Video Article

Historical View and Physiology Demonstration at the NMJ of the Crayfish Opener Muscle

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Abstract

Here we present some of the key important discoveries made with the opener neuromuscular (NMJ) preparation of crustaceans and illustrate that there is still much to learn from this model preparation. In understanding the history one can appreciate why even today this NMJ still offers a rich playground to address questions regarding pre- and post-synaptic function and plasticity. The viability and ease of access to the terminal for intracellular as well as extracellular electrophysiology and imaging are significant advantages. The mechanisms behind the modulation of vesicular kinetics and fusion within the high- and low-output terminals are begging for investigation. The preparation also offers a testable model system for computational assessments and manipulations to examine key variables in theoretical models of synaptic function, for example calcium dynamics during short-term facilitation. The synaptic complexity of active zone and statistical nature of quantal release is also an open area for future investigation both experimentally and computationally.

Video Link

The video component of this article can be found at https://www.jove.com/video/1595/

Protocol

Introduction

The neuromuscular junctions of crustaceans have provided important contributions to physiology and particularly to synaptic physiology over the years. The ease in dissection and viability are probably the key factors that promoted early anatomist and later physiologist to use crustaceans as experimental preparations. Crayfish in particular are easily obtainable from most freshwater streams and lakes as well as they are easy to maintain in a laboratory setting as compared to crustaceans requiring a cold, salt water environment.

A zoologist back in the late 1800's took to heart particular crustacean species (i.e., crayfishes) and wrote a book entitled *The Crayfish* (T.H. Huxley, 1879). This text served as the guide book on these organisms for years and today is still hailed as a comprehensive book selectively on crayfishes dealing with life history, anatomy and physiology. Huxley viewed the crayfish as a model animal to dive into the depths of zoology in all aspects; thus, the comprehensive nature of his book. The timing was advantageous as physiology was blooming in the late 1880's with Ringer's understanding of ions required for maintaining frog heart preparations (Ringer, 1882a,b). This is likely one reason that physiological experiments progressed quickly in other species as well as the crayfish. Also, a saline to maintain crustacean preparations had been described by van Harreveld in 1936.

Surprisingly the innervation of the opener muscle in crayfish legs was also being characterized around this time in history (Biedermann, 1887). But even more surprising is that physiological studies were already underway in muscles of the crayfish by Charles Richet in France. In fact, the experiments in crayfish might possibly be the first to demonstrate facilitation at the neuromuscular (NMJ) (Richet, 1879; also see Richet, 1881). Over the next few decades crayfish NMJs were being described anatomically and physiologically in respect to tension development and anatomy (Van Harreveld and Wiersma, 1936).

The advent of intracellular recording, with sharp electrodes (Ling and Gerard, 1949), revitalized the field to address different sets of questions. Crustacean muscles were known to produce graded contractions (Katz & Kuffler 1946; Katz, 1949; Wiersma, 1949), but it was not until 1953 that Fatt and Katz recorded transmembrane potentials of short term facilitation in crab muscle fibers.

The opener muscle in the limbs of crayfish was again highlighted in 1961 when Dudel and Kuffler demonstrated facilitation in this muscle and showed for the 1st time the phenomena of presynaptic inhibition (1961a,b; Dudel, 1963, 1965a). They also reported on the quantal nature of synaptic transmission at this NMJ (1961b). In the last 50 years there has been quite a bit of attention given to the preparation and various techniques used to monitor synaptic physiology. For a brief over view of investigations using this preparation, we start with the noting that the entire muscle is innervated by one excitatory and an inhibitory axon which could be selectively stimulated. Atwood (1964) demonstrated with trains of stimulation the excitatory postsynaptic potentials facilitated and produced muscle tension. Iravani (1965) reported on regional

differences in synaptic responses depending on the region of the muscle. Soon afterwards Dudel (1965a,b) recorded potentials along the nerve terminals on the opener and demonstrated that the neuromodulator serotonin enhanced synaptic transmission by increasing mean quantal content.

