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Role for the Skeletal Muscle Action Potential in Non-Hebbian Long-Term Depression at the Amphibian (*Bufo Marinus*) Neuromuscular Junction

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KEY WORDS synaptic plasticity; retrograde signaling; polyneuronal innervation; nitric oxide

ABSTRACT Retrograde signaling from skeletal muscle cells to motor nerve terminals is a recognized mechanism for modulating the strength of neuromuscular transmission. We recently described a form of long-term depression of transmitter release at the mature neuromuscular junction that is dependent on the production of nitric oxide, most likely by the muscle cell (Etherington and Everett [2004] J Physiol (Lond) 559:507–517). We now show that the depression is blocked by treating neuromuscular preparations with μ-conotoxin G111A, an antagonist of skeletal muscle voltage gated sodium channels, indicating that the depression requires postsynaptic action potential firing. Experiments on dually-innervated sartorius muscles revealed that propagation of action potentials generated by low-frequency stimulation of one nerve branch gives rise to nitric-oxide mediated depression at unstimulated nerve terminals located many millimetres away on the same muscle fiber. The non-Hebbian pattern of expression of the depression, as well as its reliance on postsynaptic action potential firing, distinguish it from forms of synaptic depression described at immature neuromuscular synapses, and may provide a mechanism for coregulation of the strength of motoneurons innervating the same postsynaptic cell. **Synapse 00:000–000, 2008.**

INTRODUCTION

Skeletal muscle fibers have the capacity to modulate the strength of the synaptic inputs they receive in an activity-dependent manner (Connor and Smith, 1994; Etherington and Everett, 2004; Fitzsimonds and Poo, 1998). This can be seen during development and re-innervation of muscle when individual fibers receive transient innervation from two or more motoneurons (Wyatt and Balice-Gordon, 2003). Differences emerge in the strength of convergent inputs during the subsequent loss of the polyneuronal innervation of the muscle cell, and a role for activity-dependent retrograde modulation of synaptic strength in this process has been postulated (Colman et al., 1997; Kopp et al., 2000).

At the immature neuromuscular junction, retrograde signaling is involved in several forms of activity dependent synaptic depression that obey Hebbian learning rules (Hebb, 1949). That is, depression is induced by low-frequency asynchronous activity of the pre- and postsynaptic cells but is not seen when the nerve and muscle are synchronously active (Cash et al., 1996; Dan and Poo, 1992; Lo et al., 1994; Lo and Poo, 1991, 1994). Lo and Poo (1991), for example, induced sustained depression of transmitter release from a neuron synapsing with a cultured myocyte by delivering a train of electrical stimuli to a different neuron coinnervating the same postsynaptic cell. It has been suggested that Hebbian depression at the immature neuromuscular junction, which leads to suppression of transmitter release from less active synaptic inputs, may contribute to the disparity in transmitter release from competing inputs that is observed during synaptic elimination (Colman et al., 1997; Kopp et al., 2000).

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We recently reported a form of depression at synapses of singly innervated fibers in mature amphibian skeletal muscle induced by 20 min of low-frequency nerve stimulation (Etherington and Everett, 2004). Our results implicated postsynaptic calcium elevation in the production of nitric oxide, believed to act as a retrograde messenger to induce depression at the nerve terminal. In contrast to Hebbian forms of depression described at the immature neuromuscular junction, the depression we described was seen when both the pre- and postsynaptic cells were synchronously active.

These findings suggest that the regulation of synaptic strength at mature neuromuscular connections might be quite different from the mechanism that has been reported at immature connections. To further investigate the expression of synaptic depression at the mature neuromuscular junction, we measured synaptic strength at synapses on dually innervated sartorius muscles. This muscle has the uncommon property of maintaining a stable polyneuronal innervation, allowing us to monitor synaptic transmission at low-frequency stimulated and unstimulated nerve inputs to the same muscle cell. We now show that the depression is mechanistically distinct from forms of synaptic depression previously described at this synapse, both in terms of its dependence on firing of skeletal muscle action potentials (APs) and its non-Hebbian pattern of expression. It is proposed that this novel form of depression may contribute to coregulation of the strength of nerve terminals stably innervating the same muscle fiber (Costanzo et al., 1999).

In addition, this identification of a postsynaptic AP-dependent form of depression suggests that the physiological relevance of studies of neuromuscular plasticity performed under conditions where postsynaptic action potential firing is prevented should be re-evaluated, particularly at mature neuromuscular synapses.

MATERIALS AND METHODS

Some methods have been described previously (Etherington and Everett, 2004).

Isolation of nerve-muscle preparations

Experiments were performed on young cane toads (*Bufo marinus*) captured in the wild in Queensland, Australia, and maintained in an animal holding facility for up to 6 months before use. Animals were sacrificed prior to experimentation by double pithing of the central nervous system, in accordance with recommendations outlined in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th edition, 2004). Sartorius and iliofibularis muscles were isolated with their nerve supplies intact and connective tissue was cut away from the surface of the muscles to facilitate electrophysiological recording. The preparations were maintained in a modified aerated amphibian Ringer solution containing (mM) NaCl (114), KCl (2), glucose (5), $NaHCO_3$ (1.8), Mops (10), and $CaCl_2$ (1.5). The pH of the solution was adjusted to 7.4 with NaOH. All experiments were performed at room temperature (21–24°C) on freshly dissected nerve-muscle preparations.

Induction of long-term synaptic depression and electrophysiological recordings

Synaptic depression was induced by stimulating the nerve supply to an isolated muscle at 1 Hz for 20 min (1 ms square pulses at optimum voltage, ~4.5 V) using a platinum-iridium suction electrode.

Iliofibularis muscles

Methods for endplate potential (EPP) recordings were the same as have been described previously (Etherington and Everett, 2004). Briefly, both iliofibularis-sciatic nerve preparations from an animal were mounted in organ baths and the nerve to one muscle was stimulated at 1 Hz for 20 min, while the other preparation was used as a control and not stimulated during this period. Both preparations were then bathed in a low-concentration of *d*-tubocurarine chloride hydrate (*d*-tubocurarine, 0.9–5 μM) to partially block postsynaptic ACh receptors and prevent muscle AP firing during EPP recording. The exact concentration of *d*-tubocurarine was varied throughout the year to maintain median EPP amplitudes in control muscles within a workable range (1.4–4 mV) despite seasonal variations in release probability (Bennett and Lavidis, 1991). Recording of EPPs started after 30 min incubation in *d*-tubocurarine, and was stopped 30 min later (1 h after termination of the low-frequency stimulation routine).

