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Topological differences along mammalian motor nerve terminals for spontaneous and alpha-Bungarotoxin-induced sprouting

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Summary. Spontaneous sproutings can be observed in end plates from normal adult vertebrate muscles and motor end plates develop increased growth signs and sprouts when target muscle cells become less active or paralysed. Nevertheless, very little is known about where in the motor nerve terminal arborization spontaneous and experimentally induced sprouts originate, their similarities and differences and also about their final maturation or elimination. In this study we investigate the topological properties of both spontaneous and alpha-bungarotoxin-induced sprouts (during different periods of intoxication and after recovery) along the motor nerve terminal branches of the Levator auris longus muscle of Swiss mice (between 48-169 day old).

Muscles were processed for immunocytochemistry to simultaneously detect postsynaptic AChRs and axons. This procedure permits us to make an accurate identification of the fine sprouts and a morphometric study of the presynaptic branching pattern profile in control muscles, during the toxin action and after recovery from paralysis.

The results show that in normal muscles, the initial and trunk segments (those between branch points) of the terminal arborization sprouted proportionally more branches when taking their relative lengths into account than the distal free-end segments. In contrast, every micrometer of alpha-bungarotoxin-treated muscles throughout the full terminal arborization have the same probability of generating a sprout. Moreover, the toxin-induced sprouts can consolidate as new branches once recovered from the paralysis without changing the total length of the nerve terminal arborization.

Key words: Nerve terminal sprouting, Neuronal plasticity, Neuromuscular junction, Neurotoxins, Mice

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Introduction

In the neuromuscular synapses of adult mammals, the motor nerve terminal and the corresponding postsynaptic muscle membrane can form a very stable and lasting junction in normal conditions (Lichtman et al., 1987; Balice-Gordon and Lichtman, 1991). Nevertheless, the motor nerve endings continuously change their shape on the micrometer scale (Hill et al., 1991; Robbins, 1991) and these ongoing movements would be the substrate for more intense synaptic plasticity and remodelling in response to altered use, ageing or changes in locomotor functional demands (Brown et al., 1981; Rosenheimer, 1985; Andonian and Fahim, 1987; Tomas et al., 1989; Deschenes et al., 1993).

Motor nerves retain the ability to sprout throughout adult life (Diamond et al., 1976). The existence of some motor nerve terminal sprouting, in otherwise normal adult muscles, has been known for many years (Tello, 1917; Ramón y Cajal, 1929; Barker and Ip, 1966; Tuffery, 1971), and is generally interpreted as being part of normal activity-dependent remodelling (Betz et al., 1980; Wernig and Herrera, 1986; Connor and Smith, 1994) and ageing (Robbins and Fahim, 1985; Andonian and Fahim, 1987; Cardasis and LaFontaine, 1987) or simply due to microtrauma in nerves or muscles (Wernig et al., 1991). Nerve terminal sprouting can also be induced to a greater extent in different experimental or pathological conditions. For instance, drug- or toxin-induced paralysis of the muscle by a pre- or postsynaptic mechanism is always followed by a sprouting reaction of the motor nerve terminal branches (Brown et al., 1981). The nerve terminal sprouts have the molecular machinery for nerve evoked calcium-dependent acetylcholine release (Juzans et al., 1996) and also for calcium-independent exocytosis (Angaut-Petit et al., 1998).

In the present work we have studied the morphological adaptations of the mouse motor nerve terminals to a decrease in muscle cell activity after a toxin-induced paralysis with alpha-bungarotoxin (α-btx).

The aim of this study is to describe the topological properties of both spontaneous and α-btx-induced sprouts along the motor nerve terminal branches. We choose to use α-btx as a stimulus for sprouting because this particular method of postsynaptic neuromuscular blockade is a relatively poor stimulus, evoking few sprouts compared to that evoked by for instance, presynaptic blockade using botulinal toxins (Duchen and Strich, 1968; Juzans et al., 1996). Using α-btx as sprouting inducer, the overall appearance of the motor nerve endings is preserved being possible to make an accurate identification of the fine sprouts and a morphometric study of the presynaptic branching pattern profile in control muscles, during the toxin action and after recovery from paralysis. Questions about the existence of a specific nerve terminal topology for sprouting and also the development of the sprouting reaction after the cessation of the growth stimulus have been addressed. We have also described the relative share of the different sprouting forms (nodal, preterminal, terminal and ultraterminal) in both normal and α-btx-treated Levator auris longus (LAL) muscles.

We used the LAL muscle of Swiss mice, described by Angaut-Petit and colleagues (1987, 1990; see also Velasco and Pécot-Dechavassine, 1993). This is an excellent mammalian nerve-muscle preparation for applying drugs or toxins in vivo, because it is very thin and flat, lies just under the skin and can be stained as a whole mount preparation. A preliminary report has been previously communicated (Lanuza et al., 1993).