By this time it was established that crustacean muscles responded to glutamate and various amino acids as well as GABA (Van Harreveld and Mendelson, 1959; Robbins, 1959; Kerkut *et al*., 1965). The inhibitory responses of GABA was identified by Florey (Bazemore *et al*., 1956, 1957) and others (Boistel and Fatt, 1958). Later GABA was isolated and confirmed by Kravtiz (Kravitz and Potter, 1965; Kravitz *et al*., 1963a,b; Kravitz, 1962) from the axons of lobster opener preparations.

The crayfish muscles offered not only easily accessible preparations but allows one to study how single identifiable motor neurons can result in various postsynaptic responses at a physiological and structural level. In particular the opener muscle is innervated by a single excitatory motor neuron, but the excitatory postsynaptic potentials (EPSPs) on differing locations can vary over 50 fold in dorsal superficial fibers (Bittner, 1968a,b) and as much as 8 fold in ventral superficial fibers (Iravani, 1965).

With the seminal discover that the opener NMJ in crayfish exhibited long-term facilitation (LTF) (Sherman and Atwood, 1971), in addition to short-term facilitation, the mechanistic underpinnings for these phenomena needed to be addressed. As a side note, long-term potentiation (LTP) was discovered in the vertebrate brain two years later (Bliss and L mo, 1973) without citation to the original discovery of the phenomenon at the crayfish NMJ. From this period on many investigators focused on the attributes of STF and LTF using the opener NMJ of crayfish to study the cellular mechanisms (Atwood, 1973, 1976, 1982; Atwood *et al*., 1994; Zucker, 1973, 1974a,b; Bittner and Sewell, 1976; Parnas *et al*., 1982a,b,c,d; Dudel *et al*., 1983; Vyshedskiy and Lin, 1997a,b,c). Also a focus point has been to understand how a single motor neuron innervating various muscles fibers on the opener muscle can give rise to such varied synaptic responses (Linder, 1974; G¨nzel *et al*., 1993; Govind *et al*., 1994; Iravani, 1965; Atwood, 1967; Bittner, 1968a,b; Sherman and Atwood, 1972; Zucker, 1974a; Parnas *et al*., 1982a; Zucker and Haydon, 1988; Dudel, 1989a,b,c,d).

Synaptic structure to account for the differential synaptic responses can be investigated via ultrastructural analysis (Jahromi and Atwood, 1974). Measures of ionic differences due to activity is able to be investigated with axonal injections of Ca2+ and Na+ indicators as well as Ca2+ buffers (Mulkey and Zucker 1993; Winslow *et al*., 2002), and these fluxes can be modeled within the terminal (Winslow *et al*., 1994; Cooper *et al*., 1996b). Activity dependent adaptations (Atwood *et al*., 1991) and the pharmacological identification of neuromodulator receptor subtypes (Dropic *et al*., 2005; Ruffner *et al*., 1999; Sparks and Cooper, 2004; Sparks *et al*., 2004; Tabor and Cooper, 2002;) that influence synaptic vesicle pools and kinetics (Logsdon *et al*., 2005; Southard *et al*., 2000; Sparks *et al*., 2003) has also been examined which is leading the way to new questions to be addressed. The concepts of calcium's role during STF versus membrane depolarization in synaptic transmission at the opener NMJ lead to some differences in opinion (Mulkey and Zucker, 1991; Hochner *et al*, 1989).

Relatively recently the regional differentiation in synaptic strength and facilitation from the single motor neuron, has been addressed and appears to be due to differences from local presynaptic changes in synaptic structure and physiology (Atwood *et al*., 1994; Atwood and Cooper, 1995, 1996a,b; Cooper *et al*., 1995b, 1996a,b). Ultrastructural analysis from electron micrographic studies has shown that the varicosities contain the majority of the synaptic contacts (Florey and Cahill, 1982; Cooper *et al*., 1995b). The strength of synaptic transmission decreases along the length of a single terminal which appears to be due to complexity of the synaptic structure (Cooper *et al*., 1996a; Govind *et al*., 1994). The differences in the synaptic structure may in part explain the differences in the Ca2+ influx during stimulation at various frequencies (Cooper *et al*., 1995b, 1996b).

