# FUNCTIONAL MORPHOLOGY OF THE NEUROMUSCULAR SYSTEM OF THE OCTOPUS VULGARIS ARM

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CEPHALOPODS MUSCULAR HYDROSTAT MUSCLE FIBER OBLIQUELY STRIATED MUSCLE NEUROMUSCULAR SYSTEM

ABSTRACT. – To better understand the neural control and behavioral output of the highly flexible octopus arm, we have characterized the arm's neuromuscular system using light and electron microscopy to examine the organization of muscle groups and pattern of innervation. Simulations and indirect calculations were used to analyze the morphometric results. Transverse and longitudinal muscle cells have similar dimensions (~1.2 mm x ~8-10  $\mu$ m) and shape (fusiform). The longitudinal muscle cells are closely packed and embedded in a 3-dimensional collagenous mesh. In the transverse muscles, bundles of muscle fibers are arranged inside loose connective tissue but, contrasting with previous reports, the orientation of these bundles is not necessarily restricted to the transverse plane of the arm. Trabeculae crossing the longitudinal muscles are transversely organized muscle fibers between two thick collagenous layers. As predicted from physiological studies, and differing from other invertebrates, the muscle cells are innervated via localized synaptic junctions. The functional significance of this organization is discussed.

# INTRODUCTION

The highly mobile and active predatory lifestyle of octopuses and other modern cephalopods (Coleoidea) differs from that of other mollusks. In the octopods this evolution of mobility and predation was accompanied by the molluscan foot evolving into eight long and flexible arms. The arms are equipped with a row of suckers possessing elaborate tactile and chemical sensory systems, as well as active attachment capabilities (Packard 1972, Wells 1978). The arm neuromuscular system combines extreme flexibility with the ability to perform highly sophisticated tasks (Gutnick *et al.* 2011, Huffard 2006, Mather 1998).

The body and behaviors of the octopus thus represent a special, complex embodiment (Pfeifer *et al.* 2007) that may involve not only a large and complex brain, but also a unique interaction with environment through adaptive neuromuscular development and elaborated sensory systems. Because of this, the octopus arm is an exciting inspiration for natural solutions to the complex engineering problem of control and generation of movement in flexible structures (Gutfreund *et al.* 1996, 1998, Sumbre *et al.* 2005, 2006: Flash & Hochner 2005). Thus, a detailed analysis of the arm neuromuscular system is of special interest.

The octopus arm, like other cephalopod tentacles, the elephant trunk and vertebrate tongue, lacks a rigid external and internal skeleton. Instead, the muscles provide skeletal support, as well as creating movements. Kier & Smith (1985) have termed this type of structure a *muscular hydrostat*, because it is mainly composed of closely packed, incompressible muscle tissue organized longitudinally, transversely and obliquely. Because the volume remains constant, the longitudinal and transverse muscle fibers are functionally antagonistic, their activation shortening and elongating the arm, respectively. Co-activation causes stiffening, while contraction of the oblique muscles causes torsion of the arm. This design principle of closely packed muscle fibers contracting in opposing directions is also used in other muscular structures in cephalopods, such as the fin, mantle and sucker (Kier 1989, Ward & Wainwright 1972, Packard &Trueman 1974, Gosline *et al.* 1983, Kier & Smith 1990, Bone *et al.* 1995).

Exploring the principles of motor control in the flexible arms of the octopus has revealed several strategies which reduce the complexity of planning movements in a structure with practically unlimited degrees of freedom (Gutfreund et al. 1996, 1998, Sumbre et al. 2001, 2005, 2006). We also have previously described some of the physiological properties of the arm neuromuscular system, particularly the passive and active electrical properties of the electrically compact (isopotential) muscle fibers and their polyneural innervation by three distinct excitatory cholinergic synaptic inputs (Matzner et al. 2000, Rokni & Hochner 2002, Gutfreund et al. 2006). We also showed that the muscle cells in the antagonistic longitudinal and transverse muscles have similar properties. Here we characterize these muscle cells morphologically using light and electron microscopy.