Materials and methods

Animals and experimental procedures

We studied the morphology and the branching pattern of motor nerve terminals in both normal (controls) and toxin-paralysed LAL muscles of male Swiss mice (Charles River, UK Limited). At all stages animals were cared for in accordance with national guidelines for the humane treatment of laboratory animals. The muscles were functionally denervated by postsynaptic blockade using α-btx, a purified fraction of the venom of the snake *Bungarus multicinctus* that binds specifically and irreversibly to acetylcholine receptors (AChRs) (Pestronk and Drachman, 1978; Ravdin and Berg, 1979). Subcutaneous injections of α-btx ((SIGMA) 1 mg/ml in Ringer solution, 50 ml) were placed in vivo over the external surface of the right LAL muscle every 48 hours from day 41 to day 60 of the animal's life. Animals were killed on day 48 (after one week of paralysis), day 60 (after 19 days of paralysis) and day 169 (after 19 days of paralysis followed by a recovery period of 109 toxin-free days). Normal animals and animals of the same age receiving Ringer injections (50 ml) served as controls. However, no significant differences were observed between these two control groups and so the data obtained from normal animals was used as the control in this study. A total of ten right LAL muscles per day was used both for control and α-btx-treated animals.

We dissected out the right LAL muscle of ether anaesthetised mice under the dissecting microscope. After removal, the muscles were pinned onto Sylgard and the effectiveness of the α-btx paralysis was assessed in all toxin-treated muscles from 48- and 60-day-old animals by conventional intracellular recording of evoked endplate potentials, as previously described (Haimann et al., 1981). In all the electrophysiologically analysed muscles, several muscle fibres contracted de visu when indirectly stimulated by a suction electrode, whereas most of the evoked end plate potentials (always more than 70%) registered in the muscle cells of the superficial layer were subthreshold in normal Ringer perfusion. Electrophysiological analysis of the synaptic transmission in α-btx-treated muscles from 169-day-old animals indicates a complete functional recovery with normal-sized evoked endplate potentials and synaptically-evoked action potentials in all muscle fibres observed (ten per muscle).

Immunocytochemical and morphological study

Muscles were processed for immunocytochemistry to simultaneously detect postsynaptic AChRs and axons. AChRs were stained with rhodaminated α-btx (Molecular Probes, Leiden, The Netherlands) and axons were labeled with antibodies against 200 kD neurofilament protein (Sigma, used at 1:500). FITC-conjugated goat anti-rabbit antibody (Jackson Immuno-Research, West Grove, PA) was used to visualize the primary antibody. For immunocytochemical studies, muscles were fixed 30 min in 2% paraformaldehyde in phosphate-buffered saline (PBS). Following rinsing with 0.1M glycine in PBS, the muscles were permeabilized with 0.5% TRITON X-100 in PBS/BSA, and incubated with primary antibody diluted in PBS/BSA, washed in PBS, reincubated with second antibody, washed again and mounted in toto with paraphenyldiamine and mowiol to retard fading. Muscle were viewed with a Reichert fluorescent microscope to study the incidence of the different types of sprouts and their topological position in the motor nerve terminal arborization. Controls without primary antibody were made. The motor endplates from the superficial layer of the LAL muscles (about 150 nerve terminals/muscle) were observed. The simultaneous pre- and postsynaptic fluorescent stain was selected because of its ability to show all branches of the terminal arborization and to reveal easily and completely the existence of fine sprouts (Fig. 1) in the large number of neuromuscular junctions that have to be studied when average values of morphometric parameters are evaluated. We considered as sprouts the characteristic fine terminal branches (usually 1-2 mm in diameter) emerging from the terminal arborization, with (60-85%) or without a growth cone at the tip and projecting outward of an AChR-enriched postsynaptic area.

We used Hoffman's classical nomenclature (Hoffman, 1950) to describe the sprouts (Fig. 2A) but also extended it to accurately describe the topological origin along the motor nerve terminal of sprouts (Fig. 2B). Thus, sproutings were classified into the following four types based on their topological origin and extension as: 1) nodal sprouts formed at the last Ranvier node; 2) preterminal sprouts formed in the initial segment of the terminal arborization (the unmyelinated axon before the first branch point of the terminal arborization); 3) terminal sprouts formed in the trunk segments between branch points (t) or in the distal free-end segments (d) of the motor nerve terminal; and 4) ultraterminal sprouts formed at any location in the nerve terminal but going out of the originally innervated muscle cell site.

Morphometrical study

To see the topological position of the sprouts at the nerve terminal, the motor nerve endings were morphometrically analysed as previously described elsewhere (Tomas et al., 1989, 1992, 1993). In this analysis we quantified 500 neuromuscular junctions in each control and α-btx treated series. These synaptic areas were displayed on a color TV monitor and processed with a semiautomatic image analysis system (Visilog) which measured the metric parameters. We systematically dissected the nerve terminals in segments as can be seen in Fig. 2B. (t) are the trunk segments contained between branch points, and (d) are the distal free-end terminal segments; (i) is the initial segment from the end of the myelin sheath to the first branch point. Both (t) and (d) segments could be first, second or third order depending on their origin; order 0 corresponded to the free-end distal segments that emerged from the first branch point of the terminal arborization. In the control LAL muscle there are no segments which were fourth order or more, although fourth order segments could appear in some growing nerve terminals after a week of α-btx-induced paralysis. We called trunk length the total length of all t segments in a synapse and distal length the total length of all d segments in a junction. The global length of the motor nerve terminal from the first branch point. We also took into account in this work the number of the branch points of a terminal, and the complexity index of the terminal arborization (Complexity index = total length x branch points/100) (Tomas et al., 1989; Deschenes et al., 1993) and the muscle cell diameter.

Statistical analysis

The distribution of the values was tested with the Kolmogoroff-Smirnow test and, when required, a *t*-test or U-test (Mann-Whitney) were used to compare the mean values. The Chi-square test was used to compare the percentages. Statistical significance was tested at a level of $p<0.05$; values below 0.05 were considered to be significant.

