and cellular recording techniques while using both genetic and pharmacological manipulations; thus, this system is ideal for examining the physiological roles of specific signaling molecules in complex pathways.

In *Drosophila*, FMRFamides are present in neurosecretory cells and have been shown to modulate muscular contractions (Hewes et al., 1998; Kaminski et al., 2002; Eade and Allan, 2009). The *Drosophila* FMRFamide gene encodes eight neuropeptides, the most abundant of which is DPKQDFMRFamide (Schneider

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et al., 1993). Many *Drosophila* GPCRs sensitive to peptides structurally related to FMRFamide have been orphaned using cell culture assays (Cazzamali and Grimmelikhuijzen, 2002; Nichols, 2006), but their *in vivo* functions have not been investigated and little is known about their downstream effectors in synapses.

Here, we have examined the roles of the ligand DPKQDFMRamide and two GPCRs in the modulation of synaptic transmission and behavior in *Drosophila*. We found that the *Fmrf Receptor* (*FR*) and the *Dromyosuppressin Receptor-2* (*DmsR-2*) are both required for DPKQDFMRamide-induced enhancement of transmitter release. Optophysiological recordings revealed that the peptide increased the amplitude of Ca^{2+} signals and that modulation of Ca^{2+} -induced Ca^{2+} release was required for this effect. We also found a role for both the *FR* and *DmsR-2* in regulating a locomotor response to intense light exposure. Additionally, application of exogenous DPKQDFMRamide to semi-intact larval preparations enhanced fictive locomotion. Thus, DPKQDFMRamide modulates both synaptic function and behavior through coincident detection mediated by *FR* and *DmsR-2*.

Materials and Methods

Fly stocks. Male and female flies were raised on standard cornmeal medium at 25°C and 60–70% relative humidity, except for larvae used in RNA interference (RNAi) knockdown experiments, which were raised at 29°C. Early wandering third-instar larvae were selected for the experiments. All stocks were obtained from Bloomington Stock Center (Indiana University, Bloomington, IN).

The *Drosophila* lines used included *Canton-S* (CS) and a conditional dominant negative temperature-sensitive dSERCA (*Drosophila sarco/endoplasmic reticulum Ca²⁺ATPase*) mutant, *CaP60A^{Kum170}*, obtained from an ethyl methanesulfonate screen using CS. The *CaP60A^{Kum170}* mutants, which fail to pump calcium back into the endoplasmic reticulum (ER) at high temperatures, were used to deplete calcium stores in the ER. Calcium ATPase function on the endoplasmic reticulum can be disrupted in these mutants by incubating them in Ca^{2+} -free HL3 at 40°C for 8 min, preventing the refilling of the calcium stores (Sanyal et al., 2005). Two inositol 1,4,5-trisphosphate (IP_3) receptor mutants were used, *P{EPgy2}Itp-r83A^{EY02522}* and *P{PZ}Itp-r83A⁰⁵⁶¹⁶ry506*. These two lines were kept balanced over TM3-green fluorescent protein (GFP) and crossed into a *w¹¹¹⁸* background for testing. The ryanodine receptor mutant *P{lacW}Rya-r44F^{K04913}* was kept balanced over CyO-GFP and was crossed into a *w¹¹¹⁸* background for testing. We also used mutants for the three G-protein-coupled receptors, *Mi{ET1}FR^{MB04659}*, *Mi{ET1}DmsR-2^{MB05984}*, and *P{EPgy2}DmsR-1^{EY03017}*. UAS regulatory sequences were used to drive the expression of inverted repeats to interfere with both the *Fmrf Receptor* (γ^1v^1 ; *P{TRiP.JF01879}attP2*) and the *Dromyosuppressin Receptor* (γ^1v^1 ; *P{TRiP.JF01850}attP2*) using either a muscle-specific (*24b-GAL4*) or neuron-specific driver (*elav-GAL4*).

Real-time reverse transcription PCR. To examine transcript levels of IP_3 and NMDA receptors, we used real-time PCR. RNA was extracted from *w-;P{PZ}Itp-r83A⁰⁵⁶¹⁶ry506/+*, *w-;P{EPgy2}Itp-r83A^{EY02522}/+*, and *w¹¹¹⁸* flies using TRIzol (Invitrogen). RNA was treated with amplification grade DNaseI (Invitrogen) and then reverse transcribed using the SuperScript II kit (Roche). cDNA was amplified using Roche Light Cycler 480 real-time PCR and SYBR Green-1 reagents. Copy number alterations were assessed by relative quantification methods that compensate for differences in target and reference amplification efficiencies. Expression levels of the *rp49* transcript were used as an internal control among genotypes, and relative mRNA levels were calculated using the comparative ΔCt (cycle threshold) method. The *P{PZ}Itp-r83A⁰⁵⁶¹⁶* insertion maps within both the *Itp-r83A* and *Nmdar1* genes, while the *P{EPgy2}Itp-r83A^{EY02522}* maps 17 base pairs from the 5' end of *Nmdar1* (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Thus, reverse transcription PCR was performed to assess mRNA levels of both the IP_3 and the NMDA receptor in each of the P-element insertion lines. Transcript levels of *Itp-r83A* in *w-;P{EPgy2}Itp-r83A^{EY02522}/+* were 0.49 ± 0.04 relative to *w¹¹¹⁸*; similarly,

w-;P{PZ}Itp-r83A⁰⁵⁶¹⁶ry506/+ levels were 0.53 ± 0.04 , relative to controls. *Nmdar1*-RA transcript levels in *w-;P{EPgy2}Itp-r83A^{EY02522}/+* were 0.81 ± 0.21 relative to controls, which was not a significant reduction, while in *w-;P{PZ}Itp-r83A⁰⁵⁶¹⁶ry506/+*, levels were significantly reduced to 0.52 ± 0.1 of controls. Thus, *P{EPgy2}Itp-r83A^{EY02522}* showed no significant decrease in NMDA transcript, but it was significantly decreased in *P{PZ}Itp-r83A⁰⁵⁶¹⁶*.

The following primers were used: IP_3 receptor, 5'-GCTGCTCATTAAGAACCTGT-3' (forward) and 5'-GGTATTGAACGATTCTTGG-3' (reverse); NMDA receptor, 5'-GGAGCTCTCCAACATGTATC-3' (forward) and 5'-GAGATGAATCCCAGATGAAG-3' (reverse); RP49, 5'-AGT-GCGTCGCGCTTCAAGG-3P (forward) and 5'-AGAACGCAGCGCAC-CGTTGG-3' (reverse).

Locomotion assay. We developed a behavioral stress response assay that mimics conditions of direct sun exposure on larvae. The larvae were placed on the inside lid of a plastic dish that had moistened tissue paper covering it. Larvae were first observed for 2 min under minimal light conditions at room temperature. Then, a bright, full spectrum light was shone directly onto the larvae through the plastic dish. The distance between the light and the larvae was kept constant. The temperature was measured between 34 and 36°C. Wandering paths were digitized and measured using ImageJ (National Institutes of Health; rsb.info.nih.gov/ij/).

Fictive locomotion assay. Intact larvae were restrained on a Sylgard dish with dissecting pins bent to resemble horseshoes. The number of peristaltic waves were counted over a 2 min period and used as a measure of locomotor activity. Semi-intact larvae were dissected up the midline to allow replacement of the hemolymph with the hemolymph-like saline HL3 (Stewart et al., 1994). HL3 contained the following (in mM): $CaCl_2$ (1.25 mM), $MgCl_2$ (20.0), KCl (5.0), NaCl (70), $NaHCO_3$ (10.0), *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (5.0), trehalose-2H₂O (5.0), sucrose (115.0). The nervous system and body wall muscles remained intact. In some experiments DPKQDFMRamide (5×10^{-6} M) was added to the saline.

Physiological procedures. For physiological experiments, the dissection was performed in Schneider's Insect Medium (Sigma-Aldrich) to prevent contractions and minimize the chance of damaging the preparation during the dissection. The larva was cut up the dorsal midline, pinned open, and cleaned of its internal organs, leaving the nervous system and body wall muscles intact. Physiological recordings were obtained from preparations that were continually superfused with HL3 solution. During the imaging of evoked Ca^{2+} responses, 7 mM L-glutamate was added to the solution to desensitize glutamate receptors and prevent muscle contractions (Macleod et al., 2002). In both electrophysiological and calcium imaging experiments, the distal cut ends of the nerves were sucked into a glass stimulating electrode and stimulated at 2 Hz with a Grass S48 stimulator or the A-M Systems isolated pulse stimulator model 2100 using impulses having a duration of 0.2–0.5 milliseconds and suprathreshold stimulus intensity (4–6 V).