Since there are regional differences in muscle phenotype and biochemistry among the muscle fibers of the opener (G¨nzel, *et al*., 1993; Mykles *et al*., 2002) which are divided up into regions, could explain a developmentally regulated muscle phenotype that influences and maintains the regional differences of the motor neuron (Mykles *et al*., 2002) . The idea of retrograde influences has been investigated in frog skeletal muscle (Nudell and Grinnell, 1983), in the lobster (Katz *et al*., 1993), and in crayfish (Lnenicka and Mellon, 1983) with reasonable convincing evidence. The local regulation of terminals in a single neuron without influencing other terminals is spatially quite possible in crustacean motor neurons because the terminals can measure from 1cm to 10cm in distance from each other. Unlike vertebrates, a motor unit may include more that one muscle in invertebrates (see review by Atwood, 1973). The excitor motor neuron that innervates the entire opener muscle also innervates the stretcher muscle in a more proximal leg segment. Facilitation measurements between muscle fibers of the opener muscle showed that there are differences which may be related to resting levels in Ca2+ ions (Cooper *et al*., 2005b) and/or possibly cooperatively of release (Parnas *et al*., 1982a,b)

The differences in structural complexity among high- and low- output synapses along the terminals on the opener muscle were investigated for quantal signatures with respect to recruitment of active zones among synapses during STF but this has proven to be difficult to ascertain (Lancaster *et al*., 2007; Viele *et al*., 2003, 2006). Possible the pools of vesicles among high- and low-output synapses will prove to be regulated differentially in kinetics as it is known neromodulators have differential effects on low and high-output terminals (Logsdon *et al*., 2005; Sparks and Cooper, 2004; Cooper *et al*., 2003).

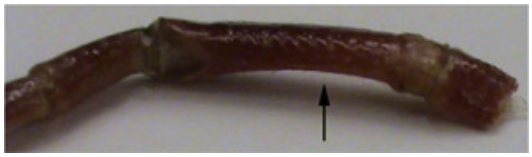
The future use of the opener muscle preparation in crayfish is as rich as it has been 50 or a 100 years ago. The preparation is still very hardy in comparison to many other synaptic preparations. Quantal responses can be electrophysiological recorded directly at the synaptic contacts as well as imaged for vesicle dynamics in various types of well defined terminals. The preparation has not lost its charm in having single identifiable neurons for the excitatory and inhibitory inputs. Despite the crayfish not being practical for genetic manipulation, studies are possible to address the role of synaptic proteins as for *Drosophila*. There are many similarities in synaptic function to *Drosophila* NMJs (Atwood and Cooper, 1995, 1996a,b) that can be examined by protein injection studies (He *et al*., 1999). The regulation of synaptic vesicle pools within motor nerve terminals is also a rich area for future investigation as well as mechanistic studies to understand calcium regulation during STF (Desai-Shah *et al*., 2008; Desai-Shah and Cooper, 2009) to explain many of the remaining mysteries in the fundamentals of synaptic transmission.

Methods

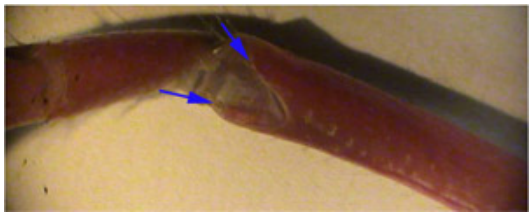
Dissection

Crayfish, *Procambarus clarkii*, measuring 6-10 cm in body length (Atchafalaya Biological Supply Co., Raceland, LA) are induced to automize the first or second walking leg by forcefully pinching at the ischiopodite segment.

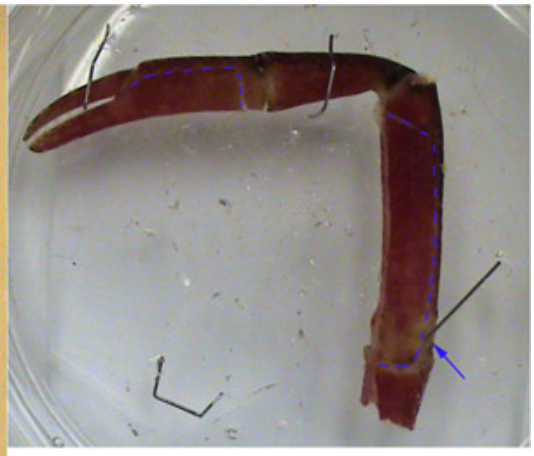
Turn the leg around until one can be sure the outside (lateral side) is facing up on the dissection plate. This is usually the arched side up. Placing the leg on a piece of tissue paper helps so the preparation can be turned easily while making these cuts.