Results

Morphology of the normal LAL muscle nerve terminals

Table 1 shows the values of the parameters measured

Table 1. Motor nerve terminal morphometric parameters in normal and α-btx treated LAL muscle.

	48 DAYS (1)	60 DAYS (2)	169 DAYS (3)	1 vs 2 (p)	2 vs 3 (p)	1vs 3 (p)
Preterminal length						
Normal	10.22±8.12	10.33 ± 7.82	13.78 ± 10.41	0.823	0.037	0.000
α-btx	9.28±6.39	11.85±9.14	13.60±11.83	0.031	0.337	0.008
Trunk length						
Normal	25.86±20.42	31.49±19.97	34.19±22.79	0.467	0.256	0.106
α-btx	31.62±18.77	34.98±21.44	36.97±22.09	0.243	0.618	0.067
Distal length						
Normal	82.31±28.64	100.97±28.89	100.21±34.29	0.000	0.823	0.000
α-btx	93.76±32.10 *	97.67±26.32	106.49±40.59	0.450	0.050	0.009
Total length						
Normal	118.24±39.90	138.43±37.52	145.06±48.08	0.001	0.149	0.000
α-btx	130.70±36.86**	141.42±39.10	151.68±56.31	0.042	0.108	0.001
Branch points						
Normal	3.06±1.42	3.35±1.47	3.31±1.60	0.165	0.486	0.344
α-btx	3.38±1.48	3.99±1.61*	3.88±1.86*	0.003	0.364	0.036
Muscle cell diameter						
Normal	50.50±14.80	52.20±8.60	50.40±8.90	0.382	0.060	0.963
α-btx	45.14±8.79*	37.28±7.64*	48.05±10.74**	0.000	0.000	0.019
Complexity index						
Normal	3.71±2.86	4.78±3.29	4.95±4.07	0.013	0.736	0.028
α-btx	4.58±3.27**	5.59±3.55**	6.29±5.42**	0.016	0.929	0.021

Values are expressed in micrometers (except for the complexity index and the branch points) as mean ± SD. p is the p value from the t-test or U-test. For each parameter and day a significant difference between normal control and α-btx values is indicated by * ($p<0.01$) or ** ($p<0.05$).

in all the morphometrically analysed neuromuscular junctions of the LAL muscle both in the normal controls and in α-btx-treated animals. To determine the growth and stabilization of the mature motor nerve terminals we studied the LAL muscle in 48- 60- and 169-day-olds male Swiss mice. Computer-aided morphometry of normal LAL muscle nerve terminals indicated that between 48- and 60-day-olds, motor nerve terminals were still growing by increasing their total length while the muscle cell diameter had reached its adult mean size by day 48. Moreover, by analyzing how dynamic preterminal, trunk and distal segments of the motor nerve endings were, we found that the increase in the branching pattern complexity index between 48 and 60 days was mainly due to an increase in the mean length of the distal free-end segments. Thereafter, between 60 and 169-day-olds, the only significant change observed was a 33% elongation of the preterminal length. Thus, it seems reasonable to conclude that by day 60, LAL motor nerve terminals have attained their mature branching pattern morphology.