Intracellular recordings were simultaneously recorded from muscles 6 and 13 using glass microelectrodes filled with 1 M KCl, with a tip resistance of ~ 40 M Ω . Stimulus intensity was constantly monitored to ensure recruitment of only type 1b nerve terminals. Extracellular focal recordings were made from single 1b boutons of the motor neuron MN6/7-1b (Hoang and Chiba, 2001). Focal macropatch electrodes with tips having an inner diameter of 5 μ m were used to enclose single 1b boutons that were identified using a 40 \times water-immersion lens and Nomarski optics. Quantal content (mean number of quanta released per impulse) was calculated for individual boutons by measuring the amplitude of their stimulus-evoked excitatory junction currents (EJCs) and dividing that by the mean amplitude of their spontaneous miniature excitatory junction currents (mEJCs) (Dason et al., 2009).

Calcium measurements. Calcium kinetics were examined at high temporal resolution using the calcium-sensitive fluorescent indicator Oregon Green 488 (1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA)-dextran (OGB). The indicator was loaded into the cut end of the motor axon, a procedure that does not alter calcium regulation (Macleod et al., 2002; Klose et al., 2008). Line scanning through individual type 1b nerve terminals was done using a Bio-Rad 600 confocal scan head (Bio-Rad MRC-600 software) on a Nikon upright microscope

(Optiphot-2) with a 40× Olympus water immersion objective [0.7 numerical aperture (NA)]. Images were acquired using a BHS filter set [exciter filter, 488 nm DF10; emission filter, OG 515 nm low pass (LP); dichroic reflector, 510 nm LP]. Fluorescence (F) is reported in arbitrary units with background subtracted. Nerves were stimulated at 2 Hz in 2.0 mM external calcium concentration unless stated otherwise.

Measurements of Ca^{2+} concentration in type 1b nerve terminals were obtained using the calcium indicator Fura-dextran (10 kDa; Invitrogen). Type 1b nerve terminals have a larger volume than their type 1s counterparts and evoke smaller initial postsynaptic responses. During high-frequency stimulation the 1b terminals facilitate while 1s terminals depress (Lnenicka and Keshishian, 2000). Fluorescence from the nerve terminals of Fura-dextran-loaded neurons was detected with an intensified charge-coupled device camera (PTI, model IC-100) connected to a Lightning 2000 frame grabber controlled by Axon Imaging Workbench 2.2 (AIW 2.2) (both from Molecular Devices). Emitted light was collected by an Olympus water immersion objective (40×, 0.7 NA) through a 530 ± 35 nm bandpass filter, both mounted on an upright Nikon (Optiphot-2) microscope. The loaded neurons were excited with light from a mercury arc lamp alternately through 340 ± 5 and 380 ± 5 nm bandpass filters on a rapidly switching filter wheel (Omega Optical). Measurements of fluorescence in response to excitation at these two wavelengths allow ratiometric calculation of intraterminal $[\text{Ca}^{2+}]$. In the experiments which used Fura-dextran, images were taken for 200 milliseconds, once every 5 s, throughout a 15 s period at rest.

The value for the dissociation constant (K_d) was taken as 865 nM following previous work by Macleod et al. (2002) and Bronk et al. (2005). Briefly, the maximum (R_{max}) and minimum (R_{min}) fluorescence values for the preparation in the experimental chamber are obtained and used to calculate the K_d of the indicator (Gryniewicz et al. 1985). For further details on calibration of the indicator see Klose et al. (2008).

ImageJ software (<http://rsb.info.nih.gov/ij/>) was used to convert PIC image files to TIFF format, allowing measurement of average pixel value within selected image regions or within lines of a line scan image. A region not containing any calcium indicator fluorescence or autofluorescence was randomly selected beside each fluorescent nerve terminal, and the average pixel intensity was measured in this region to give a background value. This value was subtracted numerically from the average pixel value of a region or line containing the fluorescent element of interest to generate the value F . $\Delta F/F$ is defined as the change in F during stimulation, normalized to F before nerve stimulation. The amplitudes of the calcium responses were measured from baseline to peak and averaged for each nerve terminal over four consecutive pulses 0.5 s apart. Two to four boutons were tested in each preparation. Only boutons with initial response amplitudes >0.125 arbitrary units in 2 mM extracellular calcium were used for further testing. Each data point represents averaged values from one preparation.

Depletion of ER calcium stores. Depletion of ER calcium stores was performed in $\text{CaP60A}^{\text{Kum170}}$ mutants following the OGB loading procedure. Larval $\text{CaP60A}^{\text{Kum170}}$ preparations were heat shocked to inactivate their temperature-sensitive sarco/endoplasmic reticulum Ca^{2+} (SERCA) pumps to prevent reuptake of calcium into the ER. Heat shock was administered by placing the preparation in a sealed Petri dish with moist paper; the dish was then placed for 8 min in an oven set at 40°C. ER Ca^{2+} was depleted by stimulating the nerve for 4 min at 30 Hz following the temperature-induced inactivation of SERCA.

Pharmacology. DPKQDFMRamide at 5.0 × 10⁻⁷ mM was used in each of the experiments. The combination of DPKQDFMRamide and L-glutamate (7 mM) caused an increase in muscle tonus and twitching in the muscle (Clark et al., 2008) that made fluorescence imaging unreliable. The spontaneous muscle twitching was blocked by 10 μM nifedipine. Xestospongine B (100 nM) was used to block release of calcium from IP₃ receptors, while 100 μM ryanodine was used to block the release of calcium from the ryanodine receptor. KN-93 (1 μM) was used to disrupt calmodulin-dependent protein kinase II (CaMKII), while the same concentration of the inactive analog KN-92 was used as a control.

Statistical analysis. Significance of $p < 0.01$ (unless stated otherwise) was assessed with a t test or two-way ANOVA with the appropriate *post hoc* test (Tukey) where applicable. All values are reported as mean ±

SEM. Time constants of signal decay were determined with nonlinear regression analysis using the equation $y = ae^{-bx}$ (Sigmaplot 11 software). Only the single exponential component was used in the analysis.

Results

Modulation of transmitter release

We examined the synaptic effects of DPKQDFMRamide at the well characterized larval body wall neuromuscular junction using a combination of electrophysiological and optophysiological recording techniques. Excitatory junction potentials (EJPs) evoked from type 1b nerve terminals (Fig. 1A) increased in amplitude by 22.9 ± 6.1% within 5 min of application of the peptide (Fig. 1B–D). In addition, we found that the effect on EJP amplitude persisted for many minutes after washing the peptide out of the bath (Fig. 1D). We also recorded synaptic currents directly from type 1b nerve terminals using extracellular recording electrodes before and after peptide application (Fig. 2A,B). The quantal content in CS larvae was 4.5 ± 0.3 before the addition of peptide and increased significantly to 6.1 ± 0.6 following its application (Fig. 2C, Table 1). EJC amplitude in CS preparations increased significantly following peptide application, whereas there was no effect on the amplitude of spontaneously released mEJCs (Fig. 2Cii,Ciii). We tested for DPKQDFMRamide-induced synaptic effects in mutants of the *FR* and *DmsR-2* receptors to determine what role the two receptors play in the observed synaptic effects. No significant change in quantal content, EJC amplitude, or mEJC amplitude was observed in either $\text{FR}^{\text{MB04659}}$ or $\text{DmsR-2}^{\text{MB05984}}$ larvae following DPKQDFMRamide application (Fig. 2C, Table 1). The frequency of mEJCs was not altered by the peptide in any synapses (Table 1). Thus, both the *FR* and the *DmsR-2* are necessary for DPKQDFMRamide-induced enhancement of quantal content.