EPPs were evoked at a frequency of 0.2 Hz by supramaximal stimulation of the sciatic nerve with a platinum-iridium suction electrode. Impalements were made with borosilicate glass microelectrodes filled with 3 M KCl (R = 5–20 MΩ). EPPs were amplified with an AM-Systems preamplifier (10× gain) connected to a Powerlab2/20 (ADInstruments) and recorded using Scope software (version 3.6.11, AD Instruments). Measurements of synaptic potential

Abbreviations

AP	action potential
DHPR	dihydropyridine receptor
EPP	endplate potential
depression	long-term depression
MEPP	miniature endplate potential
NO	nitric oxide

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amplitudes were made using the Peak Parameters extension for the Chart program (ADInstruments).

Sartorius muscles

Experiments were performed on sartorius muscles because most sartorius muscle fibers in mature amphibia receive innervation from two or more nerve axons (Bennett et al., 1985; Katz and Kuffler, 1941). In our muscles, the nerve supply to the sartorius split into two or more branches 1–2 mm before it entered the muscle. The sartorius nerve was cleared of connective tissue and the two largest branches of the nerve, referred to here as the proximal (pelvic) and distal nerve branches due to their relative position along the length of the sartorius muscle were teased apart using entomological pins. Sartorius muscles were pinned out flat in a Sylgard-lined dish containing normal Ringer solution and the two nerve branches were each taken up into a platinum-iridium suction electrode for delivery of electrical stimulation.

We confirmed that it was possible to independently electrically stimulate the two nerve branches by recording EPPs evoked by stimulation of the proximal nerve branch and then applying supramaximal stimulation to the distal nerve branch without changing the position of the recording electrode. In a sample of 20 muscle fibers, the postsynaptic response to distal nerve stimulation was smaller and of more variable time course than EPPs evoked by stimulation of the proximal nerve itself (summarized in Fig. 1A and see representative traces of the postsynaptic response to stimulation of each nerve in Fig. 1B). As the recording electrode was positioned close to proximal nerve terminals throughout these recordings, this result suggests that stimulation of the distal sartorius nerve branch does not evoke transmitter release from proximal nerve terminals.

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Recording of EPPs from sartorius muscles was performed as described above for iliofibularis muscles. The amplitude of EPPs evoked by distal nerve stimulation was measured in both sartorius muscles from an animal; one muscle was exposed to 20 min of 1 Hz proximal nerve stimulation, while the other muscle was used as a control and not stimulated. Miniature endplate potentials (MEPPs) were recorded in the vicinity of distal nerve endplates (see below) before and after a period of low-frequency proximal nerve stimulation.

For muscle AP recordings, sartorius muscles were exposed to a solution of 50 μM *N*-benzyl-*p*-toluene sulfonamide (BTS), a myosin ATPase inhibitor that prevents skeletal muscle contraction (Shaw et al., 2003). Preliminary experiments in our laboratory suggested that BTS does not have a significant effect on the frequency of spontaneous transmitter release at the amphibian neuromuscular junction; in two experiments where muscles were exposed to BTS, the MEPP frequencies after application of the drug were 106 ± 24% (n = 10 fibers) and 86 ± 12% (n = 10 fibers) of baseline, respectively. As both control and low-

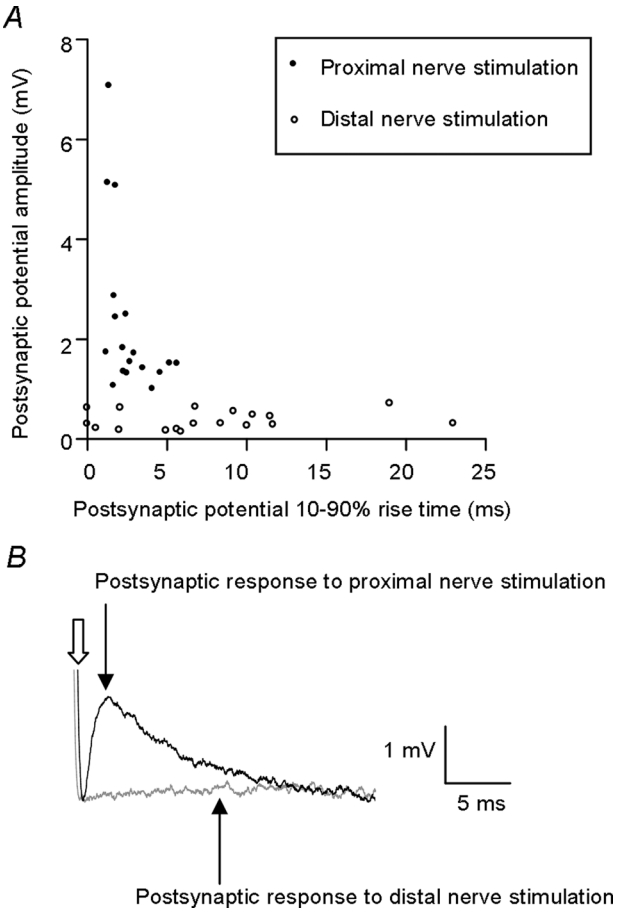

Fig. 1. Sartorius nerve branches can be independently electrically stimulated. **A**: Amplitudes and rise times of the maximum depolarizing deflection in muscle cell membrane potential in the 25 ms following proximal (filled circles) and distal nerve (open circles) stimulation, recorded in 18 muscle fibers. To obtain these data, impalements were targeted to the region of proximal nerve endplates and the proximal and distal nerves were stimulated without changing the position of the recording electrode; impalements in which an EPP was not observed in response to proximal nerve stimulation (amplitude of postsynaptic potential shift was <1.5 mV) were excluded from analysis. Stimulation of the proximal nerve generated EPPs that had relatively large amplitudes and short rise times (filled circles, average amplitude 2.3 ± 0.4 mV, average rise time 2.7 ± 0.3 ms). In contrast, stimulation of the distal nerve branch produced very little deviation in the postsynaptic potential and those potential shifts that were observed had very variable rise times (open circles, average amplitude 0.3 ± 0.2 mV, average rise time 7.7 ± 1.7 ms). These potential shifts may be related to transmitter release from distantly located distal nerve terminals, or to normal temporal variation in resting membrane potential, however their time course and amplitude would indicate that distal nerve stimulation does not depolarize proximal nerve terminals to cause transmitter release. **B**: Representative traces of the postsynaptic membrane potential of a sartorius muscle fiber from (A) after delivery of a supramaximal stimulus to the proximal nerve (black trace) and the distal nerve (gray trace), showing that simulation of the distal nerve does not lead to transmitter release from proximal nerve terminals.

