Frequenin in Crustaceans:
Cloning and Immunolocalization

By

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A thesis submitted in conformity with the requirements for the Degree of the Master of Science in the University of Toronto

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Abstract

Frequenin in Crustaceans: Cloning and Immunolocalization, Master of Science, 1998,

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Crustaceans provide many advantages in the study of synaptic transmission and plasticity. Neuromuscular preparations have been well characterized in terms of long-term facilitation, synaptic depression and long-term adaptation. In addition, crustacean motor neurons are excellent models to study the differentiation into 'phasic' and 'tonic' motor neurons. These two types of motor neurons differ substantially in their transmitter release properties. Although the ultrastructural and biochemical differences between these neurons have been well studied, very little is known about possible molecular factors underlying this differentiation.

The neuronal calcium-binding protein frequenin has been shown to enhance synaptic efficacy at neuromuscular junction in Drosophila and Xenopus and could serve as such a molecular factor. Standard molecular biology techniques were used to clone frequenin from lobster and crayfish. The lobster and crayfish frequenins were found to be closely related to originally identified Drosophila frequenin. In addition, immunolocalization of frequenin at the crayfish neuromuscular junction revealed that frequenin is heavily expressed in 'phasic' and less strongly in 'tonic' motor neurons.
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List of Abbreviations

BSA  	Bovine serum albumin

dATP  	Deoxyadenosine triphosphate

dCTP  	Deoxycytidine triphosphate

dGTP  	Deoxyguanosine triphosphate

dTTP  	Deoxythymidine triphosphate

EPSP  	Excitatory postsynaptic potential

LTA  	Long-term adaptation

NGS  	Normal goat serum

NMJ  	Neuromuscular junction

PFA  	Paraformaldehyde

PCR  	Polymerase chain reaction

SSC buffer  	Sodium chloride/ sodium citrate
Introduction

Synaptic transmission is one of the basic processes of neuronal communication. This process is a dynamic activity-dependent phenomenon which is influenced by a variety of factors, including the development and differentiation of the presynaptic terminal, and the overall level of nerve impulse activity carried by the neuron (Atwood and Cooper, 1996).

At the neuromuscular junction in crustaceans, synaptic communication between the presynaptic motoneuron and the postsynaptic muscle is highly differentiated to accomplish specific physiological functions. Past work has defined many features of the physiology of these neurons, but relatively little is known about the molecular differences responsible for their physiological differentiation. The general goal of the present study was to further define the molecular basis for this synaptic differentiation.

Crustacean motor neurons have provided useful experimental models for investigating synaptic transmission and synaptic plasticity (Atwood and Wojtowicz, 1986; Atwood, Cooper, and Wojtowicz, 1994; and Atwood and Cooper, 1995). Individual motor neurons can be identified, are large and easily accessible for experimental manipulation, and show many of the physiological properties observed in other species such as short-term
and long-term facilitation, and depression. In crustaceans, two types of motor neuron have been identified that differ fundamentally in their physiological activity and transmitter-releasing properties. These motor neurons are generally termed ‘phasic’ and ‘tonic’ (Kennedy and Takeda, 1965 a and b). For a single nerve impulse the ‘phasic’ motor neuron can release up to 1000-fold the amount of transmitter released by the tonic motor neuron onto a single muscle fiber (Atwood et al. 1991).

In searching for molecular differences which might account for neuronal differentiation in crustaceans, Atwood et al. (1995c) found that a frequenin-like molecule is differentially expressed in crayfish motoneuron terminals. This neuronal calcium binding protein has been implicated as a molecule that modulates synaptic efficacy (Pongs et al., 1993). Overexpression of frequenin in Drosophila led to increased transmitter release under conditions of repetitive stimulation (Pongs et al., 1993; Rivosecchi et al. 1994). Olafsson et al. (1995) reported that infusion of Xenopus frequenin into Xenopus motor neurons increased spontaneous quantal transmission and evoked transmitter release. These observations suggest that frequenin has a role in regulating the strength of synaptic transmission. Its differential expression in crustacean motoneurons raises the interesting possibility that some of the observed physiological differentiation might be due to this molecular difference.
In the following, I will review the general features of synaptic transmission at crustacean neuromuscular junctions and the known properties of frequenin, relating these to the physiological properties of the 'phasic' and 'tonic' motor neuron, and develop the hypothesis that frequenin plays a role in the differences in transmitter release of these two types of motor neuron.

**Synaptic transmission at the neuromuscular junction:**

The release of the neurotransmitter glutamate from the presynaptic terminal and its subsequent binding to its postsynaptic receptors on the muscle surface is the basic process of synaptic communication between the motor neuron and the muscle in crustaceans and other arthropods. The arrangement of a presynaptic release site separated from the opposing specialized postsynaptic membrane by a small cleft is defined as a (chemical) synapse. Freeze-fracture photomicrographs of the presynaptic extracellular surface of crustacean synapses (Govind et al., 1994) show clusters of large intramembranous particles, representing putative Ca\(^{2+}\) and Ca\(^{2+}\)-dependent K\(^+\) channels (e.g. Cooper et al., 1996), in close association with vesicle fusion points mediating the release of neurotransmitter (Heuser & Reese, 1979). Where synaptic vesicles cluster near the presynaptic thickening, transmission electron micrographs show a localized accumulation of electron-dense material, the dense body or presynaptic dense bar. Such structures are commonly seen at synapses of crustacean neuromuscular junctions (Jahromi and Atwood, 1974; Walrond et al., 1993).
Molecular biological and biochemical efforts have revealed that vesicular exocytosis is mediated by a regulated network of protein-protein interactions (Scheller, 1995; Sudhof, 1995). A picture is emerging of the molecular machinery by which vesicles are docked at release sites and membrane fusion is triggered in response to calcium influx. It has become clear that synaptic vesicle docking and fusion can be viewed as a special case of membrane trafficking (for review, see Bennett and Scheller (1993). Selective targeting of a particular vesicle to a particular membrane destination is thought to be mediated by specific combinations of vesicle and target membrane proteins (called SNAP receptors or SNAREs).

According to the current model, synaptic vesicles dock and then proceed through a partial fusion reaction, priming, to make them competent for the final Ca\textsuperscript{2+}-triggered step (Sudhof, 1995). During priming, a complex called the core complex is assembled from the 3 synaptic proteins, two from the plasma membrane (syntaxin and SNAP-25) and one from synaptic vesicles (snaptobrevid/ VAMP). The core complex forms the anchor for a cascade of protein-protein interactions required for exocytosis to occur. Once the trimeric core complex has formed, it serves as a receptor for SNAP and the N-ethylmaleimide-sensitive factor (NSF). NSF is an ATPase, while the SNAPs are soluble NSF-attachment proteins. Assembly of the SNAPs into the core complex creates a high-affinity binding site for NSF which then disrupts the complex under ATP hydrolysis. Thus, an ordered sequence of protein-protein-interactions leads to the assembly of a multimeric complex which is then disrupted by NSF.
To trigger the final reaction of the fusion reaction, a Ca\textsuperscript{2+} sensor is required at the site of exocytosis (see below). Synaptotagmin, which occurs in various tissue-specific isoforms and has Ca\textsuperscript{2+}-binding domains, has been identified as such a sensor (for review, see Sudhof (1995). It binds Ca\textsuperscript{2+} cooperatively and undergoes a Ca\textsuperscript{2+}-dependent conformational change which completes the fusion reaction.

Two proteins that are not an integral part of the SNAREs underlying the (final) docking and fusion steps are the synapsins and dynamin. Synapsin has been identified as a molecule important for vesicle mobilization (Pieribone et al. 1996) whereas dynamin, a GTPase, has been shown to be essential for the recycling of fused vesicles (endocytosis) (Estes et al. 1996).

Exocytosis of neurotransmitter occurs in response to a presynaptic action potential which leads to a transient increase in the calcium concentration within the nerve terminal through the opening of voltage-gated calcium channels and their interaction with synaptic vesicle proteins probably including synaptotagmin and possibly also syntaxin and SNAP-25. At all synapses, including the crustacean neuromuscular junction, the release of neurotransmitter occurs in discrete multimolecular packages, i.e. is quantal in nature (Dudel & Kuffler, 1961). The morphological counterpart to the quantum is thought to be the single synaptic vesicle (Atwood & Wojtowicz, 1986). At the crayfish neuromuscular junction, it is believed that there are approximately 6000 molecules of glutamate per vesicle (Atwood & Wojtowicz, 1986).

Depolarization-induced Ca\textsuperscript{2+} entry through voltage-gated calcium channels of the presynaptic terminal has been shown to be a factor for the induction of
neurotransmitter release. Katz and Miledi (1967 and 1968) demonstrated that altering the concentration of external Ca$^{2+}$ affected the amount of transmitter released by nerve terminal depolarization. Direct evidence for a positive relationship between Ca$^{2+}$ influx and the magnitude of neurotransmitter secretion has been shown in studies of the giant synapse in squid (for review, see Augustine et al. 1991).

Current anatomical, physiological and theoretical findings indicate that calcium channels are clustered close to specific sites, the active zones, at which vesicles release their contents and have reinforced the concept that Ca$^{2+}$ entering thorough voltage-gated calcium channels exists transiently in a localized spatial domain, the so-called Ca$^{2+}$ microdomain (for review, see Stanley et al. 1997). It is becoming clear that the spatial-temporal dynamics of these Ca$^{2+}$ microdomains determine the amount of transmitter released. The interaction between these Ca$^{2+}$ microdomains by diffusion is affected by mobile and immobile Ca$^{2+}$ buffer present and could present a mechanism by which more and more active zones become recruited during repetitive firing of nerve impulses (Cooper et al. 1996)

As a calcium-binding protein, frequenin could alter the dynamics of Ca$^{2+}$ in these microdomains or its entry through voltage-gated channels. Although frequenin is unlikely to act as mere Ca$^{2+}$ buffer for reasons outlined below, it will be very informative
to study the dynamics of Ca$^{2+}$ in the presynaptic terminal in which the expression of frequenin has been altered.

**Crustacean synaptic differentiation:**

Two broad classes of motor neurons have been shown to exist in the crustaceans. These have been designated as 'phasic' and 'tonic' types (Kennedy and Takeda 1965 a,b). Since these motor neurons are physiologically and morphologically distinct from one another, they serve as suitable models for studying factors underlying synaptic differentiation (Cooper and Atwood, 1995).