Modulation of presynaptic calcium regulation

Quantal content can be modulated by altering cytosolic calcium levels or by altering sensitivity to calcium (Zucker, 1996; de Jong and Verhage, 2009). Thus, we examined the effects of DPKQDFMRamide on presynaptic Ca^{2+} regulation by recording fluorescence from individual type 1b boutons loaded with the calcium-sensitive Oregon Green 488 BAPTA-dextran. These nerve terminals were scanned every 4 ms during 2 Hz stimulation. Presynaptic calcium responses (transients) resulting from single stimuli applied to the nerve were recorded in nerve terminals using the calcium-sensitive calcium fluorophore Oregon Green BAPTA-488 (Fig. 3A, red arrow). Increasing the extracellular calcium concentration ($[\text{Ca}^{2+}]_e$) significantly increased the amplitude of calcium transients but did not alter the time constant of decay (Fig. 3B), indicating that the level of Ca^{2+} entering the nerve terminal under these conditions does not affect the nerve terminal's ability to clear free Ca^{2+} from the cytosol (Tank et al., 1995). Application of DPKQDFMRamide (0.5 μM) to the physiological recording solution caused an increase in muscle tonus and spontaneous muscle twitching that made recording of calcium transients difficult. To prevent this disruptive effect, nifedipine (10 μM) was added to the physiological solution to block the postsynaptic effects of the peptide in the recording solution (Clark et al., 2008). Nifedipine, which blocks L-type calcium channels, did not alter the evoked Ca^{2+} responses recorded in the presynaptic terminals (Fig. 3B). DPKQDFMRamide reversibly increased the amplitude of evoked Ca^{2+} responses in presynaptic terminals without affecting time constant of decay (Fig. 3C). At 1.25 mM extracellular Ca^{2+} , the amplitude of the normalized evoked response was 0.142 ± 0.010, and after peptide application

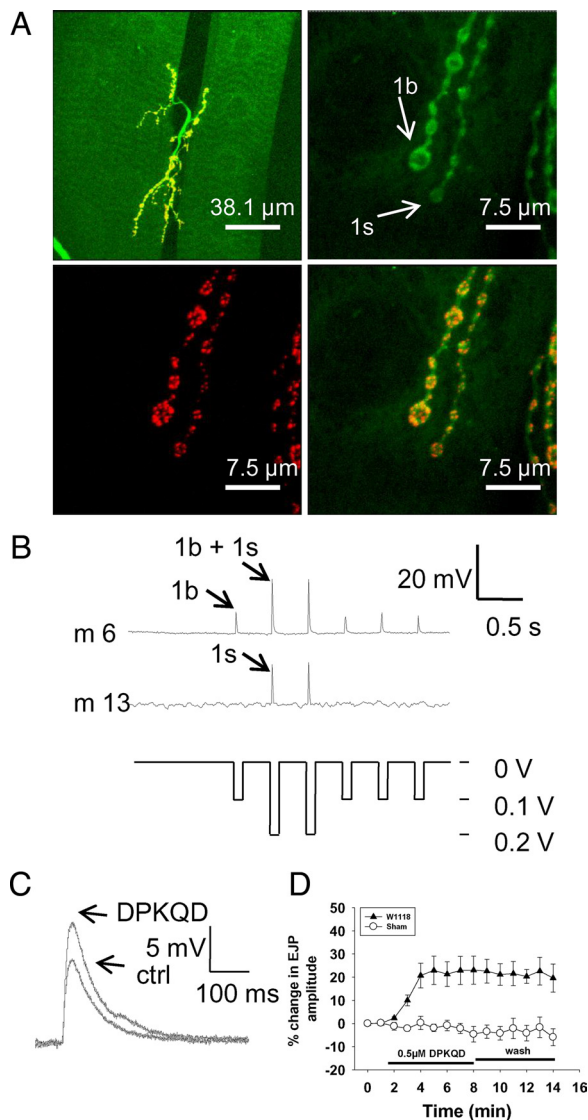


Figure 1. EJP modulation by DPKQDFMRamide. **A**, Neuromuscular junctions and synapses of larval *Drosophila*. At the top left is a low-power view of the neuromuscular junctions on abdominal muscles 6 and 7 formed by two motor axons that supply type 1b and 1s innervation. The boutons are stained with anti-horseradish peroxidase antibody (top right, green) and antibody nc82 against the active zone structural protein Bruchpilot (bottom left, red) to illustrate multiple synapses on each bouton; the merged image is also shown (bottom right). **B**, Excitatory junctional potentials recorded intracellularly from abdominal muscles 6 and 13. A stimulus intensity of 0.1 V initially recruits the MN6/7–1b (RP3) axon, which innervates only muscle 6 and 7 with tonic-like type 1b nerve terminals. Increase in stimulus intensity to 0.2 V additionally recruits the MNSNb/d-1s axon, which innervates muscles 6, 7, 12, and 13 with phasic-like type 1s terminals. Muscle 13 was therefore used as a monitor to detect accidental recruitment of type 1s terminals during the experiment. **C**, Application of 0.5 μM DPKQDFMRamide increased the amplitude of EJPs recorded in muscle 6 with stimulation of only type 1b terminals. ctrl, Control. **D**, Application of 0.5 μM DPKQDFMRamide induced an increase in amplitude of ~20% and did not significantly change following washout (wash) of the peptide ($n = 6$; $p = 0.7$). “Sham” indicates application of hemolymph-like solution containing no peptide.

it increased significantly to 0.169 ± 0.008 (Fig. 3*Ci*). Washout of the peptide restored the amplitude to 0.143 ± 0.009 within 4 min, which was significantly less than with the peptide and not different than before peptide application (Fig. 3*Ci*). In 2.0 mM extracellular Ca^{2+} , the normalized amplitude of the evoked response was 0.176 ± 0.012 ; this increased significantly with peptide application to 0.206 ± 0.013 and reversed with washout of the peptide (Fig. 3*Cii*). Decay time constant was not altered by the

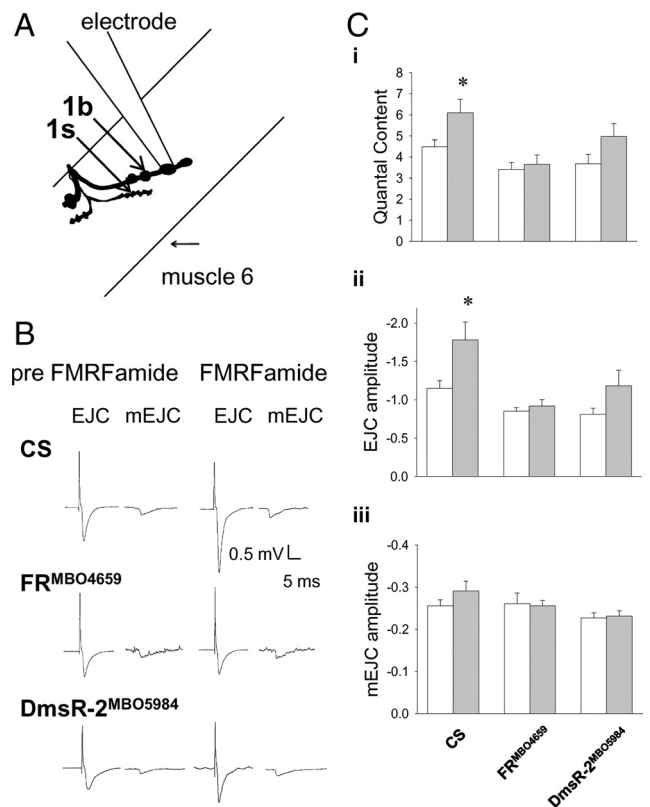


Figure 2. Modulation of excitatory junctional current recordings from type 1b boutons disrupted in *FR* but not *DmsR-2* receptor mutants. **A**, Schematic of experimental set-up used to record quantal synaptic currents from type 1b nerve terminals on muscle 6 of the *Drosophila* abdominal wall. **B**, Traces of evoked EJCs and mEJCs before and after application of peptide. **Ci**, Quantal content in CS preparations ($n = 6$) increased significantly following peptide application from 4.5 ± 0.3 to 6.1 ± 0.6 (two-way ANOVA, $p > 0.05$), but there was no significant effect of peptide application in either *FR*^{MBO4659} ($n = 6$; $p = 0.7$) or *DmsR*^{MBO5984} ($n = 8$; $p = 0.06$) preparations. **Cii**, The amplitude of EJCs in CS preparations ($n = 6$) increased significantly from $-1.15 \text{ mV} \pm 0.1 \text{ mV}$ to $-1.8 \pm 0.2 \text{ mV}$ following peptide application (two-way ANOVA, $p > 0.05$), but the peptide had no significant effect in *FR*^{MBO4659} ($n = 6$; $p = 0.7$) or *DmsR*^{MBO5984} ($n = 8$; $p = 0.06$) preparations. **Ciii**, The amplitude of spontaneously released mEJCs, measures of postsynaptic sensitivity, did not significantly change following peptide application in CS ($n = 6$), *FR*^{MBO4659} ($n = 6$), or *DmsR*^{MBO5984} ($n = 8$) preparations (two-way ANOVA, $p = 0.4$). White bars are before the peptide was added and grey bars are following DPKQDFMRamide application. Asterisks (*) indicate significant difference.