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frequency stimulated preparations were exposed to the drug, any effects on baseline transmission would not have contributed to the outcome of our experiments, therefore further investigation of the effect of BTS on baseline synaptic transmission was not undertaken. After a 90 min incubation in BTS, sartorius muscle APs were recorded in response to either direct stimulation of the muscle belly, with a pair of platinum-iridium wire electrodes, or stimulation of sartorius nerve branches with platinum-iridium suction electrodes. The postsynaptic response to 10 stimuli delivered at a frequency of 0.2 Hz was recorded in a sample of at least 10 cells before, and 10 cells after, 20 min of 1 Hz stimulation of the proximal sartorius nerve branch to a sartorius muscle. Hardware for recording of muscle APs was the same as for recording of EPPs (described above).

Solutions

Stock solutions of *N*-benzyl-*p*-toluene sulfonamide (BTS, Sigma Rare Chemicals, St Louis, MO, USA) and 2-(4-carboxyphynyl)-4,5-dihydro-4,4,5,5-tetramethyl-Ihimidazol-1-yloxy-3-oxide potassium salt (cPTIO, Sigma) were prepared in DMSO at concentrations of 100 and 200 mM, respectively. The drugs were diluted in normal Ringer immediately before use to the final working concentrations of 50 and 200 μM, respectively. Stock solutions of μ-conotoxin G111A (0.4 mM, μ-CTX, Bachem, Bubendorf, Switzerland) and *d*-tubocurarine chloride hydrate (1 mM, *d*-tubocurarine, Sigma) were prepared in Ca^{2+}-free Ringer solution and then diluted in normal Ringer solution to working concentrations of 10 μM and 0.9–5 μM, respectively.

Statistics

Results are expressed as mean ± SEM and *P* values less than or equal to 0.05 were considered statistically significant. Statistical significance for experiments involving synaptic potential (EPP or MEPP) recordings was determined with paired two-tailed Student's *t*-test using Prism software (v 3.02, GraphPad). The mean EPP amplitude was calculated for at least 5 fibers from each muscle, and the median of these cell means was then used as the estimate of amplitude for that muscle. Comparisons were always made between a control muscle and a low-frequency stimulated muscle taken from the same animal and exposed to identical concentrations of *d*-tubocurarine during EPP recording. Median MEPP amplitudes were calculated by recording from at least 5 muscle fibers before, and 5 fibers after, a period of low-frequency nerve stimulation.

Statistical analysis of sartorius muscle AP experiments was performed using the mixed procedure in SAS software (version 9.1, SAS Institute, Cary, NC). Differences between least squares means were calculated for each combination of factors, and two-tailed tests of least squares mean differences were performed where appropriate. The dependent variable was the level of muscle AP firing (expressed as a fraction of stimuli delivered), which was modeled as a function of condition (control vs. low-frequency stimulated), site (proximal nerve vs. distal nerve stimulation), and time (pre or post 20 min time interval) as fixed factors, and muscle and its interactions with condition and time as random factors. *F*-tests for fixed effects were carried out using the muscle by condition term as the denominator for the condition test, the muscle by condition by time term as the denominator for the time and time by condition interaction tests, and the mean square error term for all other fixed effect tests. A second block of experiments had the fixed factors site (proximal nerve vs. muscle belly stimulation) and time (pre vs. post 20 min time interval), with random factors muscle and muscle by time. *F*-tests for fixed effects were carried out for time with the muscle by time term as the denominator and for site and the site by time interaction using the mean square error term as the denominator.

RESULTS

Prolonged low-frequency nerve stimulation at the amphibian neuromuscular junction (1 Hz for 20 min) induces lasting (>1 h) depression of transmitter release from motor nerve terminals, manifest as a decrease in EPP amplitudes and in the frequency, but not the amplitude, of spontaneous synaptic potentials (Etherington and Everett, 2004). Interestingly, the depression of transmitter (a presynaptic phenomenon) was blocked by an antagonist of postsynaptic skeletal muscle ACh receptors (Etherington and Everett, 2004). In the present study we show that, unlike forms of depression previously identified at this synapse, this form of depression dependent on skeletal muscle AP firing and can therefore spread between widely spaced nerve terminals coinnervating the same muscle fibers.

Postsynaptic action potential firing is necessary for the induction of depression in singly innervated iliofibularis muscles

Studies of synaptic plasticity at the neuromuscular junction have traditionally involved the continuous recording of synaptic potentials in a single skeletal muscle fiber before and after an induction protocol, usually under conditions where postsynaptic ACh receptors are partially antagonized to prevent muscle AP firing and therefore contraction. Such an approach was inappropriate here because of the dependence of depression on postsynaptic ACh receptor activation; the induction of depression by low-frequency stimula-

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tion was blocked if neuromuscular preparations were incubated in the postsynaptic acetylcholine receptor antagonist prior to the stimulation (Etherington and Everett, 2004). Instead, an alternative experimental design, wherein EPPs were recorded from a sample of fibers in a low-frequency stimulated muscle and from a sample of fibers in a control (nonstimulated) muscle from the other leg of the same animal, was utilized. Any pharmacological manipulations were applied simultaneously to both control and stimulated preparations, so the potential effects on baseline transmission were common to both groups of muscles (see Fig. 2A for schematic representation of experimental design); similarly for the random variation in other muscle and nerve properties such as muscle fiber input resistance or the nonlinear summation of EPPs.

Under these conditions, 5 min of low-frequency nerve stimulation was not sufficient to produce significant depression of EPP amplitudes (bars labeled *a*, Fig. 2B), however, a large change in synaptic efficacy occurred between 5 and 10 min of low-frequency nerve stimulation, when the EPP amplitude relative to nonstimulated controls was reduced by 25% (bars labeled *b*, Fig. 2B). After 20 min of low-frequency nerve stimulation, EPP amplitudes were depressed on average by 46% relative to unstimulated control muscles (bars labeled *c*, Fig. 2B, and see traces of representative EPPs, Fig. 2C).

The robust depression of EPP amplitudes observed after 20 min of low-frequency nerve stimulation was completely blocked if low-frequency stimulated muscles (and control muscles) were preincubated for 120 min in 10 μM μ-conotoxin G111A (μ-CTX, bars labeled *d*, Fig. 2B and see also traces of representative EPPs in Fig. 2D). The μ-CTX is a selective antagonist of skeletal muscle voltage-gated sodium channels that does not have a presynaptic activity at amphibian neuromuscular junctions (Shon et al., 1998). Consistent with this view, electrophysiological recordings in 45 muscles fibers from 5 iliofibularis muscles exposed to the toxin for 2 h showed an EPP in response to all of the 226 nerve stimuli delivered. At the same time the muscle twitch amplitude was reduced by 80 ± 7% (n = 5 muscles). Thus, this result suggests that blocking the postsynaptic acetylcholine receptor per se is not a factor in the induction of depression, rather, depression requires firing of the postsynaptic AP.