The differences between these motor neurons are related to the physiological demands of the muscle fibers that they innervate. 'Phasic' neurons innervating the fast-acting muscles of the abdomen in crustaceans are generally silent and are recruited for rapid swimming and escape responses. The terminals of the 'phasic' motor neurons produce large excitatory post-synaptic potentials (EPSPs) which lead to evoked action potentials and hence contraction of the muscle fibers. Repetitive stimulation of the 'phasic' neurons leads to a rapid depression of EPSPs. These physiological effects are attributable to an initially large quantal output of transmitter and its subsequent decline (for review, see Atwood and Cooper, 1996).
‘Tonic’ neurons innervating the slow-acting postural muscles of the abdomen are usually active much of the time. Repetitive stimulation of the ‘tonic’ motor neurons at high frequency leads to facilitation rather than depression. The muscle responsible for the opening of the claw (hence called the claw opener muscle) receives its only excitatory input from a single axon with ‘tonic’ properties while the claw closer muscle and carpopodite extensor muscle of the walking limb is conjointly innervated by both ‘phasic’ and ‘tonic’ axons. In the claw closer muscle, the rapidly contracting fibers receive strong input from the ‘phasic’ axon while the slowly contracting fibers receive strong ‘tonic’ input (Atwood, 1982).

It is known from previous studies that there is an overall morphological difference in the nerve terminal morphology of these two types of axons. ‘Phasic’ axon terminals are relatively filiform and non-varicose, while ‘tonic’ axon terminals are larger in diameter and distinctly varicose (Lnenicka et al., 1986, 1991; Bradacs et al., 1997). At the ultrastructural level, the terminals of the ‘phasic’ motor neurons have uniformly-distributed synapses, whereas the synapses in the ‘tonic’ nerve endings are less widely dispersed and more confined in their localization to varicosities. Recordings from single boutons with focal extracellular electrodes have demonstrated differences in the quantal content of ‘phasic’ and ‘tonic’ motor neurons with the ‘phasic’ terminals releasing 50-2000 times
more transmitter per nerve impulse than their 'tonic' counterparts. While on average there is more synaptic surface area per unit length of the 'tonic' terminal (King et al. 1996), the release of quanta per single action potential is much greater in the filiform 'phasic' nerve endings (Cooper et al., 1995b; Bradacs et al., 1996). These findings suggest that the observed outstanding ultrastructural differences alone cannot account for the much greater transmitter output of 'phasic' nerve terminals when compared to their 'tonic' counterparts.

Differences in electrical and biochemical properties rather than gross ultrastructural differences are more likely to play important roles in synaptic differentiation of 'phasic' and 'tonic' terminals. Previous studies have indicated that there are differences in mitochondrial content and metabolic activity in these two types of motor neurons (Lnenicka et al., 1986; Nguyen and Atwood 1992a; Nguyen and Atwood, 1994). Furthermore, glutamate levels are higher in 'tonic' nerve endings (Shupliakov et al., 1995). This could possibly explain the capacity of these neurons for facilitation upon high-frequency stimulation by mechanisms which may involve making progressively more glutamate-containing synaptic vesicles available for release. The higher quantal content of the 'phasic' motor neuron cannot be explained by a higher glutamate content, since the glutamate concentration is actually lower in the 'phasic' terminals.
Previous studies by Cooper et al. (1995) have shown that a higher concentration of free Ca$^{2+}$ ions is generated in high-output terminals than in low-output terminals for the crayfish opener motor neuron ('tonic' type) at low frequencies of stimulation. Although only relative changes in Ca$^{2+}$ using a fluorescent indicator were measured, the authors reported that the high-output terminals had relatively larger changes in fluorescence during stimulation. Whether the differences in Ca$^{2+}$ signals are due to differences in the number of Ca$^{2+}$ channels in the presynaptic terminals or differences in the intraterminal Ca$^{2+}$ handling or extrusion remains to be seen.

In summary, molecular differences other than the glutamate content are likely to play an important role in determining the release properties of the 'phasic' and 'tonic' motor neuron. Neuronal calcium binding proteins could be potential candidate molecules.

Frequenin: A neuronal calcium-binding protein

The neuronal calcium-binding protein frequenin has been implicated in the modulation of synaptic efficacy (Pongs et al., 1993; Olaffson et al., 1995). Frequenin was originally discovered in a mutant in Drosophila. This mutant fly (V7) overexpresses frequenin as a consequence of a chromosomal rearrangement.
At the protein level, frequenin consists of four structural alpha helical elements, the so-called EF-hands, which are highly conserved among the different members in the superfamily of calcium-binding proteins. These “EF-hands” are the structural hallmarks for calcium binding and were first described in the calcium-binding protein calmodulin (Nakayama et al. 1994). Drosophila frequenin has been shown to bind calcium and to shift its mobility upon calcium-binding (Pongs et al., 1993).

Another structural feature of this class of neuronal calcium-binding proteins is their N-terminal myristoylation via an N-terminal glycine-residue. Studies of recoverin, a related member of the calcium-binding protein superfamily, have shown that myristoylation of recoverin decreases the affinity of calcium-binding, but introduces cooperativity of calcium binding (for review see Ames et al., 1996). Whether a similar mechanism is involved with frequenin remains to be elucidated. At the structural level, studies of recoverin have shown that myristoylation provides a conformational switch (a “myristoyl switch” ) which could be of importance for membrane-targeting and protein-protein interaction (Ames et al., 1996). Knowing the rate constants and affinity of frequenin for calcium will undoubtedly be helpful in correlating its binding characteristics to the calcium concentration measured in presynaptic terminals. Such studies will help to provide the basis for understanding the structure-function relationships of frequenin.
Studies in *Drosophila* have shown that the overexpression of frequenin leads to an enhancement of transmitter release under conditions of paired-pulse stimulation (Pongs et al., 1993). Studies in *Xenopus* nerve-muscle co-cultures have provided evidence that the infusion of *Xenopus* frequenin into motor neurons not only enhances frequency-dependent neurotransmitter release, but also alters the basal release properties (Olaffson et al., 1995). The authors showed that the infusion of the protein increased the frequency of spontaneous (quantal) synaptic currents, as well as their size. These functional studies predict a strategic localization of frequenin to the sites of transmitter release.

Frequenin expression has been localized to the developing nervous system of mammals both by in-situ hybridization and immunocytochemical means (Olaffson et al., 1997). Recent cellular and sub-cellular localization studies in mammalian brains have demonstrated that frequenin is expressed primarily in neurons and presumptive astrocytes. In cultured hippocampal neurons, frequenin has been co-localized with the dendritic marker MAP-2, and the synaptic vesicle marker SV2 (Olaffson et al., 1997). Although these authors have shown that frequenin is primarily localized to synaptic compartments, they have also found that frequenin is present in post-synaptic densities. The functional significance of this observation requires further investigation. Although the modulation of
transmitter release by frequenin has been documented, the molecular mechanisms for this modulation are still unclear.

Frequenin has initially been described as a regulator of guanylate cyclase (Pongs et al. 1993). Activation of frequenin upon calcium-binding could therefore potentially stimulate cGMP-dependent signaling pathways. Recent evidence suggested that the mammalian isoform of frequenin, called neuronal-calcium-sensor-1 (NCS-1), can substitute for calmodulin in Paramecium in vivo and in vitro (Schaad et al., 1996). Furthermore, these authors have observed that non-myristoylated recombinant mouse frequenin directly activates two Ca2+/calmodulin-dependent enzymes, 3’-5’ cyclic nucleotide phosphodiesterase, and protein phosphatases such as calcineurin in vitro. Its exact downstream signaling pathways, however, remain to be investigated.

Rivosecchi et al. (1994) proposed that a frequenin-dependent regulation of the presynaptic Na⁺-Ca²⁺ exchanger could account for the enhancement of facilitation in the frequenin-overexpressing flies. Although the exact molecular basis for this modulation remains to be investigated, these authors suggest that a stimulation of the exchanger via cGMP is responsible for this effect.
In summary, studies so far have consistently suggested a role for frequenin in the modulation of synaptic efficacy although the molecular mechanism for this modulation remains to be examined.

**Objective of the present study:**

Preliminary studies have indicated a differential expression of frequenin in phasic vs. tonic motor neuron terminals (Atwood et al. 1995). This was shown using a polyclonal antiserum raised against *Drosophila* frequenin. The major objective of this thesis was to establish the identity of molecules in the crustacean nervous system which are responsible for the immuno-reaction of the nerve terminals. In order to define precisely the molecules present in crustacean motor neurons, I have further extended the original study and have cloned the cDNA sequences for crayfish and lobster frequenin. The crustacean frequenins were identified as closely related members of the family of invertebrate frequenins.

In addition, the localization of frequenin in motor neuron terminals was further investigated by direct co-labeling studies. For this purpose, cross-reacting antibodies against *Drosophila* vesicle proteins were used in conjunction with the anti- *Drosophila* frequenin antibody. These co-labeling studies revealed that frequenin is heavily expressed in 'phasic', but less strongly in 'tonic' terminals.
Materials & Methods:

Chemicals:

If not otherwise specified, laboratory chemicals of the highest grade available (Sigma) were used for all experiments.

Animals:

The spiny lobster nerve cord library was prepared from the nerve cord, including the brain, of Panulirus interruptus, while the crayfish nerve cord library was prepared from Procambarus clarkii. For the immunocytochemistry, crayfish species Procambarus clarkii and fruit flies species Drosophila melanogaster Canton S strain were used.

Cloning of crayfish frequenin:

In order to clone frequenin-related sequences from lobster and crayfish, I have first isolated an internal DNA fragment by the polymerase-chain reaction (PCR) using degenerate primers and then used these internal fragments of frequenin cDNAs for screening of the crayfish and lobster nerve cord cDNA libraries under high-stringency conditions as outlined (Fig. 1).

The cDNA libraries used for the cloning of crayfish and lobster frequenin were provided by Greg Lnenicka (SUNY, Albany) and Wulf-D. Krenz (Salk Institute, San Diego) respectively.
a.) PCR amplification of an internal DNA fragment of frequenin:

5 ul of the cDNA library and degenerate primers against the conserved EF-hands of frequenin were used for the PCR. As a given amino acid can be encoded by more than one codon (degeneracy of the genetic code), primers were designed that vary in the third position of the codon (degenerate primer) to account for all possible codons that code for each amino acid. The sequence of the primers used were Frq F1 5' AAR ATH TAY AAR CAR TTY TTY CC 3' and Frq B1 5' CCR TCB TTR TTY TCR TCR AA 3'. The forward primer Frq F1 anneals shortly 3' to the first EF-hand, while the backward primer Frq B1 anneals 3' to the third EF-hand of frequenin (Fig. 2).

