Mechanisms of synaptic modulation by neuropeptides in crayfish and *Drosophila*

by

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A Thesis
submitted to the Department of Biological Sciences
in partial fulfillment of the requirements
for the degree of
Master of Science

February, 2003
Brock University
St. Catharines, Ontario
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Abstract

Modulation of chemical synapses is an important function in the nervous system because it underlies learning, memory, development and alteration of behaviour. Understanding the intracellular mechanisms of synaptic modulation by neuropeptides is important for understanding their functional roles. This thesis examines the mechanisms underlying synaptic modulation by neuropeptides in two invertebrates, fruitflies (*Drosophila melanogaster*) and crayfish (*Procambarus clarkii*).

In crayfish, DRNFLRFamide (DF₂), a FMRFamide-like peptide, increases transmitter release from nerve terminals at neuromuscular synapses (Skerrett et al. 1995) in a temperature-dependent manner (Friedrich et al. 1994). This thesis examines the mechanisms underlying such temperature-dependence. The enhancement of transmitter output by DF₂ increases as temperature decreases. Reducing temperature lowers transmitter output, which could potentially increase the store of transmitter available for release. Thus, the peptide might be more effective at low temperature because there is more transmitter available to be mobilized and released. This possibility is addressed by reducing transmitter release with other methods. One alternative method of reducing transmitter output, specifically reducing extracellular calcium concentration, also increases the effectiveness of the peptide. Data from these experiments, in which temperature and extracellular calcium were varied, show that the degree of modulation by DF₂ is inversely related to the strength of synaptic transmission. However, a third method of reducing transmitter release, induction of “low frequency depression”, failed to enhance the ability of the peptide to modulate chemical synapses. Thus, the
temperature-dependent effect may not be the result of increasing transmitter store, but it may be a consequence of altering calcium influx.

In fruitfly larvae, the FMRFamide-like peptide DPKQDFMFamide enhances synaptic current in body wall muscles (Hewes et al. 1998). The effectiveness of this peptide was compared between low-output and high-output synapses. DPKQDFMFamide enhances synaptic potentials elicited by stimulating a low-output motor neuron but not the synaptic potentials elicited by stimulating a high-output motor neuron. Others (Rathmayer et al. 2002) have reported that DF2 selectively modulates responses from a high-output motor neuron and not from a low-output motor neuron. Thus, motor neuron-specific modulation is not dependent on transmitter output per se, and is probably not related to the calcium-dependent and temperature-dependent effects in crayfish. The effects of DPKQDFMFamide on synaptic transmission in Drosophila require the activity of calcium/calmodulin-dependent protein kinase, IP3 receptors, and calcium ions from intracellular stores.

These studies demonstrate the importance of calcium in modulation of chemical synapses by neuropeptides.
Acknowledgements

I would like to thank my supervisor Dr. A. Joffre Mercier for guidance, mentorship, and invaluable insight. I would like to thank Dr. G. Spencer and Dr. M. Bidochka for their helpful criticism and patience during many long committee meetings.

I thank Dr. Satpal Singh and Dr. G. Gu (SUNY Buffalo) for their help with the *Drosophila* larval preparation, and their hospitality while I was at SUNY. I would also like to thank Dr. Leslie Griffith (Brandeis University, Mass) for her generous donation of the *Drosophila* stocks l(4)16 and ala1.

I thank Dr. Mary Kate Worden for helpful discussions with respect to Chapter 2 of this thesis. I am also grateful to Mr. Peter Orth and Dr. Milton Charlton for helpful comments on Chapter 2.

I would also like to thank my lab mates Mike Lowe, Anjali Kundi, and Jill Murray, who over the past two years provided insightful conversation on the subject of this thesis.

Chapter 2 of this thesis was accepted to the Journal of Neurophysiology and Chapter 3 has been submitted to the Journal of Neuroscience.
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Chapter 1. Introduction
Chemical synapses are points of communication between nerve cells (neurons) and from nerve cells to effector targets, such as muscles and glands. Synapses are critical points where the strength or efficacy of communication can either be altered or maintained. Changes in synaptic efficacy can alter the functioning of entire neural circuits and produce changes in behaviour. Thus, understanding how chemical synapses are modulated is fundamental to understanding important processes such as learning and memory.

Neuromodulation

Neuropeptides are oligopeptides capable of affecting a wide range of targets when systemically released by neurosecretory cells (or organs). They can affect local synaptic transmission and can be released as transmitters (co-transmitters). Neuromodulation of a variety of targets by the global release of neuropeptides may underlie some forms of behaviour. Alteration of synaptic efficacy by small peptides is a prominent means of neuromodulation at chemical synapses, with literally hundreds of different neuroactive peptides identified to date (Hokfelt et al., 2000). A prevalent class of neuropeptides found in every animal phylum investigated, with hundreds of variations, are the FMRFamide-related peptides (FaRPs) (Espinoza et al., 2000). Price and Greenberg (1977) first isolated the cardioexcitatory tetrapeptide, FMRFamide (Phe-Met-Arg-Phe-NH$_2$) from a bivalve mollusc. This discovery has led to the identification of a novel class of peptides containing a similar C-terminal amino acid sequence, generally F(M/L)RFamide. Inter and intraspecific variations in FaRP sequence occur at the N-terminal amino acids, many of which are less than a dozen amino acids in length. FaRPs
in invertebrates modulate heart rate, neuromuscular junctions, and synapses in the CNS (Fossier et al., 1999; Skerrett et al., 1995). FaRPs can affect central (Baux et al., 1992) and peripheral synapses (Skerrett et al., 1995; Worden et al., 1995) by directly modulating ion channels (Cottrell, 1997; Man-Son-Hing et al., 1989) or by activating intracellular second messenger systems (Mercier et al., 2001; Willoughby et al., 1999b; Willoughby et al., 1999a).

**FMRFamide-like peptides**

This thesis focuses on the mechanisms through which FaRPs modulate chemical synapses. Much is known about these mechanisms in molluscs, where FMRFamide directly gates a sodium channel in the central nervous system (Cottrell, 1997). In other tissues and in other organisms (e.g. squid optic lobe, Aplysia sensory neurons, and Lymnaea heart), FaRPs have been linked to intracellular second-messenger systems and G-proteins (Chin et al., 1994; Volterra and Siegelbaum, 1988; Willoughby et al., 1999a). At the crayfish neuromuscular junction FaRPs are known to be excitatory and require kinase activity (Friedrich et al., 1998; Hewes et al., 1998; Noronha and Mercier, 1995; Skerrett et al., 1995).

To date, a variety of protein kinases have been implicated as essential to FaRP function at the crayfish neuromuscular junction. Much work has been done with a crayfish FaRP, DRNFLRFamide, DF₂. This peptide enhances transmitter release from motor neurons onto muscle cells. DF₂ requires CaMKII, PKC, cyclic nucleotide activated kinases, and N-type calcium channels in order to modulate neuromuscular
synapses (Friedrich et al., 1998; Mercier et al., 2001; Noronha and Mercier, 1995; Rathmayer et al., 2002). DF$_2$ can activate a vertebrate FaRP receptor (GPR54), a G-protein coupled receptor that leads to the activation of a G$_{\alpha_3}$ subunit upon DF$_2$ binding (Clements et al., 2001). The effects of DF$_2$ are temperature-dependent in crayfish, with the peptide being more effective at modulating synaptic transmission at lower temperatures (Friedrich et al., 1994). The implication of kinase activity in FaRP pathways has previously been achieved with chemical inhibitors, which are not always convincing, as specificity is not fully known.

*Drosophila melanogastor* (the fruit fly) has a fully sequenced genome and literally thousands of different genetic mutants. This allows us to combine genetic and pharmacological methods to identify essential pathway proteins. Though FaRP expression has been well studied in *Drosophila* (Benveniste and Taghert, 1999; Nichols et al., 1995; Nichols et al., 1999; Schneider and Taghert, 1990), the physiological functions of these peptides and the mechanisms through which they act are largely unknown.

The purpose of this thesis is to elucidate the mechanisms through which FaRPs modulate neuromuscular junctions. Two model preparations are used to investigate the mechanisms of FaRP modulation. The crayfish *Procambarus clarkii* is used to investigate temperature-dependent effects of FaRPs. *Drosophila* larvae are used to further investigate the intracellular messengers through which FaRPs enhance synaptic transmission. Established methods of measuring quantal content (an estimate of presynaptic transmitter release) and excitatory junctional potentials (EJPs- a measure of
synaptic efficacy) are used to quantify the effects of FaRPs on the neuromuscular junction.

This thesis will provide evidence that the temperature-dependent effects of DF$_2$ are likely calcium-dependent effects. I will present evidence that a reduction in calcium influx increases the effectiveness of the peptide and, thus, mimics the effect of lowering temperature. Furthermore, the modulatory effects of the peptide (DF$_2$) are not altered by all treatments that reduce transmitter output. Thus, the increased effectiveness of the peptide at low temperature and low Ca$^{2+}$ does not simply reflect an increase in the reserve of transmitter available for release. I also show that the *Drosophila* FaRP, DPKQDFMRFamide, modulates larval neuromuscular junctions by activating the calcium-dependent kinase, CaMKII. Furthermore, IP$_3$ receptors (calcium channels) and intracellular calcium stores are involved in synaptic modulation in *Drosophila*, indicating that calcium dynamics play an important role in modulation by FaRPs. Finally, I show that the modulatory effects of DPKQDFMRFamide are neuron-specific.

**Transmitter Release at Chemical Synapses**

The release of neurotransmitter from presynaptic nerve terminals is quantal in nature. Neurotransmitter is released in discreet packets, which are vesicles containing transmitter. While recording muscle fibre membrane potential close to motor nerve synapses in the frog leg, Fatt and Katz (1952) noticed spontaneous depolarizations of roughly equal amplitude and with a time-course similar to recordings of nerve evoked depolarizations. These spontaneous depolarizations were termed miniature end plate potentials (m.e.p.p.) and each represents the post-synaptic effect of the release of a single
quantum or vesicle of transmitter (Fatt and Katz, 1952). Depolarization of presynaptic
terminals opens voltage-dependent calcium channels leading to an influx in calcium in
presynaptic terminals and calcium triggers the release of transmitter producing the end
plate potential (e.p.p.) or excitatory junctional potential (EJP). The amplitude of the
e.p.p. can be reduced by gradually lowering extracellular calcium and by raising the
magnesium concentration by the same amount. The smallest amplitude observed by Fatt
and Katz (1952) was equal to the amplitude of the m.e.p.p.'s. This suggested that the
m.e.p.p. was the smallest unit of transmitter release, representing one quantum of
transmitter, and that the EJP results from the release of many such quanta (Fatt and Katz,
1952). Each quantum corresponded to the release of the contents of one synaptic vesicle.
Del Castillo and Katz (1954) proposed that the probability of any one vesicle being
released is less than one. Quantal content (m), the average number of quanta released per
nerve impulse, is determined by two variables: n, the number of vesicles available for
release (this variable may be better described as the number of active release sites with
docked and primed vesicles) and p, the probability of release, which may vary between
release sites on a common terminal. This is shown as:

\[ m = n \cdot p \]

Alterations in quantal content reflect changes in the probability of release and/or the
number of vesicles available for release.

**Estimates of Transmitter Release**

EJP (e.p.p.) amplitude at the frog neuromuscular junction can be predicted by a
binomial equation for quantal content at the frog neuromuscular junction, as the
distribution of the number of quanta released per impulse is binomial (Del Castillo and Katz, 1954). Using this equation requires estimates of both \( n \) and \( p \), which are not available for most synaptic preparations.

Del Castillo and Katz (1954) determined that when the probability of release was lowered by reducing calcium, the number of quanta released per nerve impulse followed a Poisson distribution, and quantal content could be estimated by counting the number of failed quantal currents. When transmitter release is low, quantal content \( (m) \) equals the natural logarithm of the number of stimuli \( (N) \) divided by the number of failures (no synaptic current) \( (n_0) \):

\[
m = \ln\left(\frac{N}{n_0}\right)
\]

The failures method for estimating quantal content uses the above equation. Estimates of quantal distribution at the crayfish neuromuscular junction determined that transmitter release follows a Poisson distribution of quanta when \( m \leq 1 \) and a binomial distribution when \( m > 1 \). Thus, the failures method can be used to estimate quantal content in crayfish preparations when the rate of quantal failures is high.

Equation 2 above for estimating quantal content can be used experimentally to determine whether substances that modulate synaptic efficacy do so by increasing transmitter release from the synaptic terminals. Another method of identifying a presynaptic locus of neuromodulation requires the calculation of the coefficient of variance (CV) in EJP amplitude. Variation in EJP amplitude occurs through variation in the binomial parameters of quantal release and is independent of changes in quantal size (Faber and Korn, 1991). A presynaptic modulatory effect will change CV, and the change will be inversely proportional to the change in EJP amplitude. A postsynaptic
modulatory effect, such as increasing the sensitivity of the postsynaptic cell to transmitter, does not affect the CV of EJP amplitude.

**Crayfish FaRPs**

In the crayfish, FaRPs have been identified throughout the nervous system, in the pericardial organs, and the hindgut (Mercier et al., 1991; Mercier et al., 1997). The pericardial organs, which are neurosecretory glands just outside the heart, have the highest level of FMRFamide-like immunoreactivity in the entire nervous system (Cooke and Sullivan, 1982; Mercier et al., 1991). The crayfish FaRPs, DF2 and NF1, were first isolated from the pericardial organs of P. clarkii and were found to modulate neuromuscular junctions, heart rate, and muscle contractions (Mercier et al., 1993). Analysis of changes in quantal content and muscle fibre input resistance for the deep abdominal extensor muscles (DEM) with both of these FaRPs found that NF1 affects EJP amplitude and contraction amplitude presynaptically and postsynaptically, while DF2 acts only presynaptically at these synapses (Quigley and Mercier, 1997; Skerrett et al., 1995). Both peptides increase quantal content (the number of quanta of transmitter released per nerve impulse). NF1 increases muscle fibre input resistance by about 15%, which would increase the EJP by about 15% for a given synaptic current. DF2, however, does not alter input resistance and does not alter the sensitivity of muscle fibres to the transmitter, glutamate (Mercier et al., 2001).

At the superficial extensor muscles (SEM) of the crayfish abdomen, DF2 application results in postsynaptic modulation of muscle contraction which requires an influx of extracellular calcium (Quigley and Mercier, 1997). The calcium requirement of
these postsynaptic effects may reflect modulation of calcium channels in the plasma membrane or calcium-dependent release of calcium from internal stores or may alter the sensitivity of the contractile machinery to calcium influx (Quigley and Mercier, 1997).