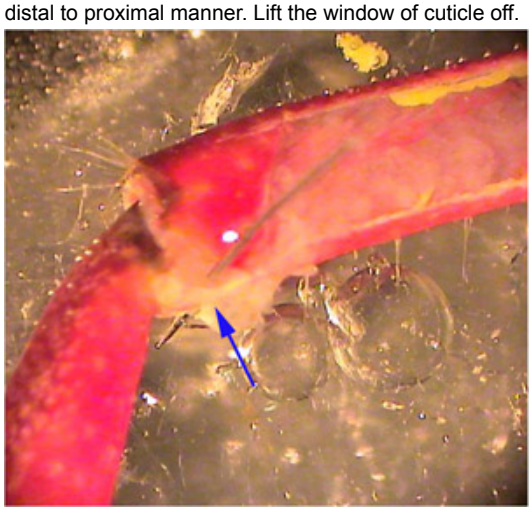
With a scalpel blade breaker and holder a sharp razor blade is used to etch the cuticle until just cutting through in the pattern shown in this figure for the meropodite segment. Care needs to be used not to cut to far distal on the dorsal to ventral cut by the meropodite - carpopodite joint. Leave the cuticle in place for now.

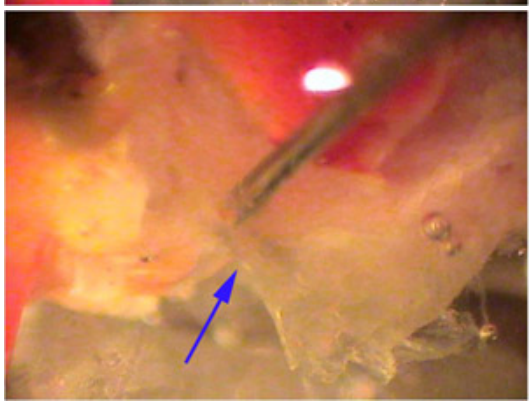


With the razor scalpel blade etch the cuticle on the propodite until just cutting through in the pattern shown in above figure for the propodite segment on one side and then repeat on other side joining the proximal cuts. Care needs to be used not to cut into the opener muscle. This can be done by keeping the blade leaning to the closer muscle when cutting through the cuticle. Also for the dorsal to ventral cut, connecting around the ventral side, be careful not to cut too proximal as the joint connection is narrow and easily broken. Leave the cuticle in place for now.

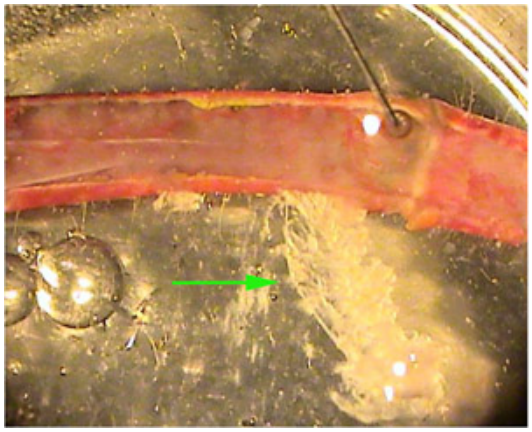


The preparations should be put into saline. This dissection dish should have a Sylgard (Dow Corning) coating on the bottom (1cm thick).The Sylgard is used so that insect pins can be stuck into it for holding the preparation still. At this point stick a pin in the dorsal caudal corner, within the cut, of the window made in the meropodite.
With fine tweezers (#5) lift slightly the cuticle from the distal end and with the razor, cut the flexor muscle fibers away from cuticle, cutting in a distal to proximal manner. Lift the window of cuticle off.