These primers were synthesized by the automatic oligo-synthesis service of the Samuel Lunenfeld Research Institute of the Mount Sinai Hospital, Toronto.

The PCR was performed in a 50 ul volume consisting of:

5 ul nerve cord phage cDNA library
2 ul of each primer Frq F1 and Frq B1
1 ul of 10 mM PCR nucleotide mix (Boehringer)
5 ul of 10x reaction buffer (Promega)

36.5 ul of sterile H2O and

0.5 ul (corresponding to 2.5 units) of Taq Polymerase
(Promega).
The PCR reagents were mixed on ice, and then the Taq Polymerase was added. The reaction was overlaid with mineral oil (Sigma), and a 'hot-start' in the Perkin-Elmer PCR machine was performed.

For amplification of the frequenin cDNA fragment from the crayfish and lobster nerve cord libraries, the following profile was used:

- initial denaturation at 94°C for 3 min,
- 94°C for 1 min, 45°C for 1 min and 72°C for 1.5 min for 35 cycles followed by
- a final extension step at 72°C for min.

25 μl of the PCR reaction was run on a 1.8% agarose gel (Life Technologies). The fragment of the expected size of 240 bp was excised from the gel, purified using the GeneClean kit (Bio101) and subcloned into the pCR II vector (Invitrogen). The insert-containing DNA was purified using the Quiagen Mini-Prep kit (Quiagen) and subjected to automatic di-deoxy-sequencing (Biotechnology Service Centre, Banting Institute, University of Toronto).

In the following, the cloning of crayfish frequenin will be described in more detail as this is the major focus of the thesis. The cloning of the lobster frequenin followed identical procedures: an internal fragment of the frequenin cDNA was first amplified by PCR using the degenerate primers Frq F1 and Frq B1. This amplified fragment was then used to screen the lobster nerve-cord cDNA library under high-stringency conditions and subsequent procedures were identical to those described for crayfish frequenin.
Fig. 1 Schematic diagram of the procedure used for the isolation of the crayfish and lobster frequenin full-length cDNA clones
Fig. 1

**PCR amplification of the frequenin cDNA fragment**

↓

**High stringency screening of the nerve-cord cDNA library**

↓

**Isolation and sequencing of a full-length frequenin cDNA clone**
b.) Screening of the crayfish nerve cord nerve cord cDNA library:

I.) Plating of the library and transfer to membranes

The library was plated on C600 bacteria (Invitrogen) at about $10^5$ plaques per large (15 cm in diameter) Petri dish. The plates were incubated at 37 °C for 8 hrs until the plaques had reached about 1 mm in diameter and the plates were subsequently chilled at 4 °C.

Each plate was transferred to Hybond N+ membranes (Amersham) according to the manufacturer’s instructions. The membrane of corresponding size was placed on the agar surface. After 1 min the membrane was removed and placed colony side up on a pad of absorbent filter paper (Whatman) soaked in denaturing solution. After 7 min, the membrane was transferred to a pad of absorbent filter paper (Whatman) soaked with neutralizing solution, and then after 3 min transferred to a fresh pad soaked with the same solution. The filter was washed in 2 x SSC (sodium chloride/ sodium citrate 20x : 3M NaCl, 0.3 M Na$_3$citrate 2H$_2$O, the pH was adjusted to 7.0 with 1 M HCl), transferred to dry filter paper and air-dried or dried at 80 °C in an oven for 10 min (Ausubel et al., 1987).

II.) Random-primed labeling of the internal PCR fragment

Labeled frequentin DNA for the high-stringency screening of the nerve cord libraries was prepared using the random primed DNA labeling kit (Boehringer). This protocol of random primed DNA labeling developed by Feinberg and Vogelstein (1994) is based on the hybridization of a mixture of all possible hexanucleotides to the DNA to be labeled.
To carry out the labeling reaction, the purified frequenin cDNA fragment was denatured by boiling resulting in linear DNA molecules. These were then annealed to random-sequence hexanucleotides and incubated with the Klenow fragment (the large fragment of DNA polymerase I, lacking 5'→3' exonuclease activity) in the presence of nucleotides, including a $^{32}$P-labeled nucleotide. In this way, the hexanucleotides prime the frequenin DNA at various positions along the template and are extended to generate double-stranded DNA that is uniformly labeled on both strands.
Fig. 2 Positions of the two degenerate primers Frq F1 and B1 used for PCR amplification of the crayfish and lobster cDNA fragments. The protein sequence of *Drosophila frequenin* with its EF-hands in bold are shown. The positions where the two primers anneal are underlined.
Figure 2. Protein sequence of *Drosophila* frequenin (Pongs et al., 1993). The EF-hands are shown in bold and the regions where the primers anneal are underlined.
According to the protocol, 25-50 ng of frequenin DNA was denatured by heating for 10 min at 100 °C and subsequent cooling on ice. The following reagents were then added to a microtube on ice:

- 25-50 ng denatured DNA
- 3 ul dATP, dGTP and dTTP
- 2 ul reaction mixture
- 5 ul = 50 uCi dCTP, 3000 Ci/mmol, aqueous solution, ddH$_2$O to make up the reaction volume to 19 ul and
- 1 ul Klenow Enzyme (4 u/ul).

The reaction was then incubated for 30 min at 37 °C and stopped by adding 2 ul 0.2 M EDTA (pH 8.0). This reaction mixture was then passed over a Sephadex 50 column (Boehringer) to separate the labeled DNA from the unincorporated nucleotides. The amount of radioactivity was then determined by scintillation counting: 3-5 $10^6$ cpm of the labeled probe were added to each hybridization tube in order to standardize the amount of radioactive probe added.

### III.) High-stringency hybridization in aqueous solution

In order to identify DNA sequences that match the sequences of the frequenin cDNA fragment, I screened the crayfish nerve-cord library transferred to membranes as described
in I.) under high stringency conditions in aqueous solution as described by Church & Gilbert (1984). All steps were carried out at 65 °C in a rotating hybridization oven.

The membranes were prehybridized in hybridization solution II (1 % crystalline solution BSA, 1 mM EDTA (Fluka), 7 % SDS (Boehringer), 0.5 M NaHPO4, pH 7.2,) for at least 1 hr. 3-5 mill. cpm of the labeled probe was then added and the hybridization was carried out for at least 16 hr. The hybridization solution was then poured out and the membranes were washed twice with low-stringency wash buffer II (0.5 % BSA, 1 mM Na2EDTA, 5 % SDS (Boehringer), 40 mM NaHPO4, pH 7.2) followed by multiple (5-8) quick washes and a final wash for 20 min in high-stringency wash buffer II (1 % SDS (Boehringer), 1 mM Na2EDTA, 40 mM NaHPO4, pH 7.2). The membranes were then removed from the hybridization tubes, briefly dried on filter paper (Whatman) and exposed to an autoradiographic film (Kodak X-Omat) for 16-24 hrs. Areas of the plates corresponding to areas of hybridization signs were eluted from the corresponding plate and replated at lower density until a single plaque could be identified. Single plaques were then subcloned into the pCR vector (Invitrogen) and the largest insert (1.5 kb) subjected to automatic di-deoxysequencing (Biotechnology Service Centre, Banting Institute, University of Toronto).
Immunocytochemistry:

a.) Animals:

I.) Crayfish:

Freshwater crayfish, *Procambarus clarkii* Girard, of intermediate size (6 cm) from rostrum to telson, mass 5-6g) were obtained from Atchafalaya Biological Supply Co., Louisiana, USA, and maintained communally in dechlorinated aerated tapwater at 16 °C on a 12 hr light/dark cycle. The animals' diet consisted of lentils, carrots and fishfood (Tetramin).

II.) *Drosophila melanogaster*:

*Drosophila melanogaster* larvae (Canton-S wild-type) were reared on standard cornmeal medium (Stewart et al, 1995). Wandering 3rd instar larvae were used for all experiments.

b.) Preparations:

I.) Crayfish muscle dissections:

For the dissection of the abdominal extensor muscles, the shell was cut through along the lower lateral border of the abdomen on each side. The lower half of the abdomen together with the flexor musculature was then readily separated from the upper half of the abdomen and the extensor musculature. The preparation was then anchored with the ventral (muscle) side up by putting a pin through the shell at each end into a Sylgard-dish. The preparation was kept in crayfish solution and processed as described below. This
preparation of the abdominal extensor muscles is described in more detail in (Atwood and Parnas, 1966).

For the dissection of the leg extensor muscle, the first (or occasionally the second) pair of walking legs was used. The muscle was exposed by removing the cuticle on the lateral aspect of the meropodite along with the entire flexor muscle and also the main leg nerve. The preparation was then superfused with crayfish solution until pre-fixation.

For preparation of the abdominal slow flexor muscle, the abdomen was pinned ventral-side up in a Sylgard dissecting dish and bathed in crayfish solution. The superficial exoskeleton was then carefully removed from the abdominal segments (1-5) exposing the slow (superficial) flexor muscles (Kennedy and Takeda, 1965 b).

II.) Preparation of Drosophila 3rd instar larval fillets:

3rd instar larvae were dissected by making a longitudinal mid-dorsal incision and pinning the cuticle flat using insect pins as described in Stewart et al. (1996). The internal organs were carefully removed to expose body-wall muscles and the nervous system. These 3rd instar fillets were then fixed in the dish and processed for staining as described below for the crayfish muscle specimens.

c.) Solutions:

I.) Crayfish saline:

The composition of the crayfish physiological solution used is as follows (concentrations in mM; modified after van Harreveld, 1936): NaCl 205.3, KCl 5.3, CaCl₂ 2H₂O 13.5, MgCl₆H₂O 2.5 and Hepes buffer, 0.5 mM, adjusted to pH 7.4.
The different crayfish muscle preparations were pre-fixed in 3% paraformaldehyde (PFA) and 4% sucrose in phosphate-buffered saline (PBS) containing (in mM): 137 NaCl, 2.7 KCl, 4.3 Na$_2$HPO$_4$ 7H$_2$O, 1.4 KH$_2$PO$_4$, pH ~ 7.3.