Since our aim is to unravel the functional organization of the arm as a biomechanical device, our morphological research has two main objectives. As Kier & Smith (1985, and Kier & Stella, 2007) pointed out, the mechanical function of muscular hydrostats depends, firstly, on the organization of muscle cells and connective tissue with respect to each other, and secondly, on the dimensions of the muscle fibers and their arrangement into muscle groups. All these factors influence movement and force generation by determining the interactions between the muscles. Examining these factors is, therefore, the first aim of our morphological analysis. The second is to use electron microscopy to determine the type and pattern of muscle innervation to complement previous physiological data (Matzner *et al.* 2000, Gutfreund *et al.* 2006).

## MATERIALS AND METHODS

All octopuses (*Octopus vulgaris*) used in this study were maintained and anaesthetized as previously described (Matzner *et al.* 2000). Briefly, subjects were anaesthetized in cold seawater containing 2 % ethanol. One arm was amputated close to its base. The arm was measured and cut transversely at designated locations to obtain several segments of the arm ~ 0.5 centimeters thick.

*Fixation protocol*: These segments were immersed in freshly prepared fixative containing a mixture of 3 % glutaraldehyde (AGAR Scientific LTD, Stansted, Essex U.K.) and 4 % para-



Fig. 1. – Vibratome cross-section (500  $\mu$ m) of the octopus arm showing the gross organization of the intrinsic muscles. A bundle of transversely cut muscle cells appears dark - transparent, while those cut longitudinally are bright - opaque. The arm musculature is divided into four parts: dorsal (D), ventral (V) and two lateral (LT) muscles. The main muscle groups are composed of transverse (T), longitudinal (L), oblique (O) and trabeculae (TR) muscles. The axial nerve cord (N) is divided into a dorsal axonal tract (dark-transparent) and a ventral ganglionic part (bright-opaque). (The horizontal diameter of the section is ~7 mm).

formaldehyde in artificial seawater for 4 hours at room temperature (RT). The fixed pieces were sectioned at 200  $\mu$ m on a vibratome and allowed to fall into phosphate buffer at pH 7.4. The sections were postfixed in 1 % osmium tetroxide and 1.5 % potassium ferricyanide in 0.1 M cacodylate buffer for 1 hour at RT. After rinsing in buffer, the tissues were dehydrated in ascending concentrations of ethanol, infiltrated with epoxy resin (AGAR Scientific LTD), embedded and polymerized for 48h at 60°C.

Ultrathin sections were cut with a diamond knife on an LKB 3 ultratome and picked up on 200-mesh thin-bar copper grids. The sections were contrasted with aqueous uranyl acetate and Reynolds lead citrate solutions and viewed under the electron microscope (Tecnai 12, Philips). The digital images were captured by MegaView 2 (CCD camera for transmission electron microscope). For light microscopy, semi-thin sections (2  $\mu$ m) were mounted on glass slides and stained with 1 % methylene blue. These were photographed with a digital Nikon Coolpix camera 950.

Synaptic vesicle and junction dimensions were measured using a Soft Imaging System (analySiS 3.0). Cell and nuclei diameters were calculated from the cross-sectional area of the structure measured directly from digital images using Photoshop (Adobe) software.

Single muscle cell dissociation: Dissociated muscle cells were obtained as described by Rokni and Hochner (2002). A



Fig. 2. – Orientation of muscle fibers in different muscle groups. Low magnification light micrograph of a lateral area from a transverse section of the arm stained with 1 % methylene blue. Muscle groups labeled as in Fig. 1. Note that the transverse muscle group is organized in bundles oriented in various directions.



