POLYNEURONAL INNERVATION OF SINGLE MUSCLE FIBERS IN CAT EYE MUSCLE: INFERIOR OBLIQUE

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Running title: Polyneuronal innervation

Keywords: motor unit, oculomotor, extraocular, cat

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ABSTRACT

Single muscle fibers with multiple axonal endplates (multiply innervated fibers) are normally present in adult extraocular muscles (EOMs), while most other mammalian skeletal muscles contain fibers with a single myoneural junction. Recent findings by others led us to investigate for the presence of polyneuronal innervation (innervation of a single muscle fiber by more than one motoneuron) in the inferior oblique (IO) muscle of pentobarbital anesthetized cats. The IO muscle nerve branches, as they coursed through the orbit, were further divided for independent or simultaneous electrical stimulation with bipolar electrodes. Four out of 5 established tests for polyneuronal innervation gave positive results. The sum of the twitch (1) and tetanic (2) tensions in response to individual nerve branch stimulation was greater than that for simultaneous (whole) nerve stimulation. The summed electromyographic (EMG) responses (3) gave a similar positive result. The result for crossed tetanic potentiation (4) was negative for polyneuronal innervation while the crossed fatigue (5) test was positive. These results are consistent with recent studies. That the EOMs exhibit polyneuronal innervation further explains the eye movement system’s functional integrity during some neuromuscular disorders as well as its ability to operate with precision after the loss of numerous motoneurons.
INTRODUCTION

In contrast to most other mammalian skeletal muscles in which individual muscle fibers have a single myoneural junction, muscle fibers with multiple endplates (multiply innervated fibers) are normally present in adult extraocular muscles (EOM) (Bach-y-Rita and Ito 1966; Harrison et al. 2007, Hess and Pillar 1963; Lennerstrand 1972; Peachey 1971). It is still not known, however, if these multiple endings on EOM muscle fibers represent axonal inputs from more than one, or only one, motoneuron. If a single muscle fiber is innervated by more than one motoneuron that would be classified as polyneuronal innervation.

The multiply innervated fibers in the orbital layer of EOM exhibited different electrical properties in the end-plate zone of the muscle as opposed to areas distal to that zone (Jacoby et al. 1989). In addition, two types of nerve endings were identified, morphologically and histochemically, along the length of these fibers in rat and monkey (Jacoby et al. 1989; Pachter 1982, 1984). Therefore, it would seem likely that the multiply innervated fibers receive polyneuronal (or at least dual) innervation.

Prior to these studies, Bach-y-Rita and Lennerstrand (1975) tested for the possibility of polyneuronal innervation in adult cat lateral rectus muscle. They were not able to demonstrate it physiologically. Their approach used recordings of muscle contraction during stimulation of only two, of several, divisions of the abducens nerve innervating the lateral rectus muscle. They used a ventral approach (through the neck) to expose the VIth nerve at its exit from the brain stem. (While many nerve rootlets are present at this anatomical location, they routinely stimulated only two of those several bundles of axons.) The presence of polyneuronal innervation is suggested if the sum of
tensions produced by individual stimulation of each nerve root is greater than the tension produced by simultaneous stimulation of both nerve divisions. This approach relies on the muscle fibers’ property that any muscle fiber supplied by both nerves (simultaneous stimulation) cannot contract more strongly than when separately stimulated. However, the “whole muscle” twitch tension size in this study was only ~ 4 g compared to 10-15 g usually seen in cat lateral rectus muscle (Barmack et al. 1971; Dimitrova et al. 2002; Goldberg and Shall 1997). Judged by the small twitch tension amplitude, a possible source of error that could have lead to negative results would be a reduced stimulation current intensity (in order to avoid current spread). Using sub-maximal nerve stimulation would not activate the whole nerve and would greatly reduce the chances of identifying polyneuronal innervation.