**FaRP activated kinases in crayfish**

The mechanisms of synaptic modulation by DF2 have been investigated in the deep abdominal extensor neuromuscular preparation. Inhibition of CaMKII prevents DF2 from increasing EJP amplitude during the first 10-15 min. of peptide application (Noronha and Mercier, 1995). CaMKII activity appears to be involved in the initial modulatory effect of the peptide, as EJP amplitude increases to levels observed without CaMKII inhibition with 15 minutes after peptide application (Noronha and Mercier, 1995). Inhibition of protein kinase C (PKC) limits the duration of modulation by DF2, suggesting the involvement of this kinase in a later phase of modulation (Friedrich et al., 1998). The phosphodiesterase inhibitor, IBMX, potentiates the effect of the neuropeptide on EJP amplitude during the early phase of modulation, suggesting the involvement of cyclic nucleotides. Antagonists of the cyclic nucleotide-dependent kinases attenuate the effect of DF2, further suggesting that cyclic nucleotide-dependent kinases may be involved in the early phase of modulation (Mercier et al., 2001).

**Effects of Temperature**

In the deep abdominal extensor muscles (DEM) of the crayfish, neuropeptide DF2 increases EJP amplitude by increasing transmitter release (Skerrett et al., 1995). The effects of DF2 on the neuromuscular junction are temperature-dependent. Reducing the
bath temperature from room temperature to 8°C increases the effectiveness of the peptide, assessed as the percentage increase in EJP amplitude elicited by the peptide (Friedrich et al., 1994). Temperature also affects EJPs, which decrease in amplitude as temperature drops (Friedrich et al., 1994).

The temperature sensitivity of an event can be described quantitatively by calculating the temperature coefficient ($Q_{10}$), where a $Q_{10}$ of 2 represents a 2-fold increase in the rate of the event with a 10°C increase in temperature. Most biochemical events involving the breaking or forming of covalent bonds have $Q_{10}$'s between 2 and 3. Events involving multiple biochemical events have large $Q_{10}$'s (>5), whereas ion passage through a channel has a low $Q_{10}$ (approx. 1.5) as would be expected for diffusion through an aqueous medium (Hille, 1991; Klockner et al., 1990). Since protein kinase phosphorylation is a metabolic event, DF2 modulation (which involves kinases) should be reduced at a lower temperature. On the other hand, the effect of temperature on modulation by the peptide may be related to other factors. Since the EJP amplitude is smaller at low temperatures, there might be a greater store of synaptic vesicles available for release. Thus, at higher temperatures there might not be as much transmitter available for release, and this may limit the effectiveness of the peptide. If so, synaptic enhancement by any neuromodulatory substance may be inversely related to the initial size of the synaptic potential. These questions are investigated in Chapter 2, which demonstrates that lowering temperature reduces the amount of transmitter released from synaptic terminals and that lowered temperature and reduced extracellular Ca$^{2+}$ can both enhance the effectiveness of the peptide.
The sensitivity of modulation by DF₂ to changes in extracellular Ca²⁺ and the similarity of this effect with the effect of temperature (Chapter 2) suggests that the enhanced ability of DF₂ to modulate synaptic transmission may be related to effects on Ca²⁺ influx. At the squid giant synapse, reducing temperature results in reduced EJP amplitude by lowering transmitter release (Charlton and Atwood, 1979). The reduction in transmitter release results from a decrease in the calcium current in the presynaptic terminals, suggesting calcium influx is temperature-sensitive (Charlton and Atwood, 1979). Numerous experiments with voltage-gated calcium channels have determined that calcium influx through these channels is highly temperature-sensitive, with Q₁₀’s often above 5 (Allen, 1996; Allen and Mikala, 1998; McAllister-Williams and Kelly, 1995; McNaughton et al., 1998). Q₁₀ values for EJP amplitude and quantal content estimated in Chapter two, are used to link the temperature-dependent reduction in EJP amplitude in the crayfish preparation to a reduction in transmitter release.

**Drosophila FaRPs**

Another way to examine the relationship between synaptic modulation and the initial size of synaptic potentials is to examine effects of a peptide on neurons with inherently different levels of transmitter output. This approach is taken in Chapter 3, using neuromuscular synapses in *Drosophila* larvae, where some of the body wall muscles are innervated by motorneurons (neurons RP3 and 6/7b) that release either high or low amounts of neurotransmitter (Kurdyak et al., 1994; Lnenicka and Keshishian, 2000). The experiments tested the hypothesis that a FaRP enhances transmitter output more prominently at a low output synapse than at a high output synapse. Since virtually
nothing has been published regarding the intracellular messenger pathways mediating the effects of neuropeptides in Drosophila, the pathways that underlie synaptic modulation by a FaRP are also investigated in Chapter 3.

To investigate FaRP modulation in Drosophila, an appropriate endogenous FaRP must be chosen. Extensive examination of the Drosophila genome reveals three FaRP genes expressing 13 different neuropeptides (Vanden Broeck, 2001). The dFMRFa gene codes for eight of these peptides and two of these FaRP sequences occur multiple times on the gene (Hewes et al., 1998).

Expression of the dFMRFa gene is limited to seventeen types of neurons in the CNS, including interneurons and six Tv cells that express dFMRFa by the means of a unique enhancer (Benveniste and Taghert, 1999). These Tv cells have large somata within the thoracic ganglion, and heavily express the dFMRFa gene (Benveniste and Taghert, 1999). Tv cells have large unbranching axons that innervate a specialized group of mesodermal cells that together form the dorsal neurohemal organs. These contribute to neuroendocrine function by secreting modulators into the haemolymph (Luer et al., 1997). This expression pattern suggests that the peptide products of the dFMRFa gene are neurohormones that have targets within the CNS and in the periphery.

The expression patterns of three Drosophila FaRPs, SDNFMRFamide, TPAEDFMRFamide, and DPKQDFMRFamide have been monitored in the larval CNS using triple label immunofluorescence (McCormick et al., 1999). This work determined that each of the peptides (all expressed on the dFMRFa gene) has a unique expression pattern likely due to post-translational modification (McCormick et al., 1999). This
suggests that each FaRP has unique targets and likely different endogenous effects within the organism.

The most examined Drosophila FaRP is DPKQDFMRamide, for which there are five copies in the dFMRFa gene, more than any other FaRP in Drosophila (Benveniste and Taghert, 1999). DPKQDFMRamide slows heart rate in pupae and modulates synaptic transmission at the larval neuromuscular junction (Hewes et al., 1998; Johnson et al., 2000). Since DPKQDFMRamide is the most prominent FaRP on the Drosophila genome and has an expression pattern suggesting peripheral targets (such as muscle), it was chosen to assay modulation at the neuromuscular junction (Nichols et al., 1995).

**Genetic advantages of Drosophila**

The advantage of using Drosophila results from its wide use as a genetic model organism, with thousands of genetic mutant and transgenic lines. Drosophila mutations provide for the alteration of protein function (i.e. the mutation leads to a loss of function or temperature-sensitivity) or a loss of protein expression (i.e. the mutation leads to a null allele with no protein produced), which provides an added dimension through which to investigate protein function. Mutation of Drosophila genes can be accomplished by a variety of procedures, such as with chemicals, radiation, and P-elements (Ashburner and Novitski, 1976). The two mutant alleles used in this thesis are both null alleles, meaning that the mutation disrupts all protein production from the gene. Both recessive alleles are lethal when homozygous; therefore, heterozygotes are used to access the effects of gene mutation. Balancer chromosomes are used to maintain the lethal mutant alleles in heterozygous stocks. Balancer chromosomes generally are homozygous lethal, contain
dominant markers, and contain inversions preventing recombination. Balancer chromosomes maintain heterozygosity with minimal risk of losing the mutation to recombination.

To study the possibility that intracellular Ca\(^{2+}\) may mediate synaptic modulation by FaRPs in *Drosophila*, we selected stocks with alterations in IP\(_3\) receptors (intracellular calcium channels that release calcium from internal stores) and CaMKII (a calcium-dependent kinase). The *Drosophila* stock P\{ry[+t7.2]=PZ\}ltp-r83A\{05616\}ry[506]/TM3, ry[RK] Sb[1] Ser[1] contains one copy of a null allele of the inositol-1,4,5-triophosphosphate receptor gene ltp-r83A\(^{1664}\)\{05616\}, balanced as an heterozygote because of the lethality of the allele. The allele was generated by a P-element insertion in the 5′ untranslated region of the only known inositol-1,4,5-triphosphate receptor gene, *ltp-r83A* (Sinha and Hasan, 1999; Spradling et al., 1999). The stock y; l(4)16/ci\(^D\) contains only one functional *CaMKII* allele (Griffith et al., 1993; Joiner and Griffith, 1997). L(4)16 has a deletion which includes the gene for CaMKII, while ci\(^D\) maintains the heterozygous stock.

**Alteration of IP\(_3\)-Mediated Calcium Release**

*Drosophila* heterozygous for the null IP\(_3\) receptor allele 1664, transcribe approximately 50% less IP\(_3\) receptor mRNA in adult heads than in wild-type adult heads (Venkatesh and Hasan, 1997). *ltp-r83A\(^{1664}\)* compliments the lethality of another three *ltp-r83a* lethal alleles, confirming a reduction in IP\(_3\) receptor expression with the allele (Venkatesh and Hasan, 1997). The lethal IP\(_3\) receptor alleles have a characteristic delayed developmental phenotype, and many larvae fail to survive the molting stages
during larval development. The molting hormone, 20-hydroxyecdysone, can rescue this phenotype, suggesting that IP$_3$ is involved in larval metamorphosis and that IP$_3$-mediated signaling occurs during larval life (Venkatesh and Hasan, 1997). Unfortunately, no pharmacological agent specifically blocks IP$_3$-receptors. The sponge toxin Xestospongion C (XeC) (isolated from an Australian sponge *Xestosponge sp.* ) blocks IP$_3$-mediated calcium release by inhibiting IP$_3$-receptors and sarco(endo)plasmic reticulum calcium ATPase (SERCA) pumps (Castonguay and Robitaille, 2002; De Smet et al., 2002; Solovyova et al., 2002). SERCA pumps are one-way calcium pumps that buffer Ca$^{2+}$ from the cytosol and maintain elevated calcium levels within the ER (internal calcium stores)(for a review of SERCA pumps see (East, 2000)). IP$_3$-mediated calcium release can be amplified by recruiting further calcium release though Ca$^{2+}$-activated calcium channels to internal stores, the ryanodine receptors (see Racay et al. (1996) for a review of intracellular calcium stores in neurons). Antagonizing IP$_3$-receptors will prevent IP$_3$-mediated calcium release. Inhibiting SERCA pumps would lead to the emptying of internal calcium stores through passive leak, which would occlude subsequent IP$_3$-mediated calcium release as the ER would no longer have elevated calcium levels. As XeC is known to affect both IP$_3$-receptors and SERCA pumps, it should effectively prevent IP$_3$-mediated calcium release.

**Ca$^{2+}$/calmodulin-dependent protein kinase II**

Ca$^{2+}$/calmodulin-dependent protein kinase II is one of the best-characterized synaptic proteins, and is essential in a variety of presynaptic and postsynaptic pathways (for review see (Lisman et al., 2002)). This kinase has an autoinhibitory domain that
associates with the catalytic domain, preventing phosphorylation. CaMKII is activated when Ca$^{2+}$ bound calmodulin binds to the autoinhibitory domain, removing inhibition of the catalytic domain. The exposed catalytic domain can then phosphorylate protein targets or its own autophosphorylation site. Autophosphorylation gives the kinase partial activity (often up to 80%) even in the absence of Ca$^{2+}$/ calmodulin (Lisman et al., 2002).

Postsynaptic CaMKII activity is required for long-term potentiation (LTP) an activity-dependent form of modulation thought to underlie some forms of learning and memory (Leonard et al., 1999). Paired-pulse facilitation, another activity dependent form of synaptic modulation, requires presynaptic CaMKII activity (Chapman et al., 1995; Silva et al., 1996). CaMKII has long been known to phosphorylate synapsin, which can lead to an increase in transmitter release (Llinas et al., 1985; Nicol, 1996). Paradoxically, CaMKII can also decrease transmitter release immediately following tetanic stimulation, as demonstrated in αCaMKII mutant mice which show an increase in transmitter release following a tetanus (Chapman et al., 1995).

**CaMKII inhibition in Drosophila**

Inhibition of CaMKII activity can be achieved with a variety of pharmacological agents that have different potencies, sites of action, and specificity. KN-93 is a potent inhibitor of CaMKII ($K_i=370\text{nM}$), as KN-93 disrupts the association of Ca$^{2+}$ bound calmodulin to the kinase, with no significant effects on cAMP-dependent protein kinase, Ca$^{2+}$/ phospholipid-dependent kinase, myosin light chain kinase, or Ca$^{2+}$-activated phosphodiesterase (Sumi et al., 1991). Though KN-93 is highly selective in its inhibitory action on kinases, recent evidence demonstrates that this compound also affects K$^+$
channels, which can affect synaptic transmission. KN-93 inhibits voltage-dependent $K^+$ channels in myocytes in a dose-dependent manner. This selectivity problem can be overcome by using the inactive analog of KN-93, KN-92. KN-92 also affects $K^+$ channels, but does not inhibit CaMKII activity (Ledoux et al., 1999). The KN-93 analog KN-92 does not affect CaMKII activity; therefore, it is a valuable control agent for at least one non-specific effect of KN-93.

The *Drosophila* mutation l(4)16 deletes the *CaMKII* gene, and is homozygous lethal (Hochman, 1971; Joiner and Griffith, 1997). The fly stock y; l(4)16/Ci is a balanced heterozygous stock that has 50% less *CaMKII* gene product with 60% the CaMKII activity of wild-type flies (Joiner and Griffith, 1997; Pla et al., 1997). This mutant provides a unique method of CaMKII inhibition, reducing protein density rather than affecting kinase catalytic activity.