Table 1. Effect of DPKQDFMRamide on parameters of quantal synaptic recordings

	Quantal content		EJC amplitude (mV)		mEJC amplitude (mV)		mEJC frequency (Hz)	
	Pre	FMRF	Pre	FMRF	Pre	FMRF	Pre	FMRF
CS								
Average	4.49	6.10*	-1.15	-1.78*	-0.26	-0.29	0.032	0.026
SEM	± 0.32	± 0.64	± 0.11	± 0.23	± 0.01	± 0.02	± 0.007	± 0.007
FR								
Average	3.41	3.66	-0.85	-0.92	-0.26	-0.26	0.009	0.014
SEM	± 0.30	± 0.40	± 0.04	± 0.08	± 0.02	± 0.01	± 0.004	± 0.003
DmsR-2								
Average	3.68	4.97	-0.81	-1.18	-0.23	-0.23	0.014	0.016
SEM	± 0.44	± 0.63	± 0.08	± 0.20	± 0.01	± 0.01	± 0.003	± 0.004

The averages and SEM quantal content, EJC amplitude, mEJC amplitude, and mEJC frequency are shown for wild-type CS as well as for *FR* and *DmsR-2* mutants. Both quantal content and EJC amplitude in CS larvae was increased following peptide application; however, there were no significant effects on either receptor mutant. There were no significant effects of the peptide on mEJC amplitude or mEJC frequency in any lines. Bold numbers with an asterisk reveal a statistically significant increase resulting from peptide application. Pre, Pre-DPKQDFMRamide.

peptide at 1.25 or 2.0 mM [Ca^{2+}]_e (Fig. 3*Ciii,Civ*). The time constant of decay in terminals bathed in 2.0 mM [Ca^{2+}]_e was 42.6 ± 8.0 ms in controls and 39.7 ± 10.4 ms following peptide application, revealing no significant change (Fig. 3*Cii*).

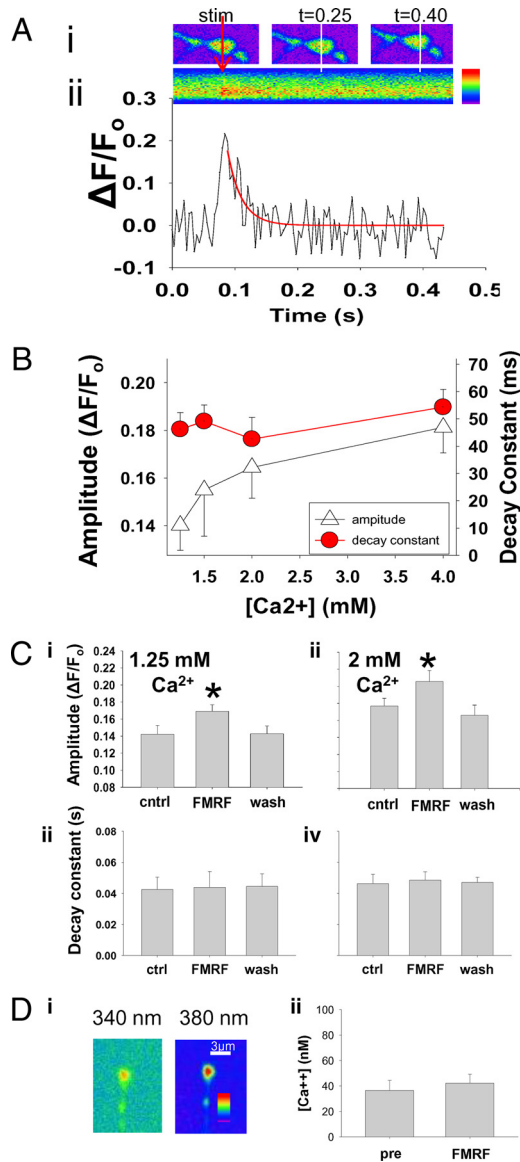


Figure 3. DPKQDFMRamide reversibly increases amplitude of evoked Ca^{2+} responses in nerve terminals. **A**, The upper images are of an OGB-loaded type 1b nerve terminal at time = 0 [stimulation (stim)], 0.25, and 0.4 s. The red arrow and white lines indicate the location of the line scans through the nerve terminal. The lower image is a composite of all of the line scan images through the same location of the nerve terminal. **Aii**, The fluorescence of the sequential line scans is graphed below the composite image. Decay of the calcium transient response was fit with a single exponential using the equation $y = ae^{-bx}$ (red line). Scale to the right of the scan represents increasing fluorescence intensity. Red arrow indicates time of stimulation. **B**, Increasing extracellular $[\text{Ca}^{2+}]$ increases amplitude of single impulse Ca^{2+} responses. Normalized amplitudes of single impulse Ca^{2+} responses increased significantly ($p = 0.01$; $n = 6$) with progressively increasing extracellular Ca^{2+} concentration (open triangles): 0.138 ± 0.010 at 1.25 mM Ca^{2+} , 0.160 ± 0.015 at 1.5 mM Ca^{2+} , 0.176 ± 0.012 at 2.0 mM Ca^{2+} , and 0.194 ± 0.019 at 4.0 mM Ca^{2+} . The time constant of decay recorded in preparations bathed in 1.25 mM $[\text{Ca}^{2+}]_o$ was 46.2 ± 6.1 ms and was not significantly different when recorded in 4.0 mM $[\text{Ca}^{2+}]_o$, which was 54.3 ± 6.5 ms. **C**, DPKQDFMRamide reversibly increases the amplitude of evoked calcium responses in presynaptic terminals. **Ci**, At 1.25 mM extracellular Ca^{2+} , amplitude of the normalized evoked response significantly increased after peptide application ($p < 0.05$; $n = 10$). Washout (wash) of the peptide restored the amplitude, which was significantly less than with the peptide ($p < 0.05$). **Cii**, In 2.0 mM extracellular Ca^{2+} , the normalized amplitude of the evoked response increased significantly with peptide application ($p < 0.05$; $n = 10$) and reversed with washout of the peptide ($p > 0.05$). **Ciii**, Decay time constant was not altered by the peptide at 1.25 mM Ca^{2+} ($p = 0.8$). **Civ**, Decay time constant was not altered by the peptide at 2.0 mM Ca^{2+} ($p = 0.9$). ctrl, Control. **D**, DPKQDFMRamide does not alter steady-state free intracellular $[\text{Ca}^{2+}]$. **Di**, Ratiometric recordings of Fura fluorescence were made at 340 and 380 nm every 5 s for 15 s, allowing quantification of $[\text{Ca}^{2+}]$ at rest. **Dii**, No difference in resting or steady-state calcium levels is detected between control and DPKQDFMRamide-exposed nerve terminals ($n = 4$; $p > 0.6$). pre, Pre-DPKQDFMRamide. Asterisks (*) indicate significant difference.

Resting levels of calcium (cytosolic steady-state free calcium concentration) in nerve terminals were recorded using the fluorescent ratiometric calcium indicator Fura-dextran at 340 and 380 nm (Fig. 3Di). Ratiometric recordings were taken every 5 s for 15 s, allowing quantification of Ca^{2+} concentration at rest. Resting Ca^{2+} concentration was 36.5 ± 8.0 nM and, following application of the peptide, 42.2 ± 7.0 nM, which was not significantly different from the control (Fig. 3Dii). Thus, the peptide did not affect resting $[\text{Ca}^{2+}]$, which is consistent with the lack of effect of the peptide on mEJC frequency (Table 1).

Role of Ca^{2+} -induced Ca^{2+} release

Calcium-induced Ca^{2+} release (CICR) occurs following the activation of calcium-sensitive receptors on the ER membrane. CICR can modulate synaptic function during prolonged high-frequency activity in which Ca^{2+} influx through voltage-activated Ca^{2+} channels accumulates to levels that activate ryanodine and/or IP_3 receptors (Llano et al., 2000).