The technique itself, applied in earlier studies, has proved to be of accurate value in elucidating the possibility of polyneuronal innervation in other skeletal muscles (Bagust et al. 1973; Brown and Matthews 1960). No consistent evidence for the existence of polyneuronal innervation was obtained by Brown and Matthews (1960) in adult cat hind-limb muscles, but the possibility of its occurrence was not excluded. The same methodological approach gave positive results in kittens up to 2-weeks old (Bagust et al. 1973). In addition to developing neuromuscular systems, where polyneuronal innervation is widespread, polyneuronal innervation is also seen during nerve regeneration after injury. This was suggested by Hoffman (1951a, b) and later confirmed by a number of morphological and physiological studies (Gillingwater et al. 2004; Gorio et al. 1983; Guntinas-Lichius et al. 2005; Ito and Kudo 1994; Jansen and Van Essen 1975; Letinsky et al. 1976; McArdle 1975; Rich and Lichtman 1989; Rotshenker and McMahan, 1976;
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Tate and Westerman 1973; Werle and Herrera 1987, 1988, 1991). The non-selectively formed synapses are then, in time, pared back to result in the usual single motor endplates on single muscle fibers (Navarrete and Vrbova 1993).

It is also now known, for extraocular muscles, that there is continuous remodeling of individual myofibers throughout life (McLoon et al. 2004; McLoon and Wirtschafter, 2002). Therefore, it is reasonable to suggest that other properties (e.g. polyneuronal innervation) seen during development and regeneration would also be retained in normal adult extraocular muscles (McLoon et al. 2004; McLoon and Wirtschafter 2002).

In addition, multiple end-plates exist in some other mammalian muscles and recent electrophysiological evidence strongly suggests polyneuronal innervation of adult human skeletal muscle fibers (Lateva et al. 2002). The approach in the Lateva et al. (2002) study was based on the electromyographic (EMG) potential of individual motor units (found by electrical decomposition) of the EMG activity of brachioradialis muscle. Assuming polyneuronal innervation of a motor unit, the single unit EMG potential should remain the same regardless of which neuron had discharged to cause the contraction. Also, in the case of simultaneous neuronal discharge close in time, the two components of the motor unit response would collide and one of them then would be blocked in the muscle fiber.

In light of all this recent evidence, the possible existence of polyneuronal innervation in normal adult EOM needed to be revisited. We have employed most of the electrophysiological tests developed by previous experimenters. We searched for a tension and/or EMG excess in the sum of muscle responses to separate versus simultaneous stimulation of the two main nerve branches of cat inferior oblique (IO)
muscle. The tension related tests included (1) twitch tension excess, (2) tetanic tension excess, (3) cross-potentiation (post-tetanic potentiation of one IO nerve branch after tetanic stimulation was delivered to the other branch), and (4) cross-fatigue (decreased tension in one branch after fatigue of the other branch). Also, an (5) EMG excess was examined. All but one test (cross-potentiation) gave positive results in relation to polyneuronal innervation.

METHODS

Electrophysiological tests for polyneuronal innervation were performed on 8 eye muscle/nerve preparations in adult cats (2.5-3.5 kg) according to procedures and protocols approved by the Animal Care and Use Committee of Virginia Commonwealth University.

Surgical preparation

The cats were initially anesthetized with 45 mg/kg sodium pentobarbital I.P. Supplemental doses of sodium pentobarbital were administered I.V. throughout the experiment to maintain deep anesthesia, indicated by the absence of withdrawal reflex to paw pinch. Following topical anesthesia of the larynx with 4% lidocaine, the animals were intubated with a 3-5 mm endotracheal tube. A heating pad was used to keep rectal temperature at 38-40 °C. Respiratory rate, end-tidal CO₂, heart rate and body temperature were continuously monitored and maintained within normal ranges.

The animal’s head was fixed in a Kopf stereotaxic frame and a midline incision from the nose to the posterior neck was made. The superior eyelid and portions of the superior and lateral aspects of the bony orbit were removed to expose the inferior oblique (IO) muscle and tendon including the insertion on the sclera. The IO muscle/nerve
Polyneuronal innervation preparation was chosen due to the long, accessible course of the whole nerve from near its origin through the cat orbit. The IO muscle’s tendon was looped with 5-0 silk suture in order to detach the tendon and muscle from the globe. The temporalis muscle was retracted and both the lateral and superior rectus muscles were detached from the globe to gain access to the optic nerve. The optic nerve and ophthalmic artery were tied off, but leaving the blood supply to the IO muscle and nerve intact, and the eye was enucleated to expose the IO nerve as it courses through the orbit. The IO muscle and main IO nerve, including those distal nerve branches entering the middle third of the muscle (endplate zone), were isolated from the surrounding tissues. Similar surgical preparations have been previously used (Nelson et al. 1986; Russell-Mergenthal et al. 1986; Shall and Goldberg 1992; Shall et al. 1995). Additional isolation of the two main IO branches was performed, as described below, to avoid current spread from one IO nerve branch to the other. To keep the muscle/nerve preparation moist and warm during the experiment it was frequently bathed in warm mineral oil. After all data was collected, each animal was euthanized with an overdose of sodium pentobarbital delivered intravenously.