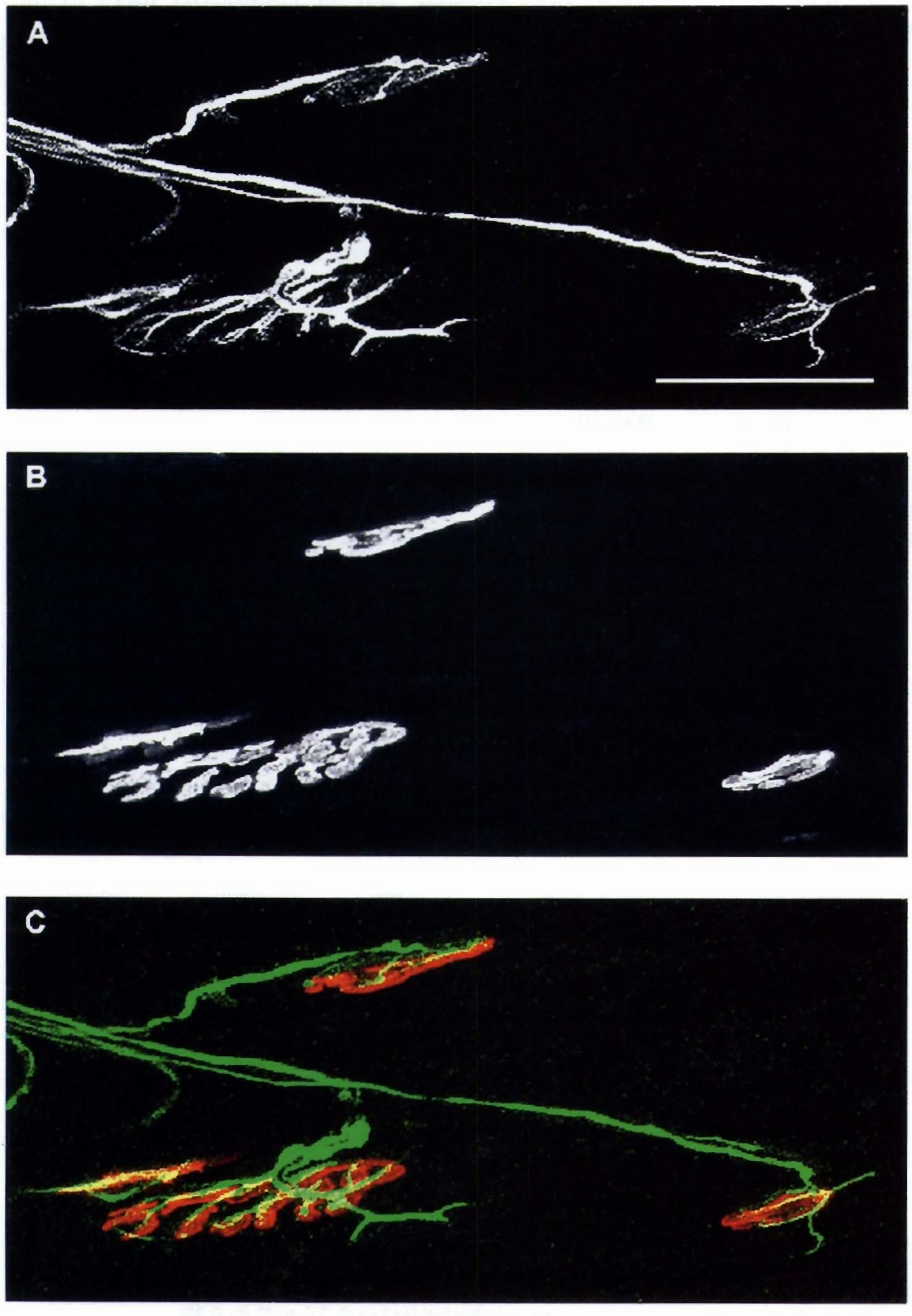

Fig. 1. α-btx-treated LAL muscle after 19 days of paralysis. Motor endplates processed for immunocytochemistry to simultaneously detect postsynaptic AChRs and axons. AChRs are stained with rhodaminated alpha-bungarotoxin **(B)** and axons are labeled with antibodies against 200 kD neurofilament protein **(A)**. Images in A and B are superimposed in **C**. We consider as sprouts the characteristic very fine terminal branches (usually 1-2 mm in diameter) emerging from the terminal arborization, with or without a growth cone at the tip and projecting out of an AChRs-enriched postsynaptic area. Bar: 25 μm.

Spontaneous sproutings in normal LAL muscle

The mean number of branch points on nerve terminals in the normal LAL muscle remained, on average, unchanged throughout the observation period covered by this study (see Table 1). Furthermore, in most endplates an exact morphological correspondence between the pre- and postsynaptic components could exist. However, a small population of LAL control motor nerve terminals showed sproutings (Fig. 3A). The existence of motor nerve sprouting under physiological conditions has also been described in other normal adult vertebrate neuromuscular models. We observed that the incidence of sprouts rase significantly during the age period considered ($p<0.05$, between 48-169 days both for preterminal and terminal sprouts). Nerve endings with terminal sprouts increased five-fold, whereas endings with preterminal sprouts largely triplicated. No ultraterminal sprouts were observed in the control muscles of the different ages, and only a very small incidence of nodal growth was observed on day 48 (1.04±0.04 %, not shown in Fig. 3A).

It should be pointed out that the initial high percentage of end plates with distal sprouts (Fig. 3B; day 48, $p<0.05$ with respect to the percentage of end plates with trunk sprouts) otherwise originated proportionally from all distal segments (see Fig. 3D; order 0, 1 and 2), changed by day 60 to a prevalence of trunk sproutings

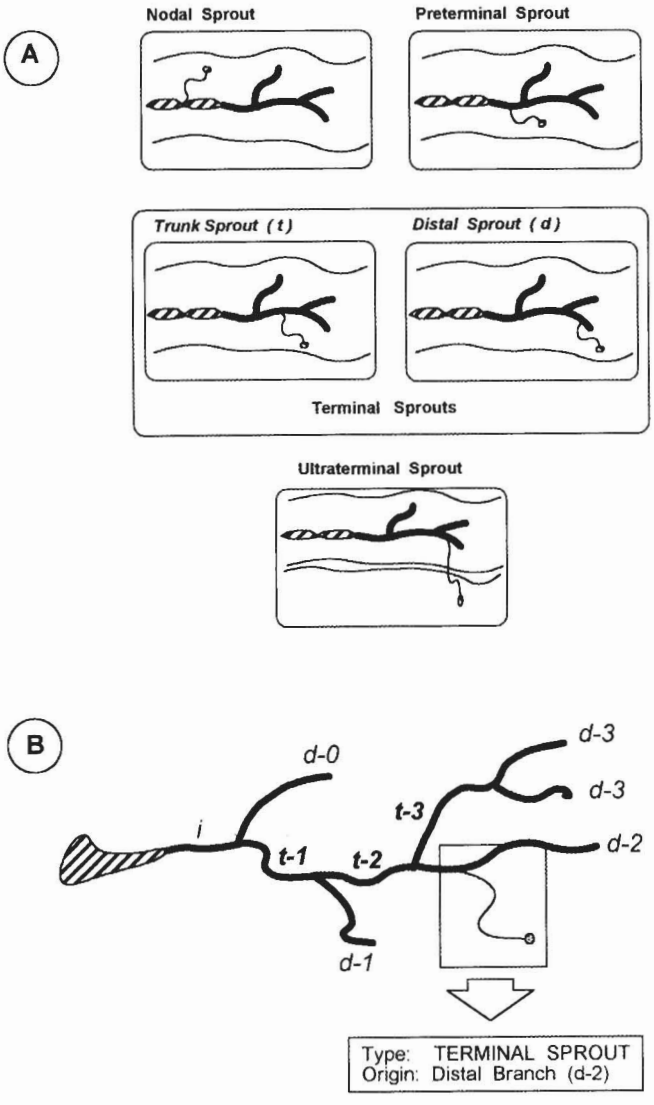

Fig. 2. A. Schematic drawings illustrating the sprouting classification used in this work. **B.** A schematic drawing of a motor nerve terminal to show the different segments of the arborization. The initial segment (i), and the trunk (t) and distal (d) segments are classified according to their centrifugal order. The inset in B shows an example sprout branch.