The transgenic fly stock ala1 (w P[w⁺ ala]) is homozygous for a P-element insertion containing the sequence encoding a peptide inhibitor of CaMKII on chromosome 1. The inhibitory peptide was fashioned after a sequence containing the autoinhibitory region and part of the calmodulin-binding domain of the rat αCaMKII (a.a. 273-301) (Griffith et al., 1993). An exception occurs where the would-be autophosphorylated threonine (a.a. 286) is replaced with an alanine; this slightly increases the inhibitory affinity of the peptide from $IC_{50}=40\mu M$ to $13\mu M$ (Griffith et al., 1993) (Figure 1.1). The alanine inhibitory peptide is selective for CaMKII, with an $IC_{50}=109\mu M$ for protein kinase C and $IC_{50}>5mM$ for cAMP-dependent protein kinase. The alanine inhibitory peptide is under the control of the *hsp70* heat-shock promoter, which was inserted on the same p-element to allow for inducible inhibition of CaMKII
Figure 1.1 CaMKII is composed of two general domains, the catalytic domain and the autoinhibitory domain. In the inactive state, the pseudosubstrate sequence on the autoinhibitory domain prevents the catalytic S site from being exposed to substrate. Ca$^{2+}$/calmodulin binding to the autoinhibitory domain removes inhibition from the S site and exposes the T site, where autophosphorylation of the kinase can occur to enhance Ca$^{2+}$ independent activity. The alanine inhibitory peptide is fashioned after the pseudosubstrate sequence, thereby inhibiting the catalytic domain even in the presence of Ca$^{2+}$/calmodulin. The alanine inhibitory peptide contains the T site, except that threonine is replaced with alanine. Image modified from Lisman et al. (2002).
(Griffith et al., 1993). At 25°C the average concentration of alanine inhibitory peptide in 
ala1 flies is 3.9μM, which is below the IC50 for CaMKII (Griffith et al., 1993).

Therefore, some basal inhibition of CaMKII is constantly occurring even at room 
temperature. Ala1 flies have a substantial reduction (50-70%) in the phosphorylation 
levels of two proteins of 30kD and 33kD as compared to untransformed siblings. Heat-
shock of ala1 flies for 1 hour at 37°C reduces the phosphorylation of these two proteins 
by a further 40-60% (Griffith et al., 1993). Chronic inhibition of CaMKII in ala1 larvae 
(three days after an hour of heat-shock) results in an increased number of active zones, 
terminal size, and abnormal terminal spouting of neurons RP3 and 6/7b in muscles of 
third instar larvae at room temperature (Wang et al., 1994). The ala2 larvae contain the 
same alanine inhibitory peptide at a different insertion site (on chromosome 2) which 
alters its expression at room temperature(Griffith et al., 1993). Ala2 larvae have greater 
basal expression (at 25°C) of the alanine inhibitory peptide and show the above 
alterations in terminal morphology even without heat-shock(Wang et al., 1994). The 
lower levels of basal expression in ala1 flies make them more useful than ala2 for the 
purposes of this thesis, as most of the major consequences to synaptic development 
caused by chronic CaMKII inhibition are avoided, and induced expression of the 
inhibitor with heat-shock would likely be more pronounced.

Inhibition of CaMKII with the mutant or with ala1 (ala2) both result in 
impairment of the learning phase of courtship conditioning, an assay of associative 
learning (Joiner and Griffith, 1997). However, only inhibition with the alanine inhibitory 
peptide affected the memory phase of courtship conditioning (Joiner and Griffith, 1997). 
The levels of alanine inhibitory peptide expressed in these experiments were likely well
Known

level of expression after heat-shock (e. the concentration of the inhibitor) is vaguely
conceivably affect complex pathways. The dual peptides is specific but unfortunately the
protein during development may have consequences to synaptic function, which could
remain due to the wild-type allele in heterozygotes. Furthermore, the loss of CAMKII
selective means of reducing the enzyme's activity, but functional CAMKII protein
but not as specific as the alanine inhibitory peptide. The CAMKII mutant is the most
Each method of inhibiting CAMKII has its pros and cons. KN-93 is very potent,
other CAM kinases.
more specific to the gene product of CAMKII, whereas inhibition with glutarimide
strokes have reduced CAMKII activity. Inhibition of the kinase in the mutant is likely
Prosopisphaera, CAMKII is involved in memory, and that both the mutant and the transgenic
nonassociative learning and sensitization (Githih, et al., 1993). It seems likely that in
mal and alas these show improvements in levels of acoustical priming, a measure of
derependent protein kinases (Githih, et al., 1993; Jones and Githih, 1997). Furthermore,
below the ranges of PKC inhibition, but they may be affecting other C± / calmodulin

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Chapter 2

Synaptic Modulation by a Neuropeptide Depends on Temperature and Extracellular Calcium

T.W. Dunn & A.J. Mercier
Abstract

The crayfish neuropeptide DRNFLRFamide increases transmitter release from synaptic terminals onto the deep abdominal extensor muscle fibres. As temperature decreases from 20°C to 8°C, the size of excitatory junctional potentials (EJPs) decreases, and the peptide becomes more effective at increasing EJP amplitude (Friedrich et al., 1994). The goal of the present study was to determine whether the enhanced effectiveness of the peptide is strictly a temperature-related effect, or whether it is related to the fact that the EJPs are smaller at low temperature, allowing a greater range for EJP amplitude to increase. Decreasing temperature reduced the number of quanta of transmitter released per nerve impulse (assessed by recording synaptic currents) and increased input resistance in muscle fibers. As in earlier work, the ability of the peptide to increase EJP amplitude was enhanced by decreasing temperature. However, the peptide was also more effective at increasing EJP amplitude when transmitter output was lowered by reducing the ratio of calcium to magnesium ions in the bath. Thus, the effectiveness of the peptide may be related to the level of output from the synaptic terminals.
Introduction

Neuropeptides play important roles in mediating and modulating transmission at chemical synapses. One major "family" of neuropeptides consists of those structurally related to FMRFamide (Phe-Met-Arg-Phe-NH$_2$), a tetrapeptide originally isolated from a mollusk (Price and Greenberg, 1977). FMRFamide-like peptides are present in every animal phylum (Greenberg and Price, 1992) and have a variety of physiological effects, including modulating chemical synapses (e.g. (Baux et al., 1992; Cottrell et al., 1992; Jorge-Rivera et al., 1998; Man-Son-Hing et al., 1989).

The FMRFamide-like peptide, DF$_2$ (DRNFLRFamide) is found in the pericardial organs of crayfish and is thought to be released into the circulation as a neurohormone (Mercier et al., 1993). This peptide increases the amplitude of excitatory junctional potentials (EJPs) in crayfish muscles (Mercier et al., 1993; Skerrett et al., 1995). This effect has been studied most extensively in deep extensor muscles (DEMs) of the crayfish abdomen. The neuropeptide increases the number of quanta of transmitter released per nerve impulse but does not alter muscle fiber input resistance or responsiveness to ionophoretic application of glutamate (Mercier et al., 2001; Skerrett et al., 1995). Thus, the enhancement of EJP amplitude appears to involve presynaptic rather than postsynaptic mechanisms. Pharmacological studies suggest that the effect requires the activity of calcium/calmodulin-dependent protein kinase (Noronha and Mercier, 1995), protein kinase C (Friedrich et al., 1998) and cyclic nucleotide-dependent protein kinases (Mercier et al., 2001).

One interesting aspect of DF$_2$'s ability to modulate transmitter release is its apparent temperature-dependence. In the crayfish DEMs, DF$_2$ is more effective at
enhancing EJP amplitude at lower temperatures (Friedrich et al., 1994). The functional
significance of this temperature dependence and the mechanisms that underlie it are not
known. As temperature drops, EJP amplitude decreases in the DEMs (Friedrich et al.,
1994) and in the deep abdominal flexor muscles (Czernasty and Bruner, 1980). The
enhanced capacity for DF₂ to increase EJP amplitude might help to compensate for
reduced synaptic transmission at low temperatures.

Here we investigate mechanisms underlying the temperature-dependence of DF₂’s
effect on synaptic transmission. Because lowering the temperature reduces EJP
amplitude, it seems likely that the number of quanta of transmitter released also decreases
under these conditions. Such a reduction in transmitter output may simply increase the
range through which the peptide can increase transmitter release. The present study
confirms that quantal content, a direct measure of transmitter release, decreases with
temperature. It also shows that reducing the Ca²⁺/Mg²⁺ ratio, which would decrease
calcium influx, enhances DF₂’s ability to modulate synaptic transmission.
Methods

Crayfish (*Procambarus clarkii*), approximately 5-8cm long, were obtained from Atchafalaya Biological Supply Co. (Raceland, La.) and were kept in large holding tanks at 14-16°C. The freshwater in the tanks was aerated, circulated and filtered. The crayfish were fed Tender Vittles™ dry cat food. Immediately before dissection, the crayfish were cooled in ice and euthanized by destruction of the cerebral and thoracic ganglia. The dorsal part of the abdomen, containing the DEMs (Parnas and Atwood, 1966), was dissected away and was secured in a 1.0 ml recording chamber that was perfused continuously with crayfish saline. Temperature was regulated by cooling the saline and monitored with a digital thermometer (±0.05°C). The crayfish saline was modified from that of Van Harreveld (1936). Most experiments were performed in a standard “low calcium, high magnesium” saline to prevent muscle twitching (Mercier and Atwood, 1989). This saline had the following constituents (in mM): NaCl 200.7, KCl 5.36, CaCl$_2$ 6.5, MgCl$_2$ 12.3, HEPES 5.0; pH 7.4. In other experiments, the concentration of CaCl$_2$ was decreased to 4.2 mM, and the MgCl$_2$ concentration was raised to 14.9 mM to reduce transmitter output more drastically.

EJPs were elicited in muscle L1 of the fourth abdominal segment by stimulating excitatory axon 3 in the third abdominal segment, using methods described elsewhere (Mercier and Atwood, 1989; Skerrett et al., 1995). Stimuli were applied at 0.2 Hz using a Grass S88 stimulator and a Grass stimulus isolation unit (SIU). Postsynaptic potentials were recorded with glass microelectrodes filled with 3M KCl. EJPs were monitored on a digital storage oscilloscope and were acquired on an IBM-compatible computer using a computerized data acquisition system designed and constructed by the Electronics
Division at Brock University. The sampling frequency for the acquisition system was 10 KHz. Signals were averaged every 30 s, so that the EJP acquired represented the average of 6 responses. EJP amplitudes were measured and compiled using software developed by Mr. Tom MacDonald at Brock University. EJPs were corrected for non-linear summation (Martin, 1955) as in previous experiments e.g. (Mercier and Atwood, 1989; Skerrett et al., 1995).

Quantal synaptic currents were recorded using glass macropatch electrodes approximately 10 µm in diameter, as described elsewhere (Dudel, 1982; Mercier and Atwood, 1989). Electrodes were placed on the surface of the muscle fibers at locations that optimized the clarity and size of a single quantal current. Because it was impossible to count individual quanta at higher temperatures, quantal content was determined using the “failures method” described by del Castillo and Katz (1954). According to this method, quantal content (m) can be estimated as:

\[
m = \ln \left( \frac{N}{N_0} \right),
\]

where \(N\) is the number of stimuli, and \(N_0\) is the number of failures. The assumption underlying this method is that the frequency of quantal releases can be fitted by a Poisson distribution. This assumption is valid only when transmitter output is low. When quantal content is higher (>1.0), quantal release is fitted better by a binomial distribution, and the failures method underestimates quantal content (e.g. (Johnson and Wernig, 1971). For these reasons, recording sites were selected based on failure rate. In experiments where temperature was raised, sites were selected if the quantal failure rate was >70% at the low starting temperature; in experiments where temperature was lowered, sites were chosen if failure rate was <30% at the high starting temperature. Thus, the temperature sensitivity
of quantal content was assessed from temperatures where m<1. Simultaneous intracellular recording in the muscle fibre allowed verification of the post-synaptic response to stimulus pulses, so that a failed quantal current represents failed release at the monitored bouton and not sub-threshold stimulation of the axon. The failures method was used in preference to methods that involve comparing averaged responses to spontaneous quanta because spontaneous releases are infrequent in the DEMs and in at least some cases appear to indicate damage to the nerve terminals in this preparation. To insure that estimates of quantal content were accurate, the results from at least 50 stimuli were used.

Johnson and Wernig (1971) showed that at crayfish neuromuscular junctions, the Poisson calculation overestimates m by 4-39% when estimated values of p, the release probability, are between 0.1 and 0.5. Although we sought conditions where p < 0.1, we could not estimate p reliably in the present experiments. One can approximate p from the relation:

$$m = np,$$

where m is the number of quanta released per nerve impulse, and n is the number of active zones. Morphological studies indicate that crustacean synapses can have 20-100 active zones in the vicinity of a macropatch electrode of the size we used (Atwood and Tse, 1988; Mgshina et al., 1998). Using these estimates, a quantal content of 0.36 (which corresponds to ln(N/No) with a 70% failure rate), would yield p values in the range of 0.004 to 0.02. A 70% failure rate, therefore, was considered sufficient to satisfy the criterion that p is sufficiently low to estimate m from the Poisson relation. Lower failure rates (as occurred at 17.5°C in the present study) overestimate m (see Discussion).
Another way to address the question of whether the synaptic changes occur presynaptically or postsynaptically involves calculating the coefficient of variation (CV) associated with EJP fluctuations. This method does not assume that the data fit a Poisson distribution. CV is calculated from the relation

\[ CV = \frac{s_{\text{EJP}}}{\text{mean EJP}}, \]

where \( s_{\text{EJP}} \) is the standard deviation associated with EJP fluctuations. As \( m \) increases, CV decreases (Martin, 1966). Factors that increase EJPs through presynaptic mechanisms decrease CV. Presynaptic and postsynaptic effects of an experimental treatment can be distinguished by comparing two ratios:

(4) \[ r = \frac{CV^2_{\text{before}}}{CV^2_{\text{after}}}, \]

and

(5) \[ \pi = \frac{M_{\text{after}}}{M_{\text{before}}}, \]

where \( CV^2_{\text{before}} \) and \( CV^2_{\text{after}} \) are, respectively, the squares of the coefficients of variation before and after treatment, and \( M_{\text{after}} \) and \( M_{\text{before}} \) are, respectively, the mean EJP amplitudes after and before treatment (Faber and Korn, 1991). A plot of \( r \) vs. \( \pi \) will yield a positive slope if the treatment increases EJPs through presynaptic mechanisms. The same plot, however, will produce a horizontal line where \( r = 1 \) if EJPs increase only through postsynaptic changes (Faber and Korn, 1991).