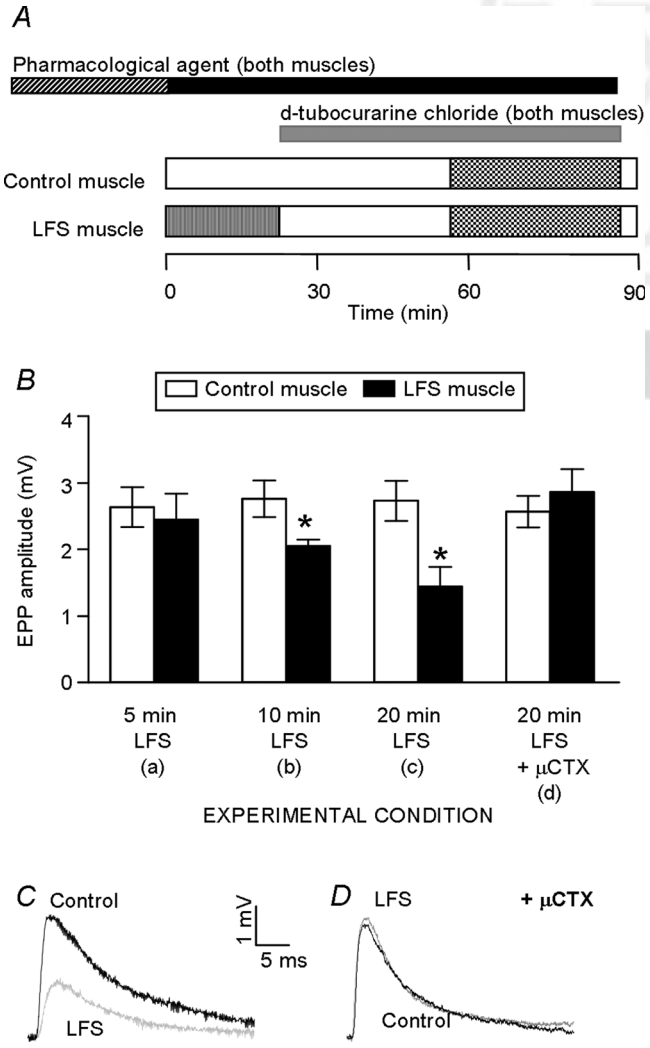

Fig. 2. Long-term depression of transmitter release induced by low-frequency nerve stimulation is dependent on postsynaptic action potential firing. **A**: Experimental design schematic showing design of experiments for recording EPPs from control and low-frequency stimulated (LFS) muscles; the time scale is shown relative to the start of the period of low-frequency nerve stimulation, represented by the vertical line pattern. The period of exposure to the ACh receptor antagonist *d*-tubocurarine is shown by the gray bar and the time window for EPP recording is indicated by the checkred pattern. The black horizontal bar reflects the period of exposure to the relevant drug or solution, with the diagonal white lines on this bar indicating the preincubation period, which varied between 30 and 120 min depending on the drug used. **B**: Average amplitude of EPPs recorded from control muscles (open bars) and muscles exposed to low-frequency nerve stimulation of varying durations (filled bars). Five minutes of low-frequency stimulation was not sufficient to induce lasting depression of EPP amplitudes (bars labeled *a*, P = 0.576, n = 5 pairs of muscles, Student's two-tailed paired t-test), however 10 min of low-frequency stimulation significantly reduced EPP amplitudes relative to nonstimulated controls (bars labeled *b*, *, $P < 0.05$, n = 6 pairs of muscles, Student's two-tailed paired t-test). Twenty minutes of low-frequency nerve stimulation also produced significant depression of EPP amplitudes when experiments were performed in normal Ringer solution (bars labeled *c*; *, $P < 0.05$, n = 5 pairs of muscles, Student's two-tailed paired t-test) but there was no difference in the average amplitude of EPPs recorded from control and low frequency stimulated muscles after incubation with 10 μM μ-conotoxin G111A, a selective antagonist of skeletal muscle voltage-gated sodium channels (bars labeled *d*, P = 0.278, n = 5 pairs of muscles, Student's two-tailed paired t-test). **C**: Representative traces of EPPs from a control muscle (black trace) and a muscle exposed to 20 min of low-frequency nerve stimulation (gray trace) in normal Ringer solution. **D**: representative traces of EPPs from a control muscle (black trace) and a muscle exposed to 20 min of low-frequency nerve stimulation (gray trace) after incubation in 10 μM μ-conotoxin G111A. Data in bars labeled c, part (B), and part (C) reproduced (from Etherington and Everett., 2004, p 510).

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Depression spreads between active and inactive synaptic contacts on dually innervated sartorius muscle fibers

The evidence described earlier suggests that the trigger for this low frequency stimulation-dependent form of depression is postsynaptic AP firing. As APs are propagated undiminished along the length of skeletal muscle fibers, we tested whether propagation of the postsynaptic AP during low-frequency stimulation of one nerve input to a muscle fiber would induce depression at inactive synaptic connections distantly located on the same muscle fibers.

Experiments to address this issue were performed on sartorius muscles and their associated nerve supplies, because previous studies have shown that most sartorius muscle fibers in adult amphibia retain innervation from more than one motoneuron (Bennett et al., 1985; Katz and Kuffler, 1941) at synaptic sites located several millimetres apart along the long axis of the muscle fibers (Ypey, 1978). Electrophysiological recordings from the cane toad sartorius muscles used here confirmed that the majority of muscle fibers received electrically independent synaptic contacts from both proximal and distal branches of the sartorius nerve (Fig. 3A). On average 61 ± 7% of fibers within a sartorius muscle produced a postsynaptic potential (EPP or AP) in response to stimulation of both nerve branches (left most bar in figure), with a small proportion of fibers responding only to stimulation of the proximal or distal nerve branch (black and gray bars, respectively). It is possible that the proportion of dually innervated fibers in the sartorius muscles may even be slightly higher than 61%, if subthreshold synaptic potentials were too distant from the recording electrode to be detected in some fibers. Examples of APs recorded from a dually innervated sartorius muscle fiber in response to stimulation of the proximal and distal sartorius nerve trunks are shown in Figure 3B (black and gray traces, respectively).