**Stimulation and recording protocol**

A bipolar stainless steel electrode (~2 mm between poles) was hooked under each IO nerve branch approximately 8 mm from their muscle entry. Usually, two main natural nerve branches were visible and they are referred to as the lateral and medial branches. IO muscle tension and electromyographic (EMG) activity were recorded in response to separate or simultaneous stimulation of these two nerve branches. Simultaneous stimulation of both nerve branches would be equivalent to whole nerve stimulation. Pulses of 0.2-ms duration, 0.5-3 mA current intensity and variable frequencies were
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produced by a programmable pulse generator (AMPI Master-8) and delivered to the stimulating electrodes. The twitch characteristics of the IO muscle and muscle portions innervated by each IO nerve branch were analyzed in response to single pulses delivered at a rate of 1/s (10 stimuli). IO tetanic contractile properties were evaluated in response to constant frequency pulse trains of 200 ms duration using a frequency range from 50 to 220 Hz, delivered at 5-s intervals (3 trials for each frequency). In addition to supra-maximal IO nerve stimulation, in several cases sub-maximal stimulation was tested by an appropriate reduction of the stimulation current intensity until the evoked tension dropped to ~50% of that evoked by maximal stimulation. In these cases we used sub-maximal stimulation to determine if the results related to polyneuronal innervation depend on the stimulation current intensity.

The IO muscle suture was attached to a force transducer (MLT050, ADInstruments) and the muscle was set at an optimal length for maximal isometric twitch tension. EMG electrical activity was recorded, simultaneously with muscle tension, using a bipolar electrode consisting of 2 fine wires (25 μm pole diameter), separated by about 5 mm, inserted into the belly of the IO muscle (Shall and Goldberg 1995; Shall et al. 1995). One electrode pole was located about 4 mm distal to the central endplate zone and the other pole was about 9 mm distal to the zone. We wanted to record the electrical response of many IO muscle fibers, but no attempt was made to differentiate responses from the muscle’s global and orbital layers as was done in previous studies. The fine wires did not move in the muscle (fixed at isometric tension) and EMG recordings were stable and consistent.
Current spread control

The following measures controlled for the possibility of current spread from the stimulating electrode of one nerve branch to the other nerve branch. 1) Muscle contraction was monitored and its absolute size was observed online. Increasing the intensity of stimulation to one nerve branch resulted in a gradual increase in muscle tension until it reached a plateau. A supra-maximal level of stimulation was thus achieved. Further increases of stimulus intensity could lead to current spread to the other (non-stimulated) nerve branch. This current spread was seen as a sudden step increase of evoked tension amplitude. 2) The two ‘natural’ IO nerve branches were further split apart to impose a larger distance between stimulating electrodes and the muscle and thereby avoid direct stimulation of the muscle. One nerve branch (usually lateral) was detached from the whole IO nerve (where it divided from the other branch) and pulled away from the main nerve trunk. Each nerve branch could then be positioned in a separate plastic bath and soaked in mineral oil. This technique for complete physical isolation of the two nerve branches basically eliminated stimulation current spread. 3) Finally, after all other recordings were completed, one nerve branch (usually the lateral) was cut proximal to the muscle and completely removed from the preparation. Muscle responses to stimulation of the remaining branch were compared before and after the cut to assure that none of that response amplitude came from stimulation of the other branch via current spread. Muscle response to stimulation of the remaining IO nerve branch did not differ significantly in comparison with the response recorded just before the cut. Muscle tension in response to 200 Hz supra-maximal stimulation of the remaining IO branch was $9.7 \pm 1.1 \text{ g}$ in comparison with the tension of this branch before the cut ($10.4 \pm 1.3 \text{ g}$, $p>0.2$). The EMG
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peak-to-peak amplitude was also similar: $1.7 \pm 0.5 \text{ mV}$ before and $1.6 \pm 0.4 \text{ mV}$ after the branch was cut ($p>0.2$). These insignificant changes represent $4 \pm 5 \%$ change in tension and $1 \pm 9 \%$ change in EMG amplitude, showing that eliminating one nerve branch could result in either excess or deficit in muscle tension and EMG activity. This test confirmed that current spread was successfully eliminated during the data collection in this experiment.