II. Solution used for *Drosophila* larval dissection:

The third instar larval fillet dissections were performed in Schneiders medium (Life Technologies).

d.) Antibodies:

The anti-*Drosophila* frequenin antibody was directed against the full-length *Drosophila* frequenin (Pongs et al., 1993). It recognizes a 21 kDa protein in crayfish ganglia and also cross-reacts with the recently-cloned crayfish frequenin (see Appendix). The antibody was a gift from O. Pongs (Hamburg, Germany) and was used at a dilution of 1:100. The anti-*Drosophila* synapsin antibody (SYNORF1) was used at a dilution of 1:20 and was the generous gift of E. Buchner (Klagges et al., 1995). The anti-*Drosophila* dynamin antibody (Estes et al. 1996) was a gift of R. Kelly and J. Roos and was used at a dilution of 1:100. The anti-*Drosophila* cysteine-string protein antibody was kindly provided by K. Zinsmaier and used at 1:5 (Zinsmaier et al., 1994). It showed only weak immunoreactivity and was not characterized further. The anti-*Drosophila* synaptotagmin was provided by H. Bellen (see Cooper et al., 1995 a). This antibody was raised in rabbits and therefore was not used for the double-labeling experiments with the anti-*Drosophila* frequenin antibody which was also raised in rabbits. A second anti-synapsin antibody (G-304) was donated by A.
Czernik and P. Greengard and used at 1:100. It produced a staining similar to the one using the anti-\textit{Drosophila} synapsin (SYNORF1) mouse monoclonal antibody (Klagges et al., 1995). For the double-labeling shown here however, only the mouse monoclonal anti-synapsin antibody was used.

Except for the anti-\textit{Drosophila} synapsin monoclonal antibody which was used as hybridoma supernatant, all polyclonal antisera were used as affinity-purified antibodies.

e.) Fixation and staining:

I. Prefixation of crayfish muscle preparations:

The crayfish nerve muscle preparations were prefixed in 3 \% PFA and 4 \% sucrose in PBS.

After this prefixation, which lasted 30 min at room temperature, the specimens were transferred briefly to the crayfish standard solution. Then, thin layers of the lateral parts of the (deep) abdominal extensor muscles were sliced off the surface with a razor blade scalpel. After prefixation for 30 min at room temperature, the leg exensor muscle and the slow flexor muscle were also transferred briefly to the standard crayfish solution, separated from the exoskeleton, and then cut into smaller thin pieces. These 3 different nerve-muscle preparations were then further fixed and processed as described below.

II. Fixation and staining:

Crayfish nerve-muscle preparations, prefixed and dissected into small pieces as described above, were fixed in PBS solution containing 3\% paraformaldehyde and 4\% sucrose for an additional 1hr at room temperature.
The 3rd instar larval fillets were only fixed for 1 hr in PBS containing 3% paraformaldehyde and 4% sucrose. No further dissection was necessary for this preparation.

The fixed specimens (both the *Drosophila* larval fillets and the crayfish nerve-muscle preparations) were then transferred to individual wells of a multi-well plate (Nunc), washed three times at one-hour intervals with PBS, and permeabilized in PBS containing 2% normal goat serum (NGS) (Jackson Immunochernicals), 2% bovine serum albumin (BSA) (Sigma), and 4% Triton-X-100 (Sigma) (hence called 4% PBT) for one hour at 4 °C. These preparations were incubated with the primary antibodies in 0.4% PBT overnight. Following incubation in the primary antibody, the specimens were washed three times as above and incubated with secondary antibodies in 0.4% PBT for one hour. Secondary antibodies were then removed and specimens washed three times in PBS and mounted in Permafluor (Immunon) mounting medium.

Anti-rabbit or anti-mouse secondary antibodies were obtained from Jackson Immunolabs and were used at 1:400 dilution in 0.4% PBT. For the double-labeling experiments in which both frequenin and synapsin immunoreactivity were demonstrated, goat anti-mouse-cy3- and donkey anti-rabbit-cy5-conjugated secondary antibodies (Jackson Immunochernicals) were used. The goat anti-mouse cy3-conjugated secondary antibody was used for the mouse monoclonal anti-*Drosophila* synapsin antibody (SYNORF1), while the donkey anti-rabbit cy5-conjugated secondary antibody was used for the polyclonal anti-*Drosophila* frequenin antibody raised in rabbits. Cy3 and cy5 are both cyanine dyes. Cy5 has been used with a variety of other fluorophores due to the wide separation of its
emission spectrum from that of shorter-wavelength-emitting fluorophores. Cy5 is excited maximally near 650 nm and fluorescences maximally near 670 nm. Cy3 can be maximally excited near 550 nm with a peak fluorescence near 570 nm.

III.) Imaging:

The mounted specimens were imagined with a MRC 600 confocal microscope (Bio-Rad, Mississauga, ON) equipped with a Krypton/Argon laser using either a 40x or 63x oil-immersion objective and the COMOS software. For excitation of cy3-conjugated secondary antibodies, the YHS filter block was used, while for the excitation of cy5-conjugated secondary antibodies the RHS filter block was used. The YHS filter block consists of the 568 nm DF10 excitor filter, the dichroic reflector 585 DRLP and the emission filter 585 EFLP. The RHS filter block consists of the excitor filter 647 DF10, the dichroic reflector 660 DRLP and the emission filter 680 EF32.

To ensure confocality, the pinhole was set to -0.5 in all experiments. If z-scans were taken, the distance between individual planes was set to 0.5 μm. The individual z-scans were projected and saved into a single projected image file. These projected z-scans were then imported into Confocal Assistant (Bio-Rad) and Adobe Photoshop for generation of the red-green overlays shown in the figures.
**Results:**

I have cloned frequenin cDNAs from crayfish and lobster nerve cord libraries and analyzed the primary sequences coded by these cDNAs. Antibodies against two synaptic proteins, synapsin and dynamin, were used to reveal the localization of synapses in different crayfish nerve-muscle preparations and to compare these with the location of the sites of expression of frequenin immunoreactivity, as revealed by the anti-\textit{Drosophila} frequenin antibody.

a.) Cloning and analysis of crayfish and lobster frequenin cDNAs:

Crayfish frequenin was cloned from a nerve-cord library using a combination of PCR and high-stringency screening (Fig. 1). As the EF-hands at the conserved regions were considered to be conserved among members of the frequenin family, two degenerate primers (Frq F1 and B1) was designed to amplify a fragment from the nerve cord library (Fig. 2). This crayfish frequenin cDNA fragment was then used to identify a full-length frequenin cDNA clone using the procedures described in the “Material and Methods” section (Fig. 1).
Following identical procedures to those described for crayfish frequenin, I amplified an internal lobster frequenin cDNA fragment from the lobster nerve cord library (Fig. 4) and used it to screen the lobster nerve cord cDNA library under high stringency conditions.

A PCR fragment of the expected size was amplified from the lobster nerve cord (Fig. 4) and crayfish nerve cord cDNA library (Fig. 3) using the primers Frq F1 and B1. These fragments were used for high-stringency screening of the corresponding library in order to isolate a full-length lobster and crayfish frequenin cDNA clone.

The crayfish cDNA frequenin clone with the largest insert of 1.5 kb identified (Fig. 5) was subjected to automatic di-deoxysequencing. The sequence obtained is shown in Fig. 6. The analysis of all possible open reading frames (a, b and c) of the crayfish cDNA sequence was performed using the MAP program (GCG package, Wisconsin, Madison). MAP translates the DNA sequences into all possible open reading frames ((a), (b) and (c)). The longest open reading frame for crayfish frequenin that could be identified by MAP is reading frame (a). This reading frame starts at position 204 (marked by an arrow) and ends at position 797 (Fig. 6). The crayfish frequenin open reading frame is preceded and followed by several stop codons demonstrating that this open-reading frame is a full-length open reading frame (Fig. 6).
Fig. 3 Gel electrophoresis on a 1.8 % agarose gel of the PCR products amplified from the crayfish nerve-cord library using the degenerate primers Frq F1 and Frq B1. The PCR fragment of the expected size (at the arrow) was excised and subcloned.

A: 1 kb ladder molecular weight marker (Life Technologies), B: First amplification from the crayfish nerve-cord library, C: 10 ul of the first amplification reaction was amplified for a second time, D: PCR amplification from *Drosophila* frequenin (positive control) using the Frq F1 and Frq B1 primers.
Fig 3
Fig. 4 Gel electrophoresis on a 1.8 % agarose gel of the PCR products amplified from the lobster nerve-cord library using the degenerate primers Frq F1 and Frq B1. The PCR fragment of the expected size (at the arrow) was excised and subcloned.

A: 1 kb ladder molecular weight marker (Life Technologies), B: First amplification from the lobster nerve-cord library, C: 10 ul of the first amplification reaction was amplified for a second time, D: PCR amplification from Drosophila frequenin (positive control) using the Frq F1 and Frq B1 primers.
Fig 4
Reading frame (a) encodes for a protein of 193 amino acids (Fig. 8). The extracted protein sequence of crayfish frequenin with its highlighted structural features, the EF-hands and the possible N-myristoylation motif, is shown in Fig. 8.

The analysis of the lobster frequenin cDNA clone identified with the largest insert (1.2 kb) (Fig. 5) revealed, using MAP (GCG package, Wisconsin, Madison), a single open reading frame starting at position 58 and terminating at position 610 of reading frame (a) (Fig. 7). This open reading frame is also preceeded and followed by stop codons (Fig. 7). This longest open-reading frame that could be identified encodes for protein of 188 amino acids in length (Fig. 9). The extracted protein sequence with its structural features, the EF-hands and the N-terminal glycine residue that could serve as a myristoylation site, is shown in Fig. 9.

A pairwise comparison of crayfish, lobster and Drosophila frequenin cDNA sequences using GAP (GCG software package) revealed that the three sequences are about 67 % identical to each other. GAP aligns two sequences in their entirety by introducing gaps into the sequence. Gaps are introduced into each of the two sequences to be aligned, until a maximum of sequence similarity is reached. At the protein level, a pairwise alignment using GAP showed that the amino acid sequences of crayfish and lobster frequenin are about 74
and 80 % identical to that of Drosophila frequenin, respectively (Fig. 10 and 11). Lobster and crayfish frequenin share 74 % identity at the amino acid level (Fig. 12).
Fig. 5  Analysis of the insert-size of purified single plaques of crayfish and lobster frequenin cDNAs identified from the high-stringency screen by electrophoresis on a 1.2% gel.