Fig. 3. – Low power electron micrograph demonstrating the organization of muscle fibers and connective tissue in three different areas of the intrinsic muscles. All sections were taken from the lateral group of muscles ~2.5 cm from the base of the second left arm (arm length 292 mm). In the animals used here, weighing about 400 gm, arm diameter was ~13 mm at this location. A: Transverse section of the longitudinal muscle group. Muscle fibers of the trabeculae crossing the longitudinal muscle are cut longitudinally. \* marks collagenous tissue. B: Transverse section of muscle cells of the trabecular group. The longitudinal muscles on both sides of the trabecula are cut obliquely. Thick layers of collagenous tissue (\*) separate the longitudinal and trabecular muscles. C: Muscle cells of the transverse group (cut in the transverse plane of the arm). The muscle cells are organized in bundles within a mass of connective tissue.

small piece of arm intrinsic musculature was taken from either a transversal or longitudinal muscle group (see Fig. 1) under a dissecting microscope. The tissue was incubated at 25-30° C for 4-6 h in 0.2 % collagenase (Sigma type I) dissolved in L15 culture medium (Biological Industries, Bet Haemek, Israel) adjusted to the concentration of salts in seawater. The enzymatic treatment was terminated by rinsing with L15. The tissue was then triturated manually until an appreciable concentration of dissociated muscle cells could be detected in the supernatant. These cells were kept at 17° C and an aliquot of the cells was transferred to a plastic Petri dish mounted on an inverted microscope. The cells settled on the bottom of the dish within a few minutes and were subsequently photographed using phase contrast optics.

## RESULTS

#### Gross morphology based on LM of arm cross-section

The intrinsic muscle system is the main generator of

arm movements. Figure 1 shows an unstained cross-section of the intrinsic muscles, obtained by removing the subdermal and sucker muscle systems. The cross-section shows the general structural organization, as described previously for the Octopus vulgaris arm (Graziadei 1971), and for other species of octopus (Kier & Smith 1985, Kier 1988, Kier & Stella 2007). We divide the intrinsic muscles into four main groups: dorsal (D), ventral (V) and two lateral groups (LT). Each of the main muscle groups are composed of transverse (T), longitudinal (L) and 3 sets of oblique muscles (O). The transverse muscles surround the axial nerve cord (N) that runs along the arm. The planes of the muscle fibers of this transverse mass are generally thought to lie perpendicular to the long axis of the arm. Surrounding the transverse muscles, longitudinal muscles run parallel to the long axis of the arm between the trabeculae (TR) formed by transverse muscle fibers.

Figure 2 shows the general organization of the three different muscle groups as seen in a low magnification LM image of a transverse section. As expected, the longitudinal and oblique muscle groups were cut close to



Fig. 4. – High power electron micrograph of collagen fibers oriented in two directions in a cross-section of a longitudinal muscle group. A: Striated structure typical for collagen. B: Possible association between the collagen fibers and muscle cells. Note fingers of dense material (arrows) close to the Z line structure.

perpendicular to their longitudinal axis. The transverse (T) muscles are composed of loosely assembled but distinct groups of muscle fibers, segregated according to the orientation of the muscle fibers within the muscle, which may vary from almost orthogonal to the transverse orientation of the section (circled in red) to almost parallel (yellow). In contrast, Kier (1988, Kier & Stella 2007) reported an almost uniform transverse orientation of fibers in the transverse muscles. If this were the case here, all the transverse muscle fibers would have been cut close to longitudinally in our section, like the group labeled in yellow in Fig. 2. However, Kier and his coworker studied mainly *Octopus bimaculoides*, and our findings may thus represent a species-specific morphological organization.



Fig. 5. – Electron micrograph of a transverse section of the right group of the longitudinal muscle. A:The muscles show typical obliquely striated fibers with mitochondria (M) in the core of the cells surrounded by myofilaments (F). Only one of the muscle cells is cut at the nucleus (N) and two bear a synaptic junction. B: Greater magnification of the area within the square in A. C: Greater magnification of the area within the rectangle in A. The synapses contain clear vesicles and dense junctional membrane (J).