Data analysis

The possible existence of polyneuronal innervation in cat IO muscle was explored by five different approaches.

1 & 2. **Twitch and tetanic tension excess.** The tension elicited by simultaneous stimulation of both IO nerve branches (therefore the whole nerve) was compared with the sum of the tensions evoked by separate stimulation of each nerve branch. For each experimental session the response of the whole nerve was obtained first, then for each nerve branch and finally the whole nerve was stimulated again. This was done as a control. No significant differences were found between the first and the control whole nerve tests for both twitch and tetanic stimuli. The whole nerve response was evaluated as a mean of the tension maximal amplitudes of the first and the control tests. The sum of tensions evoked by separate stimulation of the IO branches was calculated by adding the maximal amplitudes of each nerve branch.

3. **Cross-potentiation.** This test is based on the increase in twitch tension following tetanic stimulation (post-tetanic potentiation). In the presence of polyneuronal innervated muscle fibers, an increase in twitch tension of one IO nerve branch could occur after tetanic stimulation is delivered to the other IO nerve branch (cross-
potentiation) (Bach-y-Rita and Lennerstrand, 1975; Bagust et al. 1973). The twitch
tension response (average of 10 trials) to stimulation of one nerve branch was done after
the other branch had been subjected to tetanic stimulation consisting of our sequence of
200 ms trains ranging from 50 to 220 Hz (5 sec interval between trains), repeated three
times. (Fig. 2A illustrates the sequence.)

4. Cross-fatigue. This test explored the possibility that fatigue of the muscle fibers
in response to stimulation of one nerve branch is present in muscle fibers innervated by
the other nerve branch. Fatigue of each IO nerve branch was evaluated by comparing
tetanic tensions in response to 200 Hz stimulation frequency of supra-maximal current
intensity before and after a 2-min fatigue protocol delivered to one of the IO nerve
branches. A randomly selected IO nerve branch (data from 4 medial and 3 lateral
branches was collected) was presented with 150 Hz frequency, 500-ms train duration, 1
train/sec stimulation to fatigue the neuromuscular compartment innervated by this
branch.

5. EMG excess. The EMG activity of the IO muscle recorded during simultaneous
stimulation of both IO nerve branches (whole nerve) was compared with the sum of the
EMG activity produced by separate stimulation of each nerve branch (EMG excess
similar to the tension excess). The individual EMG signals were zeroed using the mean of
100 ms baseline EMG prior to the stimulus and then aligned by the artifact for summing.
In some cases the EMG signals of the two branches stimulated separately had different
latencies. Therefore they were first summed and then the peak-to-peak amplitude of the
sum was compared with the whole nerve peak-to-peak amplitude. The first 1 ms of the
EMG response was considered as an artifact and was excluded from the analysis.
The results were statistically evaluated using Student's t-test at probability level $\alpha=0.05$. The data is presented as mean ± SE for the group in both absolute values and percentages. Any excess of the sum of the two IO branches over the whole nerve values was expressed as a positive number.

RESULTS

Tension excess

Twitch contractions. The sum of the twitch tensions elicited by each of the two IO nerve branches stimulated separately exceeded the twitch tension elicited by stimulation of the whole IO nerve (simultaneous stimulation of both IO nerve branches). Examples of IO muscle responses to single pulse supra-maximal stimulation of each IO nerve branch separately or simultaneously (whole IO nerve stimulation) are given in Fig. 1A in comparison with the algebraic sum of the two IO nerve branches twitch tensions (dashed line). Group means ± SE of twitch tensions for each IO nerve branch, the whole IO nerve and the calculated sum of twitch tensions are presented in Fig. 1B (supra-maximal stimulation). The sum of IO nerve branches twitch tensions for the two IO nerve branches (3.4 ± 0.2 g) significantly exceeded the whole nerve twitch tension (3.0 ± 0.2 g) by 16 ± 4 % on average (paired t-test: $p<0.005$, $n=8$). In several cases ($n=5$) the stimulation intensity for each branch was intentionally reduced (sub-maximal stimulation). This current intensity resulted in 40-50% decrease of IO muscle tension in comparison with the corresponding tensions of the branches stimulated supra-maximally. Whole nerve sub-maximal stimulation was achieved with both branches stimulated at equivalent current intensities. In contrast to supra-maximal IO stimulation, sub-maximal twitch stimulation of IO nerve and its branches did not reveal any significant twitch tension
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excess (Fig. 1B). Additionally, any twitch tension excess found during sub-maximal stimulation was quite variable, averaging $2 \pm 11\%$ (SD 24\%) as it ranged from negative to positive values.