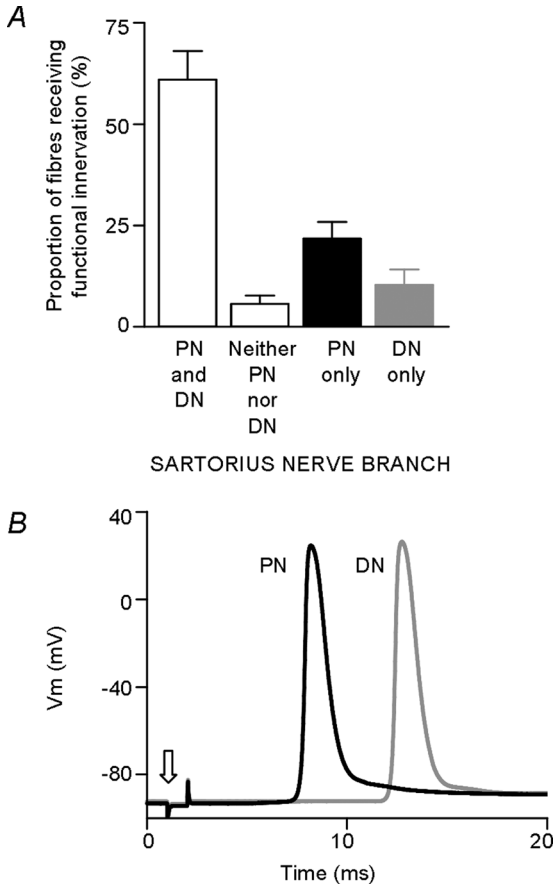

Fig. 3. Characterization of dual innervation of amphibian sartorius muscles. **A**: Average proportion of sartorius muscle fibers that received functional innervation from both proximal and distal nerve branches (left most open bar), neither the proximal or distal nerve branch (right hand open bar), the proximal nerve only (black bar) and the distal nerve only (gray bar). Fibers were considered to receive functional innervation from a nerve if stimulation of the nerve branch produced a postsynaptic potential (EPP or AP) in that fiber (n = 63 muscle fibers sampled from 6 sartorius muscles, at least 10 fibers per muscle). **B**: overlay of APs recorded from a single sartorius muscle fiber in response to stimuli delivered to the proximal (PN, black traces) and distal (DN, gray traces) nerve branches. The latency between delivery of the stimulus (artifact indicated by arrow) and recording of the AP differs between the two nerves due to differences in positions of the motoneuron terminals relative to a recording electrode positioned at the proximal end of the muscle. V_m, muscle cell membrane potential.

The difference in the latencies of APs evoked by stimulation of the two nerve branches (Fig. 3B) reflects differences in the position of the proximal and distal nerve terminals relative to the recording electrode, which was positioned at the proximal end of the muscle during these recordings. We observed an average latency difference of 5.1 ± 0.4 ms (n = 11 fibers from 4 sartorius muscles), which corresponds to an average distance of 8.2 mm between proximal and distal endplates (based on an estimated muscle AP conduction velocity of 1.6 ± 0.3 ms^{-1}, derived from compound AP recordings in four sartorius muscles).

Thus most cane toad sartorius muscle fibers are innervated by both proximal and distal nerve branches at synapses located several millimetres apart, providing an ideal preparation for testing whether low-frequency stimulation of one nerve input leads to the spread of depression to other synaptic connections on the same muscle fibers. In these experiments, the proximal nerve branch was exposed to the low-frequency stimulation routine because electrophysiological recordings showed that out of the 76% of fibers receiving innervation from the distal nerve branch (left most open bar and gray bar, Fig. 3), at least 85% also received innervation from the proximal nerve branch (left most open bar only, Fig. 3).

To allow concurrent monitoring of synaptic efficacy at both proximal and distal nerve synapses in individual muscle fibers, the level of nerve-evoked muscle

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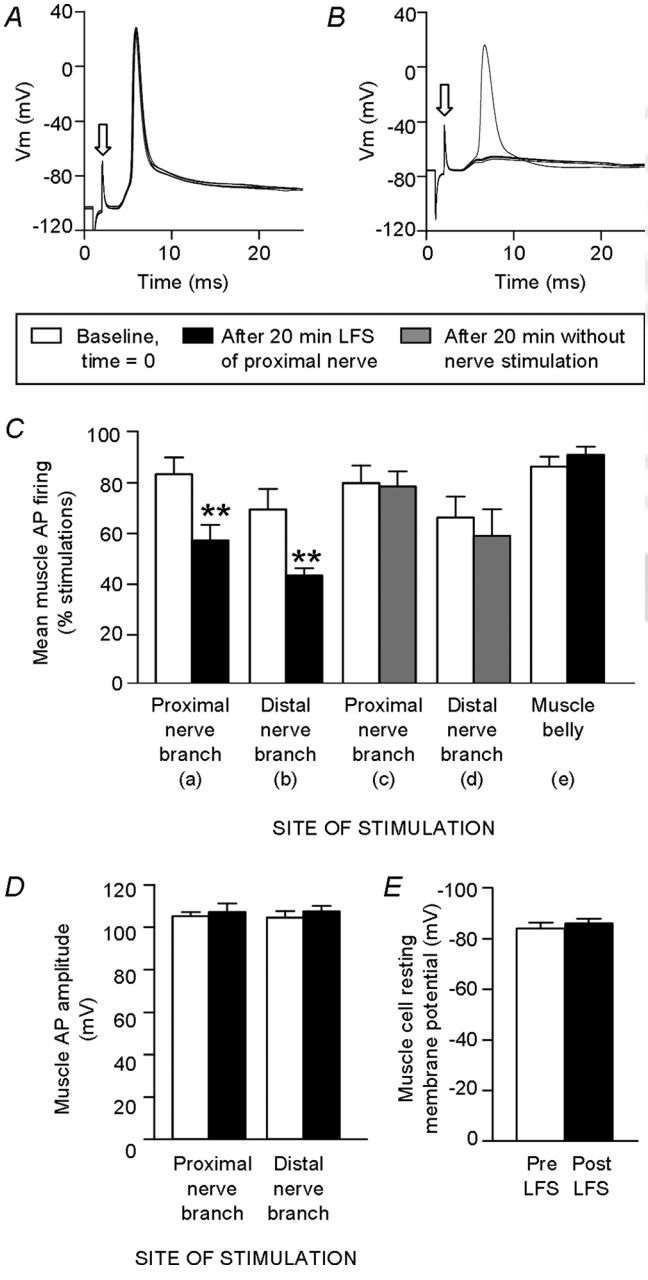
AP firing was used to quantify depression. This was possible because a period of low-frequency nerve stimulation causes sufficient depression of transmitter release to produce failure of the usually reliable transmission at the somatic neuromuscular junction. Figure 4 shows an example of synaptic transmission failure in a representative muscle fiber during a period of low-frequency nerve stimulation; the level of nerve-evoked AP firing in this fiber decreased from 100% at the beginning of the stimulus train (Fig. 4A), to only 20% of nerve stimulations at the end of the 20 min stimulation period, with subthreshold EPPs generated in response to four of the five stimuli (Fig. 4B).