## Gross morphology of the organization of the transverse and longitudinal muscles as shown by EM

We explored the structure and organization of muscle and connective tissue at the EM level, examining longitudinal muscles (L in Fig. 1), transverse muscles (T) and the trabeculae (TR). These muscles are thought to be important in bending and stiffening of the arm, as in arm extension and fetching, movements we are studying behaviorally and physiologically (reviewed in Flash & Hochner 2005).

Low power EM cross-sections showed that the longitudinal muscles are densely packed with muscle fibers (Fig. 3A), while the variously aligned muscle cells in the transverse muscle group (Fig. 2) are more dispersed (Fig. 3C), and the trabecular muscle fibers are organized in dense groups (Fig. 3B). The nature of the loose extracellular material of the transverse muscles is not yet defined. Embedded within the longitudinal and trabecular muscles is a mesh of opaque tissue (marked by asterisk in Fig. 3A,B). In the trabeculae, this material is concentrated as a continuous sheet at the border with longitudinal mus-



cles (Fig. 3A,B). Its striated appearance in longitudinal section is typical of collagen (Fig. 4).

### Ultrastructure of single muscle cells

All the arm muscle cells have the obliquely striated structure typical for cephalopod muscle fibers (Kier 1988, 1996, Bone et al. 1995, Budelmann et al. 1997). The core of the muscle cells is occupied by mitochondria (M), while myofilaments (F) are organized along their periphery (Fig. 5, see also Fig. 4B). The nucleus (N) lies in the wider part of the cell. Typical for cephalopod muscles (Bone et al. 1995), the cells have no transverse tubular system, and a sarcoplasmic reticulum system is organized as a set of sub-sarcolemmal cisternae (marked SR in Fig. 4B). These are the source of tubules that penetrate the myofilaments at the Z line area. It has been suggested that this system mediates the activation of the myofilaments by releasing Ca<sup>++</sup> following muscle excitation (Bone et al. 1995).

penetrate or dent the cell (thick arrows Fig. 4B), may adhere the cells to the extracellular scaffold. Dimensions of the muscle cells The distribution of the areas of muscle cell cross-

The densely packed fibers of the longitudinal muscles

are separated only by the collagenous extracellular material. The electron micrograph in Fig. 4B shows a typical

relationship between the collagen fibers and the sarcolemma. Thin finger-like processes, which seem to either

sections should reflect the cells' fusiform shape and the organization of adjacent cells. Such distributions varied widely, as in the typical examples shown in Figs. 3 and 5. Only few cells were cut at the location of the nucleus (N in Fig. 5). The histogram in Fig. 6A gives the distribution of cell diameters as calculated by approximating the mainly polygonal cross-section of the cell to circular. The distribution of the diameters of 424 cells measured from 3 sections, obtained from near the base of the same arm,

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Fig. 6. - The muscle cells are fusiform, i.e., half cylinder, half cone. A: Histogram showing the skewed distribution of the diameters of 424 longitudinal muscle cells (open squares). The broken line (open circles) gives the estimated normal distribution calculated from the peak (9.85 µm) and the standard deviation (± 1.54) derived from the distribution of cells with diameters larger than the peak. B: Simulations (see text) of the expected diameter distribution of fusiform cells (inset) plotted for different ratios of cylinder to cone parts (in percent). A good resemblance to the real distribution (black squares) was obtained with cells that were 50 % cylinder (open diamonds).

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Fig. 7. –The dimensions of enzymatically dissociated muscles cells were similar to those measured in the EM micrographs. A: Example of a long intact muscle cell (arrow) together with a cell that was damaged (but most likely healed) in the preparation process (i.e. during preparation of a small  $\sim 3 \times 3 \times 3$  mm muscle tissue sample). The integrity of the cell was shown by the tapering ending (inset, a). Inset (b) shows a representative population of intact (arrow) and damaged cells. **B**: Histogram showing the length distribution of dissociated intact cells from muscle samples taken from an area of c. 8 mm diameter at the beginning of the distal third of the arm. The histogram combines results from 4 samples from 4 octopuses