To test for a role of intracellular Ca^{2+} stores in peptide-induced modulation of the Ca^{2+} response, we used *Drosophila* with a temperature-sensitive SERCA mutation that allows depletion of calcium stored in the endoplasmic reticulum. Evoked Ca^{2+} response amplitude in CaP60A^{Kum170} mutants was dramatically increased following temperature-induced inactivation of SERCA (Fig. 4A). Control CaP60A^{Kum170} response amplitude was 0.164 ± 0.007 , and the time constant of decay was 51.2 ± 7.3 ms. Following the inactivation of SERCA function with a heat shock treatment, but before Ca^{2+} stores were depleted, Ca^{2+} response amplitude increased significantly to 0.416 ± 0.029 , and the time constant of decay increased significantly to 124.1 ± 24.2 ms (Fig. 4B,C). Preparations were subsequently stimulated at high frequency for several minutes to deplete the ER of remaining Ca^{2+} stores. Following this depletion, both amplitude and time constant of decay were restored to control levels, and subsequent application of DPKQDFMRamide did not significantly increase the amplitude of evoked responses at 2 Hz stimulation (Fig. 4C). Thus, release of intracellular Ca^{2+} from the ER stores is necessary for DPKQDFMRamide-induced modulation of presynaptic Ca^{2+} responses.

Calcium release from the ER can occur through the calcium-sensitive ryanodine receptor or through the IP_3 receptor (Streb et al., 1983; Bardo et al., 2006). Inositol 1,4,5-trisphosphate is a second messenger made by hydrolysis of phosphatidylinositol 4,5-bisphosphate, a phospholipid that is located in the plasma membrane (Venkatesh and Hasan, 1997). IP_3 binds to and activates the IP_3 receptor on the membrane of the ER, opening a Ca^{2+} channel which results in the release of Ca^{2+} into the cytoplasm. This increase in Ca^{2+} can activate the ryanodine receptor-operated channel on the ER, leading to a further increase in the Ca^{2+} response. Xestospongins B is a membrane-permeable selective antagonist of IP_3 receptors (Bardo et al., 2006). The amplitude of evoked responses at 2 Hz in preparations bathed in xestospongins B was 0.180 ± 0.009 and was not different from that of controls (Fig. 5A). DPKQDFMRamide, in the presence of xestospongins B, had no significant effect on response amplitude (Fig. 5Aii). Two IP_3 receptor mutants, $P\{PZ\}Itp-r83A^{05616}ry506$ (IP_3R^{05616}) and $P\{EPgy2\}Itp-r83A^{EY02522}(\text{IP}_3R^{EY02522})$, were also tested for sensitivity to the peptide. Both the IP_3R^{05616} and $\text{IP}_3R^{EY02522}$ mutants exhibited a decrease in IP_3 receptor transcript levels (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Nerve terminals from both IP_3 receptor mutant lines revealed a loss of the measured response to the peptide (Fig. 5Ai). Ryanodine (a plant alkaloid that interferes

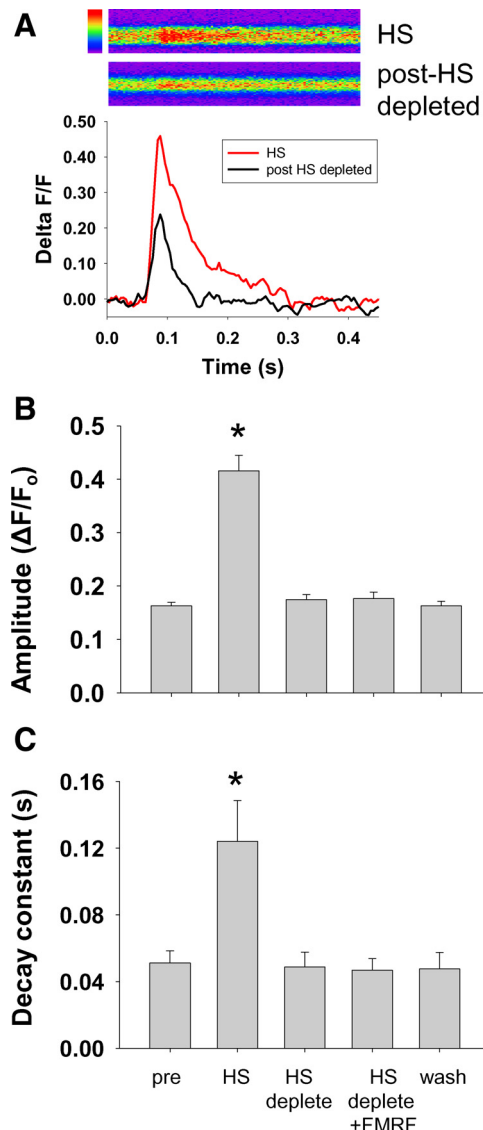


Figure 4. Depletion of ER Ca^{2+} stores prevents peptide-induced modulation. *Drosophila* larvae with a conditional dominant negative temperature-sensitive SERCA mutation were used to test for a role of intracellular Ca^{2+} stores in DPKQDFMRamide-induced modulation of evoked Ca^{2+} release. **A**, Normalized amplitude of evoked Ca^{2+} responses in *CaP60A^{Kum170}* mutants under control conditions (black) and with inactivated SERCA (red) following an 8 min heat shock (HS) at 40°C. Each trace is the average of four consecutive responses during a 2 Hz train and averaged every 20 ms. **B**, Following inactivation of SERCA function, normalized amplitude increased dramatically ($p < 0.001$; $n = 5$). ER Ca^{2+} stores were depleted after ~2 min of 30 Hz stimulation. Following this depletion, response amplitudes reduced to control levels ($p = 0.001$). Subsequent application of DPKQDFMRamide did not significantly alter the amplitude of evoked responses ($p = 0.9$). **C**, The decay constant was significantly increased following inactivation of SERCA but was restored to control levels following the depletion of calcium stores from the ER. Application of the peptide had no effect on decay constant in terminals with depleted ER calcium stores. pre, Pre-DPKQDFMRamide. Asterisks (*) indicate significant difference.

with the receptor responsible for calcium-induced calcium release) at 100 μM was used to block Ca^{2+} -induced calcium release from the ER as done previously in *Drosophila* type 1b nerve terminals (Shakiryanova et al., 2007). Stimulus-evoked Ca^{2+} responses in the nerve terminals of both *w⁻;P{lacW}Ryarr44Fk04913/+* larvae and those exposed to ryanodine were not significantly altered by DPKQDFMRamide (Fig. 5*Ai,Aii*). The time constant of decay was not altered by either ryanodine or

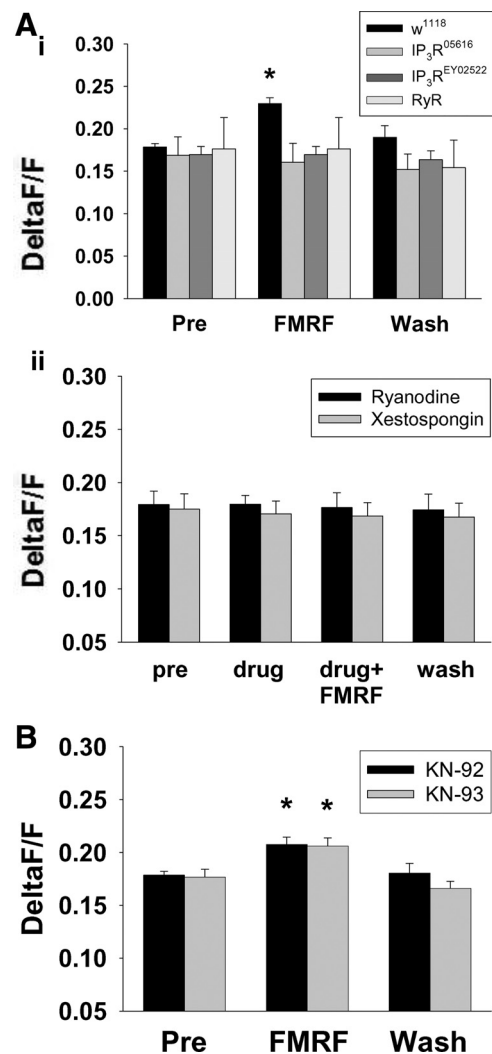


Figure 5. Peptide-induced modulation of calcium response mitigated by disruption of IP_3 or ryanodine receptor but not CaMKII. **Ai**, Genetic disruption of IP_3 (*IP₃R⁰⁵⁶¹⁶* and *IP₃R^{E02522}*) or ryanodine receptor (RyR) prevented the DPKQDFMRamide-induced increase in calcium response amplitude observed in *w¹¹¹⁸* controls. **Aii**, Pharmacological disruption of IP_3 receptors with 100 nM xestospongine B or disruption of ryanodine receptors with 100 μM ryanodine also mitigated the peptide-induced increase in calcium response amplitude observed in controls (see Fig. 2). **B**, Pharmacological disruption of CaMKII with 1 μM KN-93 had no effect on peptide-induced modulation of calcium response amplitude. The inactive analog KN-92 also had no effect on calcium responses. Pre, Pre-DPKQDFMRamide. Asterisks (*) indicate significant difference.

xestospongine B, revealing that calcium clearance was not altered (data not shown). Disrupting release of calcium from internal stores using either IP_3 or ryanodine receptor mutants also prevented the peptide-induced modulation of synaptic transmission (data not shown). Thus, release of calcium from intracellular stores through both the ryanodine and IP_3 receptors is required for the DPKQDFMRamide-mediated modulation of synaptic function.