When nerve-evoked APs were recorded from a sample of muscle fibers before and after the period of low-frequency proximal nerve stimulation, an overall decrease in the average level of both proximal and distal nerve-evoked muscle AP firing was observed (bars labeled a and b, Fig. 4C). The proportion of proximal nerve stimulations that triggered a muscle action potential decreased by 40 percentage points following stimulation of the proximal nerve branch at 1 Hz for 20 min (that is, from 88 to 48% of proximal nerve stimulations producing a postsynaptic AP, bars labeled a, Fig. 4C) consistent with activity-dependent depression of transmitter release at these synapses (Fig. 2B). Stimulation of the proximal nerve branch caused a similar decrease (38%) in the capacity of the distal nerve branch to trigger a muscle AP (bars labeled b, Fig. 4C). The figure shows that 88 ± 5% of proximal nerve stimulations and 69 ± 8% of distal nerve stimulations evoked an AP in normal sartorius muscles (n = 4 muscles, open bars labeled a and b respectively, Fig. 4C). This level of AP firing was below 100% because APs in a small proportion of fibers could not be evoked by stimulation of a particular nerve branch, possibly because these fibers were not innervated by that nerve branch (see Fig. 3A).

There was no change in the capacity of either nerve to elicit muscle APs over the time course of the experiment if neither nerve branch was stimulated at low-frequency (bars labeled c and d, Fig. 4C). The

Fig. 4. Low-frequency stimulation of one nerve input to dually innervated sartorius muscles causes failure of both stimulated and unstimulated nerve branches to trigger muscle action potential firing. Overlay of five postsynaptic potentials recorded in response to five stimuli delivered to the proximal sartorius muscle nerve during continuous recording from a single muscle fiber, at the beginning (**A**) and end (**B**) of 20 min of 1 Hz nerve stimulation, showing failure of nerve-evoked AP firing. The decrease in membrane potential and AP amplitude in part (B) reflects slight displacement of the recording electrode during continuous recording from a single muscle fiber and is not a product of the depression itself (see part *E* below). Stimulus artifacts are indicated by open arrows. V_m, muscle cell membrane potential. **C**: average level of sartorius muscle AP firing at the start of experiments (open bars) and after a 20 min period of either low-frequency proximal sartorius nerve stimulation (black bars) or no nerve stimulation (gray bars). Stimulation of the proximal sartorius nerve branch at 1 Hz for 20 min caused a significant decrease in the capacity of both the proximal and distal nerves to trigger a muscle AP (black bars labeled *a* and *b*) relative to the prestimulation baseline for each nerve branch (open bars labeled *a* and *b*; **, $P < 0.01$, n = 6 muscles, difference of least squares means for prestimulation vs. poststimulation). There was no change in the capacity of either nerve to evoke muscle APs if preparations were left for twenty minutes without stimulation (bars labeled *c* and *d*; $P = 0.472$, n = 8 muscles, difference of least squares means for pre no-stimulation vs. post no-stimulation). Low-frequency proximal nerve stimulation did not affect muscle AP firing in response to direct stimulation of the muscle belly (bars labeled *e*; $P = 0.631$, n = 4 muscles, difference of least squares means for prestimulation vs. poststimulation). There was also no difference in the amplitude of muscle cell APs (**D**) or the resting muscle cell membrane potential (**E**) observed when distal and proximal nerves were stimulated before (open bars) and after (filled bars) the period of low-frequency proximal nerve stimulation.

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capacity of sartorius muscle fibers to fire APs in response to direct muscle belly stimulation was unaffected by a period of low-frequency proximal nerve stimulation (bars labeled e, Fig. 4C), as was the amplitude of skeletal muscle APs (Fig. 4D) and the resting muscle cell membrane potential (Fig. 4E). The results indicate that failure of the distal and proximal nerve-evoked muscle APs does not result from a change in the excitability of the postsynaptic membrane. Together, these findings suggest that propagation of postsynaptic APs during low-frequency stimulation of a nerve input to skeletal muscle fibers is sufficient to induce depression at unstimulated synaptic contacts on the same muscle fiber.

Depression at unstimulated synaptic contacts onto sartorius muscle fibers, induced by low-frequency stimulation of convergent nerve inputs, is expressed presynaptically and mediated by nitric oxide

The muscle AP recordings above demonstrated that a decrease in synaptic efficacy is induced at unstimulated synapses exposed to a period of low-frequency postsynaptic AP firing. To determine whether transmission failure at these unstimulated distal nerve synapses occurs through a similar, presynaptic mechanism to depression at low-frequency stimulated synapses (Etherington and Everett, 2004), the effect of low-frequency proximal nerve stimulation on synaptic potentials at distal nerve synapses was measured.

The general position of distal and proximal nerve endplates along the length of sartorius muscles was determined by making impalements at various positions along the long axis of sartorius muscles and recording APs in response to both distal and proximal nerve stimulation. It was possible to establish which nerve endplate was closest to the impalement site by measuring the relative latency of proximal and distal nerve-evoked APs; in general, impalements at more proximal sites on the muscles were closest to terminals of the proximal nerve branch (black bars, Fig. 5) while more distal impalements were closer to terminals of the distal nerve branch (gray bars, Fig. 5). The figure reveals that all impalements made at sites in the distal third of the muscles (beyond the entry point of the distal nerve branch, indicated by gray dots at bottom of figure) were closer to distal than to proximal nerve terminals; this information was used to target impalements for the purpose of selectively recording synaptic potentials at distal nerve terminals.

F5

EPPs generated by distal nerve stimulation after 20 min of low-frequency proximal nerve stimulation were on average 29 ± 12% (filled bar, Fig. 6A) smaller than distal nerve EPP amplitudes in nonstimulated muscles from the other leg of the same animals (open bar, Fig. 6A, $n = 5$ pairs of muscles). The true magnitude of depression may be even higher than 29%, because a small proportion of fibers innervated by the distal nerve do not receive innervation from the proximal nerve branch (and would not be exposed to low-frequency firing of postsynaptic APs). Thus, firing of APs during low-frequency proximal nerve stimulation is sufficient to depress transmission at inactive distal nerve terminals.