A: 1 kb ladder molecular weight marker (Life Technologies), B: insert from a single plaque identified from the crayfish nerve-cord library. C: insert from an independent plaque identified from the crayfish nerve-cord library. D: insert from a single plaque identified from the lobster nerve-cord library. E: insert from a single plaque identified from the crayfish nerve-cord library. This insert is identical to B, as it was derived from the same plaque.

The longest inserts from each screen, (B) from the crayfish nerve-cord library and (D) from the lobster nerve-cord library, were subjected to sequencing.
Fig. 6 Translation of the crayfish frequenin cDNA into the possible open reading frames using **MAP** (GCG software package) This program translates the nucleic acid sequence in any of the possible translation frames. The three possible reading frames are shown as (a), (b) and (c). The translation initiation site, the initial methionine, of the crayfish frequenin is marked by an arrow (position 204). The open reading frame (a) of the crayfish frequenin ends at position 797 as depicted by an asterisk.
Fig. 6

```
1 60
GCAGAGAGAAGCGGCTAGCAGACAAAGAGACGAGAGATATTATATAAAACAAAAAGGTGAA
CGTCTCTCTGGCCTACTGTCTCTCTGGCCTCTCTAAAAATTATTGTTTTCCCCACTTT

a A E R S G S R Q E T Q R Y Y I N K R G E
b Q R E A V A D K R R R D I I * T K G V K
C R E K R * Q T R D A E I L Y K Q K * S

61 120
GCATCGGTATGGAAACCATGATCTGGATGAATCATGCACCCCAACCACTCTCAGTGTT
CGTAGGCAAATAACTTATGTACTAGACCTACTTTAGTGACCCGGGTGGAGTACATCAAC

a A S V I E Y H D L D E S C P N H L L S S G
b H P L L N T M I W M N H A P T T S V V
C I R Y * I P * S G * I M P Q P P Q * W C

121 180
GTGAGTCTGTGCTAATCTCTCTCTCTCCTCTCTCTACCTCTCTCTCTCTCGCC
CACTGACACAGATTAAGGAGGGAAAGAGGAGAGAGGAAAGTGAGTTGGAGGAGAGAGCGGG

a V S L C * F L P S P P L S S P L P S P S P
b * V C A N S S L L L L F H H L S L P R P
C E S V L I P F P S S F I T S P F L A Q

181 240
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TCTGAGTCTGTTGTTGGAGCTTTGTACCTCTCTCTCTGAGGTTTGTGGAGGTTGCTCTTGA

a R R H Q P P A N M G K K N S K L K L Q E T
b D V T N H L Q T W E R R T P N S N R K L
C T S P T T C K H G K E E L Q T G T N Y

241 300
ATCCAGGAAGACTGTTGAAAGAAACCTATTATTTTCTGAACAAAAAATCAAGCAATGGACAAAG
TAGGTCTTTTGCACACTCCTCTCTGGAATAAAAAGACTGTCTTTTGATTGTGACCTGTTTC

a I Q K L C E E T Y F S D K E I K Q W H K
b S R N C V K K L I F L T K K S S N G T S
C P E T V * R N L F F * Q R N Q A M A Q A

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GTTACCGTGTTTCTCCAAAGAGGATTTCTACTACAGGTTTACCGGAAACTGCTCCTGCCCAAAG

a Q W H K G F L K D C P N G L L L T E T G F
b N G T R G S S K I V Q M A F * R R R G S
C M A Q G V P Q R L S K W P F D G D G V H

361 420
ATCAAGATATAACAAAGCAGTTTCTTCCCAAGGAGACCCCAACCAAGGATTGTTCCTCTCTGTTG
TAGTTCTATATGTTGCTAAAGGGGGTTCCCTGCTGGGTGTTCAACGGAGAGACCAC

a I K I Y K Q F F P Q G D P T K V A S L V
b S R Y T S S S S S P R E T P P P R L P L W C
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Fig. 7 Translation of the lobster frequenin cDNA into the possible open reading frames using MAP (GCG software package). This program translates the nucleic acid sequence in any of the possible translation frames. The three possible open reading frames are shown as (a), (b) and (c). The translation initiation site, the initial methionine, of the lobster frequenin is marked by an arrow (position 58). The open reading frame (a) of the lobster frequenin ends at position 688 as depicted by an asterisk.
Fig. 7

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| AAGGATGACAACGAGTGGCAGACAAAGGATTCTCAAGAGCTGTCCGAAATGGCTACTGAC  | -
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| KRSNSGKTFSKTVMAY*P  | -
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| HPSALHHRERG*KATMGF  | -
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| FKL VDNDGIFITRREMYS  | -
| SSCMTTMMVSSSPERRCIV  | -
| QAV*RQRWFHPHRPGDV*YS  | -
As in *Drosophila* frequenin (Pongs et al., 1993), the first and fourth EF-hand are most likely non-functional based on the analysis of the primary structure. In the first EF-hand of crayfish and lobster frequenin, the CPNG amino acid motif is found (Fig. 8 and 9, respectively). The cysteine and proline residues are believed to impair the α-helical arrangement of this EF-hand (Ames et al., 1996). The fourth EF-hand of crayfish and lobster contains a number of charged amino acid residues as does the fourth EF-hand of *Drosophila* frequenin. The third EF-hands are almost 100 % identical between crayfish, lobster and *Drosophila* frequenins except for a single amino acid change (Y→F) in third EF-hand of both crayfish and lobster frequenins (Figs. 10 and 11). The second EF-hand is 100 % conserved in lobster frequenin compared to *Drosophila* frequenin (Fig. 11). A single amino acid substitution has occurred in the second EF-hands of crayfish frequenin (A→S) when compared to *Drosophila* frequenin (Fig. 10).

The N-terminal myristoylation consensus sequence (MGXXS) is also found in crayfish frequenin (Fig. 8), whereas this motif is absent in lobster frequenin (Fig. 9). Whether the glycine-residue immediately downstream (YRGIQ) (Fig. 9) can function as myristoylation site in lobster frequenin remains to be determined.

It is apparent from the sequence comparison that the lowest degree of sequence conservation is found in the N- and C-terminus (Fig. 11 and 12). While the N-terminus of
crayfish and *Drosophila* frequenin is rather similar, the C-terminus is not (Fig. 11.). The opposite pattern of sequence conservation is found for lobster and *Drosophila* frequenin (Fig. 11). Between these two proteins, the C-terminus is almost identical except for 5 amino acid changes, while the N-terminus is less well conserved.
Fig 8 Amino acid sequence of crayfish frequenin, as extracted by MAP (GCG software package). The EF-hands are shown in **bold**, the N-terminal glycine residue that might be myristoylated is also shown in **bold** and marked by an asterisk.
Crayfish Frequentin

MGKKMSKLKQETIQKLCEETYFSDKKEIKQWHKGFLKDCPNGDLLTE
TGFIKIQKFFPQGDPTKVASLVFRVFDENNDGAIEFEEFIRALSITSR
GNVDEKLLWAFRLYDVNDGFITREEYISIVDAIYQMVGQAPEAE
DENTPQKRVDKIFSMRRGSNAFAFYPGRNTHKSRIPAHWRPLLVD
PSSVPbars*  193
Fig. 9  Amino acid sequence of lobster frequenin, as extracted by MAP (GCG software package).

The EF-hands are shown in **bold**, the N-terminal glycine residue that might be myristoylated is also shown in **bold** and marked by an asterisk.
Lobster Frequentin

1    *
MKTYRGIQEKQTSLNILVTKEIKQWHKGFLKDCPNGLLTETGFKI
YKQFFQPQGDPTKFASLVFRVFDEENDDGSEIFEEFIRALSITSRGNVDE
KLLWAFKLYDVNDGFTREEMYSIVDAIYQMVGAPEAADENTP
QKRVDKIFSQMDKNHDEKLTLEFKEGSNADPRIVQALSLGDN*
Fig. 10 Pair-wise alignment of crayfish and *Drosophila* frequenin protein sequences using **GAP** (GCG software package). The protein sequence of crayfish frequenin is shown in the upper and *Drosophila* frequenin is lower panel. **GAP** generates the best alignment of two sequences in their entirety by introducing gaps in either of the sequences to effect better alignment. The parameters used for each alignment and the percent identity and similarity are shown above each sequence alignment. Identical amino acids are marked by lines, while similar amino acids are marked by dots. The four EF-hands are marked by the arrows.
Pairwise-alignment of crayfish and *Drosophila* frequenin protein sequences

Crayfish (upper panel) vs. *Drosophila* frequenin (lower panel)

Gap Weight: 3.000   Average Match: 0.540
Length Weight: 0.100 Average Mismatch: -0.396
Quality: 217.8   Length: 192
Ratio: 1.171   Gaps: 2
Percent Similarity: 85.405   Percent Identity: 73.514