Role of CaMKII

Previous studies have suggested that CaMKII is involved in peptide-mediated modulation of synaptic function (Dunn and Mercier, 2005; Shakiryanova et al., 2007). We tested CaMKII for a role in DPKQDFMRamide-induced synaptic modulation. The peptide-induced increase in excitatory junctional potential amplitude can be suppressed by the CaMKII blocker KN-93

(supplemental Fig. 3, available at www.jneurosci.org as supplemental material). We also tested whether CaMKII has a role in DPKQDFMRamide-induced modulation of presynaptic Ca^{2+} responses by applying the pharmacological blocker KN-93 and its inactive analog KN-92 to the preparations. Neither agent modulated presynaptic Ca^{2+} response amplitude alone, but when DPKQDFMRamide was applied in combination with either KN-93 or KN-92, Ca^{2+} response amplitude increased significantly (Fig. 5B). Thus, the peptide-induced modulation of evoked presynaptic Ca^{2+} response amplitude is not sensitive to CaMKII disruption.

Modulation of behavior

Drosophila larvae respond to bright light by moving away from the source (Caldwell et al., 2003), and they crawl more often and faster in response to temperature elevations (Klose et al., 2005) (Fig. 6*Ai,Aii*). This escape response protects larvae from desiccation. We used a bright high temperature light (34–36°C) and measured locomotor activity to determine whether DPKQDFMRamide plays a role in the escape response. Distance traveled under room temperature and low light conditions by w^{1118} larvae was 2.83 ± 0.43 cm and not different from a mutant of the *Fmr* Receptor ($FR^{MBO4659}$), which traveled 2.70 ± 0.36 cm in the same time period. However, under bright light conditions the mutant larvae traveled 4.20 ± 0.59 cm, which is significantly less than controls 6.71 ± 0.65 cm (Fig. 6*Ai,Aii*). The escape response (percentage increase in locomotor activity induced by intense light exposure) was $194.6 \pm 60.2\%$ in the background control w^{1118} . Both of the GPCRs known to be sensitive to DPKQDFMRamide, the *Fmr* Receptor ($FR^{MBO4659}$) and the *Dromyosuppressin* receptor-2 ($DmsR-2^{MBO5984}$) (Johnson et al., 2003), were tested for roles in responses to the bright light. In $FR^{MBO4659}$ the locomotor response was $66.08 \pm 20.71\%$, while in $DmsR-2^{MBO5984}$ it was $53.6 \pm 8.5\%$, both of which were significantly less than in the control (Fig. 6*Bi*). We also examined the *Dromyosuppressin* receptor-1, which belongs to the same neurotensin/thyrotropin-releasing factor receptor family as the *FR* and *DmsR-2* (Hewes and Taghert, 2001) but has only been shown to be sensitive to dromyosuppressin and not DPKQDFMRamide (Johnson et al., 2003). In these mutants ($DmsR-1^{EYO3017}$) the light-induced increase in distance traveled was $195.4 \pm 51.7\%$ and was not different from w^{1118} larvae (Fig. 6*Bi*). The data reveal that both the *FR* and the *DmsR-2* receptors, but not the *DmsR-1* receptor, are necessary for the escape response and suggest that DPKQDFMRamide is regulating the response.

The reduced behavioral phenotype was also observed with targeted knockdown of either the *Fmr* receptor or the *Dromyosuppressin* receptor-2. The normal increase in locomotor activity occurring during the escape response was significantly reduced in

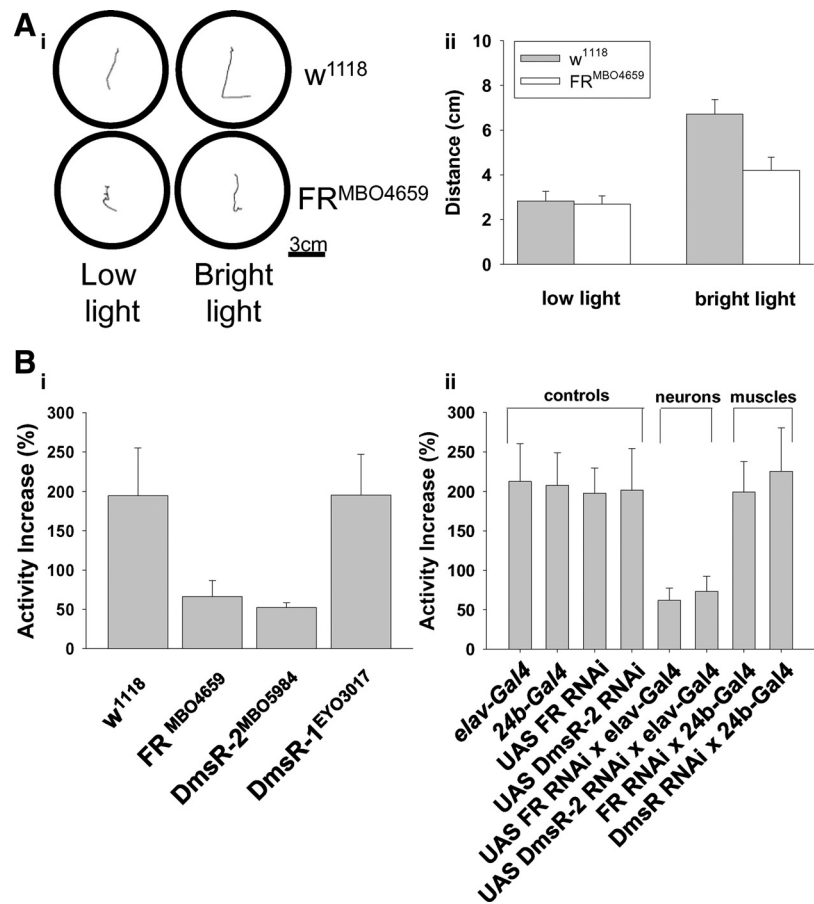


Figure 6. DPKQDFMRamide enhances larval locomotion. **Ai**, Sample wandering paths from one w^{1118} and one $FR^{MBO4659}$ mutant larvae during a 2 min recording period under dim light (room temperature) conditions and then subsequent bright light conditions (34–36°C). **Aii**, Distance traveled in mutant $FR^{MBO4659}$ ($n = 10$) and wild-type background (w^{1118}) ($n = 10$) over a 2 min recording period at low light and then a subsequent 2 min recording period at bright light ($p = 0.01$). **Bi**, The stress-induced increase in locomotor activity ((bright light – low light)/low light) was significantly reduced in the mutants $FR^{MBO4659}$ [$MI[ET1]CG2114^{MBO4659}$] ($p = 0.02$; $n = 7$) and $DmsR-2^{MBO5984}$ [$MI[ET1]CG13803^{MBO5984}$] ($p = 0.01$; $n = 7$), but not in $DmsR-1^{EYO3017}$ ($p = 0.02$; $n = 7$). **Bii**, Disruption of the normal behavioral stress response through knockdown of either the *FR* or the *DmsR-2* receptor using targeted RNA interference in neurons. The percentage increase in locomotor activity resulting from bright light exposure was significantly reduced in both $UAS FR RNAi \times elav-Gal4$ ($n = 6$; $p < 0.01$ for both) larvae and $UAS DmsR-2 RNAi \times elav-Gal4$ ($n = 6$; $p < 0.05$ for both) compared with their respective controls, $UAS FR RNAi$ ($n = 6$), $UAS DmsR-2 RNAi$, and $elav-Gal4$ ($n = 6$). The activity increase when RNAi knockdown of *FR* was targeted to muscles, $FR \times 24b-Gal4$ ($n = 6$), was not different from the controls $24b-Gal4$ ($n = 6$; $p = 0.3$) or FR ($n = 6$; $p = 0.4$), $FR, DmsR-2$. $DmsR-2 \times 24b-Gal4$ ($n = 6$; $p = 0.7$).