**Tetanic contractions.** Similar to the twitch tension, the sum of tetanic tensions for the two IO nerve branches exceeded the whole IO nerve tetanic tension ($p<0.05$). Tetanic tension excess was consistent with all the frequencies used and gradually increased with the higher stimulation frequencies. Figure 2A illustrates maximal tetanic tensions at different stimulation frequencies for each IO nerve branch and the whole IO nerve. It can be seen that the sum of tetanic tensions of the two IO branches (dashed line) always ‘exceeds’ the whole IO nerve maximal tetanic tension. For the lowest stimulation frequency (50 Hz) tetanic tension excess was $17 \pm 3\%$, similar to the twitch tension excess ($16 \pm 4\%$ at 1 Hz in Fig. 2A). Maximal tetanic tension excess was achieved at 140 Hz ($28 \pm 1\%$) and remained at this level regardless of further stimulation frequency increase (Fig. 2A).

Sub-maximal current intensity at 200 Hz stimulation frequency showed $8.4 \pm 1.2$ g summed maximal tetanic tension of the two IO branches stimulated separately vs. $7.7 \pm 1.1$ g tetanic tension of the whole IO nerve stimulation (Fig. 2B). Whereas supra-maximal IO nerve stimulation at 200 Hz gained $28 \pm 1\%$ tension excess ($p<0.05$), sub-maximal stimulation at the same stimulation frequency resulted in statistically insignificant tension excess of $8 \pm 2\%$ ($p>0.1$).

**Post-tetanic potentiation**

Tetanic stimulation (200-ms trains of 50 to 220 Hz) of one IO nerve branch caused post-tetanic potentiation in the same branch, but not in the other branch (cross-
potentiation). The twitch size in the tetanus-stimulated branch increased to 2.6 ± 0.9 g after the tetanic stimulation in comparison with 2.2 ± 0.7 g before the tetanic stimulation (p<0.05), presenting 19 ± 6 % of post-tetanic potentiation. The twitch size of the other, not tetanus-stimulated, IO nerve branch did not change significantly (-1 ± 2 %). Thus, the cross-potentiation test for polyneuronal innervation did not produce a positive result.

**Summation of muscle electrical activity**

The summed EMG activity of IO muscle when the two IO nerve branches were stimulated separately significantly exceeded the IO EMG activity of the whole IO muscle (simultaneous stimulation of the branches). Examples of EMG signals from each IO nerve branch during separate or simultaneous stimulation with supra-maximal current intensity are shown in Fig. 3A. The summed EMG activity following separate branch stimulation (dashed line in Fig. 3A) exceeds the EMG activity of the whole IO nerve. The peak-to-peak EMG amplitude for the sum of separately stimulated nerve branches was 5.8 ± 0.4 mV, significantly larger in comparison with the EMG amplitude for simultaneously stimulated IO nerve branches, 4.8 ± 0.3 mV (p< 0.05) as also illustrated in the bar graph of Fig. 3B. The average percentage EMG excess for the group was 19 ± 5 %. Interestingly, sub-maximal IO branch stimulation also showed EMG excess similar to supra-maximal stimulation (20 ± 7 %). Separate IO branch stimulation summed to 1.6 ± 0.2 mV, whereas simultaneous IO branch stimulation resulted in 1.4 ± 0.2 mV EMG amplitude of IO muscle.

**Cross-fatigue**

Muscle response to 200 Hz stimulation of the branch that had received fatiguing stimulation decreased to 56 ± 4 % of the response prior to fatigue (Fig. 4 left). Muscle
response to 200 Hz stimulation frequency of the other (non-fatigued) branch should remain unaltered if no polyneuronal innervation exists (cross-fatigue test). However, the non-fatigued IO nerve branch stimulation with 200 Hz frequency showed smaller but significant reduction in tetanic tension to $88 \pm 3\%$ ($p<0.05$) of the initial response (Fig. 4 right). The EMG data simultaneously collected with the tension also showed significant reduction in peak-to-peak amplitude to $67 \pm 7\%$ for the fatigued IO branch and $93 \pm 3\%$ for the non-fatigued IO branch compared to the EMG size before the fatigue protocol was applied ($p<0.05$). The results of the cross-fatigue test suggest that some muscle fibers innervated by the non-fatigued branch were already fatigued without being subjected to the fatiguing protocol.