F6

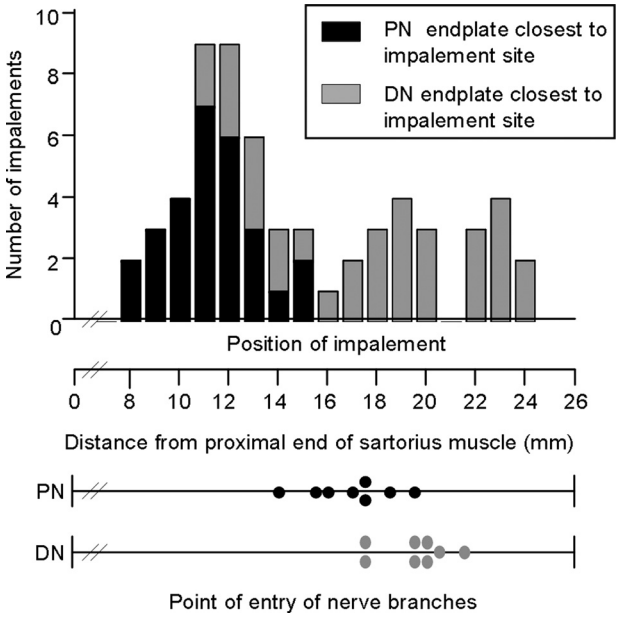

Fig. 5. Characterization of dual innervation of amphibian sartorius muscles. The upper part of the figure shows the number of muscle fiber impalements that were closer to proximal (black bars) or distal (gray bars) nerve terminals respectively, at various sites along the long axis of the muscles. All impalements were from sartorius muscle fibers that fired APs in response to electrical stimulation of both proximal and distal nerve branches; the relative latency of APs evoked by stimulation of the two nerve branches was used to determine whether each impalement was closer to a distal or proximal nerve terminal ($n = 61$ impalements from 8 sartorius muscles). Note that impalement sites were not systematically varied to sample all positions along the length of the muscles, but instead represent a sample of the impalement positions normally used during electrophysiological recordings from sartorius muscles. The black and gray dots on the lines at the bottom of the figure represent the sites of insertion of the proximal and distal nerve branches respectively, on the eight sartorius muscles sampled.

Similar to the depression observed in singly innervated iliofibularis muscles after low-frequency nerve stimulation (Etherington and Everett, 2004), low-frequency stimulation of the proximal sartorius nerve did not affect the sensitivity of the postsynaptic membrane at distal nerve synapses. There was no difference in the amplitude of MEPPs recorded in the region of distal nerve terminals before and after a period of low-frequency proximal nerve stimulation (Fig. 6B), supporting a presynaptic change in transmitter release as the mechanism for depression.

We then tested whether depression at unstimulated distal nerve terminals occurs through a similar nitric

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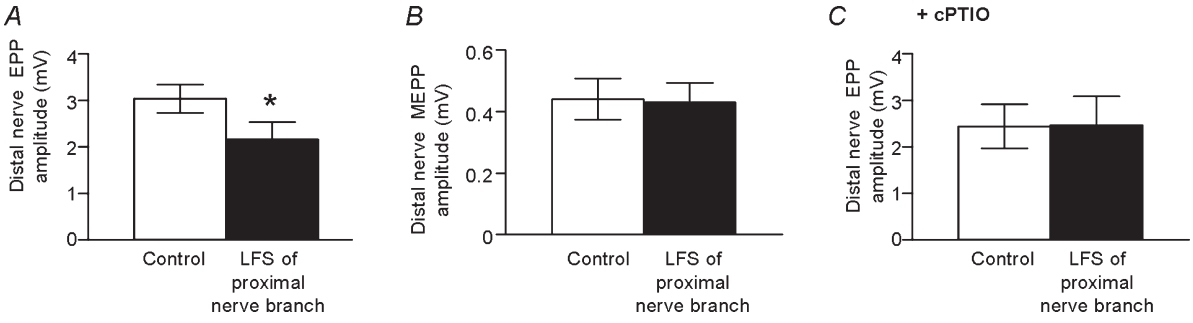

Fig. 6. Low-frequency stimulation of the proximal nerve branch induces nitric oxide-mediated long term depression of transmitter release from unstimulated distal nerve terminals on dually innervated sartorius muscles. **A**: low-frequency stimulation of the proximal sartorius nerve branch caused significant depression of EPPs evoked by distal nerve stimulation (the distal nerve itself was not exposed to a period of low-frequency stimulation; *, $P < 0.05$, $n = 5$ pairs of muscles, Student's two-tailed paired t-test). **B**: MEPP amplitudes recorded in the region of distal nerve endplates after a period of low-frequency proximal nerve stimulation (filled bar) were the same as amplitudes recorded in the same muscle before the stimulation period (open bar; $P = 0.922$, $n = 5$ pairs of muscles, Student's two-tailed paired t-test). **C**: exposure to the NO scavenger cPTIO prevented depression of distal nerve EPP amplitudes in response to a period of low-frequency proximal nerve stimulation ($P = 0.929$; $n = 4$ pairs of muscles, Student's two-tailed paired t-test).

oxide (NO)-mediated mechanism to depression at synapses directly exposed to a period of low-frequency nerve stimulation (Etherington and Everett, 2004). Exposure to the membrane impermeant NO scavenger cPTIO completely blocked the induction of depression at unstimulated distal nerve synapses after a period of low-frequency proximal nerve stimulation (Fig. 6C). Although NO synthase is expressed both pre- and postsynaptically at amphibian neuromuscular junctions (Descarries et al., 1998), the average distance of 8 mm between distal and proximal nerve endplates on the sartorius muscles used here (see Methods) is far beyond the diffusion distance of NO in biological tissues (~500 μm, Blottner and Luck, 2001), making it unlikely that NO produced locally at stimulated proximal nerve terminals could diffuse to, and depress transmitter release at, unstimulated distal nerve endplates. Thus, it seems more probable that the postsynaptic process which leads to NO production at low-frequency stimulated synapses (Etherington and Everett, 2004) also leads to localized production of NO at unstimulated synaptic contacts on the same muscle fibers, by propagation of the postsynaptic AP.

DISCUSSION

The present study investigated the role of postsynaptic skeletal muscle fibers in long-term depression of transmitter release at the mature amphibian neuromuscular junction. The experiments showed that depression induced by low-frequency nerve stimulation is triggered by firing of the postsynaptic AP. Furthermore, we have shown here that the depression is expressed at both low-frequency stimulated and non-stimulated nerve terminals coinnervating the same postsynaptic cells. On the basis of these findings, in combination with our earlier observations about the mechanism for depression (Etherington and Everett, 2004), it is proposed that muscle AP firing induces NO-dependent depression of transmitter release from widely separated nerve terminals, irrespective of whether these terminals are active or not. This dependence of the depression on muscle AP firing and its novel, non-Hebbian pattern of expression distinguish it from forms of activity-dependent depression previously identified at this synapse.