Input resistance in the muscle fibers was measured using two microelectrodes inserted approximately 50\( \mu \text{m} \) apart into the same fibre. One electrode was used to inject electrical current while the other was used to record voltage.
The temperature sensitivity of EJP amplitude, quantal content, and muscle fibre input resistance was assessed by calculating a $Q_{10}$ value for each of these variables. $Q_{10}$, the temperature coefficient was calculated using the van't Hoff equation:

$$Q_{10} = \left(\frac{k_2}{k_1}\right)^{10/(T_2-T_1)},$$

where $k_1$ and $k_2$ are mean values for a given variable at temperatures $T_1$ and $T_2$. Thus, a $Q_{10}$ of 1 represents no temperature sensitivity in variable $k$, and a $Q_{10}$ of 3 indicates a three-fold increase in $k$ with an increase in 10°C.

All values represent means ±SEM, and statistical comparisons were made using a Mann-Whitney U test unless otherwise noted.
Results

EJP amplitude and quantal content (measured at a stimulus frequency of 0.2 Hz) were both affected by changes in the bath temperature between 8°C and 20°C. Figure 2.1A displays a representative trial in which the temperature was reduced from 20°C to 8.5°C while EJP amplitude and the quantal synaptic currents were monitored simultaneously. Quantal content (determined from the percentage of failures) and EJP amplitude both decreased as temperature decreased. The effect on quantal content was more pronounced than the effect on EJP amplitude in this example. This experiment was performed with a total of six preparations. Temperature was lowered in half the preparations and raised in the other half to ensure that the reduction in quantal content with decreased temperature was not the result of changes in muscle tonus that might move the macropatch from the original recording site. Mean EJP amplitudes, the percentage of failures and mean values for quantal content for all 6 preparations are shown in Figures 2.2A, 2.2B and 2.2C, respectively. As temperature decreased from 17.5°C to 10°C, mean EJP amplitude decreased significantly (P<0.05), the mean percentage of failures decreased significantly (p<0.005), and mean quantal content decreased significantly (P<0.005). The mean Q_{10} value associated with changes in EJP amplitude (averaged from individual trials) was 3.46±0.52, and the mean Q_{10} for changes in quantal content was 7.62±1.49.

The decrease in estimated quantal content suggests that the effects of temperature on EJP amplitude are mainly presynaptic, attributable to changes in transmitter output from the synaptic terminals. To obtain corroborative evidence for a presynaptic effect,
Figure 2.1. The relationship between temperature and EJP amplitude, quantal content, and muscle fibre input resistance. (A): In this representative trial, EJP amplitude (corrected for non-linear summation) and quantal content (calculated using the failures method) increased as temperature increased. The large jump in quantal content above 17°C is a result of the over estimation of the failures method when m>1 (for explanation of this see (Johnson and Wernig, 1971)). The inset box shows representative traces of single EJPs at two temperatures 20°C (upper trace) and 10°C (lower trace). As observed in earlier work, reducing temperature increased the latency and slowed the time course of the EJP (Friedrich et al., 1994). Bars: 20mV, 5msec. (B): Muscle fiber input resistance decreased as temperature was increased. This representative trial shows the gradual decrease in input resistance to a 100nA hyperpolarizing current pulse as temperature is increased by 12°C.
Figure 2.2. The effect of lowering temperature on EJP amplitude, the % failures, quantal content, and the relationship between CV and EJP amplitude. (A): EJP amplitude was significantly decreased (p<0.05) by a reduction in temperature from 17.5°C to 10°C. The average EJP amplitude at 17.5°C and 10°C was measured in six preparations. (B): The average percentage of failures at two temperatures in the six preparations used to determine quantal content. The percentage of failures was significantly higher (p<0.005) at 10°C than at 17.5°C. (C): Quantal content, estimated from at least 50 stimuli using the failures method, was significantly reduced (p<0.005) between 17.5°C and 10°C (n=6 preparations). These two temperatures were chosen because quantal currents were measured at these two temperatures in all preparations and m<1. (D): The relationship of the ratio of the coefficients of variation squared for EJP amplitude at two different temperatures is plotted against the modification factor, which is the ratio of the mean EJP amplitudes at the two temperatures. Notice the points follow a positive slope and in all cases r>π.
the coefficient of variation (CV) associated with EJP amplitudes was calculated for each of the 6 trials in which temperature was altered (Table 1). In each trial, CV was lower at 17.5°C than at 10°C, suggesting enhancement of transmitter output at the higher temperature. We also plotted r (the ratio of CV² values) vs. π (the ratio of mean EJP amplitudes) for each trial (see “Methods”). The data from our six temperature trials (Figure 2.2D) fell within an area where the slope is greater than 1 (i.e. r > π). This result is predicted when EJP amplitude is enhanced by presynaptic mechanisms and not by postsynaptic mechanisms (Faber and Korn, 1991).

In another set of trials (6 preparations), muscle fibres were penetrated with two microelectrodes, and input resistance was measured as temperature was changed. Data from a representative trial are illustrated in Figure 2.1B. As with EJP amplitude and quantal content measurements, temperature was changed in both directions, from 20°C to 8°C and from 8°C to 20°C. The Q₁₀ value associated with changes in input resistance was −1.82±0.25; the negative value indicates that input resistance increased with decreasing temperature. Czternasty and Bruner, (1980) also reported that input resistance of crayfish muscle fibers increased when temperature was lowered from 19°C to 9°C, and they obtained a Q₁₀ value equivalent to −2.1. The increase in input resistance with decreasing temperature probably helps to compensate somewhat for the reduction in quantal content and explains why the Q₁₀ value for the effect on EJP amplitude is lower than that for quantal content.

As reported in Friedrich et al. (Friedrich et al., 1994), DF₂ is more effective at increasing EJP amplitude at lower temperatures. Figure 2.3A shows the effect of 200nM DF₂ on deep abdominal extensor muscle L1 at two different temperatures, 12°C and
Table 1. The coefficient of variation for EJP amplitude is shown for each trial at the two temperatures. In all cases the CV is larger at the lower temperature.

<table>
<thead>
<tr>
<th>Trial #</th>
<th>CV$_{17.5^\circ C}$</th>
<th>CV$_{10^\circ C}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.677</td>
<td>20.929</td>
</tr>
<tr>
<td>2</td>
<td>6.703</td>
<td>10.084</td>
</tr>
<tr>
<td>3</td>
<td>6.819</td>
<td>18.332</td>
</tr>
<tr>
<td>4</td>
<td>3.777</td>
<td>5.808</td>
</tr>
<tr>
<td>5</td>
<td>2.803</td>
<td>7.696</td>
</tr>
<tr>
<td>6</td>
<td>17.933</td>
<td>44.202</td>
</tr>
</tbody>
</table>
20°C. (In this experiment, the Ca$^{2+}$ and Mg$^{2+}$ concentrations of the saline were 4.2 mM and 14.9 mM, respectively.) EJP amplitude increased by approximately 80% at 12°C and by approximately 30% at 20°C. Actual EJP amplitudes increased from 2.6±0.5 mV to 4.2±0.7 mV at 12°C and from 9.6±3.5 mV to 11.6±6.3 mV at 20°C. The EJP amplitudes observed in the presence of DF$_2$ at 12°C were significantly lower (p<0.05) than the EJP amplitudes prior to peptide application at 20°C. The initial rate of increase in EJP amplitude appeared to be similar at both temperatures, which is surprising because decreasing temperature generally slows the rate of biochemical and physiological processes. However, the maximal increase in EJP amplitude occurred slightly earlier at 20°C (7.5 min) than at 12°C (9.5 min), and the effect of the peptide appeared to decline slightly at 20°C but not at 12°C. A decline in the effect of DF$_2$ on EJPs has been observed previously at 12°C, but only after approximately 15 min (Noronha and Mercier, 1995). The mechanisms underlying this decline are not known, but the data suggest that raising the temperature from 12°C to 20°C speeds up some of the subcellular processes activated by DF$_2$.

To further examine the possibility that the enhanced effect of the peptide at low temperatures is directly related to reduced transmitter output, EJPs were raised slightly by increasing the ratio of Ca$^{2+}$ to Mg$^{2+}$ ions in the saline. Saline containing 6.5 mM Ca$^{2+}$ and 12.3 mM Mg$^{2+}$ was used in these experiments (which were performed at 12°C). Raising the Ca$^{2+}$/Mg$^{2+}$ ratio in this manner increased the initial EJP amplitude and reduced the effectiveness of the peptide (expressed as the percentage change in EJP amplitude). The mean EJP amplitude during the 8 minute period immediately prior to peptide application was 6.3±1.5 mV in 6.5 mM Ca$^{2+}$/12.3 mM Mg$^{2+}$. This was
Figure 2.3. Increasing temperature and/or extracellular calcium levels reduced the percentage increase in EJP amplitude caused by 200nM DF2. (A): The effect of temperature. EJP amplitude is expressed as the percentage change from the average EJP amplitude prior to peptide application. The Ca\(^{2+}\)/Mg\(^{2+}\) concentrations are 4.2mM and 14.9mM respectively. (n=7 for both 20°C and 12°C; error bars are standard errors of the mean.) (B): The effect of extracellular calcium. These trials were conducted at 12°C and as in Figure 2.3A, EJP amplitude is expressed as the percentage change from the average amplitude during the pre-peptide period. (n=7 for 4.2mM Ca\(^{2+}\) saline and 6 for 6.5mM Ca\(^{2+}\) saline.)
significantly larger than the mean EJP amplitude over the same time period in 4.2 mM Ca\(^{2+}\)/14.9 mM Mg\(^{2+}\) (2.6±0.5 mV; P<0.05). Following application of DF\(_2\) in the higher Ca\(^{2+}\) saline, EJPs increased by approximately 30\% (Figure 2.3B) to an average value of 8.1±2.0 mV. A comparison of data from the two saline solutions indicates that increasing the extracellular Ca\(^{2+}/\)Mg\(^{2+}\) ratio increases the initial EJP amplitude and reduces the response to the peptide (Figure 2.4A).

The effects of DF\(_2\) at the two different temperatures and Ca\(^{2+}/\)Mg\(^{2+}\) ratios are summarized in Figure 2.4A. The percentage increase in EJP amplitude was calculated by comparing EJPs 5-8 minutes after applying DF\(_2\) with EJP amplitudes averaged over the 3-minute period immediately before peptide application. Reducing temperature and reducing the Ca\(^{2+}/\)Mg\(^{2+}\) ratio have similar effects on the ability of DF\(_2\) to enhance transmitter output. Decreasing temperature and decreasing the Ca\(^{2+}/\)Mg\(^{2+}\) ratio both significantly (p<0.05) enhanced the percentage increase in EJP amplitude elicited by DF\(_2\). DF\(_2\) elicited the largest percentage increase in EJP amplitude in the lower calcium saline at the lower temperature, when the two factors were combined.

The similarity between the effects of changing temperature and Ca\(^{2+}/\)Mg\(^{2+}\) levels suggests that the effectiveness of DF\(_2\) is inversely related to initial EJP amplitude. To test this idea, data from both experiments were combined and plotted in Figure 2.4B. There was a significant, negative correlation between the percentage increase in EJP amplitude elicited by DF\(_2\) and the initial EJP amplitude (r = -0.417; N=32; p<0.05). The data suggest that the effectiveness of the peptide is inversely related to the log of the initial EJP amplitude.
Figure 2.4. Reducing temperature and extracellular calcium levels both lead to greater modulation by DF_2. (A): Mean values of the increase in EJP amplitude measured between 5 and 8 minutes after peptide application. Amplitudes in this period are expressed as the percentage change from the average EJP amplitude during the pre-peptide period. The trials at low temperature and in the low calcium saline are significantly different from all the other trials. (Error bars depict standard errors of the mean; n=8,7,6,7 from left to right.) (B): Initial EJP amplitude is inversely related to the effectiveness of DF_2 to increase transmitter release. The initial EJP amplitude of each trial was plotted with the percent change (increase) in EJP amplitude to 200nM DF_2 as compared to the pre-peptide period. This trend is significant (r = -0.417; N=32; p<0.05), and the data are fit best by the complex function, y=-0.1393Ln(x) + 0.5984.
An alternative method for reducing the initial EJP amplitude was explored. In some crustacean muscles, stimulating the axon repeatedly at low frequencies (e.g. 0.2 Hz or lower) causes a reduction in EJP amplitude, referred to as low frequency depression (Bruner and Kennedy, 1970; Bryan and Atwood, 1981; Zucker and Bruner, 1977). Such low frequency depression is thought to be presynaptic in origin, resulting from a gradual reduction in transmitter output. Two sets of trials were conducted at room temperature to determine whether this method of reducing transmitter release would also enhance the ability of DF2 to increase transmitter output. In one set of preparations (n=6), the nerve was stimulated for approximately 30 minutes before DF2 was applied. This decreased EJP amplitude from 11.1±1.7 mV to 5.9±0.9 mV before peptide application. In a separate set of preparations (n=6), the nerve was stimulated for only 8 minutes before applying the peptide, causing very little low frequency depression. (In this case, the EJPs decreased from 9.8±2.0 mV to 9.1±0.4 mV.) The initial EJP amplitudes prior to any depression (at the beginning of each trial) were not significantly different between the two sets of trials (p>0.05). Figures 2.5 A and B show the trial with the greatest amount of LFD prior to peptide application matched with a trial of the same initial EJP amplitude (Figure 2.5A), even in this trial the effect of DF2 is unaltered (Figure 2.5B). Figure 2.5A shows actual changes in EJP amplitude (in mV), and Figure 2.5B shows the effectiveness of DF2, expressed as the percentage change in EJP compared to the average amplitude recorded 3 minutes before peptide application. In the example shown in Figure 2.5A, the peptide increased EJP amplitude from approximately 17 mV to approximately 24 mV when there was little or no low frequency depression and from approximately 8 mV to approximately 12 mV after substantial low frequency depression. The percentage
A

EJP amplitude (mV)

-40 -30 -20 -10 0 10 20 30 40
Time from DF$_2$ application (min)

- 9mV of depression
- 1 mV of depression

B

% change in EJP amplitude

-40 -30 -20 -10 0 10 20 30 40
Time from DF$_2$ application (min)

- 9mV of depression
- 1 mV of depression
Figure 2.5. Tonic stimulation gradually reduces EJP amplitude but does not alter the percentage increase in EJP amplitude caused by DF2. (A): In this figure the y-axis intersects at the point of peptide application in both trials; this is done to compare the effect of the peptide between the two trials. Both of these representative trials begin at similar EJP amplitudes and both are clearly modulated by DF2. One trial exhibits a 9mV reduction in EJP amplitude prior to DF2 application. (B): When the same trials as in 5A are compared by expressing EJP amplitude as a percentage change of the average EJP amplitude between 0-3 minutes before peptide application, there is no apparent difference in the peptide’s effects. (C): The two groups have no significant difference (P>0.05) in the percent increase in EJP amplitude 5-8 minutes after DF2 application as compared to the 3 minute control period. (n=6 for each group and the bath temperature was 22°C for all trials.)
increase in EJP amplitude between the two trials was comparable (Figure 2.5B). Data from 6 trials in each group were combined (Figure 2.5C). The percentage increase in EJP amplitude 5-8 minutes after peptide application was not significantly different between the two groups (p>0.05).
Discussion

These experiments confirm earlier observations that DF2 increases EJP amplitude more effectively (when expressed as percentage change) at low temperature (Friedrich et al., 1994). These experiments also confirm that decreasing temperature reduces EJP amplitude, and provide direct evidence that this is caused by a reduction in transmitter release presynaptically. An increase in input resistance compensates somewhat for reduced quantal content at low temperatures, but EJP amplitude remains highly temperature dependent (Figure 2.1). This suggests that one of the physiological roles of the neuropeptide DF2, may be to compensate for reduced transmitter output when temperature is reduced. This idea is supported by observations that the pericardial organs of spiny lobsters, which contain and release peptides homologous to DF2, fire impulses when temperature is reduced from 20°C to 14°C (Kuramoto and Tani, 1994).