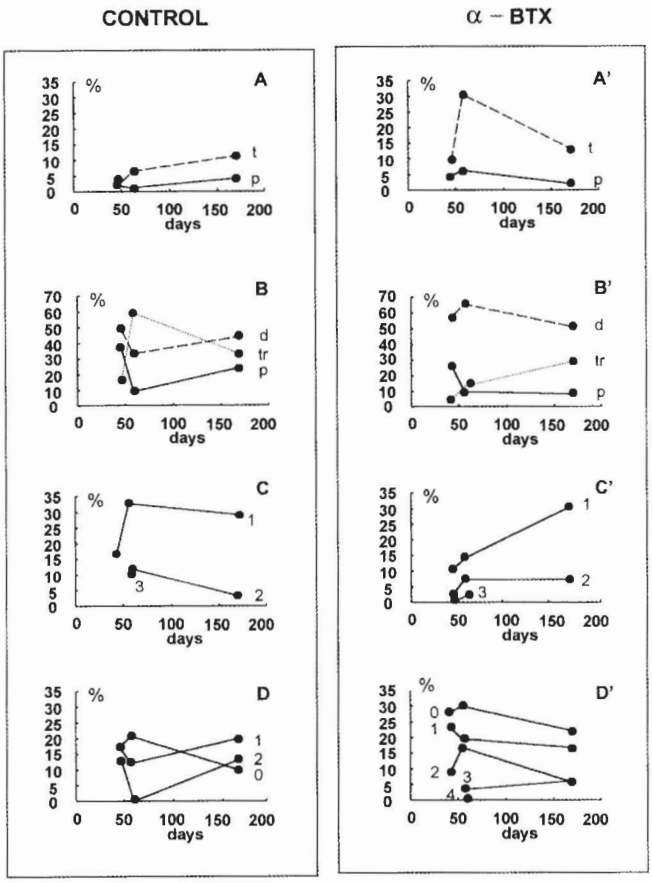

Fig. 3. Control LAL muscles (**A-D**). In A, absolute incidence of preterminal (p) and terminal (t) sprouting. The small incidence of nodal sprouting on day 48 (1.04±0.04 %) is not shown in the figure. In B, relative incidence of preterminal (p), trunk (tr) and distal (d) sprouts. C and D show the intraterminal topological origin of sprouts. In C, relative incidence of trunk sprouts according the centrifugal order (1, 2, 3) of the generating trunk segments. In D, relative incidence of distal sprouts according to the centrifugal order (0, 1, 2) of the generating distal segments; order 0 corresponds to the free-end distal segments that emerge from the first branch point (see also Fig. 2 B). The standard deviations are not represented for clarity. See text for the significance of the differences. α-btx treated muscles (**A'-D'**). The same symbols and explanation as for control except for the existence of sprouts which originate in the highest order distal segments (order 3 and 4).

($p<0.05$) which mainly came from the more proximally placed trunk segment (order 1, see Fig. 3C). By day 169 (see Fig. 3B) no significative differences were found between the percentages of distal and trunk sprouts ($p>0.05$). These data show that sprouting in the terminals of the normal animal was not a random process, it appeared that sprouting could discriminate between preterminal, trunk or distal segments for their origin be originated, particularly between days 48 and 60, when nerve endings were acquiring their mature branching pattern.

Nevertheless, absolute data of the number of endings with sproutings in a particular location in each observation period per se is not enough to describe any age-dependent topological selective origin of the sprouts along the nerve terminals because of the differences in real size between the three intraterminal topological regions. Perhaps the longer region had the highest probability of sprouting. To investigate this possibility we examined the mean length of the preterminal, trunk and distal regions of the LAL motor nerve endings (Table 1) expressed as a percentage of the total terminal length (Fig. 4, control, lined bars) in order to calculate the "expected proportion" of preterminal, trunk and distal sproutings (considering that each micrometer of the nerve terminal arborization should have, on average, the same probability of generating a sprout at each particular moment). The data about the actual "observed proportion" of sprouts is also included in this figure (filled bars). It can be seen that the "observed proportion" of sprouts in the proximal regions (preterminal and trunk segments) of the nerve endings is always equal or higher than the "expected proportion" whereas the distal free-end segments of the terminal always generate fewer sprouts than is expected because of their relative length.