**DISCUSSION**

Four out of five electrophysiological tests developed to indicate polyneuronal innervation gave consistently positive results when applied to cat IO muscle and the two main divisions of its nerve. Excision of one nerve branch did not change, significantly, IO muscle responses to stimulation of the remaining branch, indicating that any changes within a branch or the interaction between the two IO nerve branches were not due to current spread.

The sum of twitch tensions with separate stimulation of the two IO nerve branches exceeded the twitch tension of the whole IO muscle by 16%. While this tension excess could be a sign of polyneuronal innervation, it could be also due to mechanical effects of the series elastic component of the muscle instead. It has been shown by Brown and Matthews (1960) that if a spring is inserted between the isometrically contracting muscle and the force transducer, the tension excess (called in their study “tension deficit”
due to a reverse method of percent calculation) rises from 23% to 65%. However, the effect of the series elastic component of the muscle should be minimized during tetanic contractions and the individual fibers would be able to develop their full tension. Indeed, the tension excess during tetanic contractions of IO muscle was still present and even larger (up to 28% at the higher frequencies of stimulation) in comparison with the tension excess during twitch muscle contractions. The larger extent of tension excess during tetanic over twitch stimulation could also reflect the presence of non-twitch multiply innervated muscle fibers (Bondi & Chiarandini 1983; Jacoby et al. 1989) and/or non-twitch motor units (Nelson et al. 1986). Obviously, non-twitch units would not be activated using single twitch stimulation paradigms.

Both of the above tests for polyneuronal innervation use the arithmetic sum of the tensions developed upon stimulation of each IO nerve branch to compare with the muscle tension during whole nerve stimulation. These tests proved to be successful in establishing polyneuronal innervation in skeletal muscle during the early postnatal development period (Bagust et al. 1973). When the major wiring of the neuromuscular system has been accomplished as in normal adults and only minor adjustments may be required, the electrophysiological tests for polyneuronal innervation failed to produce positive results (Brown and Matthews 1960). It may be argued that the tension excess observed here in EOM and in previous studies in other skeletal muscles could be due to the complex muscle architecture (Alvarado-Mallart and Pinçon-Raymond 1976; Goldberg et al. 1997; Mayr et al. 1975; Trotter et al. 1995). The internal properties of the muscle can result in non-linear summation of motor unit forces (Goldberg et al. 1997; Monti et al. 2001; Perreault et al. 2003; Shall et al. 2003; Troiani et al. 1999). The
problem of non-linear force summation in the tests for polyneuronal innervation that rely only on tension excess can be solved by the other methods we have used.

The EMG activity of the IO muscle being free of non-linear tension summation, also showed excess of the sum of the two IO nerve branches separately activated over their simultaneous activation. The EMG excess (~20%) was smaller than the observed tension excess (~30% during tetanic stimulation). This could be an indication that a portion of the tension excess might be due to a non-linear force summation. It is also possible that the peak-to-peak analysis of IO EMG activity might miss some EMG components contributed exclusively by multiply-innervated muscle fibers.

Cross-potentiation (potentiation of twitch tension when single pulses are delivered to one IO branch following tetanic stimulation of the other IO branch) was not found in the present study. It is quite possible that the multiply innervated fibers of EOM may not be subjected to post-tetanic potentiation. Thus, the twitch tension potentiation in the neuromuscular compartment supplied by the IO branch that had received tetanic stimulation may be produced only by the singly innervated muscle fibers. Also, in our attempt to avoid any undue fatigue of the muscle, the tetanic stimulation sequence to evoke crossed-potentiation may have been too mild.

In contrast, the cross-fatigue test (decrease of muscle response to 200 Hz stimulation of one IO nerve branch after a fatigue protocol had been applied to the other branch) was always positive. Whereas both the tension and the EMG activity from the neuromuscular compartment of the fatigued IO branch were greatly reduced, a smaller but significant decrease was noted in the other, non-fatigued IO branch. Such a phenomenon could only be observed if axons from the ‘fresh,’ non-fatigued IO branch
reach muscle fibers that had already been fatigued. A recent review (Cairns and Lindinger 2008) indicates that ionic interactions may play a role in fatigue spreading to non stimulated muscle fibers. Very intense stimulation or exercise of leg muscles was used to induce the response. How this might translate to EOM using well established motor system fatigue tests is not known.