The reduction in quantal content with temperature is probably the result of reduced calcium influx through voltage-gated calcium channels in the synaptic terminals. The temperature coefficient quantifies the sensitivity of an event to a change in temperature of 10°C. Biochemical events involving changes in covalent bonding have Q10's generally between 2-3, as do many physiological processes (Purves et al., 1995). The method used for estimating m may contribute somewhat to the high mean value (Q10>7.0) and large variance in the Q10 for quantal content. The failures method tends to overestimate quantal content when release levels are high and the data do not fit a Poisson distribution. This would tend to make estimated values of m excessively high only at the higher temperatures (e.g. at temperatures above 17°C in Figure 2.1A). Such an effect would exaggerate the changes caused by temperature and probably contributes
to the high $Q_{10}$ values for $m$ and for the high variance associated with them. However, even at 17.5°C, values of $m$ approached 1.0 but did not exceed it. Using data from studies where $m$ was estimated by directly counting quanta (Mercier and Atwood, 1989; Johnson and Wernig, 1971; Zucker, 1973), $\ln(N/N_o)$ overestimates $m$ by 5-25% when $\ln(N/N_o)$ is between 0.7 and 1.0. Even a 25% overestimate of $m$ cannot account for the 200% increase in $m$ that we observe when temperature increases from 10°C to 17.5°C (Figure 2.2C). Thus, it is unlikely that the high $Q_{10}$ values are wholly the result of overestimating $m$. In fact, since input resistance increased with a drop in temperature, the effect of temperature on $m$ should be more pronounced than for EJP amplitude.

Calcium passage through the pore-forming region of an open calcium channel has little temperature sensitivity ($Q_{10}$ of roughly 1.5; Klockner et al., 1990)) as would be expected for diffusion through an aqueous medium (Hille, 1991). However, Charlton and Atwood (1979) reported a large reduction in EPSP amplitude at the squid giant synapse that corresponded to a reduction in the presynaptic calcium current. The temperature-sensitivity of the calcium currents appears to be due to temperature-dependent changes in the calcium channels. The major temperature-sensitive components are not yet fully understood, but probably involve interaction between channel subunits, interaction between calcium channels and SNARE proteins and phosphorylation state of the calcium channels (Allen, 1996; Allen and Mikala, 1998; Bunemann et al., 1999; Wiser et al., 1996; Wiser et al., 1997). Some of the kinetic parameters of calcium channels have large $Q_{10}$'s, well above 3 (Allen, 1996; McAllister-Williams and Kelly, 1995; McNaughton et al., 1998), suggesting that the high temperature-sensitivity of calcium channels results from the coupling of multiple metabolic events (Morris and Clarke, 1981). Thus, the
high Q_{10} values for quantal content in the present study probably reflect high
temperature-sensitivity of the calcium channels to some extent.

Quantal content (m) is determined by two variables (Del Castillo and Katz, 1954):
n, the average number of synaptic vesicles ready for release, and p, the average
probability of a single quantum being released (m = n·p). Thus, DF\textsubscript{2} could potentially
increase transmitter release either by increasing p or by increasing n. An increase in p
would be brought about by increasing calcium influx into the synaptic terminals, by
releasing calcium from intracellular stores or by increasing the sensitivity of the secretory
apparatus to intracellular calcium. An increase in n would be brought about by
increasing the number of vesicles that are docked at active zones or the number of active
zones.

It is well established that decreasing the extracellular Ca\textsuperscript{2+} level reduces quantal
content and EJP amplitude by reducing calcium influx (e.g. (Augustine and Charlton,
1986; Dodge and Rahamimoff, 1967; Dudel, 1981)). The similarities between the effects
of reducing temperature and reducing extracellular calcium suggest that these two
treatments enhance the ability of DF\textsubscript{2} to increase transmitter release via a common
mechanism. Since both these treatments are likely to reduce quantal content by lowering
calcium influx, it seems likely that the enhanced effectiveness of the peptide is related to
a reduction in calcium influx and, thus, to a reduction in binomial variable p. Reducing
the extracellular Ca\textsuperscript{2+} level has been shown to reduce p without affecting n at crayfish
neuromuscular junctions (Dudel, 1981). The ability of DF\textsubscript{2} to enhance transmitter output
from the fast closer excitor axon of crabs requires N-type calcium channels (Rathmayer
et al., 2002). This suggests that DF\textsubscript{2} acts by increasing calcium influx, which would
increase binomial variable p. The present data suggest that if p is reduced at low temperature or low extracellular Ca\(^{2+}\) levels, the scope for modulation by the neuropeptide may be enhanced. Experiments involving calcium imaging in nerve terminals would help to determine whether or not DF\(_2\) acts on calcium influx.

In this and a previous report (Friedrich et al. 1994), the effectiveness of DF\(_2\) in modulating chemical synapses has been expressed as the percentage change in EJP values, rather than in the actual change in mV. This was done primarily to minimize the effect of variation in EJP amplitudes between different preparations. However, it is worth noting that actual EJP amplitudes after peptide application at 12°C were still lower than those recorded at 20°C before peptide application. Thus, although the percentage change in EJP amplitude induced by the peptide is greater at the lower temperature, the actual EJP amplitudes do not even approach those at the higher temperature with no peptide. Thus, it seems unlikely that reducing temperature simply leaves a greater supply of transmitter quanta, and that the peptide is less effective at the higher temperature because there are smaller reserves of quanta available for release.

To further investigate the effect of lowering transmitter release on effectiveness of the peptide, the EJP amplitude was lowered through low-frequency depression. Tonic low-frequency stimulation (0.2Hz) of crustacean phasic excitors often leads to a reduction in EJP amplitude over time (Bruner and Kennedy, 1970; Lnenicka and Atwood, 1985; Pahapill et al., 1986; Zucker and Bruner, 1977). Although initial EJP amplitude was significantly reduced by LFD, the magnitude of the modulation by DF\(_2\) was unaffected. Thus, the mechanisms through which low frequency depression reduces transmitter output may differ from those underlying the effects of reducing temperature
and reducing extracellular calcium. The mechanisms underlying low-frequency depression are not well understood, but appear to involve the generation of nitric oxide (Aonuma et al., 2000). Treatments that reduce calcium influx, such as lowering extracellular Ca\(^{2+}\) (Czternasty and Bruner, 1980), raising extracellular Mg\(^{2+}\) (Bruner and Kennedy, 1970) and adding Mn\(^{2+}\) to the bath (Bryan and Atwood, 1981), drastically reduce transmitter release without affecting low frequency depression. Such observations suggest that this form of depression does not result from a reduction in calcium influx.

In summary, reducing extracellular calcium levels and reducing the temperature both enhance the ability of neuropeptide DF\(_2\) to increase transmitter release. The similarity of the effects of these two experimental treatments suggests common underlying mechanisms related to changes in calcium influx. The results also suggest that the mechanisms through which low-frequency depression reduces transmitter output differ from those underlying reductions in temperature or extracellular calcium. Taken together, the present data and those of Rathmayer et al. (2002) suggest that presynaptic calcium channels are involved in the modulatory effect of DF\(_2\). The functional significance of the sensitivity and calcium sensitivity of the peptide's effects are still to be determined.
Chapter 3

Synaptic Modulation by a *Drosophila* Neuropeptide is Motorneuron Specific
and Requires CaMKII and Intracellular Calcium

T.W. Dunn & A.J. Mercier
Abstract

The *Drosophila* FMRFamide-related peptide, DPKQDFMRFamide modulates synaptic transmission at the larval neuromuscular junction. The amplitude of excitatory junctional potentials (EJPs) produced by the selective stimulation of motorneuron RP3 increase following application of 1\(\mu\)M DPKQDFMRFamide. EJPs elicited by stimulating 6/7b, however, exhibit no significant increase with the same concentration of neuropeptide. The mechanisms underlying the modulatory effects of DPKQDFMRFamide were examined using a combination of pharmacological and genetic methods. Three independent lines of evidence implicate CaMKII as an essential effector protein or part of the signal transduction pathway. The effect of the neuropeptide is suppressed by 1\(\mu\)M KN-93 (CaMKII inhibitor) and by heat-shock induced expression of a CaMKII inhibitor. A heterozygous CaM kinase mutant responds poorly to the peptide. Finally, we provide evidence that modulation by the neuropeptide requires release of calcium from internal stores. An IP3 receptor mutant allele and application of a toxin that affects IP3-mediated calcium release both reduce synaptic modulation by DPKQDFMRFamide.
Introduction

Neuropeptides are important chemical signals in the nervous system, capable of acting as neurotransmitters and of modulating synaptic transmission. The mechanisms through which neuropeptides act are not completely understood. The neuromuscular system of *Drosophila* has the potential to serve as a useful model system for studying the mechanisms of neuropeptide action. *Drosophila* offers the opportunity to complement the use of pharmacological agents with genetic mutants when designing experiments to identify the essential second messenger pathways.

To date, however, little is known about the modulatory effects of neuropeptides in *Drosophila*. The *Drosophila* genome encodes sequences for at least 52 oligopeptides, based on homology with known neuropeptide families (Vanden Broeck, 2001). The *dFRMFa* gene contains sequences for eight peptides with structural similarity to FMRFamide (Phe-Met-Arg-Phe-NH2), a tetrapeptide originally isolated from clams (Price and Greenberg, 1977). FMRFamide-related peptides (FaRPs) are found in wide and diverse range of animals, both vertebrate and invertebrate, and several FaRPs have been shown to modulate chemical synapses (Espinoza et al., 2000; Fossier et al., 1999; Greenberg and Price, 1992; Jorge-Rivera et al., 1998; Mercier et al., 2001; Worden et al., 1995). Most of the gene products of the *dFRMFa* gene have been shown to modulate the neuromuscular function in *Drosophila* larvae (Hewes et al., 1998). Seven of the eight peptides encoded on the dFRMFa gene enhance nerve-evoked contractions of the body wall muscles. DPKQDFMRFAamide, the most abundant of these peptides, enhances synaptic currents, suggesting a presynaptic site of action (Hewes et al., 1998).
These synaptic currents were recorded in ventral longitudinal muscles 6 and 7, which receive dual innervation by two glutamatergic motoneurons, RP3 and 6/7b (Keshishian et al., 1996; Lnenicka and Keshishian, 2000). Hewes et al. (1998) reported an increase in the compound EJC while stimulating both motoneurons but never determined whether one or both motoneurons were modulated. They also found no increase in the peptide’s efficacy with IBMX, which suggests no involvement of cyclic nucleotide second messengers (Hewes et al., 1998).

This chapter represents a more thorough investigation of the mechanisms behind the modulatory effect of the Drosophila FaRP DPKQDFMRFamide on the larval neuromuscular junction. This chapter will provide evidence that modulation by the peptide of the motoneurons innervating the ventral longitudinal muscles is selective, to the tonic-like motoneuron, RP3. Experiments in this chapter combine pharmacology and genetics to identify proteins essential to the neuromodulatory effects of the Drosophila FaRP, DPKQDFMRFamide. Experiments with an inositol 1,4,5-trisphosphate (IP3) receptor mutant and the sponge toxin Xestospongin C (XeC) suggest that IP3-mediated calcium release is required for modulation at the NMJ by this neuropeptide. The Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII) inhibitor KN-93, a CaMKII mutant, and a transgenic fly line expressing an inducible CaMKII inhibitor implicate this kinases activity in the modulatory effects of DPKQDFMRFamide. We propose that FaRP modulation at the Drosophila neuromuscular junction requires CaMKII and IP3 receptor calcium channels.
Methods

**Flies.** Wild-type *Drosophila melanogaster* (Canton S.) were obtained from Boreal Laboratories, St. Catharines, ON. CaMKII\(^+/\) \{y; l(4)16/Ci\(^D\)\} and ala1 \{w P[w\(^+\) ala]\} lines were the generous gift of Leslie Griffith (Brandeis University, Mass.). The IP\(3\) receptor mutant \{P(ry\(^{47.2}=\)PZ)Itp-r83A\(^{05616}\) ry\(^{506}/\)TM3, ry\(^{RK}\) Sb\(^1\) Ser\(^1\)\} was obtained from the Bloomington stock center at Indiana University and is heterozygous for the null IP\(3\) receptor allele *Itp-r83A*\(^{1664}\) (this allele is also known as *Itp-r83A*\(^{05616}\)). All flies were raised on a cornmeal-based medium (from Boreal) at 21-23\(^\circ\)C on a 12:12 light/dark cycle.

All experiments were performed on wandering stage third instar larvae. Larvae were dissected in the recording saline, a low Ca\(^{2+}\), high Mg\(^{2+}\) haemolymph-like saline (HL3, (Stewart et al., 1994)) containing (in mM): 70 NaCl, 5 KCl, 1.25 CaCl\(_2\), 20.25 MgCl\(_2\), 10 NaHCO\(_3\), 5 trehalose, 115 sucrose, and 5 HEPES, pH 7.0.

**Heat-shock.** Heat-shock of ala1 and wild-type larvae was performed by placing third instar larvae in a dry incubator for 1 hour at 37\(^\circ\)C. Since the somata of RP3 and 6/7b reside in the ventral ganglion, a time delay of 2 hours after the 1-hour heat-shock was used to allow time for the alanine inhibitory peptide to reach the synaptic terminals.