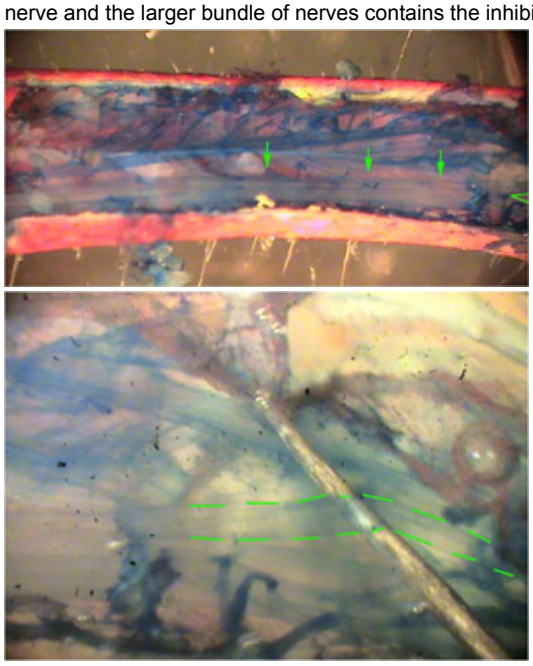


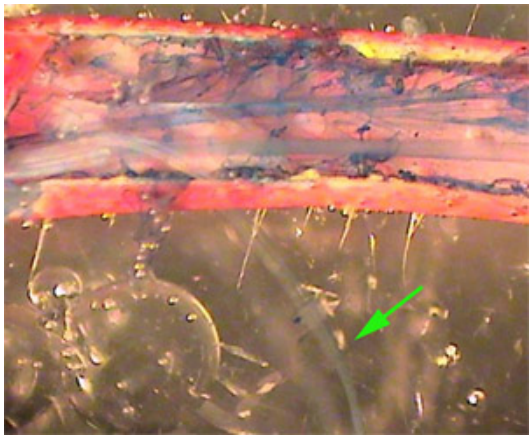
Now cut the apodeme (tendon) at the meropodite - carpopodite joint (shown below). Be very careful to pull the tendon away from the leg cavity before making the cut and to only cut the tendon and not the main leg nerve that is on the inner side of the tendon. Pinch the tendon where it was cut with tweezers and pull the flexor muscle off by lifting it in a caudal direction. Now the main leg nerve and the extensor muscle are exposed.



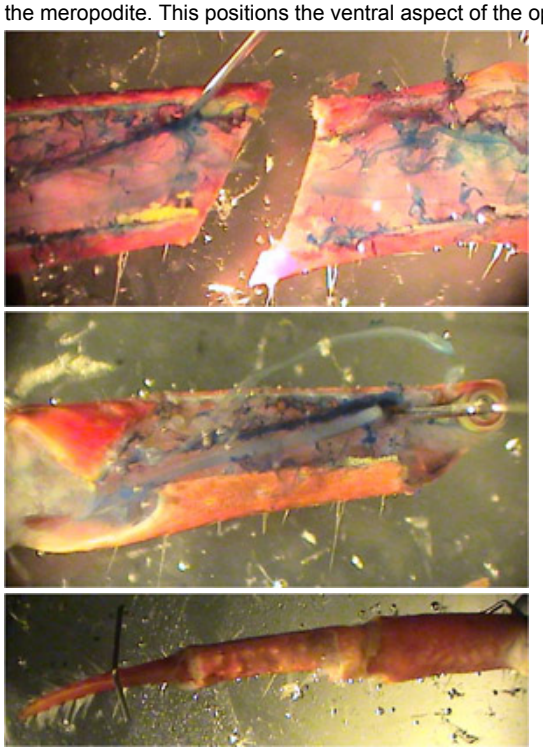
Proceed to the propodite segment and now cut at the propodite dactylopodite joint. Here the closer tendon maybe cut from the cuticle attachment. Pull the ventral (closer muscle side) segment of the propodite down and back caudally, so that the muscle attached in the caudal region can be seen. Cut these muscles with the razor. Be careful not to cut the muscle too close to the joint and risk cutting the motor nerve branch to the opener muscle. The opener muscle is now exposed to the saline.