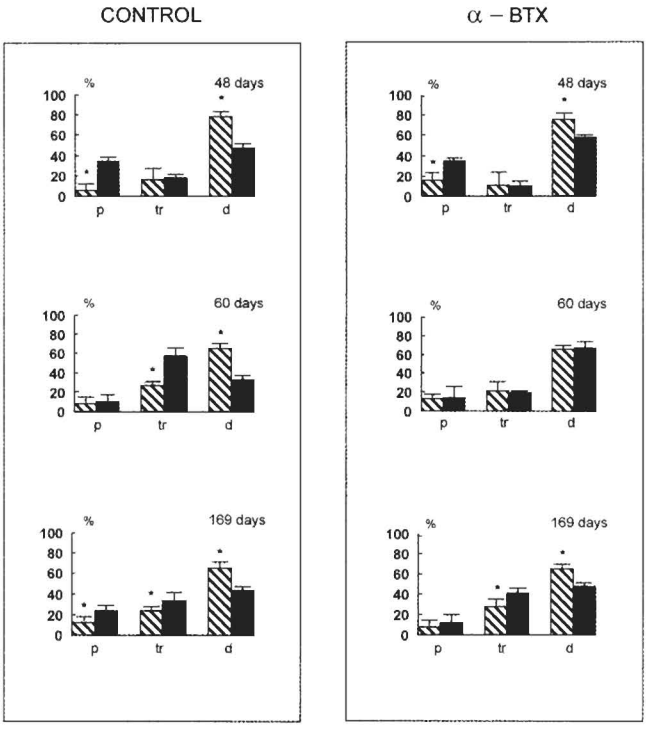

Fig. 4. Shows the extent of sproutings assessed in control and α-btx-treated muscles. Values of "expected sproutings" (lined bars) and "observed sproutings" (filled bars) at the preterminal (p), trunk (t) and distal (d) topological regions of the LAL nerve terminals are expressed as percentage mean±SD. See text for explanations. The "expected percentages" (lined bars) marked with asterisks are significantly different from the corresponding "observed percentage" (filled bar). *: $p>0.05$.

α-btx-induced sprouts

LAL muscles were paralysed by using α-btx. The neurotransmission blocking effect of α-btx was maintained for 19 days (between 41-60 days of the animals life). Nerve terminal morphology and sproutings were studied at 7 and 19 days during paralysis and also after 109 toxin-free days.

The incidence of sprouts increased during the paralysis period and by the last day of toxin presence (day 60) sprouting occured in almost 50% of the synapses. In absolute terms, this reaction was mainly supported by terminal sprouting (see Fig. 3A', day 60). Nevertheless, although the absolute number of endings with α-btx-induced preterminal sprouts was relatively small, the increase in preterminal sproutings with respect to the normal control animals was almost three folds that of the increase in terminal sproutings (compare Figs. 3A and 3A', day 60). After recovery (day 169), the percentage of synapses that had sprouts decreased. However, they did not fully return to control values because there was an increased terminal sprouting (day 169, $p<0.05$ between control and α-btx-treated muscles, Figures 3A and 3A'). Preterminal growth completely attained control value at the end of the observation time.

Interestingly, nodal sprouting did not participate either in normal or in toxin-induced growth of the axonal endings. On the other hand, during the α-btx paralysis (days 48 and 60) we found a small incidence of ultraterminal growth (<1% of the endings showing sprouts).

We also studied the precise topological distribution of the terminal sprouts and found that the percentage of endings showing trunk sprouts was below the control values ($p<0.05$) during α-btx paralysis (compare Figures 3B and 3B', days 48 and 60). Nevertheless, there was a statistically significant increase in this percentage thereafter (day 169; $p<0.05$) in relation to the control muscles which was largely due to the fact that the trunk sprouts originated almost exclusively from first order trunk segments (Fig. 3C'). On the other hand, the percentage of endings that developed distal sprouts was higher (60%) than the control during the α-btx blocking period (differing significantly from control on day 60, $p<0.01$) although, in this case, the control value was fully attained at the end of the observation follow-up period (see Figures 3B and 3B'). The results of the α-btx-induced sproutings summarised in Fig. 3 (C', D')

clearly indicate that the sprouting capacity for each topological region of the nerve terminal arborization depends on the centrifugal order of the segments, decreasing the further away from the origin.

Our results show that drug-induced paralysis cancelled out the topological differences described in the normal control between the sprouting capacity of trunk and distal regions of motor nerve endings. Furthermore, we provide evidence that after a long-term α-btx sprouting induction (see Fig. 4, α-btx 60 days) every micrometer of the full-length axonal arborization has, on average, the same probability of generating a sprout and such sprouting behaviour also differs from what is observed in the normal control. Nevertheless, the control situation is restored in the recovery period and this means that the distal free-end segments of the terminals generate fewer sprouts than is to be expected by their relative length whereas the contrary is true for the proximal segments of the nerve endings.

Effects of α-btx on the morphology of LAL muscle nerve terminals

The simultaneous observation of the postsynaptic AChRs and axons permit us to make an accurate morphometric study of the presynaptic branching pattern profile both during the toxin action and after recovery from paralysis. The results of the quantitative morphometric analysis of α-btx-treated nerve terminals are summarised in Table 1 alongside the comparative mean values of the control animals. Our results indicate that there was a clear increase in the total length of the motor nerve terminals in α-btx-treated LAL muscles after seven days of paralysis (day 48). This might be due to a general lengthening of the terminal on the disused muscles (mainly of the distal free-end segments). However, we cannot completely discard that a precocious consolidation of some toxin-induced, recently-formed sprouts might also contribute to this increase in length despite the fact that the increase in the mean number of branch points by day 48 in the α-btx-treated muscles was not significant. Thereafter, under the continuous effect of α-btx during 19 days LAL muscle nerve terminals increased their branching pattern complexity, mainly because of a significant increase in the number of branch points when some sprouts stabilized. This high level of branching pattern complexity remained unchanged long after the α-btx effect ceased (169-day-old animals). Interestingly, the mean length of the nerve terminal did not significantly increase either at the peak of the toxin-induced sprouting reaction (day 60) or after recovery (day 169).