Fig. 8. – Electron micrograph of an oblique section of a muscle cell at the site of the elongated nucleus (N). This section is a rare case where two synapses (S) were seen on the same muscle fiber. Panels A and B are enlargements of each synapse. Arrowheads mark the area of contact between the nerve and muscle. Note that, while the left synapse is embedded within the muscle cell, the other shows a more superficial contact.

was non-uniform and was skewed toward cells of smaller diameter. Since cephalopod muscle cells have been shown to have an elongated and fusiform shape (Bone *et al.* 1981, 1995, Kier 1985), such a distribution may result from the combination of the muscle fibers being sectioned at different locations along their fusiform structure together with a random organization of adjacent muscle cells.

We tested this possibility by simulating the fibers as cylinders with cones attached to each end (Fig. 6B inset). The average and the standard deviation of the cylinder diameter was estimated by fitting a normal curve to the data points equal to or greater than the peak values (9.85  $\pm$  1.54 µm) (Fig. 6A). First we used these two values to generate random cell diameters, and, then, in a second random process, the site of sectioning along the cell was



determined. The simulated diameter was then calculated according to the assumed ratio between the cylindrical and cone parts of the fiber. This diameter was held larger than 2.2  $\mu$ m as this was the level of resolution of the measurements.

The results in Fig. 6B give the actual measurements of 424 cells together with simulations of a similar number of cells using different cone-to-cylinder ratios (expressed as percent of the cylindrical part). The distribution of purely cylindrical cells (closed circles) is normal and reflects the variability expected at this sample size. Increasing the proportion of the cone part increased the number of cells showing a small diameter and reduced the relative number of cells at the peak. Symmetrical cells, where the length of the two cones was equal to that of the cylinder (open diamonds), gave a good fit to the distribution of the 424 cells. The simulations thus suggest both that the muscle fibers have a symmetrical fusiform shape, and that adjacent muscle fibers are organized randomly, emphasizing the *non-segmental* organization of the arm musculature.

#### Estimation of muscle fiber length

We estimated the length of the muscle fibers indirectly based on an assumption of random organization. The method uses the fraction of cells cut at the level of the nucleus. If the cells are randomly organized (or if the sample is very large in an organized muscle structure), then the ratio of cells cut at the nucleus to the total number of cells should be equal to the ratio of the average length of the nucleus to the average length of the cell.

Longitudinal sections of the muscle cells revealed the elongated structure of the nuclei, whose average length was  $16.3 \pm 2.7 \ \mu m \ (n = 7)$  and diameter was  $4.78 \pm 0.97 \ \mu m \ (n = 8)$  (Fig. 7). Since the fraction of cells with

Fig. 9. - Similar distributions of cell diameters in different muscle groups at the base of the arm suggest that different groups are composed of muscle fibers of similar dimensions. Longitudinal muscle fibers of the dorsal part (open diamonds); longitudinal muscle fibers of the lateral part (open triangles); transverse muscle fibers (open circles); muscle fibers of the trabeculae (open squares). The longitudinal muscles at a distal part of the arm (open circles) show a different distribution and lower peak diameter.

nuclei was 1.47 % (26 nuclei, 1769 cells), the length of the cell was estimated to be ~1.1 mm (calculated using the equation  $L_c = L_n/P_n$ , where  $L_c$  is the average length of the muscle fiber,  $L_n$  is the length of the nucleus and  $P_n$  is the fraction of cell sections containing nuclei, i.e. the probability of cutting the nucleus). This method estimates the lengths only of the parts of each cell with diam. > 2.2  $\mu$ m, the level of resolution of our measurements. Based on the average cell shape (Fig. 6), the cell length was therefore corrected to ~1.21 mm.