```
1 MKKKMSKLQDIETIQKLCEETVFSKDEIKWKGFLKDPNGLLTTETFIFIK 50
1 MKKSSKLQDIETIQKLCEETVFSKDEIKWKGFLKDPNGLLTTETFIFIK 50
51 IYKFPPQGDPPTKVASLVFRVDENNGDAIEEFEFIRALKSITRSNVDEK 100
51 IYKFPPQGDPASLVFRVDENNGDAIEEFEFIRALKSITRKNVDEK 100
101 LLWAFRLYDVNDGFTIREEYIVDAIYMQGQAEDENTPKRVDK 150
101 LLWAFRLYDVNDGFTIREEYIVDAIYMQGQAEDENTPKRVDK 150
151 IFSMRGSN,FAYYPGRNTHKSRIAPAWRPRLVDPSSVPS 191
151 IFSMQGSK,LDQMDKNOGKLTLEDFREGSKA......DPRIVQALSLLGDG 186
```
Fig. 11 Pair-wise alignment of lobster and *Drosophila* frequenin protein sequences using **GAP** (GCG software package). The protein sequence of lobster frequenin is shown in the upper and *Drosophila* frequenin is lower panel. **GAP** generates the best alignment of two sequences in their entirety by introducing gaps in either of the sequences to effect better alignment. The parameters used for each alignment and the percent identity and similarity are shown above each sequence alignment. Identical amino acids are marked by lines, while similar amino acids are marked by dots. The four EF-hands are marked by the arrows.
Fig. 11

Pair-wise alignment of lobster and *Drosophila* frequenin protein sequences

b.) Lobster (upper panel) vs. *Drosophila* frequenin (lower panel)

<table>
<thead>
<tr>
<th>Gap Weight</th>
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<th>Average Match: 0.540</th>
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</thead>
<tbody>
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<td>Average Mismatch: -0.396</td>
</tr>
<tr>
<td>Quality:</td>
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<td>Length: 190</td>
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<tr>
<td>Ratio:</td>
<td>1.249</td>
<td>Gaps: 1</td>
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<tr>
<td>Percent Similarity: 89.011</td>
<td>Percent Identity: 80.769</td>
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1 MKTNYRGIQÉQKQTSILNLIVÎ......KEIKQMHKFLKDCPNGLLTETGF 44
1 ...MGKSSKLLKDQTDRLLTDTYFTKEIRQWHKGFKDCPNGLLTTEQGF 48
45 IKIYKQFFQGDPDTKASLVFRVFDENNDSIEEFIRÁLSITSRGNVD 94
49 IKIYKQFFQGDPSKFASLVFRVFDENNDSIEEFIRÁLSVTSDKGNLD 98
95 EKLWAFKLYDVONGDGFITREEMYSIVDAIYQMGHAPEÁADENTPOKRVT 144
99 EKLQWAFLYDVONDGITYREEMYIVDAIYQMGQPOQEDENTPOKRVT 148
145 DKIFSQMDKHNHEKLTLEEFKESGNADPRÍVOQALSLGDN* 184
149 DKIFDQMDKHNHDGKLTLLEEFSKADPRÍVOQALSLGGG* 188
```
Fig. 12 Pair-wise alignment of lobster and crayfish frequenin protein sequences using GAP (GCG software package). The protein sequence of lobster frequenin is shown in the upper and crayfish frequenin is lower panel. GAP generates the best alignment of two sequences in their entirety by introducing gaps in either of the sequences to effect better alignment. The parameters used for each alignment and the percent identity and similarity are shown above each sequence alignment. Identical amino acids are marked by lines, while similar amino acids are marked by dots. The four EF-hands are marked by the arrows.
Fig. 12

**Pair-wise alignment of crayfish and lobster frequenin protein sequences**

Lobster (upper panel) vs. crayfish frequenin (lower panel)

Gap Weight: 3.000  Average Match: 0.540
Length Weight: 0.100  Average Mismatch: -0.396

Quality: 204.2  Length: 198
Ratio: 1.110  Gaps: 1
Percent Similarity: 78.333  Percent Identity: 73.889

<table>
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<tr>
<th>Lobster</th>
<th>Crayfish</th>
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<tbody>
<tr>
<td>MKTNYRGIQEKTSLINVTEIKQHKGFKLDPNGLLTETGFIK</td>
<td>MGKMKSLKQETIQKCEETYFSDEIKQHKGFKLDPNGLLTETGFIK</td>
</tr>
<tr>
<td>IYQEFFPOQPDKTFASLFRVFDENNGSIEEFEFIRALSSGTSRNVDEK</td>
<td>IYQEFFPOQPDKTVASLFRVFDENNGSIEEFEFIRALSITSRGNVDEK</td>
</tr>
<tr>
<td>LLWAFKLYVDNDGFITREEMSIVDAlYQMVHAEAMDENPTQKRDK</td>
<td>LLWAFRLYVDNDGFITREEMSIVDAlYQMVGQAEAMDENPTQKRDK</td>
</tr>
<tr>
<td>IFSQMDKN...........HDEKTLLEFKEGSNADPRIVQALSQDN*</td>
<td>IFSQMRGGSNAAFYPGRKTNSIPAHWRPLLDPSSVPSLA*....</td>
</tr>
</tbody>
</table>

Length: 198  Gaps: 1

La.
b.) Immunolocalization of synapsin, dynamin and frequenin-like molecules at crayfish and *Drosophila* neuromuscular junctions:

As described in "Materials and Methods", the antibodies used to investigate the expression of these three proteins were originally generated against the *Drosophila* isoforms, but appear to cross-react with their crayfish counterparts (Table 1). This is in agreement with a previous report using an anti-*Drosophila* synaptotagmin antibody (Cooper et al., 1995 a). These authors showed that synaptotagmin-like immunoreactivity was present in both 'phasic' and 'tonic' terminals. For comparison and to confirm the specificity of the antibodies used, the localization of synapsin, dynamin and frequenin immunoreactivity at the mature neuromuscular junction of *Drosophila* larvae was examined (Fig. 16).

In the following, the antibodies tried and the observed pattern of immunoreactivity are described. As the anti-*Drosophila* frequenin antibody is a polyclonal antibody raised in rabbits (see "Materials and Methods"), only the anti-*Drosophila* synapsin mouse monoclonal antibody (SYNORF1) was further characterized and used for the double-labeling studies. The mouse monoclonal anti-*Drosophila* synapsin antibody was used to
reveal synaptic terminals, while the anti-\textit{Drosophila} frequenin antibody was used to reveal
the localization of frequenin immunoreactivity in these direct co-labeling experiments. The
same strategy was applied to compare the localization of synapsin and dynamin
immunoreactivity. The anti-synapsin E2 domain antibody (Table 1) is polyclonal and
therefore was not further characterized. The anti-\textit{Drosophila} cysteine-string protein (csp)
antibody shows only weak immunoreactivity in crayfish and therefore was not used further
(Zinsmaier et al., 1994). Instead, the anti-dynamin antibody was used as another antibody
for labeling synaptic terminals. The anti-\textit{Drosophila} synaptotagmin antibody is also
polyclonal and therefore was not suitable for double-labeling experiments with the
polyclonal anti-\textit{Drosophila} frequenin antibody.

In the following, one representative example for each of the three different crayfish
muscle preparation is shown. The staining shown represent an example of 6-8 different
experiments.

c.) Immunolocalization at crayfish neuromuscular junctions :

I.) Synapsin :

In the three different crayfish nerve-muscle preparations examined, there was a good
immunoreactivity in the nerve terminals. This implies that a synapsin-like molecule was
expressed in the terminals of both ‘phasic’ and ‘tonic’ motor axons (Fig. 13 a, 14 a and 15 a
and 13 d, 14d and 15d). We have obtained an identical staining pattern using a polyclonal antibody against the conserved E2 domain of synapsins (G-304, data not shown). The expression pattern of synapsin immunoreactivity almost perfectly overlaps with that of dynamin in all three nerve-muscle preparations examined (Fig. 13 c, 14 c and 15 c for the overlay).
Table 1 Antibodies tested for cross-reactivity in crayfish

<table>
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<th>Immunogen</th>
<th>Species</th>
<th>Mono-/ Polyclonal</th>
<th>Cross-reactivity in crayfish</th>
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</thead>
<tbody>
<tr>
<td>Synapsin</td>
<td><em>Drosophila</em></td>
<td>Mono-</td>
<td>Yes</td>
</tr>
<tr>
<td>E2 domain of synapsin</td>
<td>Mammals</td>
<td>Poly-</td>
<td>Yes</td>
</tr>
<tr>
<td>(G-304)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dynamin</td>
<td><em>Drosophila</em></td>
<td>Poly-</td>
<td>Yes</td>
</tr>
<tr>
<td>Synaptotagmin</td>
<td><em>Drosophila</em></td>
<td>Poly-</td>
<td>Yes</td>
</tr>
<tr>
<td>Cysteine-string protein</td>
<td><em>Drosophila</em></td>
<td>Mono-</td>
<td>(Very weak)</td>
</tr>
</tbody>
</table>
II.) Dynamin:

In the abdominal slow flexor muscles, the fast abdominal extensor and the leg extensor nerve-muscle preparation, the staining pattern of the anti-dynamin antibody included both thin filiform and more varicose terminals (Fig. 13 b, 14 b and 15 b, respectively). This implies that a dynamin-related molecule is present in crayfish motor nerve terminals. The expression pattern of the dynamin immunoreactivity is almost identical to that of synapsin immunoreactivity at these crustacean neuromuscular junctions (Fig. 13 c, 14 c and 15 c for the overlay).

III) Frequenin:

In the crayfish fast abdominal extensor muscle, frequenin immunoreactivity was detected in all thin filiform terminals (Fig. 15 e). Its expression pattern was almost identical with that of synapsin (Fig. 14 d and f for the overlay).

In the abdominal slow flexor muscle, frequenin immunoreactivity was restricted to a subpopulation of terminals (Fig. 15 e and f for the overlay). These terminals appeared to be more varicose than the thin filiform terminals stained in the abdominal extensor muscle. The same features were revealed by the anti-synapsin staining (Fig. 15 d).
The dichotomy of frequenin immunoreactivity became more apparent in the leg extensor muscle. In this nerve-muscle preparation, frequenin immunoreactivity was found only in the thin filiform, but not the larger varicose terminals (Fig. 13 e and f for the overlay). In particular, in regions where thin filiform and more varicose terminals run close together as revealed by the anti-synapsin staining (Fig. 13 d), only the thin filiform terminals strongly express frequenin immunoreactivity (Fig. 13 f for the overlay). Notably, frequenin immunoreactivity was found not to be expressed in all thin filiform terminals (Fig. 13 e). This implies that not all terminals of the same axon contain the same amount of the immunoreactive molecule.

d.) Immunolocalization of synapsin, dynamin and frequenin at the Drosophila larval neuromuscular junction:

I.) Synapsin:

At the Drosophila larval neuromuscular junction, synapsin is expressed in both types of synaptic terminals (type Ib for big and Is for small) (Atwood et al., 1993) (Fig. 16 a and d). The localization of synapsin is virtually identically to that of dynamin (Fig. 16 f for the overlay).
II.) **Dynamin**:  

Dynamin expression was detected in Ib and Is terminals. Its expression pattern overlapped with that of synapsin (Fig. 16 e and f for the overlay).