In these experiments, the mean muscle cell diameter showed significant changes during the observation periods. While functionally denervated, the myofibres underwent marked and significative atrophy (from day 48 to 60, 45.14±8.79 and 37.28±7.64 mm respectively, $p<0.001$). However, the size of muscle cells reversed after 109 toxin-free days (169-day-old series, 48.05±10.74). On the other hand control muscles showed a constant muscle cell size throughout the ages considered (Table 1). Thus, there is evidence that muscle cell size does not affect nerve terminal enlargement in our conditions.

Discussion

Normal control LAL muscle

Our results show that the branching pattern of motor nerve terminals reaches adult morphological stability around day 60 in normal LAL muscles of Swiss mice. None of the parameters considered showed any significant changes thereafter, between 60- and 169-day-olds, except for the preterminal length which increased minimally. These findings agree with several in vivo studies of repeatedly observed, fluorescently labelled, normal adult neuromuscular junctions showing that there is a basic synaptic stability over time (Lichtman et al. 1989; Herrera et al., 1990; Wigston, 1990; Hill et al., 1991). Nevertheless, spontaneous sproutings can be observed in end plates from control LAL muscles as described in many normal vertebrate muscles by using silver stains (Brown, 1984; see Wernig and Herrera, 1986 for a review). Furthermore, sprouts continuously increased throughout the life period considered (48-169 days). It is possible to speculate that some sprouts can consolidate as nerve terminal branches and that these growth changes in mature end plates can cause the normally occurring ageing-related nerve terminal growth and synaptic elaboration observed in senescent muscles (Cardasis, 1983; Robbins and Fahim, 1985; Jans et al., 1986; Andonian and Fahim, 1987; Cardasis and LaFontaine, 1987). Nevertheless, there was no significant increase in the number of nerve terminal branch points during the observation period (48-169 days), and it is reasonable to conclude that most of the spontaneous sprouting observed can be a transient growth response in the context of normal activity-dependent synaptic plasticity.

In control muscles, depending on the age period considered, sprouting takes place in different regions of the motor nerve terminals. This suggests that there is a topological selection of sprout branches which leads to the mature branching pattern of the nerve terminals by the end of the second month of life. In this sense, the growth of the motor nerve terminals observed on day 48 is mainly due to the lengthening of the distal free-end branches. Thus, not surprisingly, at this age most sprouting also takes place in the distal segments. Nevertheless, by day 60, when nerve endings have already attained their mean adult complexity and have stopped growing, most sprouts occur in the axial trunk segments of the nerve terminal. Such precise age-related topological selectivity of the sprout branches along the nerve terminal disappears in completely mature animals (on day 169) (Fig. 4B).

We further investigated this age-dependent

topological differential sprouting in the control muscles by comparing the "observed" with the "expected" incidence of sprouts. This approach was used on the incidence of sproutings in control LAL muscles on day 48 and showed that the percentage of preterminal sprouts "observed" was surprisingly higher than the "expected" values of such a small region of the branching pattern. In fact, the sprouting capacity of the distal free-end segments is depressed throughout the period studied in relation with their length whereas the most proximal short preterminal segment and also the axially positioned trunk segments (that are only 1/3 of the terminal length) can generate sprouts more easily than is to be expected by their length. Nevertheless, these topological differences cannot be fully explained by the difference in proximity to motorneuronal supplies, because on day 60 the most proximal preterminal segment had a smaller outgrowth response than the more axially placed trunk segments (see Fig. 4).

In summary, we demonstrate that there is a topological lack of uniformity in the sprouting capacity of the preterminal, trunk and distal regions of the motor endings in normal animals. Complex structural or activity-dependent differences between the segments of the terminal regions can play a role. This suggestion is reinforced by the finding that α-btx-induced paralysis (see below) can turn the motor nerve terminal into a homogeneous structure, as far as sprouting capacity is concerned, in which each micrometer of the arborization has the same probability of eliciting growth signs. However, the distance between the nerve terminal arborization and the neuronal soma may affect sprouting capacity in certain conditions. In this respect, botulinum toxin-induced nerve terminal sprouting is more abundant in proximal than in distal muscles, and there seems to be a reduced capacity for sprouting in the terminals of longer axons (Pestronk and Drachman, 1988). Because of the existence of some feedback mechanisms which link the regulation of growth to the supply of new membrane in response to demand, it seems important to determine where each of the constituents of the neuronal membrane is added to the growing neurite (Futerman and Banker, 1996).