In order to check if reduced current intensity was the reason Bach-y-Rita and Lennestrand (1975) did not see tension excess in cat lateral rectus muscle, sub-maximal stimulation was applied to some of the preparations. Both twitch and tetanic IO muscle responses were very variable and quite similar to the results of Bach-y-Rita and Lennerstrand (1975), especially the twitches. Thus, the sub-maximal IO nerve branch stimulation resulted in small and non-significant tension excesses. Therefore, the ‘absence’ of polyneuronal innervation in lateral rectus muscle (Bach-y-Rita and Lennerstrand 1975) seems to be more likely due to insufficient nerve activation. With a partially activated nerve, the effects of the series elastic component of the muscle will be largely involved, including the tetanic stimulation. In contrast to the lack of consistent tension excess with sub-maximal nerve stimulation, the EMG activity excess in the IO muscle was notable and equal to the EMG excess found during supra-maximal nerve stimulation. As with the supra-maximal nerve stimulation, the EMG activity proved to be a reliable source as a test for polyneuronal innervation. The consistency of EMG activity excess regardless of stimulation current intensity also indicates that mechanical factors had prevented registration of tension excess during sub-maximal nerve stimulation.

In conclusion, multiple electrophysiological tests on cat IO muscle and the natural branches of its nerve suggest the presence of polyneuronal innervation of EOM fibers.
This result is consonant with many recent findings, both anatomical and physiological, in extraocular as well as other skeletal muscles. And the result also adds to the body of evidence showing how the eye movement system appears to maintain its functional integrity in the face of insults to the CNS (reviewed in McClung et al. 2004). That is, a loss of motoneurons does not necessarily translate into a loss of muscular control since more than one motoneuron may control the same muscle fiber (McClung et al. 2004).

REFERENCES


**Letinsky MS, Fischbeck KH, McMahan UJ.** Precision of reinnervation of original postsynaptic sites in frog muscle after a nerve crush. *J Neurocytol* **5**: 691-718, 1976.


**McArdle JJ.** Complex end-plate potentials at the regenerating neuromuscular junction of the rat. *Exp Neurol* **49**: 629-638, 1975.


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Acknowledgments: Supported by NIH grant EY 11249 AND Jeffress Memorial Trust. The authors greatly appreciate the technical support of Mary Ellen Goldberg.

Figure Legends:

Fig. 1. Comparison of twitch tension produced by stimulation of whole IO nerve and the sum of twitch tensions of each IO branch stimulated separately. A) Example of twitch tensions for the whole IO nerve and each of its branches when stimulated with supra-maximal current intensity. B) Group means ± SE of twitch tensions of the whole IO nerve and its branches evoked by supra- (n=8) and sub-maximal current intensity (n=5, *p<0.05).

Fig. 2. Comparison of tetanic tension produced by stimulation of whole IO nerve and the sum of tensions of each IO branch stimulated separately. A) Group means ± SE for each frequency of stimulation of IO with supra-maximal current intensity (twitch responses to stimulation pulses at 1 Hz and to pulse trains of 50 to 220 Hz frequency). All data point differences are significant (p<0.05). B) Group means ± SE of muscle responses to 200 Hz stimulation with supra- and sub-maximal current intensity. Supra-maximal, but not sub-maximal, current intensity resulted in significant tension excess (*p<0.05).

Fig. 3. Comparison of EMG signals in response to IO branch or whole nerve stimulation. A) Example of IO EMG activity produced by supra-maximal stimulation of whole IO nerve, each IO nerve branch stimulated separately and their sum. Note: The EMG signals are aligned by the onset to facilitate amplitude comparison. B) Group means ± SE of EMG peak-to-peak amplitudes of the whole IO nerve and the summed EMG activity of the branches (n=8, * p<0.05).
Fig. 4. Muscle tension and EMG amplitude (group mean ± SE, n=7) of one IO nerve branch after a 2-min fatigue protocol (150 Hz frequency, 500-ms train duration, 1 train/s). Muscle response to 200 Hz stimulation is used for comparison. *Fatigue:* reduction in tension and EMG amplitude in response to 200 Hz stimulation following fatiguing stimulation of the same branch (p<0.05). *Cross-fatigue:* reduction in tension and EMG amplitude although fatiguing stimulation was applied to the other branch (p<0.05).