III.) **Frequenin**:  

At the larval neuromuscular junction, frequenin is expressed in both Ib and Is terminals (Fig. 16 b). Its expression pattern almost completely overlaps with the synapsin expression pattern (Fig. 16 b and c for the overlay).
Fig. 13 Neuromuscular junction of the crayfish leg extensor muscle. A: synapsin immunoreactivity (red); B: dynamin immunoreactivity (green); C: the corresponding overlay for A and B is shown in yellow. D: synapsin (red); E: frequenin (green); the overlay of D and E is shown in F. Note that while synapsin (A,D) and dynamin (B) reveal both thin filiform and more varicose endings, frequenin localization is restricted to thin filiform nerve endings (E and the overlay in F in yellow). A heterogeneity is revealed among the thin filiform terminals, as some filiform endings shown in D are devoid of frequenin immunoreactivity (see E and F). The size of the scale bar is 10 μm.
Fig. 14  Neuromuscular preparation of the crayfish fast extensor muscles of the abdomen. Synapsin and dynamin immunoreactivity are revealed in A (red) and B (green). Synapsin and dynamin immunoreactivity reveals thin filiform nerve endings in a continuous network over the muscle (the overlay of A and B is shown in C in yellow). Frequenin immunoreactivity is shown in E (green) which almost completely co-localizes with corresponding regions as revealed by synapsin shown in D. The co-localization of synapsin (D) and frequenin (E) is shown in F in yellow. The size of the scale bar is 10 μm.
Fig. 15  Neuromuscular preparation of the crayfish slow flexor muscles. A and B reveal synapsin and dynamin immunolabeling, respectively. Small varicose terminals are stained by synapsin and dynamin and these staining pattern co-localize with one another as shown in C (regions of co-localization are shown in yellow). While synapsin stains all small varicosities (D), the frequenin labeling is restricted to a subset of the terminal varicosities (E), as illustrated for the overlay in (F). The size of the scale bar is 10 μm.
Figure 16 Mature *Drosophila* larval neuromuscular junction. The synapsin staining is shown in A, the frequenin staining is shown in B and the overlay of both labels in C in yellow. Both Ib (marked by the arrow) and Is (marked by the arrowhead) terminals are stained. In D the synapsin staining and in E the dynamin staining is revealed. The synapsin and dynamin staining almost prefectly overlap as shown in F. The size of the scale bar is 20 μm.
Discussion:

a.) Cloning of crayfish frequenin:

I. General molecular features:

I have cloned two forms of frequenin, one from lobster and one from crayfish, and identified them as closely-related members of the family of neuronal calcium sensors (Fig.4). The crustacean frequenin molecules are closely related to frequenin from *Drosophila* (Fig. 10 and 11). The highest degree of conservation at the protein level is found in the EF-hands, whereas the N- and C-terminal regions are less well conserved. This growing family of neuronal calcium sensor proteins includes the neurocalcins (Dyers et al., 1996), recoverin (see Ames et al., 1996, for review) and the frequenins (Pongs et al. 1993, Olafsson et al., 1995 and 1997). The C-terminus of lobster frequenin is remarkably similar to that of *Drosophila* frequenin (Fig. 11) while the N-terminus is not. Interestingly, the reverse pattern of sequence conservation is found for crayfish frequenin (Fig. 10). For crayfish frequenin, the N-terminus is more similar to the comparable region of *Drosophila* frequenin, while the C-terminus beyond the fourth EF-hand is less well conserved.

The genomic structure of *Drosophila* frequenin has been described (Pongs et al., 1993), but the genomic structure of other frequenins remains to be elucidated. This is of great interest from an evolutionary standpoint. With further observations, it will be possible
to determine if the frequenins arose from a common ancestor and the variable N- and C-terminus of the different members was added by exon shuffling to the core exon encoding the EF-hands (Gilbert et al., 1997).

II.) Conservation of EF-hands:

The conservation of the EF-hands underlines their importance for calcium-binding of these proteins. In crayfish and lobster frequenin as well as in other frequenins described so far (Pongs et al., 1993 and (Olafsson et al., 1995 and 1997), the first and fourth EF-hands are disabled. The second and third EF-hands, however, are remarkably well conserved except for a single amino-acid change (Y→F). Pongs et al. (1993) confirmed the importance of the second and third EF-hands of Drosophila frequenin for Ca\(^{2+}\)-binding by systematically mutating the EF-hands and performing Ca\(^{2+}\)-induced mobility shift assays to assess these mutants for their ability to bind Ca\(^{2+}\). They found that mutation of the second and third EF-hands of Drosophila frequenin critically affected the ability of the protein to bind Ca\(^{2+}\).

III.) N-myristoylation motif:

Crayfish frequenin has the classical N-terminal myristoylation motif (MGSSX) (Ames et al., 1996) at the N-terminus (Fig. 8), while lobster frequenin lacks the motif (Fig. 9).
Whether a glycine residue further downstream at the N-terminus of lobster frequenin is myristoylated remains to be shown.

For recoverin, the N-myristoylation is of functional importance as a conformational switch (Ames et al., 1996). Upon calcium-binding, the myristoyl group of recoverin becomes exposed on the protein backbone and allows the protein now to interact with its targets and the membrane. So far, Drosophila (Pongs et al., 1993), Xenopus (Olafsson et al., 1995), mouse (Olafsson et al., 1997) and crayfish frequenin molecules (this study) carry the classical myristoylation motif. Only lobster frequenin lacks this motif. It will be interesting to determine the function of myristoylation of frequenin in terms of its ability to bind Ca\(^{2+}\) and to bind to membranes.

IV.) Functional implications derived from the primary sequence:

The importance of this degree of sequence flexibility of the N- and C-terminus is currently not understood. Even though the primary structure of the N- and C-terminus is different among the family members, it might be that they all share common elements of secondary structure. It will be very interesting to study the structural dynamics of frequenin upon calcium binding and its calcium binding characteristics. The primary structure of lobster and crayfish frequenin predicts two functional Ca\(^{2+}\)-binding sites, as the first and
fourth EF-hands are predicted to be non-functional based on the primary amino acid sequence.

These studies will be of importance not only from a structural point of view, but also to better understand the physiological function of the protein and its possible regulation of the intraterminal calcium dynamics.

Modeling studies predict that the secondary structure of crayfish frequenin is similar to that of recoverin (Fig. 17) other members of the family of calcium-binding proteins (Ames et al., 1996) and contains 8 helices, 2 strands and 12 β-turns. As the C-terminal sequence 3' to the fourth EF-hand is however distinct from recoverin, this sequence was not included in the modeling of the crayfish frequenin structure. To confirm this predicted model and to resolve further structural details, structural spectroscopic studies of crayfish frequenin are required.

V.) Future work:

The cloning of crayfish frequenin will also allow us to further address and understand its precise physiological function. Injection of the recombinant protein into phasic and tonic motor neurons will help us to understand the function of frequenin in transmitter release. For this type of experiment, non- and myristoylated recombinant frequenin can be produced in *E. coli* and injected into the axons of 'phasic' and 'tonic' motor neurons close
to the transmitter release sites. It would be predicted that the injection of frequenin into axons of tonic motor neurons will enhance neurotransmitter release.

The generation of antibodies against crayfish frequenin will not only be instrumental for future comparative labeling experiments, but also complement the protein injection experiments. Injection of whole anti-crablyfish IgG molecules or Fab' fragments might functionally deplete frequenin from crayfish terminals. We will then be able to study the consequences of this depletion on transmitter release in the two different types of motoneuron terminals. If frequenin enhances synaptic efficacy, its functional depletion from synaptic terminals would be predicted to decrease the probability of transmitter release.

Such antibody inhibition experiments have been successfully carried out to probe the role of synapsin (Pieribone et al. 1996) in neurotransmitter release. These injection experiments of fluorophore-conjugated anti-synapsin antibodies also allowed the identification of two functionally distinct pools of synaptic vesicles and their movement to be followed in a live preparation. The importance of dynamin in neurotransmitter release has been most recently studied by disrupting its interaction with one of its binding partners, amphiphysin (Shupliakov et al. 1997). Amphiphysin, which occurs in various isoforms, binds dynamin via its SH3 domain near its COOH terminus. Microinjection of a
fluorophore-conjugated fusion protein containing the SH3 domain of human amphiphysin inhibited synaptic vesicle endocytosis which resulted in an activity-dependent distortion of the synaptic architecture and a depression of transmitter release.

b. ) Immunolocalization of frequenin at the NMJ :

I.) Patterns of localization :

We have used the cross-reactivity of the anti-*Drosophila* frequenin antibody as an indication that frequenin-like molecules exist at crayfish neuromuscular junctions. This does not provide final proof of the existence of frequenin at these synapses, but implies that closely-related molecules are expressed. To further strengthen the notion that crayfish frequenin is expressed at crayfish neuromuscular junctions, the anti-*Drosophila* frequenin antibody has to be tested for cross-reactivity with crayfish frequenin cloned in this study and its staining pattern compared to that revealed with the anti-crayfish frequenin antibody.

We have shown that frequenin is heavily expressed in thin filiform and less strongly in more varicose motor neuron terminals (Figs. 13, 14 and 15). These thin filiform terminals could be identified as belonging to the ‘phasic’ axon, while the more varicose type of terminals belong to the ‘tonic’ axon. The thin filiform terminals have been shown to
release much more neurotransmitter per impulse than the thicker varicose terminals, and to generate the large 'phasic' EPSPs (Bradacs et al., 1997).

In the almost exclusively 'phasically' innervated crayfish abdominal fast extensor muscle (Parnas and Atwood, 1966), frequenin was found to be expressed in a dense network of 'phasic' terminals, which completely overlapped with the expression pattern of synapsin immunoreactivity (Fig. 14 b.) and c.). In the dually innervated leg extensor muscle (Atwood and Cooper, 1995), frequenin was found to be heavily expressed in the 'phasic' terminals, but less strongly in the 'tonic' terminals (Fig. 13 b.) and c.). In the slow abdominal flexor muscle preparation, frequenin was found to be expressed in a subset of the more varicose terminals of the 'tonic' type. These terminals may belong to axon 6. Although this axon is of the 'tonic' type, it has a more intermittent pattern of impulse production and is less 'tonic' in its activity than the other motor neurons supplying this muscle (Wine et al., 1974).

Expression of synaptic vesicle proteins such as synapsin and dynamin are detected in both 'phasic' and 'tonic' nerve terminals, suggesting that there are not gross detectable differences (at the light microscopic level) between 'phasic' and 'tonic' motor neurons in these proteins. At the mature Drosophila larval neuromuscular junction (NMJ), frequenin was expressed in both Ib and Is terminals. These terminals are glutamatergic and differ in
their transmitter release properties albeit the differences are less pronounced than for the ‘phasic’ and ‘tonic’ crustacean motoneurons (Atwood et al., 1993 and Atwood and Cooper, 1995). At the *Drosophila* NMJ, synapsin and dynamin were also both expressed in Ib and Is boutons. The localization of synapsin and dynamin at the fly larval neuromuscular junction almost entirely overlapped with the expression pattern of frequenin, which is also expressed in Ib and Is boutons.