**Preparation.** Wandering stage third-instar larvae were collected from the sides of their culture vials and dissected as described in Jan and Jan, (1976). The segmental nerves were cut near the ventral ganglion and the CNS was removed. The bath volume (approx. 200\(\mu\)L) was continuously exchanged at a rate of 0.75 mL/min by perfusion with a peristaltic pump. Only muscles in abdominal segments 3 through 6 were used.

**Electrophysiology.** Excitatory junctional pontentials (EJPs) were evoked by stimulation of an abdominal segmental nerve with a suction electrode filled with saline.
EJPs were evoked in the commonly used ventral longitudinal muscles 6 or 7, and 12 of the corresponding hemi-segment with glass microelectrodes (40-60 MΩ) backfilled with 3M KCl. EJP amplitude was measured and recorded at room temperature with a data acquisition system built by Mr. Tom MacDonald at Brock University (Electronics Division). EJP amplitude was corrected for nonlinear summation (Cheung et al., 1999).

Selective stimulation of RP3 and 6/7b. Motorneuron RP3 innervates muscles 6 and 7 of the abdominal segments and motorneuron 6/7b innervates muscles 6, 7, 12, and 13 (Keshishian et al., 1996; Hoang and Chiba, 2001)(see Figure 3.1A). Stimulation of the segmental nerve will produce a compound EJP in muscles 6 and 7 from motorneurons RP3 (large boutons, Ib) and 6/7b (small boutons, Is) (Kurdyak et al., 1994). The compound EJP in muscles 6 and 7 is the summation of two EJP’s, one of which corresponds to an EJP in muscle 12 and 13 (6/7b), whereas the other does not (RP3) (Lnenicka and Keshishian, 2000). By simultaneously monitoring EJPs in muscles 7 and 12 while reducing stimulus voltage or stimulus duration, either neuron can be derecruited from contribution to the compound EJP. Figure 3.1B demonstrates the derecruitment of the EJP of 6/7b, leaving a reduced EJP (from RP3 alone) in muscle 7 and no EJP in muscle 12. Figure 3.1C shows the derecruitment of the EJP of RP3, where there is still a reduced EJP (from 6/7b alone) in muscle 7 and an EJP of unchanged amplitude in muscle 12. When recording the EJP from 6/7b alone in muscle 7 (or 6), the stimulus duration was often varied to elicit a compound EJP or a failure. This was done to be certain that the EJP being recorded was not a reduced amplitude compound EJP.
Figure 3.1. A, Diagram of the ventral longitudinal muscles 6, 7, 12, and 13 in Drosophila larvae. RP3 innervates only muscles 6 and 7, whereas 6/7b innervates all four muscle fibres. B, Example of 6/7b dropping out of the compound EJP in muscle 6 with a reduction in stimulus duration. The upper traces were recorded in muscle 6 and the lower traces in muscle 12. Stimulation of the abdominal nerve produces a compound EJP in muscle 6 and a single EJP in muscle 12 (black traces). A reduction in stimulus duration in this representative preparation causes 6/7b to drop out leaving a single EJP in muscle 6 and no EJP in muscle 12 (gray traces). C, Example, opposite to B, where reduction in stimulus duration leaves a single EJP (upper gray trace) in muscle 6 from 6/7b (upper traces) while the EJP in muscle 12 remains unchanged (lower traces).
**Chemicals and Peptide.** KN-93, KN-92, and Xestospongicin C were purchased from Calbiochem and were dissolved in saline containing 0.1% DMSO final (bath) concentration. The *Drosophila* FaRP, DPKQDFMRFamide was synthesized by Cell Essentials, Boston Mass., and was of 98% purity by reverse-phase HPLC.

**Analysis.** All error bars on graphs are standard errors of the mean, and the number of preparations is in parentheses on the graphs (n). Statistical significance was determined using Mann-Whitney U tests, unless noted otherwise (i.e. Figure 3.9). EJP amplitude for each trial was expressed as a percent change from the amplitude in the prepeptide period (1 minute prior to DPKQDFMRFamide application). This allows for comparison of trials with different sized initial EJPs.
Results

Selective modulation of RP3

The ventral longitudinal muscles 6 and 7 are dually innervated, and though it is known that DPKQDFMRFAmide increases the compound response, the effects of the peptide on each individual motorneuron was not previously determined (Hewes et al., 1998). When the EJP produced by RP3 was selectively evoked in muscle 7, as in Figure 3.1B, EJP amplitude increased significantly by 23.0±9.3% following application of 1μM DPKQDFMRFAmide (p<0.05)(Figure 3.2A). However, selective stimulation of 6/7b (as in Figure 3.1C) exhibited no significant modulation of EJP amplitude (maximal increase of 6.0±3.6%) with application of the neuropeptide (p>0.05)(Figure 3.2B). Selective stimulation of RP3 was used in all other experiments to address the mechanisms of modulation by the peptide.

Ca2+/ calmodulin-dependent protein kinase II activity is essential to FaRP modulation

Second messenger activated kinase activity has been linked to the modulatory effects of FaRPs in a variety of species (Fossier et al., 1990; Mercier et al., 2001; Willoughby et al., 1999a). FaRP modulation of transmitter release at the neuromuscular junction has been previously linked to CaMKII and PKC activity in the crayfish (Friedrich et al., 1998; Noronha and Mercier, 1995).

CaMKII becomes active when calcium-bound calmodulin associates with the autoinhibitory domain of the kinase. This association allows the catalytic domain to
Figure 3.2. Selective modulation of motor neurons RP3 (A) and 6/7b (B) by DPKQDFMRFamide. A. The EJP produced by selective stimulation of RP3 (Figure 3.1B) is significantly increased by DPKQDFMRFamide (p<0.05). B, Selective stimulation of 6/7b produces no apparent increase in EJP amplitude with application of DPKQDFMRFamide (p>0.05). (n=number of preparations).
phosphorylate serine or threonine residues on protein targets with the consensus sequence RXXS/T, including the autophosphorylation site on the kinase (for review see. (Lisman et al., 2002)). The cell permeable CaMKII inhibitor KN-93 interferes with the association of Ca\(^{2+}\)/calmodulin with the kinase and prevents activity, including autophosphorylation, with an IC\(_{50}\) = 0.37\(\mu\)M (Sumi et al., 1991). In all trials, KN-93 (or KN-92) was applied to the preparation twenty minutes before recording. KN-93 inhibited the peptide’s ability to increase EJP amplitude, and the effect was dose-dependent (Figure 3.3A and B). At a KN-93 concentration of 1\(\mu\)M, the peptide DPKQDFMRFamide had no effect on EJP amplitude (p>0.05). In contrast, the inactive analog of KN-93, KN-92 did not affect the efficacy of the peptide; there was no significant difference between the percentage change elicited by the peptide in the presence or absence of KN-92 (p>0.05)(Figure 3.3A). The complete absence of EJP modulation by the peptide with KN-93 suggests that CaMKII activity is essential for the modulatory effects of DPKQDFMRFamide.

Pharmacological agents have limitations especially with specificity. For example, KN-93 has been shown to affect K\(^+\) currents in myocytes, although KN-92 serves as a control for this possibility (Loudo et al., 1999). Other non-specific effects however, cannot be ruled out. To further implicate CaMKII activity as essential to the modulatory effects of the FaRP peptide, two different fly lines with altered CaMKII activity were acquired and the effectiveness of DPKQDFMRFamide measured on EJP amplitude.

The mutation l(4)16 deletes the CaMKII gene and flies heterozygous for the allele (l(4)16/ci\(^{b}\) have a 40% reduction in CaMKII activity and 50% less CaMKII protein than wild-type flies (Joiner and Griffith, 1997). The effect of DPKQDFMRFamide on the CaMKII mutant was attenuated compared to wild-type larvae, both in amplitude and in
Figure 3.3. The CaMKII inhibitor KN-93 inhibits EJP modulation by DPKQDFMFamide. A, All trials experienced a 20-minute preincubation period with either KN-93, KN-92, or the solvent 0.1% DMSO (control). 1μM KN-93 completely inhibits modulation by the neuropeptide, whereas the inactive analog KN-92 and the solvent do not affect modulation (n). B, KN-93 dose-dependently decreased the effect of DPKQDFMFamide on EJP amplitude. Points are average % change in EJP amplitude 1-4 min. after 1μM peptide application with points for 1μM KN-92 and 1μM KN-93 without peptide application for comparison (n=6 for all points).
duration (Figure 3.4). The maximal EJP amplitude in the presence of the neuropeptide was only 12.9±1.9% greater than without peptide, and the modulation lasted for less than 2.5 minutes after DPKQDFMRFamide application. In wild-type larvae, the maximal increase in EJP amplitude was 23.0±9.3% and the effect maintained for the six minutes of recording after peptide application. The truncated modulatory effect of DPKQDFMRFamide on the CaMKII mutant larvae is very different from that of the wild-type larvae.

The second fly line used with altered CaMKII activity is the ala1 line. The ala1 larvae contain an insertion of the alanine inhibitory peptide downstream of an hsp70, heat-shock promoter (Griffith et al., 1993; Wang et al., 1994). The alanine inhibitory peptide is similar to the autoinhibitory region of rat CaMKII, which contains the autophosphorylation site (alanine substituted for threonine) and is adjacent to the calmodulin-binding domain (Griffith et al., 1993). There is some expression of the alanine inhibitory peptide in ala1 flies at 25°C (1.9-3.9μM) and likely at room temperature (22°C), but at levels below the IC50 (IC50=13μM in Drosophila) (Griffith et al., 1993). The levels of alanine inhibitory peptide are increased following a 1-hour heat-shock (HS) (Griffith et al., 1993).

Figure 3.5A shows the time course of modulation by DPKQDFMRFamide in the ala1 transgenic flies with and without heat-shock. Without heat-shock, the ala1 larvae exhibit a short-lived response (peak is 14.6±2.2%) with a time course similar to that of the CaMKII mutant (Figure 3.5A). Heat-shock eliminates the responsiveness of the larvae to DPKQDFMRFamide, with no significant modulation of EJP amplitude during
The wild-type response (in parentheses) is significantly increased compared to peptide controls, but the amplitude and duration of modulation is reduced compared to response to DPCDFRFRamide. EJP amplitude is significantly increased from the no response to 1 μM DPCDFRFRamide.

Figure 3.4: Mutant larvae heterozygous for a null CAMKII allele show an impaired response to 1 μM DPCDFRFRamide.
Figure 3.3. Heat shock blocks EP modulation by DPIKOMDFRFAamide in all larvae.

(A) Ali I larvae with no prior heat shock have an attenuated response to 1uM DPIKOMDFRFAamide (1) after a 1-hr heat shock at 37°C. Ali I larvae experience no modulation of EJP amplitude.

(B) Ali I larvae with no prior heat shock have an attenuated response to 1uM DPIKOMDFRFAamide (1) after a 1-hr heat shock at 37°C. Ali I larvae experience no modulation of EJP amplitude.
the five minutes monitored after peptide application (Figure 3.5B). Modulation of EJP amplitude by DPKQDFMRFamide in wild-type larvae was not different after the same heat-shock treatment.

All three methods of decreasing CaMKII activity (KN-93, mutant, ala1) caused a significant decrease in EJP amplitude when preparations were stimulated for 5 minutes at 0.5 Hz in the absence of peptide (Figures 3.3-3.5), suggesting synaptic depression. No such change in EJPs from RP3 was observed in wild-type larvae and in larvae treated with KN-92 (data not shown). A similar reduction in EJP amplitude was produced by selective stimulation of the phasic–like neuron 6/7b at 0.5Hz (Figure 3.2B).

**IP$_3$ receptors are required for modulation by the peptide**

CaMKII activation by calcium-bound calmodulin occurs in response to a rise in cytosolic calcium levels. The calcium could come either from extracellular sources or from intracellular stores. The phosphoinositol pathway leads to the production of IP$_3$, which gates the IP$_3$ receptor, a calcium channel to internal calcium stores within the endoplasmic reticulum. In the *Lymnaea* heart, FMRFamide leads to the production of IP$_3$ by phospholipase C (Willoughby et al., 1999b). Since many neuropeptides act through G-protein coupled receptors, IP$_3$ is a logical second messenger to examine.

The *Drosophila* IP$_3$ receptor gene *itp-r83A* is the only known IP$_3$ receptor encoding sequence in the *Drosophila* genome. A P-element insertion in the 5’ untranslated region of the *itp-r83A* gene completely disrupts the IP$_3$ receptor protein producing the null allele *itp-r83A*^{1664} (Sinha and Hasan, 1999; Spradling et al., 1999). IP$_3$
Figure 3.6. The effect of DPKQDFMRFamide on EJP amplitude in larvae heterozygous for an IP₃ receptor mutation. EJP amplitude is not modified by the neuropeptide, as trials with and without the peptide are very similar, unlike the effect in wild-type larvae (n).
receptor mRNA levels in heterozygous adult heads (itp-r83A1664+) are approximately half that of wild-type flies (Venkatesh and Hasan, 1997). Even the partial reduction in IP3 receptor expression in the heterozygote resulted in a total loss of the modulatory effects of DPKQDFMRFamide on EJP amplitude (Figure 3.6). EJP amplitude was unaffected by DPKQDFMRFamide in this mutant, suggesting a requirement for IP3 receptors in the modulatory effects of the neuropeptide.