$UAS FR RNAi \times elav-Gal4$ larvae to $62.0 \pm 15.3\%$ when compared with the controls; $UAS FR RNAi$ increased by $197.7 \pm 31.7\%$ and $elav-Gal4$ increased by $212.6 \pm 47.5\%$. The increase in activity in $UAS DmsR RNAi \times elav-Gal4$ was also significantly reduced to $73.2 \pm 19.5\%$ compared with controls; $UAS DmsR RNAi$ increased by $201.5 \pm 52.8\%$. RNAi knockdown of *FR* in muscles, $UAS FR RNAi \times 24b-Gal4$, resulted in a $199.2 \pm 38.4\%$ increase in activity and was not different from either control: $24b-Gal4$, which was $207.8 \pm 40.9\%$, or the $UAS FR RNAi$. Likewise, RNAi knockdown of *DmsR-2* in muscles, $UAS DmsR-2 RNAi \times 24b-Gal4$, exhibited $225.4 \pm 54.7\%$ in activity, which was also not different from controls: $UAS DmsR-2 RNAi$ or $24b-Gal4$. Thus, RNAi knockdown of either receptor in nerves, but not in muscles, mimicked the behavioral phenotype observed in mutants of the same receptors, supporting a role for each receptor in regulating behavior (Fig. 6*Bii*). The data also suggest that previously observed activity of this peptide at the neuromuscular junction (Hewes et al., 1998) acts via GPCRs located presynaptically on the nerve.

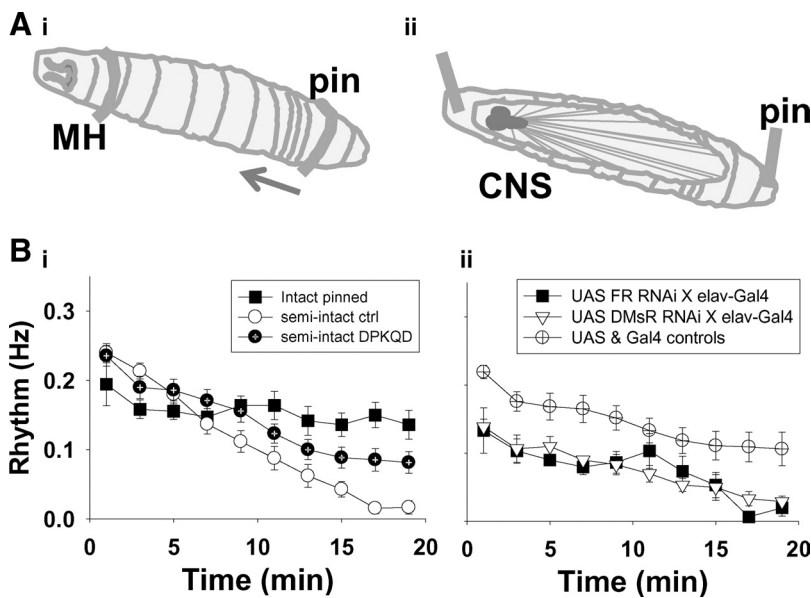


Figure 7. DPKQDFMRamide partially restores propensity for fictive locomotion. **A**, Diagram of restrained third instar wandering larvae attempting to crawl (arrow indicates direction of peristaltic wave of locomotor muscle contractions). The rhythm frequency (number of contractions per second averaged over consecutive 2 min time bins) during a 20 min recording period was examined in both intact (**Ai**) and semi-intact larvae (**Aii**). **Bi**, Rhythm frequency remained stable in pinned intact larvae ($n = 6$) but decreased steadily in semi-intact larvae following replacement of hemolymph with the hemolymph-like saline HL3 and, after 20 min, was significantly reduced ($n = 20$; $p > 0.001$). When hemolymph-like saline contained (5×10^{-6} M) peptide, the rhythm frequency in the semi-intact larvae was significantly higher than without peptide ($n = 20$; $p = 0.002$) and significantly lower than in intact larval preparations ($p < 0.05$). **Bii**, RNAi interference of either DPKQDFMRamide-sensitive receptor mitigated the peptide-induced partial rescue of fictive locomotor activity. The fictive rhythm in genetic control lines was significantly higher than both $UAS FR^{MBO4659} \times elav-Gal4$ ($p = 0.02$) and $UAS DmsR-2^{MBO5984} \times elav-Gal4$ ($p > 0.05$). UAS and Gal-4 lines were combined because they were not significantly different at any time point (circle, $n = 10$; square, $n = 5$; triangle, $n = 5$). MH, Mouth hooks.

To examine the effects of DPKQDFMRamide on locomotor activity in the absence of other neuroactive substances, we used a fictive locomotion assay that allowed replacement of the hemolymph with hemolymph-like saline (Fig. 7*Ai,Aii*). Larvae respond to elevated temperature by increasing the frequency of their rhythmic contractions, resulting in an increase in distance traveled per unit time (Klose et al., 2005). Thus, we used the frequency of peristaltic waves in pinned larvae as a measure of locomotor activity. Pinned intact larvae attempted to crawl every 5–7 s. The peristaltic frequency remained stable following pinning, beginning at 0.19 ± 0.03 Hz and remaining at 0.14 ± 0.21 Hz 20 min later, which was not significantly different. The fictive locomotor rhythm of semi-intact larvae in which the hemolymph was replaced with hemolymph-like saline was initially 0.21 ± 0.17 Hz but diminished significantly over a 20 min period to 0.02 ± 0.01 Hz, presumably due to the removal of endogenous hormones and neuromodulators. In those preparations bathed in DPKQDFMRamide (5×10^{-6} M) for 20 min, the frequency of fictive locomotor activity was partially restored to 0.08 ± 0.02 Hz, significantly higher than without the peptide. RNAi knockdown of either receptor sensitive to DPKQDFMRamide mitigated the peptide-induced enhancement of fictive locomotor activity observed in wild-type preparations. The fictive rhythm in genetic control lines was 0.106 ± 0.025 after 20 min of exposure to the peptide (5×10^{-6} M), significantly higher than both $UAS FR^{MBO4659} \times elav-Gal4$, which was 0.013 ± 0.013 Hz, and $UAS DmsR-2^{MBO5984} \times elav-Gal4$, which was 0.025 ± 0.01 Hz (Fig. 7*Bii*). Thus, our data suggest that the neuropeptide DPKQDFMRamide acts via two GPCRs, *FR* and *DmsR-2*, both expressed in neurons, to control the escape response in *Drosophila* larvae.

Discussion

We have investigated the mechanisms by which the neuropeptide DPKQDFMRamide modulates synaptic function and behavior. We show that DPKQDFMRamide signaling through both the *FR* and the *DmsR-2* is required for the peptide-induced modulation of synaptic transmission and an escape response in *Drosophila*. At peripheral synapses we show the GPCR activation by DPKQDFMRamide increases the amplitude of both Ca^{2+} transients and quantal content per nerve stimulus. Modulation of the active Ca^{2+} response occurred regardless of extracellular Ca^{2+} concentration but was abrogated by depleting intracellular Ca^{2+} stores or by interfering with Ca^{2+} release from these stores. We provide evidence suggesting that DPKQDFMRamide-induced modulation of calcium regulation results from enhanced Ca^{2+} -induced Ca^{2+} release from the endoplasmic reticulum involving the combined activity of both IP_3 receptor and ryanodine receptor function. Thus, we show that DPKQDFMRamide modulates behavior, transmitter release, and intracellular calcium handling through two GPCRs.

Several *Drosophila* G-protein coupled receptors sensitive to FMRFamides have been deorphaned using cell culture assays (Cazzamali and Grimmelikhuijzen, 2002; Nichols, 2006). For the majority of these receptors, little is known of the role they play in regulating behavior and what cellular pathways they modulate. Both the *FR* and the *DmsR-2* were necessary for the peptide-mediated modulation at the neuromuscular junction. Meeusen et al. (2002) suggested that in *Drosophila* only one FMRFamide receptor, *CG2114*, is encoded by the genome since alignment of *CG2114* with the closest related receptor genes (*CG5911*, *CG13803*, *CG8985*, and *CG5936*) yielded only 11–14% sequence identity. Subsequent analysis of these orphan receptors in cell culture using the β -arrestin GFP translocation assay revealed that both *CG2114* (*FR*) and *CG13803* (*DmsR-2*) are sensitive to DPKQDFMRamide. GPCRs can dimerize to form complex signaling molecules (Woehler and Ponimaskin, 2009), which may explain why both GPCRs, the *FR* and the *DmsR-2*, were required for DPKQDFMRamide-enhanced transmitter release. The full complement of GPCRs has yet to be thoroughly characterized. At least 13 FMRFamide-like peptides exist in *Drosophila* (Schneider and Taghert, 1988), and it is therefore conceivable that other FMRFamide receptors may be found.