Fig. 8 shows results from measurements of enzymatically dissociated muscle cells. As shown in Fig. 7 the population of dissociated cells contained both intact and broken cells. The former could be easily distinguished by the presence of the typical tapering of the muscle cell ends as shown in Fig. 7B. For the histogram in Fig. 7D only intact cells were measured (Matzner *et al.* 2000, Rokni & Hochner 2002). The average muscle cell length at a section of arm of diameter c. 0.5 cm was  $957 \pm 291 \ \mu m$  (n = 53). Note, however, that the distribution is skewed; this range correlates with the estimate above based on density and dimension of nuclei. Similar results have been obtained from squid mantle muscle (Bone *et al.* 1981, Milligan *et al.* 1997).

## Muscle fiber dimensions in the various muscle groups

Despite the different organization of the longitudinal, transverse and trabecular muscles (Figs. 2, 3), the dimensions of the muscle cells comprising each group seemed to be similar at a specific location along the arm, i.e. cell diameters of all groups showed similar distributions of cross-sectional diameter (Fig. 9). On the other hand, 20.2 cm down the 29.2 cm long arm, the peak diameter of longitudinal muscle cells reduced to ~6  $\mu$ m, com-

pared with ~7-8  $\mu$ m at the base of the arm (Fig. 9 open circles). Here the distributions suggested that the fibers are more cylindrical. The reduction in cell diameter was not proportional to the reduction in arm diameter (from 1.3 cm at the base to 0.4 cm at the distal part), nor to arm cross-section (132 mm<sup>2</sup>/ 12.5 mm<sup>2</sup> = 10.5 versus 44  $\mu$ m<sup>2</sup> / 23.7  $\mu$ m<sup>2</sup> = 1.86). This means that the cells change in both size and number along the arm, although the change in cell diameter is much less profound.

#### The neuromuscular junction and types of innervation

Muscle innervation, particularly the structure of the neuromuscular junction and the density of innervation, was studied using EM. The neuromuscular junctions showed a similar structure to that reported in other cephalopod muscles (Graziadei 1966, Bone *et al.* 1995). As shown in Figs. 5 and 8, the nerve terminals contained translucent vesicles of about 45 nm in diameter together with a few mitochondria. Larger dense-cored vesicles of about 100 nm diameter were occasionally observed in the terminal region. There was no special structure or folding of the subsynaptic membrane, the only morphological characterization of the junction is the thickening of the pre- and postsynaptic membranes at the junction area (Fig. 8A, B).

Our EM sections revealed only a small number of synaptic terminals onto muscle fibers relative to the number of muscle cells. Qualitatively, the frequency of detecting synaptic connections was comparable to that of detecting nuclei in the cross-sections (e.g. Fig. 5). More than one synaptic junction onto one cell was rarely seen and then only in longitudinally sectioned cells (Fig. 8). In a quantitative analysis, we found that only 15 of 613 muscle fiber cross-sections bore synaptic junctions (2.45 %). This is more than the percentage of cells cut at the nucleus (1.47 %, see above) but is not statistically different (Fisher's exact test; two-tailed P = 0.1484). Thus, we conclude that the muscle cells are not densely (multiterminally) innervated, instead each muscle cell is most likely innervated at a single synaptic junction (see Discussion). Recent data using rhodamine conjugated alpha-bungarotoxin labeling has confirmed this indirect inference (Nesher et al. 2011 Society for Neuroscience Abst).

Although physiological experiments have revealed three types of excitatory input to each muscle cell (Matzner *et al.* 2000), neither the shape nor diameter of the vesicles allowed classification of the synaptic terminals into different types. The rare example of two synaptic junctions onto the same muscle cell in Fig. 8 does not show overt differences in the shape of the synaptic vesicles nor in the structure of the junction. Figure 10A shows the distribution of the average diameter of the vesicles in 65 terminals. There was a large scatter but no obvious groups. The distribution is not significantly different from the estimated normal distribution (Fig. 10A, p < 0.78 chisquare for known distribution).