Xestospongion C (XeC) has been described as a selective inhibitor of IP3 receptors in some preparations and a blocker of smooth endoplasmic reticulum calcium ATPase (SERCA) Ca2+ pumps in others (IC50=358nM) (Oka et al., 2002; Castonguay and Robitaille, 2002; De Smet et al., 2002; Solovyova et al., 2002). If IP3-mediated calcium release is required for modulation, and if the toxin selectively inhibits IP3 receptor gating, then XeC should inhibit the effects of the peptide. However, if the toxin inhibits SERCA pumps then application of XeC alone should enhance EJP amplitude as calcium release from the ER would occur by passive leak (Solovyova et al., 2002). Furthermore, if XeC blocks SERCA pumps, prior exposure to the toxin should occlude DPKQDFMRFamide modulation by emptying internal calcium stores. Figure 3.7 demonstrates that XeC enhances EJP amplitude. The degree of modulation and the time course are similar to those of DPKQDFMRFamide and are not caused by the solvent, 0.1% DMSO (data not shown). The effect of the toxin (XeC) on EJP amplitude plateaued approximately 5 minutes after application. If the SERCA pumps are blocked, internal calcium stores will eventually deplete, reducing or preventing any subsequent IP3-mediated calcium release. Figure 3.8A shows the effects of DPKQDFMRFamide on EJP amplitude following a 10-
Figure 3.7. The effect of the sponge toxin XeC alone on EJP amplitude with the selective stimulation of motorneuron RP3. XeC increases EJP amplitude by about 25% five minutes following application.
Figure 3.8. Preapplication of XeC inhibits (occludes) the modulatory effect of DPKQDFMRFamide on EJP amplitude. A, In all the trials represented in this figure, XeC was applied 10 minutes before recording. B, The inhibitory effects of XeC are dose-dependent. Points are average % change in EJP amplitude 1-4 min after 1μM DPKQDFMRFamide application at various concentrations of XeC (n=4,4,6,4,4 left to right).
minute pretreatment with various concentrations of XeC. XeC reduced the modulatory effect of DPKQDFMRFamide on EJP amplitude in a dose-dependent manner (Figure 3.8B). At 500nM, XeC prevented modulation by DPKQDFMRFamide, as maximal increase in EJP amplitude was only 3.3±4.4% (Figure 3.8A). The highest concentration of XeC used was 500nM, as higher concentrations depolarized the muscle fibres.

Results from all the experiments on intracellular mechanisms are summarized in Figure 3.9. Three methods of reducing CaMKII activity and two methods of inhibiting IP3-mediated calcium release significantly reduce the ability of DPKQDFMRFamide to increase EJP amplitude. The complete loss of the modulatory effects of DPKQDFMRFamide on the EJP with 1μM KN-93 (Figure 3.3), ala1 heat-shocked (Figure 3.5), and in the IP3 receptor mutant (Figure 3.6) suggests that CaMKII and IP3 receptors function as essential components in the FaRP modulatory pathway at the Drosophila NMJ.
Figure 3.9. Summary of the inhibition of the modulatory effects of DPKQDFMRFamide. Comparison of peak increase in EJP amplitude with 1μM DPKQDFMRFamide in mutant larvae and with pharmacological inhibition of CaMKII and IP3-mediated calcium release. Each bar represents the peak increase for the respective condition. All this data appears in previous figures. Note- the 500nM XeC group is with preincubation in the toxin not the effects of the toxin itself. (*p<0.05 compared to the wild-type response with Wilcoxin signed rank test).
Discussion

The results of this study indicate that the *Drosophila* neuropeptide DPKQDFMFamide can modulate neuromuscular synapses in a neuron-specific manner. Furthermore, the modulation appears to require release of Ca$^{2+}$ from intracellular stores and CaMKII activity.

Selective modulation of transmitter release in two neurons innervating the same muscle fibre has been reported recently for a crustacean FMRFamide-related peptide (Rathmayer et al., 2002). The crayfish peptide DF$_2$ (DRNFLRFamide) selectively modulates the phasic excitor of the crab closer muscle but does not modulate the tonic excitor to the same muscle. This difference is apparently due to the presence of N-type Ca$^{2+}$ channels in the terminals of the phasic axon; blocking these N-type Ca$^{2+}$ channels partially reduces EJPs from the phasic axon and blocks the entire effect of the peptide (Rathmayer et al., 2002). The two motorneurons innervating the ventral longitudinal muscles of larval *Drosophila* have been compared to the phasic and tonic excitors of crustaceans (Kurdyak et al., 1994). RP3 has large, lower output terminals (Ib) and generates smaller EJPs that facilitate at high frequency stimulation; these are all characteristic of tonic motorneurons in crustaceans. Neuron 6/7b has smaller terminals and produces a larger EJP that depresses with high-frequency stimulation; these are characteristics of phasic motor neurons (Kurdyak et al., 1994). DPKQDFMFamide modulates the tonic-like motorneuron rather than the phasic-like neuron. Based on the findings of Rathmayer et al (2002), we speculate that an N-type current maybe involved in selective modulation of RP3 terminals.
We have not examined whether the peptide works presynaptically or postsynaptically. Motorneuron specific modulation and the observation of no apparent or consistent alteration in muscle fibre resting membrane potential both suggest that the peptide increases EJP amplitude presynaptically. However, this does not rule out postsynaptic effects that may alter synaptic efficacy through means other than increasing the post-synaptic sensitivity to transmitter.

Here we report that functional CaMKII is necessary for the *Drosophila* FaRP, DPKQDFMRFamide, to increase EJP amplitude at the neuromuscular junction. We implicate CaMKII based on three independent methods: a pharmacological approach using KN-93, a genetic approach using the CaMKII mutant, and a combination of genetics and pharmacology using the transgenic ala1 larvae. All three methods suggest that CaMKII is an essential pathway protein involved in the modulatory effects of DPKQDFMRFamide on the *Drosophila* NMJ. Partial inhibition of CaMKII, as in the CaMKII mutant and the Ala1 transgenic fly without heat shock, attenuates the response to the peptide but does not completely obliterate it. Treatment with 1 μM KN-93 and application of heat shock to Ala1 suppressed the response to the peptide more completely. CaMKII is involved in many different forms of synaptic modulation, acting both presynaptically and postsynaptically (for reviews see Fukunaga et al., (1996); Greengard et al., (1993); Lisman et al., (2002)). At crayfish neuromuscular junctions, the peptide DF2 also requires CaMKII. Thus, arthropod FaRPs may modulate neuromuscular synapses through common mechanisms.

CaMKII is activated by calcium bound to calmodulin. DPKQDFMRFamide, therefore, might lead either directly or indirectly to a rise in cytosolic calcium. Because
FMRFamide induced production of IP₃ has been reported previously (Willoughby et al., 1999b), we investigated the possibility that the effect of DPKQDFMRFamide requires the release of Ca²⁺ from internal stores. IP₃ receptors are calcium channels that release calcium from internal stores when gated by IP₃. The elevated calcium levels within the ER are maintained by unidirectional calcium pumps (Perry et al., 2001). Larvae that under express IP₃ receptors show no response to application of DPKQDFMRFamide (Figure 3.6). This lack of a detectable response suggests that either DPKQDFMRFamide requires a high density of IP₃ receptors for efficient intracellular signal transduction or the mutation leads to developmental consequences that alters the efficiency of signal transduction in the synaptic terminals. The latter possibility seems unlikely because IP₃-mediated calcium release was altered with the sponge toxin Xestospongin C (XeC).

The known inhibitory targets of XeC are IP₃ receptors and SERCA pumps (De Smet et al., 2002). The enhancement of EJPs by XeC alone (Figure 3.7) suggests that the toxin inhibits SERCA pumps in this preparation. The gradual increase in EJP amplitude may reflect the passive leak of calcium from the ER into the cytosol. Regardless of whether XeC affects IP₃ receptors, emptying of internal calcium stores would occlude the effect of subsequent exposure to DPKQDFMRFamide. Pre-treatment with XeC reduces the effectiveness of the peptide in a dose-dependent manner (Figure 3.8). Figures 3.6, 3.7, and 3.8 suggest that IP₃-mediated calcium release from internal stores is an essential step in the modulatory pathway that mediates the effects of this neuropeptide.

The apparent increase in depression of EJPs with CaMKII inhibition suggests that basal kinase activity regulates synaptic transmission when RP3 is stimulated at 0.5 Hz. Therefore, the modulatory effect of the peptide might not involve CaMKII activation, but
may require basal activity of this protein kinase. In the absence of CaMKII inhibition, stimulation at 0.5 Hz causes depression of EJPs evoked by neuron 6/7b but no detectable depression in EJPs from neuron RP3 (Figure 3.2B). These observations probably reflect differences in motoneuron subtype. *Drosophila* phasic and tonic terminal subtypes have been compared to the phasic and tonic terminals of crustaceans (Atwood et al., 1997; Kurdyak et al., 1994). EJPs produced by phasic motorneurons of crustaceans are more prone to synaptic depression, and many exhibit such depression at low stimulus frequencies (Atwood and Wojtowicz, 1986; Bruner and Kennedy, 1970; Bryan and Atwood, 1981). The greater depression in EJP amplitude with selective stimulation of the phasic-like neuron (6/7b) is likely a property of the terminal subtypes.

In summary, we show that DPKQDFMRFamide selectively modulates EJP amplitude elicited by one of two motorneurons innervating the ventral longitudinal muscles, and we provide evidence that the modulatory effects involve release of calcium from intracellular stores and CaMKII activity. It seems likely that IP$_3$-mediated calcium release activates CaMKII, as in the classical phosphoinositol pathway. The phosphoinositol pathway can be activated by G-proteins, resulting in an increase in cytosolic calcium and subsequently, CaMKII activation. CaMKII can increase transmitter release through the phosphorylation of such synaptic protein targets as synapsin, syntaxin, and synaptotagmin (Lin et al., 1990; Llinas et al., 1985; Ohyama et al., 2002; Popoli et al., 1997). However, we have not actually demonstrated that CaMKII activity increases in response to the peptide. It is still possible that basal CaMKII activity is necessary and sufficient for the enhancement of EJP amplitude. The upstream effector proteins and the downstream target proteins involved in the modulatory pathway induced
by DPKQDFMRamide will need to be investigated. Finally, calcium imaging of synaptic terminals will be required to demonstrate IP$_3$-mediated calcium release occurring upstream of CaMKII activation.
Chapter 4

Perspectives
Comparison of DF₂ and DPKQDFMRFamide modulatory mechanisms

The wide diversity of FaRP pathways identified to date makes interspecific comparison of FaRP effects difficult. *Lymnaea* FaRPs have been linked to direct sodium channel gating, IP₃ production, and cyclic nucleotide production (Perry et al., 2001; Willoughby et al., 1999a; Willoughby et al., 1999b). The FMRFamide-gated sodium channel was originally identified in *Helix* central neurons (Cottrell, 1997), and is gated by FMRFamide and FLRFamide, while another endogenous *Helix* FaRP, pQFYRFamide antagonizes this action. The *Lymnaea* FMRFamide-gated sodium channel is also found in the CNS (Perry et al., 2001). Another target tissue of FaRPs in *Lymnaea* is cardiac muscle, where both FMRFamide and pQFYRFamide increase cyclic nucleotide production, but only FMRFamide and not pQFYRFamide increases IP₃ production (Willoughby et al., 1999a; Willoughby et al., 1999b). Two generalizations can be drawn from the above information: First, modulatory pathways may be similar in closely related species (both *Helix* and *Lymnaea* are snails) and in similar tissues. Second, the extensive variation in FaRP sequence does not simply reflect redundancy, but may represent differential FaRP function. This latter idea is not new; for example, the wide variety of FaRPs found in *Drosophila* have diverse functions on the heart (Johnson et al., 2000; Merte and Nichols, 2002).

FaRP modulation of both the crayfish and *Drosophila* neuromuscular junctions requires CaMKII (Noronha and Mercier, 1995). CaMKII inhibition in both these preparations impairs EJP amplitude modulation by endogenous FaRPs. In the crayfish, modulation of EJP amplitude appears to occur in at least two phases. An early phase can be inhibited with CaMKII inhibition, while a later phase appears to be mediated by
Protein Kinase C (PKC) (Friedrich et al., 1998). The experiments with *Drosophila* presented in Chapter 3 did not involve long durations of peptide application, so the possibility of a PKC mediated late phase will need to be investigated in *Drosophila*.

A comparison of the modulatory effects of DPKQDFMFamide on *Drosophila* synapses with those of DF$_2$ (DRNFLRFamide) on crayfish synapses reveals some interesting information. Two types of glutamatergic motorneurons found in larval *Drosophila* are physiologically similar to tonic and phasic excitatory motorneurons of crustaceans (Kurdyak et al., 1994). DF$_2$ selectively modulates EJPs produced by phasic motorneurons in crabs (Rathmayer et al., 2002), while DPKQDFMFamide selectively modulates EJPs produced by tonic-like motorneurons in *Drosophila*. The important similarity is that one axon subtype is modulated while the other is not. The difference in which subtype of motorneuron is modulated may be a clue to the physiological function of FaRPs.

Another identified target of FaRP modulation in both crustaceans and *Drosophila* is the heart. The effects of DF$_2$ and DPKQDFMFamide are opposite on their respective endogenous heart preparations, with the former being excitatory and latter inhibitory (Johnson et al., 2000; Skerrett et al., 1995).

**The importance of calcium to FaRP modulation**

Calcium and proteins activated by calcium, or proteins involved in calcium mobilization appear to be necessary components of the mechanisms of FaRP modulation at arthropod neuromuscular synapses. Several lines of evidence support this conclusion. First, the neuron-specific modulation by DF$_2$ in crabs is related to N-type calcium
channels (Rathmayer et al., 2002). Second, the temperature-dependence of DF₂ appears
to involve calcium influx (Chapter 2). Third, IP₃-receptors involved in the modulatory
effects of DPKQDFMFamid are calcium channels (Chapter 3). Fourth, modulation by
FaRPs requires CaMKII in both Drosophila and crayfish; in the crayfish PKC is also
required (Chapter 3)(Friedrich et al., 1998;Noronha and Mercier, 1995).

The possible mechanisms for increasing transmitter release include increasing
calcium influx, increasing the number of vesicles available for release, or altering the
sensitivity of the release machinery to intracellular calcium.

**Calcium influx and the phosphoinositol pathway**

Chapter 2 suggests that calcium influx is important for the effect of DF₂ on EJP
amplitude, and Rathmayer et al., (2002) implicate N-type calcium currents in the
modulatory effect of DF₂. This suggests that plasma membrane calcium channels are
affected by the peptide either directly or indirectly. Direct interaction with calcium
channels is not described for a neuropeptide, but indirect modulation through G-proteins
has been described for a variety of neuromodulatory substances including FLRFamide
(Fossier et al., 1999;Yang et al., 2001).