Activation of the *FR* and *DmsR-2* by DPKQDFMRamide modulates intracellular calcium handling. Calcium triggers neurotransmitter release and plays a central role in various forms of modulation. Increased neural activity causes increased Ca^{2+} accumulation that activates target proteins, enhancing transmitter release (Zucker, 1996; de Jong and Verhage, 2009). Most investigations of presynaptic modulation have focused on calcium channel modulation (Man-Son-Hing et al. 1989; Rathmayer et al. 2002; Gundlfinger et al. 2007; Donato et al. 2008; Leal and Klein, 2009; Sánchez-Mejorada et al. 2009). Some evidence has sug-

gested that FMRFamide can alter the calcium-sensitivity of the secretory apparatus (Man-Son-Hing et al. 1989); however, effects on Ca^{2+} -induced Ca^{2+} have been scantily examined. One study examined the effect of cholecystokinin on glutamate release in hippocampal granule cells and revealed a modulatory role for IP_3 receptor function (Deng et al., 2010), but it did not examine a role for ryanodine receptors. In mammalian hippocampal and cerebellar synapses, involvement of CICR in short-term facilitation has been suggested (Emptage et al., 2001; Bardo et al., 2006), although this remains somewhat controversial (Carter et al., 2002). A body of literature indicates that peptides modulate the slow secretion of neuroactive substances from pancreatic β -cells (Zhang et al., 2007). Here, we show that initiation of CICR by a peptidergic hormone can modulate fast transmission at a glutamatergic synapse. We found that depletion of ER calcium stores prevented DPKQDFMRamide-induced modulation of Ca^{2+} transients. Thus, we suggest that the peptide activates pathways that sensitize the Ca^{2+} store to activation by residual Ca^{2+} following action potential invasion of the nerve terminal. This allows sufficient Ca^{2+} release to supersede the capacity of the SERCA pumps and local buffering proteins to activate neuromodulatory pathways.

Clusters of IP_3 receptors create local signals known as Ca^{2+} “puffs,” whereas ryanodine receptors produce Ca^{2+} “sparks.” These Ca^{2+} signals produce moderate elevations in Ca^{2+} concentration with a typically limited spatial spread (Berridge et al., 2003; Xu et al., 2009). In *Xenopus* oocytes, Ca^{2+} that is released by a cluster of receptors can diffuse to adjacent receptors, resulting in saltatoric propagation of the initial signal to trigger global Ca^{2+} waves and oscillations (Yao et al., 1995; Sun et al., 1998). It is conceivable that a similar propagation of calcium from a set of IP_3 receptors to a set of ryanodine receptors is occurring in the confines of the *Drosophila* nerve terminal. This might explain why disruption of either receptor results in the loss of the modulation rather than a partial attenuation. How CICR is modulated by DPKQDFMRamide is unclear. Because both the IP_3 and ryanodine receptors are required for the peptide’s effect, it is unclear whether one or both of these intracellular receptors is being modulated following activation of the GPCRs. Regardless, the peptide modulates Ca^{2+} -induced Ca^{2+} release, activating modulatory pathways leading to enhanced transmitter release.

CaMKII plays a role in DPKQDFMRamide-dependent modulation of transmitter release. Disruption of CaMKII disrupted the peptide-induced modulation of synaptic transmission; however, it did not prevent peptide-induced modulation of presynaptic Ca^{2+} responses. Thus, we suggest a role for CaMKII following release of internal Ca^{2+} stores and before vesicle exocytosis. The modulatory effects on presynaptic calcium handling reversed within 5 min of washing out the peptide, while the postsynaptically recorded effects persisted long afterward. CaMKII is autophosphorylated following Ca^{2+} /calmodulin activation, resulting in an increased affinity for the calmodulin complex, prolonging the time that the kinase is active (Hodge et al., 2006). In addition, autonomic activity of the phosphorylated kinase complex, even after the calmodulin complex has dissociated from the complex, further prolongs the active state (Hudmon and Schulman, 2002). Interestingly, neurotransmitter release is enhanced by ryanodine receptor-activated CaMKII activity in these nerve terminals (Shakiryanova et al., 2007), which could explain the observed postsynaptic effects of the peptide (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Several other second messengers are likely involved in conferring the effects of the peptide on transmitter release. In

Aplysia, presynaptic facilitation induced by FMRFamide in sensory neurons of the gill-withdrawal reflex and siphon-withdrawal reflex acts via cAMP (Mackey et al., 1987), while in crustaceans, FMRFamide-like peptides act through both cAMP and cGMP (Badhwar et al., 2006). IP_3 receptors are phosphorylated by cAMP-dependent protein kinase A (Soulsby and Wojcikiewicz, 2005) and could thus be involved in the peptide-induced modulation.

The increased transmitter release induced by DPKQDFMRamide at the neuromuscular junction increases muscle force by 3.5-fold (Hewes et al., 1998). At the onset of a locomotor burst during the cycle, the transition between low and high neuromuscular activity is not instantaneous but rather increases progressively before peaking at 60 Hz when maximal force of the contraction is attained (Klose et al., 2005). The observed increase in transmitter released per action potential could expedite the rate at which maximal force is achieved, resulting in the observed increase in rate of locomotion. Interestingly, during the late third instar stage when FMRF mRNA levels increase, the larvae cease feeding and enter the wandering stage, which is characterized by increased locomotor activity (Sokolowski, 1980).

Although it has been previously suggested that FMRFamide-like peptide-induced modulation of arthropod neuromuscular synapses can result from modulation of N-type calcium channels (Rathmayer et al., 2002), we provide strong evidence for an alternate modulatory pathway. An approximate third- or fourth-power relationship between transmitter release and calcium level at release sites suggests that changes in calcium influx are not responsible for the increased evoked calcium response (Jan and Jan, 1976; Robinson et al., 2002; Okamoto et al., 2005). Voltage-activated Ca^{2+} channels are located in high densities at active zones, the sites of active transmitter release; thus, if they were responsible for the increased Ca^{2+} response, the 20% increase in presynaptic Ca^{2+} signal observed would translate into a near doubling of EJP in the postsynaptic cell, which does not occur (Fig. 1). The present data suggest that DPKQDFMRamide binds to *FR* and *DmsR-2*, modulating calcium-induced Ca^{2+} release, which requires combined action of both ryanodine receptors and IP_3 receptors. Since pharmacological disruption of CaMKII did not prevent the peptide from modulating the calcium response, the increased intracellular calcium signal likely activates CaMKII, resulting in the observed enhancement in release of neurotransmitter. In addition, modulation of a behavioral stress response also requires both the *Fmrf* receptor and the *Dromyosuppressin receptor-2*. The increase in locomotor activity we observed results from a complex combination of centrally driven and peripherally regulated increases in output.

Several neuropeptides have been shown to play a role in modulating behavior in both invertebrates and mammals. Neuropeptide Y, secreted by the hypothalamus in mammals, reduces physical activity (de Bono and Bargmann, 1998), while neuropeptide F, the invertebrate homolog, plays a similar role in *Drosophila* by reducing aggressive behavior (Dierick and Greenspan, 2007). Interestingly, both peptides have also been shown to regulate food intake (Sokolowski, 2003). Disruption of another neuropeptide in *Drosophila*, tachykinnin-related peptide, gives rise to hyperactive flies (Winther et al., 2006). Activation of FMRFamide-releasing cells has been correlated with ecdysis behavior (Kim et al., 2006), and FMRF inhibits egg laying in *C. elegans* (Ringstad and Horvitz, 2008). Activation of the GPCR, NPR-1, by its ligand FLP-21 regulates hypoxia-enhanced sensory perception, revealing a role for a FMRFamide-related neuropeptide in a stress-induced behavior (Pocock and Hobert, 2010). The

present work demonstrates that DPKQDFMRamide enhances fictive locomotion via two GPCRs and that these same GPCRs mediate the escape response. *Drosophila* larvae move away from light (Caldwell et al., 2003) and increase their crawling velocity with temperature elevations (Klose et al., 2005; Luo et al., 2010) (Fig. 6*Ai,6ii*). Prolonged exposure to 36°C can activate the cellular heat shock response in *Drosophila* (Ritossa, 1962; Karunanithi et al., 1999); thus, the observed escape response to bright, high temperature light likely represents an adaptive behavioral stress response against the deleterious effects of sun exposure in the natural environment of the larvae.

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