The expression of depression at the mature amphibian neuromuscular junction is non-Hebbian

A period of low-frequency nerve stimulation at the mature amphibian neuromuscular junction produces substantial (~55%) and lasting (>1 h) depression of transmitter release from motor nerve terminals, which is dependent on activation of nitric oxide (NO) synthase and sustained production of NO (Etherington and Everett, 2004). The basis for the prolonged NO signal was proposed to be dephosphorylation of NO synthase by the calcium-calmodulin dependent protein phosphatase calcineurin. An activity-dependent calcium signal was therefore implicated in the depression, and our observation that depression was blocked by an antagonist of skeletal muscle acetylcholine receptors (AChRs) suggested that this calcium signal was postsynaptic.

A major finding of the present study is that depression is prevented by an antagonist of skeletal muscle voltage-gated sodium channels, and is therefore dependent on firing of the postsynaptic AP rather than AChR activation per se. This observation eliminated a local, AChR-mediated calcium signal, involved in depression at immature neuromuscular synapses (Cash et al., 1996), as the trigger for depression. The AP-dependence of depression also suggested that the

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spread of the depression may differ from forms of depression previously identified at this junction, a possibility that was investigated using the amphibian sartorius muscle preparation, which maintains a stable polyneuronal innervation throughout maturity.

We showed that repetitive low-frequency stimulation of only the proximal branch of the sartorius nerve induces a significant decrease in the capacity of both the proximal and distal sartorius nerves to generate a muscle AP. Thus, the form of depression at the mature neuromuscular junction is non-Hebbian, that is, expressed at both synapses that are depolarized in synchrony with the postsynaptic cell (proximal nerve synapses) and at synaptic connections that are inactive while the postsynaptic cell is depolarized (distal nerve synapses).

We observed here and in our previous work (Etherington and Everett, 2004) that MEPP amplitudes were normal in the presence of profound (45%) depression of EPP amplitudes, thus it seems reasonable to conclude that depression at both stimulated and unstimulated (distal) terminals occurs through a mechanism that is predominantly presynaptic. Although desirable, a full quantal analysis was not feasible due to the difficulty in adequately voltage clamping skeletal muscle fibers and holding the same impalement throughout the prolonged stimulation routine. Evidence for a common mechanism of depression at both stimulated and unstimulated terminals comes from the finding that they are both dependent on the passage of NO through the extracellular space.

In contrast, the forms of depression hitherto identified at the developing neuromuscular junction have obeyed Hebbian learning rules; the depression is induced by asynchronous activity of the pre- and postsynaptic cells, but is not seen when the nerve and muscle are synchronously active. For example, Lo and Poo (1991) showed that depolarisation of a myocyte by stimulating one of two convergent nerve inputs led to selective suppression of release from the nonstimulated input. Release from the stimulated input was unchanged or even potentiated. These Hebbian forms of plasticity are tightly dependent on both spatial and temporal specificity (Cash et al., 1996); application of ACh to receptors on the muscle cell surface only induced depression if it was applied within 20 μm of the presynaptic terminal (Dan and Poo, 1992) and depression was not seen if the acetylcholine pulse was accompanied by synchronous stimulation of the nerve terminal. Significant depression was observed if presynaptic stimulation and application of the acetylcholine pulse were temporally separated by more than 10 ms (Cash et al., 1996).

The depression in our work was observed under conditions of both synchronous and asynchronous pre- and postsynaptic depolarization, and at terminals that were on average 8 mm away from the site of low-frequency stimulation, thus it lacks the temporal and spatial specificity of forms of depression previously described at this synapse. A form of neuromuscular depression with these novel properties has significantly different implications for synaptic function than the Hebbian forms of depression previously described at this synapse, particularly in situations of polyneuronal innervation.

Possible implications of long-term depression for competition between multiple presynaptic inputs

Experimental investigations of polyneuronal innervation have generally focused on the transient occurrence of this phenomenon during development, or during reinnervation of the mature neuromuscular junction after injury (Bennett et al., 1985; Katz and Kuffler, 1941). However, stable polyneuronal innervation of mature neuromuscular junctions is well-supported in amphibia, where two (or sometimes more) axons form synaptic connections that are distributed along the long axis of a single muscle fiber (Lateva et al., 2002). More recently, multiple endplates, subserved by different motoneurons (Duxson and Sheard, 1995; Happak et al., 1997; Zenker et al., 1990) and separated by up to 46 mm, have been identified on mature muscles in a variety of species (Wyatt and Balice-Gordon, 2003).

It has been proposed that the expression of Hebbian forms of depression during development may assist in the organization of neuromuscular contacts by generating disparity in the strength of competing nerve inputs and contributing to the loss of polyneuronal innervation (Costanzo et al., 1999; Werle and Herrera, 1987). However, the strengths of convergent inputs onto muscle fibers receiving stable dual innervation are not disparate, rather, multiple synaptic inputs onto a particular muscle fiber are generally well matched in terms of both quantal content and EPP amplitude. Costanzo et al. (1999) showed that the strength of coinnervating synaptic terminals was equivalent in different muscle fibers with widely varying total synaptic strengths, leading the authors to suggest that the strengths of converging inputs onto a given muscle fiber are coregulated (that is, multiple inputs are strengthened, or weakened, together). These observations seem to imply the existence of non-Hebbian processes for modulating the strength of synaptic connections at multiply innervated neuromuscular junctions. On the basis of the expression pattern of, and cellular mechanism for, the non-Hebbian depression described here, we propose that it may represent one such mechanism for the coregulation of the strength of convergent synaptic inputs.

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Methodological implications of action potential-dependent plasticity for investigations of neuromuscular synaptic function

The somatic neuromuscular junction is distinct from many synaptic connections in the central nervous system because a single spike in a presynaptic neuron is often sufficient to trigger AP firing in postsynaptic muscle fibers. We have now identified a form of long-term synaptic plasticity that is dependent on, and apparently propagated by, postsynaptic AP firing. This observation has implications for the execution of future studies on synaptic function at this synapse.

Investigations of synaptic plasticity at the somatic neuromuscular junction have traditionally followed an experimental paradigm used widely in the central nervous system, where electrophysiological monitoring of synaptic function is carried out continuously in a single muscle, or even a single cell, before, during and after a manipulation. However, because firing of skeletal muscle APs can obscure recordings of synaptic potentials, at the neuromuscular junction these experiments are often performed under conditions where postsynaptic AP firing is prevented electrically or pharmacologically (see, for example, Newman et al., 2007; Redman and Silinsky, 1994; Thomas and Robitaille, 2001). The present study illustrates that firing of the skeletal muscle AP is not only a consequence of synaptic function, but can also have a significant and lasting impact on synaptic efficacy. Thus greater consideration needs to be given to preventing postsynaptic AP firing during experimental monitoring of synaptic transmission at the mature neuromuscular junction, if the results of such studies are to have relevance to the normal physiological functioning of this synapse.

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