The phosphoinositol pathway is G-protein activated, leads to IP₃ production and
can lead to the activation of the calcium-dependent kinases CaMKII and PKC. This
pathway has been heavily investigated with respect to phototransduction. The
photoreceptor protein rhodopsin is a G-protein coupled receptor coupled to a Go₃q
subunit. Stimulation of rhodopsin by light causes the release of the Go₃q subunit from the
G-protein/ receptor complex (Scott et al., 1995). The Go₃q subunits activate PLC, which
(N-type) calcium channels show greater inhibition by Gβγ subunits with longer times of 
and different effects on the α1 and α1B calcium channel subunits. Generally, N-type 
and Gβγ subunits in different combinations show different degrees of channel inhibition 
the calcium channel (Cóoper et al., 2000; Zamponi et al., 1997). Subtypes of Gβγ subunits 
these loop II binding sites by PC and antagonizes the interaction of the Gβγ subunits with 
associated with transmitter release (Zamponi et al., 1997). Phosphorylation of one of 
domains I and II of the α1(N-type) or α1B(N-type) subunits of calcium channels 
Gβγ subunits can bind to two sites on the intracellular loop between transmembrane 
prevent modulation of the channel by noradrenaline (Hertlitz et al., 1996). The 
calcium channel is not affected by the Gα subunit and inhibition by the Gβγ subunits can 
association with the pore forming α-subunit of the channel (Hertlitz et al., 1996). The 
the membrane bound Gβγ subunits, can directly inhibit N-type calcium currents through 
recently as it is also a modulator of synaptic efficacy. Activated G-proteins, specifically 
The other half of the activated G-protein (Gβγ subunits) has received attention 
pathway is involved in the modulatory effects of DP2/βARK-family.

For both IP3 receptors and CAMKII (Chapter 3), suggest that the phospholinositol
in cells unrelated to phototransduction (Alvarez et al., 1996). The apparent requirement
activation with the finding of multiple splice variations of the Gα gene and expression
modulation of synaptic transmission. This variety of G-protein signaling is confirmed in
a variety of other G-protein linked signal transduction pathways involved in the
(Alvarez et al., 1996; Scott et al., 1995). The phospholinositol pathway is also involved in
causes a release of calcium from the internal stores of the endoplasmic reticulum.
recovery from inhibition (Arnot et al., 2000). These findings not only allow for selective inhibition of calcium channels involved in transmitter release, but also for prevention of this inhibition by phosphorylation. In Aplysia, FLRFamide presynaptically activates the phosphoinositol pathway through G-proteins, which leads to the activation of PKC (Baux et al., 1992). The activation of PKC by diacylglycerol and calcium liberated from internal stores directly modulates N-type calcium channels presumably by phosphorylation (Fossier et al., 1990; Fossier et al., 1999).

As mentioned earlier in this discussion, inhibition of CaM kinase only inhibits the early phase (0-10 min. after peptide application) of modulation by DF₂. After this EJP amplitude increases to the levels seen without CaM kinase inhibition within 10-15 minutes (Noronha and Mercier, 1995), and this late phase of modulation by DF₂ can be blocked by inhibiting protein kinase C (PKC) (Friedrich et al., 1998). Furthermore, the initial phase of modulation by DF₂ is amplified with PKC inhibition (Friedrich et al., 1998). Further examination of the involvement of PKC in modulation by DF₂ found that inhibition of the kinase also highly attenuated the modulatory effects of a subsequent application of DF₂ after a wash (Bisson, 1996). Together these three observations suggest that PKC is likely involved in maintaining modulation, possibly by preventing or counteracting a mechanism that antagonizes DF₂ activity. This idea is supported by the finding that activated G-proteins (specifically the β, γ subunits) inhibit N-type calcium channels and that this inhibition can be prevented when the channel is phosphorylated by PKC (Cooper et al., 2000). This is particularly relevant in light of a recent study implicating N-type calcium channel modulation by DF₂ (Rathmayer et al., 2002).
CaMKII target proteins

A wide variety of presynaptic proteins are potential targets of CaMKII. It is tempting to suggest that CaMKII modulates N-type calcium currents, and that PKC maintains long periods of modulation by preventing inhibition by the Gβγ subunit “off switch”. However, to date no direct phosphointeraction between CaMKII and N-type calcium channels has been reported that results in a stimulatory effect. CaMKII can increase synaptic efficacy through a variety of other protein targets in the synapse.

*Drosophila* Ca$^{2+}$/ calmodulin-dependent protein kinase II (CaMKII) is heavily expressed in the adult CNS and is present both presynaptically and postsynaptically at the neuromuscular junction (Griffith, 1997). The only known presynaptic phosphorylation target of CaMKII in *Drosophila* is the K$^+$ channel *ether-à-go-go (eag)*. Alterations in CaMKII activity or mutations in *eag* have similar consequences to synaptic transmission and associative learning. Inhibition of CaMKII reduces the peak K$^+$ influx through *eag* and increases the inactivation rate (Wang et al., 2002). At synaptic terminals inhibition of CaMKII phosphorylation of *eag* K$^+$ channels results in hyperexcitability, suggesting that an increase in CaMKII activity would be inhibitory. CaMKII phosphorylation of *eag* would reduce transmitter release by shortening the duration of synaptic terminal depolarization, which decreases the terminal calcium influx. Thus, it is unlikely that *eag* is involved in the modulatory effect of DPKQDFMRFamide.
Increasing the number of vesicles available for release: Synapsin

At the presynaptic terminals of other organisms, CaMKII activity has been found to have excitatory effects by phosphorylating a variety of proteins. The classic synaptic CaMKII target is the vesicle/cytoskeletal cross-linking protein Synapsin I (Llinas et al., 1985). When dephosphorylated synapsin I cross-links synaptic vesicles with the cytoskeleton by binding to actin, this excludes these vesicles from the readily-releasable pool (Bahler and Greengard, 1987; Dearborn, Jr. et al., 1998; Hirokawa et al., 1989). The phosphorylation of synapsin by CaMKII or PKA decreases the affinity of the protein for actin and synaptic vesicles, and this allows these liberated vesicles to enter the readily-releasable pool (Lin et al., 1990; Llinas et al., 1985; Nah et al., 1993). This action would tend to increase the number of vesicles available for release and would therefore be excitatory. The *Drosophila* synapsin gene (*syn*) shows high homology in the conserved C domain of rat and human synapsin Iα and Iβ (Klagges et al., 1996). The D domain of synapsin Iα and Iβ is the modulatory domain, the region phosphorylated by CaMKII in rat and humans. There is little homology between the *Drosophila syn* gene and the rat synapsin Iα domain D, but the inferred protein has numerous CaMKII consensus sites (R-X-X-T/S) which could be phosphorylated (Klagges et al., 1996). Phosphorylation of the *syn* gene product has yet to be demonstrated in *Drosophila*, but it is probably modulated by CaMKII considering that synapsin I is a highly conserved protein found in a wide range of animals and having a similar synaptic function (Greengard et al., 1993). Thus, transmitter release may be increased through synapsin phosphorylation, which increases the number of vesicles available for release.
Increasing transmitter release through SNARE proteins

Other than synapsins, proteins thought to be involved in vesicle docking and/or fusion such as synaptotagmin and syntaxin may alter transmitter release following CaMKII activation (see (Gerst, 1999) for review of SNARE hypothesis). Fast, synchronous exocytosis of synaptic vesicles occurs at synaptic terminals in response to local calcium influx through voltage-gated calcium channels (Zucker and Haydon, 1988).

The actual fusion machinery has yet to be conclusively determined, though a variety of proteins (many of which are found only in neurons) have been implicated as being essential to evoked release of transmitter in neurons (Chen et al., 1999). The t-SNARE proteins (syntaxin and SNAP-25) and v-SNARE proteins (synaptobrevin or VAMP) are found on the presynaptic membrane and the vesicular membrane respectively. It is thought that these proteins form a complex (with a variety of other proteins) to localize or dock the vesicle to the presynaptic membrane (Weber et al., 1998). The Ca\(^{2+}\) dependence of transmitter release has been investigated in a number of preparations, and release is proportional to Ca\(^{2+}\) current raised to an exponent of 3-4. This suggests that the fast, synchronous release requires the binding of 3 to 4 Ca\(^{2+}\) ions to a protein with relatively low affinity for Ca\(^{2+}\) (Andreu and Barrett, 1980; Augustine et al., 1985; Littleton et al., 1994).

The vesicle-associated and neuron specific protein synaptotagmin has received a lot of attention as it has two repeated domains capable of binding phospholipids when stimulated by the binding of 4 Ca\(^{2+}\) ions (Brose et al., 1992). Studies with a variety of synaptotagmin mutants in Drosophila have shown that the Ca\(^{2+}\) dependence and sensitivity of transmitter release can be altered by mutating this protein (Littleton et al.,
synaptotagmin has been shown to alter transmitter release. Phosphorylation of
synaptotagmin by CaMKII facilitates the interaction between syntaxin and
synaptotagmin, suggesting that this kinase may increase at least the efficiency of vesicle
priming or docking to the presynaptic membrane (Verona et al., 2000).

Other SNARE proteins such as syntaxin, SNAP-25, and synaptobrevin have not
been shown to have altered function following CaMKII phosphorylation (Risinger and
Bennett, 1999; Verona et al., 2000). However, CaMKII binding to syntaxin (not
phosphorylation) can modulate transmitter release. A search for a protein that bound to
the core SNARE complex at micromolar Ca$^{2+}$ concentrations in an ATP-dependent
manner found CaMKII (Ohyama et al., 2002). Though CaMKII binding did not change
kinase activity, exocytosis in neurons was decreased when binding was inhibited by
microinjection of the binding fragment (Ohyama et al., 2002). CaMKII binds only to
open syntaxin (able to bind other SNARE proteins) and therefore may function
structurally to promote SNARE protein association (Ohyama et al., 2002). Furthermore,
synaptotagmin lacking the CaMKII phosphorylation site showed similar binding
increases with CaMKII binding to syntaxin (Ohyama et al., 2002). Therefore, the effects
of activated CaMKII binding to syntaxin are not the same as the effects of
phosphorylation of synaptotagmin by the kinase.

**CaMKII mediated modulation**

A further possibility is that CaMKII mediates the action of another protein,
indirectly participating in the modulatory effects of DF$_2$ and DPKQDFMRFamide. The
apparent increase in EJP amplitude depression with CaMKII inhibition in Chapter 3 during the control trials suggest an involvement of basal CaMKII activity, which when inhibited may prevent subsequent modulation by the peptide. For example, the secretion of cholecystokinin requires the activation of L-type calcium channels (Mangel et al., 1996). The modulation of these L-type calcium channels by cAMP-dependent protein kinases (PKA) requires functional Ca\(^{2+}/\) calmodulin-dependent protein kinase II (CaMKII), and the authors suggest that CaMKII phosphorylation primes the channel for PKA phosphorylation (Basavappa et al., 1999). This possibility cannot be ruled out, especially since PKA activity may accompany the early phase of DF\(_2\) modulation in crayfish synapses (Mercier et al., 2001).

**Kinase Anchoring**

The serine/threonine protein kinases are involved in a wide range of intracellular signal transduction pathways, often having opposite modulatory effects. Distinct modulatory effects may be achieved by activating distinct pools of kinase, localized by anchoring proteins.

Studies of synaptic modulation over the past few decades have implicated a few second messenger activated kinases that appear to act on a wide range of targets, many of which have very different effects. The specificity of kinase effects with different stimulation protocols may be the result of kinase anchoring to or near the specific targets. For instance, the catalytic subunits are soluble when active, and are bound to a regulatory subunit when inactive. A-kinase anchor proteins (AKAPs) bind the PKA regulatory domain and have variable regions for insertion into lipid membranes, and for attachment
to the cytoskeleton. These proteins likely localize the actions of PKA to very specific targets, which would allow for distinct actions of the kinase, depending on which localized pools were stimulated. This would also apply specificity in the localization of the effector proteins upstream of kinase activation, such as adenylate cyclase for the above example.

The targeting of CaMKII to distinct subcellular locals occurs through a variety of mechanisms. The anchoring of CaMKII to NMDA channels was the first demonstration that CaMKII was also anchored near phospho-targets (Leonard et al., 1999). Recently an anchoring gene within the gene for CaMKII has been shown to assemble with, localize, and regulate the activity of CaMKII holoenzymes. The αKAP transcript is missing the kinase domain of CaMKII and is posttranscriptionally processed differently (Bayer et al., 1998). The presence of the CaMKII association domain in αKAP allows incorporation into the CaMKII holoenzyme and the variable N-terminal region is capable of membrane insertion and targeting for at least the sarcoplasmic reticulum (Bayer et al., 1998; Takeuchi and Fujisawa, 1997). Dense clustering and tight association of CaMKII to synaptic vesicles may also have to do with αKAP anchoring or a similar mechanism. The investigation of αKAP targeting of CaMKII to the SR found opposite effects, depending on whether the kinase was anchored or soluble (Hain et al., 1995). This is one of the more important findings of CaMKII targeting as it demonstrates that anchoring not only limits the mobility of the kinase to specific subcellular domains, but it also restricts or guides phosphorylation by the kinase at specific target proteins. CaMKII is also capable of binding to cytoskeletal elements like F-actin as well as target proteins that may already be docked such as myosin-V, actinin, and synapsin (for review see (Bayer and
Furthermore, calmodulin anchoring to calcium channels and other targets may add another dimension of modulation (Peterson et al., 1999). The ability of CaMKII to act over short and long durations, to be inhibitory or excitatory seems to depend on the nature of activation and the target of phosphorylation.

Conclusions

Chapters 2 and 3 provide added insight into the mechanisms of FaRP modulation at the neuromuscular synapse. Most notable is the dependence of calcium and calcium related proteins to FaRP modulation. The temperature-dependent effects of DF2 appear to be related to an altered calcium influx at lower temperatures, and prior activity-dependent modulation in the form of low-frequency depression does not alter subsequent modulation by the peptide, a finding that likely has functional significance (Chapter 2). Chapter 3 confirms neuron-specific modulation by FaRPs, recently described with DF2 in the crab (Rathmayer et al., 2002). Finally, Chapter 3 indicates CaMKII and IP3-receptors/ intracellular calcium stores are essential to FaRP modulation of the NMJ in Drosophila, further highlighting the similarity in FaRP modulation at the crayfish and Drosophila neuromuscular junctions. This provides the possibility of using both preparations for the study of FaRP action on the neuromuscular junction.

The general conclusion of this thesis is that calcium is very important for the modulatory effects of DPKQDFMRFamide and DF2 at neuromuscular junctions. Further experimentation will be required to better understand the complex calcium dynamics involved. Calcium imaging of nerve terminals will be required to verify that reducing temperature reduces calcium influx in crayfish, that calcium influx is altered by DF2, and
that intracellular calcium is released by DPKQDFMFamide. Calcium imaging experiments will be crucial for determining if one calcium related event, such as activation of CaMKII or N-type calcium channels, is dependent on another event such as the release of calcium from intracellular stores. Furthermore, experiments will be required to address the modulatory pathway involved in FaRP modulation.
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