The only clue for different types of synapses may come from the nature of the contact the presynaptic terminals make with the sarcolemma of the muscle cells. While some junctions showed superficial contact with the muscle fiber, others were deeply embedded within the muscle membrane, although not "engulfed" by the muscle as described by Graziadei (1966) in the lip of the cuttlefish. An example for this difference is shown in Fig. 7C where arrowheads mark the area of contact between the two membranes. However, measuring the proportion of nerve terminal enwrapped by postsynaptic sarcolemma (contact length/terminal circumference) gave no clear separation into distinct groups (Fig. 10B). Nonetheless, statistical analysis showed a significant difference from an estimated normal distribution (Fig. 10B closed circles, p < 0.027, chi-square for known distribution). It is thus possible that there are synaptic types differing in their contact area with the muscle cell.

We found no morphological indication for electrical coupling, such as gap junctions between muscle fibers. Physiological experiments have similarly found no indication for significant electrical coupling (Matzner *et al.* 2000).

#### DISCUSSION

We shall discuss the current anatomical results in the context of our previous physiological studies (Matzner *et al.* 2000, Rokni & Hochner 2002, Gutfreund *et al.* 2006) in order to better understand the functional organization of the special neuromuscular system of the octopus arm. This will improve our modeling approaches (Yekutieli *et al.* 2005) and may provide biologically inspired ideas for a new type of robotics (Walker *et al.* 2005).

The gross anatomy of the octopus arm is basically similar to that of other cephalopod arms (see also Kier 1985, 1988, 1996, Bone *et al.* 1995, Budelmann *et al.* 1997). The muscle cells show an obliquely striated organization of their myofilaments, typical for cephalopod arm, mantle and other muscles but different from the cross-striated muscles of the rapidly elongating tentacles of the squid (Kier 1985, 1996). As suggested by Kier and colleagues, this may be one of the interesting morphological foundations for the different behaviors and lifestyles seen in cephalopod species.

Octopus arms are unique muscular hydrostats in which muscle tissue is utilized both for force generation and skeletal support. Such a muscular hydrostat classically consists of a set of antagonistic groups of muscles which consist of densely packed muscle cells. We show here that, although each muscle group of the octopus arm is composed of muscle cells with the same ultrastructure and dimensions, they differ in their density, orien-



Fig. 10. - Morphometric characterization of the neuromuscular junctions. A: Histogram of average diameters of clear vesicles measured in 65 terminals. Closed circles show the expected normal distribution calculated from the total average and standard deviation. There was no significant difference between the observed and the expected normal distribution (chi-square test for known distribution  $\vec{p} < 0.78$ ). **B**: Histogram showing the relative area of contact between 56 presynaptic terminals and the sarcolemma as defined by the fraction of the terminal membrane length in contact with the muscle cell. The distribution diverges significantly from a normal distribution (closed circles, chi-square test for known distribution p < 0.027), suggesting a non-uniform class of synaptic contact.

tation and interaction with the surrounding connective tissue. Because their physiological properties are similar (Matzner *et al.* 2000, Rokni & Hochner 2002), these anatomical differences may play an important functional role in constraining the biomechanical properties of each muscle group.

The four longitudinal muscle groups are composed of densely packed muscle cells (Fig. 3). Unique to this muscle group, a collagenous tissue is embedded as a 3 dimensional mesh inside the muscular mass. This flexible collagenous matter may serve as a scaffold for the longitudinal muscle cells and may be viewed as a collagenous sponge-like structure packed with muscle tissue (Fig. 4B). The cells in the trabeculae of transverse muscles are also densely organized but here, in contrast, connective tissue occupies much of the muscle volume; a layer of relatively thick collagen tissue runs along the border between the longitudinal and trabecular muscle groups. This suggests that the antagonistic mechanical relations between the longitudinal and trabecular muscles are based not only on the mechanical and passive force of the muscle fibers, but also on the different elastic forces passively generated by the connective tissue. This has also been suggested for the cephalopod mantle and fin (Bone *et al.* 1981, Johnsen & Kier 1993).