Return to the meropodite region to isolate the nerve bundles containing the excitatory and inhibitory motor neurons to the opener muscle. In the most caudal region of the meropodite segment the leg nerve bundle usually contains a separated nerve bundle. This short region where two bundles can be seen is where the dorsal bundle can be transected with fine scissors. The cut end can then be picked up with # 5 tweezers and gently pulled distally until about half the length of the meropodite segment is reached. This long nerve branch contains the excitatory opener nerve and the larger bundle of nerves contains the inhibitory motor neuron of the opener muscle.

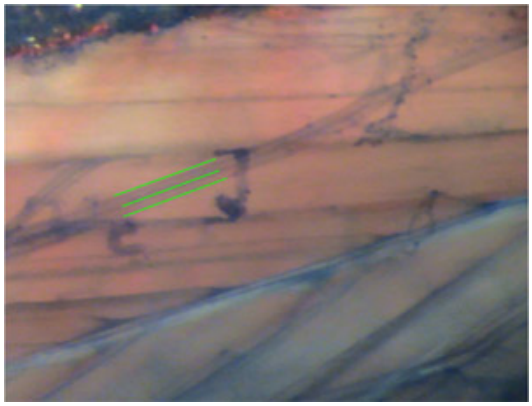




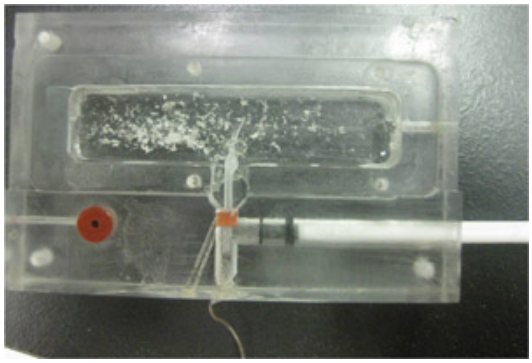
The preparation in the meropodite segment is now cut in a diagonal manner such that an insect pin can be placed through the dorsal aspect of the meropodite. This positions the ventral aspect of the opener muscle up so that it faces the observer (as shown below).



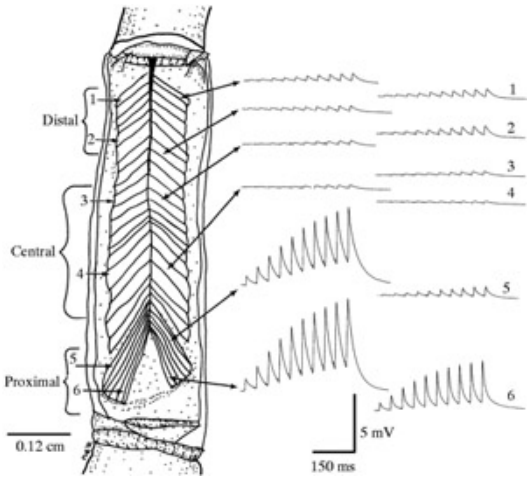
The residual fibers of the closer muscle that blocks the view of the opener muscle can now be removed by pushing the fibers against the cuticle and out of the propodite cavity. Sometimes a connective tissue covers the opener which can be removed by carefully using the #5 tweezers. The main leg nerve that runs along the opener muscle and goes into the dactylopodite can be either cut in the start of the dactylopodite joint or just pulled up with the fine tweezers. This main leg nerve and sometimes the obvious associated blood vessel can now be pulled gently in a proximal direction for the length of the opener muscle and then cut away.



Now the opener muscle is exposed without any tissue to get in the way of an intracellular electrode or a focal macropatch electrode.



In order to stimulate the excitatory nerve to the opener muscle the preparation is now moved to a recording chamber designed with a plastic suction electrode. Having the stimulating electrode built into the chamber avoids having to use a micromanipulator to place a stimulating electrode. Pin the preparation down in the recording dish and place the branch of the nerve that contains the excitatory nerve in the suction electrode.