### II.) Functional implications: physiology

As mentioned above, frequenin was heavily expressed in ‘phasic’ terminals and less strongly in ‘tonic’ terminals in the leg extensor muscle preparation. Frequenin expression was not found in all small and filiform ‘phasic’ terminals however. This could potentially reflect the heterogeneity of release properties that exists among ‘phasic’ terminals. In the limb extensor muscle, Bradacs et al. (1997) described a large variation in the quantal content of individual visualized terminal structures. The excitatory phasic and tonic postsynaptic potentials (EPSPs) recorded on the exposed inner surface of the muscle also varied in their amplitude depending on the recording position along the muscle (Bradacs et al. 1997).
It will be interesting to carry out focal recordings from individual ‘phasic’ nerve terminals along the muscle fiber and to label these recording sites by fluorescent beads (Wojtowicz et al., 1994). The measured quantal content of these tagged terminals can then be correlated to the expression level of frequenin at the light microscopical level and ultimately quantified via quantitative immuno- electron microscopy (EM).

III.) Functional implications: morphology

A large body of evidence now indicates that neurons adapt to different levels of activity (Atwood and Nguyen 1995). Studies in crustaceans in particular have shown that pre-synaptic nerve endings undergo a wide range of structural and functional changes when their electrical activity is altered (see Atwood and Nguyen, 1995, for review). Long term adaptation (LTA) comprises a series of activity-induced alterations that persist for days or weeks (Lnenicka and Atwood, 1986; Atwood et al., 1991). Particularly dramatic changes occur at ‘phasic’ neuromuscular junctions in crayfish. The persistently-stimulated ‘phasic’ motor neuron responds by adapting to a more ‘tonic’ phenotype. Physiologically, the initial depression in transmitter release of the ‘phasic’ motor neuron induced by continuous 5 Hz stimulation is diminished in neurons previously conditioned with extra stimulation. Also, post-stimulus potentiation analogous to long-term facilitation is decreased in the conditioned neurons (Lnenicka et al. 1985 (b); Pahapill et al., 1985). Morphologically, the ‘phasic’ terminals are transformed from their thin filiform appearance into thicker and more varicose ‘tonic’-like structures. This morphological transformation is accompanied by an increase in mitochondrial cross-sectional area and enhanced mitochondrial
branching. These persistent physiological and morphological changes are independent of synaptic transmission (Lnenicka and Atwood, 1988), but dependent on protein synthesis (Nguyen et al, 1990).

We would predict that the expression of frequenin gradually declines under these conditions of long-term adaptation, as the ‘phasic’ motor neuron converts into a more ‘tonic’ phenotype. It will be interesting to see if the alteration of frequenin expression under conditions of LTA is just a consequence of the reprogramming of the overall pattern of protein expression, or actually an initial factor that induces the conversion of the ‘phasic’ motoneuron into a more ‘tonic’ phenotype. If the latter is the case, down-regulation of frequenin protein expression, for example by anti-sense DNA application, should induce a structural conversion similar to that seen with long-lasting electrical stimulation. Frequenin might thereby indirectly sense electrical activity and convert it into a biochemical signal.

Frequenin has also been implicated in the plasticity of the mature Drosophila (larval) neuromuscular junction. Besides the synaptic enhancement in the frequenin-overexpressing fly mutant (V7), the geometry of motor neuron terminal arborization is also altered in these animals (Angaut-Petit et al., 1993). Using an axonal membrane marker, these authors show a significant reduction in number and length of motor terminal branches in these V7 mutants. We have further extended the analysis of the geometry of the NMJ in larval transgenic animals expressing multiple copies of frequenin under control of an inducible promotor and confirmed the earlier results (Jeromin et al., 1997). It remains to
be seen whether this reduction in the number of boutons and length of the junction is a homeostatic mechanism of the fly to compensate for the larger synaptic output.

c.) Possible cellular mechanisms

The molecular basis for the frequenin-mediated synaptic enhancement still remains a mystery. It was initially suggested that frequenin stimulates guanylate cyclase (GC) in a Ca\(^{2+}\)-dependent manner (Pongs et al., 1993). We have reanalyzed this effect and could not detect any modulatory effect of frequenin on GC activity (Jeromin A. Palczewski K, Atwood HL, unpublished observations). Rivosechhi et al. (1994) suggested that frequenin modulates the activity of the Na\(^+\)-Ca\(^{2+}\) exchanger, as they observed that perfusion of the sodium channel blocker tetrodotoxin or of low Na\(^+\) abolished the enhancement of the facilitation in the frequenin-overexpressing flies. From this, the authors concluded that internal Na\(^+\) accumulation can enhance facilitation of transmitter release at larval NMJs of frequenin-overexpressing flies and that this effect is possibly dependent on the modulation of the activity of the Na\(^+\)-Ca\(^{2+}\) exchanger by frequenin. Additionally, Poulain et al. (1994) observed that the Ca\(^{2+}\)-dependent modulation of the fast-inactivating K\(^+\) current is absent in the mutant flies. It remains to be seen whether or not this lack of modulation of the fast-inactivating K\(^+\) current by calcium is related to the proposed increased activity of the Na\(^+\)-Ca\(^{2+}\) exchanger. These different models can, however, be directly tested in the crayfish and Drosophila neuromuscular junction preparations.

At present, the molecular basis for the synaptic enhancement by frequenin remains unclear. Although some models support the notion that frequenin alters the electrical
properties of the terminal ('electrical model') (Poulain et al., 1994), it cannot be excluded that frequenin might participate in vesicle release or recycling ('biochemical model'). It will be very important to study the subcellular distribution of frequenin in the presynaptic terminal and identify molecules that interact with frequenin in order to distinguish between the 'electrical' and 'biochemical' models.
Figure 17  Predicted model structure of crayfish frequenin. The secondary structure was modeled using the Swiss-Model protein modeling package (Peitch M.C., 1995) and the modeled structure displayed by RasMol, v2.5 (1994), written by Roger Sayle. Glaxo Research and Development, UK. The predicted structure for crayfish frequenin is based on the NMR structure of Ca\textsuperscript{2+}-bond recoverin (see Ames et al., 1996). The model is oriented so that the N-terminus is pointing up and the C-terminus is shown in lower right corner.

Like recoverin, crayfish frequenin is predicted to be a compact protein made of two domains separated by a narrow cleft. Each domains contains a pair of EF-hands with each EF-hand being made of two \( \alpha \)-helices. Following an \( \alpha \)-helix at the NH\textsubscript{2} terminus, the first EF-hand consists of two \( \alpha \)-helices separated by a \( \beta \)-sheet. The following two \( \alpha \)-helices of the second EF-hand are also separated by a short \( \beta \)-strand. Each of two \( \alpha \)-helices of EF-hand 3 and 4 are separated by loops.
d.) General conclusions:

In summary, the cloning of crayfish frequenin has provided a tool that will allow us to not only study its precise role in synaptic transmission, but to also investigate its role in different conditions of synaptic plasticity. The differential expression of frequenin in the 'phasic' terminals may be causally linked to the larger transmitter release of this type of terminals.

Frequenin provides a molecular link between function and structure, i.e. its overexpression not only modulates synaptic function, but also the structure of the neuromuscular junction. The crayfish neuromuscular junction is uniquely designed for further functional experiments, as the single-bouton recordings in combination with injection experiments are feasible, and different forms of plasticity have been well documented.
Summary

Crustaceans provide many advantages in the study of synaptic transmission and plasticity. Neuromuscular preparations have been well characterized in terms of short- and long-term facilitation, synaptic depression and long-term adaptation (Atwood & Wojtowicz, 1986).

Additionally, crustacean motor neurons are excellent models to study the differentiation into ‘phasic’ and ‘tonic’ motor neurons. These two types of motor neurons differ substantially in their transmitter release properties.

Although the ultrastructural differences between these neurons have been well studied (Atwood and Cooper, 1995), very little is known about possible molecular factors underlying this differentiation. This thesis constitutes an effort to implicate the differential expression of the neuronal calcium-binding protein frequenin as such a molecular factor.

Standard molecular biology techniques were used to clone frequenin from lobster and crayfish nerve cord. The lobster and crayfish frequenins were found to be closely related to originally identified Drosophila frequenin. There is sequence conservation in the Ca\(^{2+}\)-binding domains of the second and third EF-hands. The N-terminal myristoylation motif in crayfish frequenin could also be important for the function of this molecule.

Immunolocalization of frequenin at the crayfish neuromuscular junction using a cross-reacting antibody against Drosophila frequenin revealed its expression in synaptic terminals and sub-terminal regions of axons. Frequenin was however not expressed in all terminals. In the almost exclusively phasically innervated abdominal fast extensor muscle, frequenin was expressed in the thin filiform ‘phasic’ terminals. In the leg extensor muscle,
which is dually innervated by a 'phasic' and a 'tonic' neuron, the expression of frequenin was restricted to the filiform 'phasic' terminals and not found in the more varicose 'tonic' terminals. In the abdominal slow flexor muscle, innervated by 'tonic' neurons with varying physiological properties, frequenin was found to be expressed in at least one of the neurons. Possibly, this neuron could be axon 6, which fires in more 'phasic'-like behaviour.

In conclusion, the cloning of crayfish frequenin and its immunolocalization have shown that frequenin-like molecules occur in crustaceans. This finding provides the basis to study frequenin's precise physiological function in transmitter release at the neuromuscular junction. Analysis of the immunolocalization of frequenin at the crayfish neuromuscular junction has shown that frequenin is heavily expressed in 'phasic' and less strongly in 'tonic' motor neurons. The differential expression is correlated with physiological and morphological differences in these neurons, but a causal relationship remains to be determined.
References


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Appendix

Western Blot of *Drosophila* and crayfish fusion protein were run on 15 % SDS-PAGE gel and blotted onto Nitrocellulose membrane (Schleicher & Schuell). The blot was then probed with the anti-*Drosophila* frequenin antibody (1:500), incubated with the goat anti-rabbit peroxidase-conjugated antibody (Bio-Rad, 1:3000) and developed using the ECL kit (Amersham). *Drosophila* frequenin fusion protein was run in lane A and crayfish fusion protein in lane B. In each lane, the antibody recognizes the uncleaved protein (upper band), partially cleaved (middle band) and cleaved fusion protein (lower band at the arrow). The position of the 21 kDa band as revealed by a molecular weight marker is shown. The cleaved crayfish frequenin fusion protein (lower band) runs at a slightly higher molecular weight than the cleaved *Drosophila* fusion protein.
IMAGE EVALUATION
TEST TARGET (QA-3)