The four longitudinal muscle groups (two lateral, dorsal and ventral, Fig. 1) may serve as cores of dynamic skeletal beams when the group is stiffened by co-contraction of the longitudinal and trabecular muscles (and probably also the oblique muscles that wrap around the two, Fig. 1). In this case, the elastic forces generated by the fortified connective tissue in the trabeculae would contribute much of the force opposing shortening of the longitudinal muscles, thus contributing a passive component to the stiffening. The trabecular muscles may function mainly during elongation, while the trabecular connective tissue may resist arm shortening, amplifying stiffening. Indeed, behaviorally, arm elongation is much more common than arm shortening (relative to a rest length). For example, fetching and pulling movements involve the formation of two stiffened segments but do not involve their shortening (Sumbre et al. 2005, 2006). Also, reaching movements involve various combinations of bend propagation and arm elongation (Hanassy & Botvinnik et al. unpubl).

The transverse muscle group, which surrounds the

axial nerve cord, displays yet another structural variant. These muscles are neither densely packed nor lie exclusively in a transverse direction. Instead, the cells are organized in relatively small bundles of a few tens of muscle fibers, each oriented in one out of several typical directions. They are embedded in loosely packed connective tissue. This structure suggests that the transverse muscle group may create different bending forces acting on the longitudinal beams, rather than acting as muscular hydrostatic structure.

## Implications for arm muscle physiology

The length of the muscle cells estimated here (~1.2 mm) is considerably shorter than the electrotonic space constant of ~ 8 mm estimated physiologically (Matzner *et al.* 2000). This implies that cell voltage can be controlled by a localized synaptic input, and this conclusion is supported by the low density of synaptic junctions found here. There does not appear to be any electrophysiological need for the multi-terminal innervation common in other invertebrates, where cell length is usually several times the space constant (Bullock & Horridge1965).

This electrical compactness also suggests that fast action potentials are not necessary for propagating electrical activity along the muscle fiber, as in vertebrate striated muscle (Matzner *et al.* 2000). Indeed, we found that L-type Ca<sup>++</sup> current is the sole voltage-dependent inward current in the cells (Rokni & Hochner 2002), suggesting that the purpose of the active Ca<sup>++</sup> current is to activate the contractile machinery. This active current may be sufficiently efficient for introducing Ca<sup>++</sup> into the core of these thin cells, with no need of a TTS-like system, which is absent in these muscle cells (Bone *et al.* 1995).

The three physiologically different classes of synaptic input to each muscle cell are all cholinergic and excitatory, but they are segregated into two very different quantal postsynaptic responses - a distinct "fast" and unusually large mEPSP (5-25 mV/2-5 ms rise-time) and "slow" and small EPSPs (1-5 mV/10-25 ms) (see Matzner et al. 2000). These differences do not appear to originate from differently structured synaptic junctions, since morphological characterization of the synaptic junctions at the EM level did not show segregation into a distinct synaptic structure. Similarly, in Crustacea, no structural differences between fast (phasic) and slow (tonic) synaptic junctions have been found (Msghina et al. 1998). Nevertheless, in the octopus arm, the area of contact of the nerve terminal with the plasmalemma did not display a normal statistical distribution as would be expected if synapses belonged to a single population (Fig. 10). Different engulfment by the postsynaptic membrane may lead to more efficient activation of the postsynaptic receptors, slower diffusion rates and faster transporter action.

The muscle cells comprising the different muscle groups have similar passive and active membrane prop-

erties and their synaptic inputs show a simple neuromuscular transformation dynamics (Matzner *et al.* 2000). We have shown here that they have similar structure, dimensions and probably also morphological patterns of innervation. All this evidence suggests that the octopus arm is composed of *similar functional motor units*. Basing the structure of the flexible arm on similar motor units with similar properties is probably an evolutionary adaptation to the complex problem of controlling a flexible arm with unlimited degrees of freedom. At the same time, this similarity stresses the importance of morphological organization and interaction with non-muscular tissue for the arm's global biomechanical function.

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