(taken from: Mykles, D.L., Medler, S.A., Koenders, A., and Cooper, R.L. (2002) Myofibrillar protein isoform expression is correlated with synaptic efficacy in slow fibres of the claw and leg opener muscles of crayfish and lobster. Journal of Experimental Biology 205 (4): 513-522.)

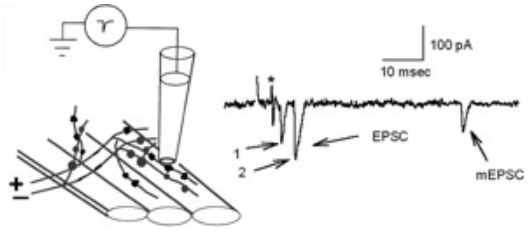
Saline

Dissected preparations are maintained in crayfish saline, a modified Van Harreveld's solution (in mM: 205 NaCl; 5.3 KCl; 13.5 CaCl2.2H2O; 2.45 MgCl2.6H2O; 5 HEPES adjusted to pH 7.4).

Recording intracellular EPSPs

To elicit an evoked response, the excitatory axon is selectively stimulated by a Grass stimulator. A region of opener muscle is impaled with sharp intracellular electrode (20 to 30 mOhm resistance) filled with 3 M KCl. A standard head stage and amplifier for intracellular recording can be used; however we used a model Axonclamp 2B (Molecular Devices, Sunnyvale, CA, USA) amplifier and 1 X LU head stage. Short term facilitation (STF) or various other type of responses desired can be obtained by varying the stimulus conditions. STF is obtained by giving a train of 10 or 20 pulses at 10 or 20 second intervals, respectively, to the excitatory nerve. The frequency of stimulation within the train can be varied (40, 60 and 80 Hz). Intracellular EPSP recordings are routinely performed by these standard procedures (Crider & Cooper, 1999, 2000; Cooper *et al*. 1995b; Dudel, 1983; Sparks and Cooper, 2004; Desai-Shah and Cooper, 2009).

The opener muscle is divided in three general regions: distal, central and proximal. Even though the entire open muscle is innervated by a single motor neuron, the NMJs are structurally different and have regional specific differences in synaptic efficacy in these three general regions (Cooper *et al*. 1995a,b). The muscle fiber phenotype type has also been shown to be different in these regions (Mykles *et al*. 2002). For these reasons, the most distal fibers are used, since they are easily demarcated for consistency among preparations.



Recording focal quantal EPSPs directly over identifiable regions of the nerve terminal

The synaptic varicosities are visualized with the vital dye 4-Di-2-Asp (Magrassi *et al*., 1987), which does not affect synaptic transmission, at the concentrations and times employed (5 µM, 5-min treatment, Cooper *et al*., 1995b). With fluorescence microscopy, the lumen of a macro-patch recording electrode (Cooper *et al*., 1995c; St¨hmer *et al*.,1983) could be placed directly over a single isolated varicosity. To evoke the nerve terminal, the excitatory motor nerve is stimulated as mentioned above. Spontaneous as well as evoked quantal responses can be recorded along the string of visualized varicosities, by gently lowering the lumen and raising it over each varicosity.

The synaptic potentials are recorded through a macro-patch electrode essentially as described by Dudel,1981; Wojtowicz *et al*. (1991) and Mallart (1993). Kimax glass (outer diameter: 1.5 mm) was pulled and fire-polished to produce patch tips with inside diameters ranging from 10 to 20 µm. The lumen of the electrode is filled with the bathing medium. The amplifier is the same as that used for the intracellular recordings mentioned above. Electrode and seal resistance can be determined by passing test current pulses through the electrode. Seal resistances ranged from 0.3 to 1.0 M0hm and the electrode resistance ranged from 0.5 to 1.0 M.0. Seal resistance can be monitored throughout the recording.

Direct counting of quantal events is possible with low stimulation frequencies. For each evoked response, the number of quantal events can be determined. For a series of responses, the total numbers of quantal events are counted to then estimate mean quantal content based upon these direct counts. One approach to calculate mean quantal content is taking the total number of quanta and divide by the total number of responses (del Castillo and Katz, 1954). There are other approaches one can use as well based on the peak amplitude or the area of the EPSPs (Cooper *et al*., 1995b).

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