Effects of α-btx on the morphology of LAL muscle nerve terminals and sprouting

The synaptic transmission was blocked with α-btx, which binds almost irreversibly to the acetylcholine receptors on the postsynaptic membrane and prevents them being activated by acetylcholine (Pestronk and Drachman, 1978). Experiments performed with α-btx in this work (using α-btx 1 mg/ml in Ringer solution, 50 ml; every 48 h via s.c. for 7 to 19 days) never actually produced a myasthenia gravis syndrome, which can be induced by 3-5 mg of α-btx every 48 h via s.c. for 3-4 weeks (Plomp et al., 1994). After the α-btx treatment not all LAL motor nerve terminals showed sprouts and not all muscle fibres became permanently paralysed. The toxin was applied to the external surface of the LAL muscle and the level of block seemed to decrease deeper in the muscle. Nevertheless, at the end of the period of toxin application, 2/3 of the superficial synapses showed subthreshold activity whereas the maximal incidence of sprouting affected almost 1/2 of the superficial junctions. This lack of correspondence indicates that some junctions were not continuously blocked during the period of the toxin application, so sprouting would either not occur or not be maintained once (if) generated. Because the normal AChRs turnover only a part of the α-btx binding sites are occupied (Turney et al., 1996) when the postsynaptic membrane is stained with rhodaminated α-btx to study the endplate morphology.

The morphology and extension of the sprouts generated in our experiments with α-btx were similar to those observed in control muscles. The relative weakness of the sprouting induction procedure we used may explain the almost total absence of other more vigorous growth signs, i.e., ultraterminal and collateral (nodal) sprouting which, in contrast, can be observed after longer and more intensive presynaptic paralysis with botulinal neurotoxins (Hopkins et al., 1981; Comella et al., 1993; Juzans et al., 1996; Angaut-Petit et al., 1998). On the other hand, other factors independent of muscle inactivity must contribute to nerve terminal outgrowth in type-A botulinal neurotoxin because the sprouting process and the extent of the total nerve terminal arborization continued to increase after functional recovery (Molgó et al., 1990).

When we studied the topological origin of sproutings in α-btx-paralysed LAL muscles, we detected a higher and significant incidence of preterminal and terminal sprouts during the period of the toxin action. α-btx specifically blocks transmission and paralyses muscle fibres without blocking axoplasmic transport (Anderson and Edström, 1973). So, like all chemical agents that block neuromuscular transmission, the toxin on its own caused terminal sprouting (Holland and Brown, 1980). Previous results that indicate the sprout-inhibiting action of α-btx may be due to the fact that an insufficiently purified toxin was used (Pestronk and Drachman, 1978).

Our results provide evidence that in the control nerve endings, preterminal segments (the unmyelinated axon before the first branch point of the terminal arborization) and trunk segments (those between branch points) generate more sprouts than is to be expected when their length is compared to that of the distal free-end segments. In contrast, every micrometer of the full-length terminal arborization in α-btx-treated muscles has, on average, the same probability of generating a sprout wherever it is located in the nerve terminal tree. Skeletal muscle may use different molecules to encourage axonal sprouting after paralysis and activity changes in normal controls. This probably reflects different underlying mechanisms involved in repair and maintenance, respectively (Funakoshi et al., 1995).

In α-btx-treated 169-day-old animals (109 toxin-free

days after 19 days of paralysis), the percentage of synapses with sprouts did not fully recover control values because there was an increased percentage of sprouts. We do not have any particular explanation for this residual remodelling. However, the main acute changes which occur in the nerve terminals during α-btx action are reversible. In this respect, immobilization experiments indicate that changes in neuromuscular junctions (i.e., enlargement of the ACh-sensitive area and sprouting of the terminal branches) revert to normal within 20-50 days of the termination of immobilization (Fischbach and Robbins, 1971). Moreover, nerve terminal growth dissapears in muscles once recovered from partial denervation (Brown et al., 1981) or blockade of axonal conduction (Tsujimoto and Kuno, 1988).

Nevertheless, we believe that some α-btx-induced growth can subsequently consolidate as a new nerve terminal branch because there is also an increase in the number of branching points after recovery from the toxin action (day 169). However, the total length did not change, which indicates two things: firstly, the great stability, once generated, of the toxin-induced sprouted branches, and secondly, the existence of an effective mechanism limiting excessive elongation of distal branches. These findings suggest a precise morphological adaptation of each particular segment of the motor nerve terminals so as not to exceed a limited synaptic length.

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References

Anderson K.E. and Edström A. (1973). Effects of nerve blocking agents on fast axonal transport of proteins in frog sciatic nerves in vitro. Brain Res. 50, 125-134.

Andonian M.H. and Fahim M.A. (1987). Effects of endurance exercise on the morphology of mouse neuromuscular junctions during aging. J. Neurocytol. 16, 589-593.

Angaut-Petit D., Molgó J., Connold A.L. and Faille L. (1987). The levator auris longus muscle of the mouse: a convenient preparation for studies of short- and long-term presynaptic effects of drugs or toxins. Neurosci. Lett. 82, 83-88.

Angaut-Petit D., Molgó J., Comella J.X., Faille L. and Tabti N. (1990). Terminal sprouting in mouse neuromuscular junctions poisoned with botulinum type A toxin: morphological and electrophysiological features. Neuroscience 37, 799-808.

Angaut-Petit D., Molgó J., Faille L., Juzans P. and Takahashi M. (1998). Incorporation of synaptotagmin to the axolemma of botulinum type-A poisoned mouse motor endings during enhanced quantal acetylcholine release. Brain Res. 797, 357-360.

Balice-Gordon R.J. and Lichtman J.W. (1991). The plasticity and stability of neuromuscular synapses in living mice. In: Plasticity of motoneuronal connections. Wernig A. (ed). Elsevier. Amsterdam. pp 71-84.

Barker D. and Ip M.C. (1966). Sprouting and degeneration of mammalian motor axons in normal and de-afferentated skeletal muscle. Proc. Royal Soc. B 163, 538-554.

Betz W.J., Caldwell J.H. and Ribchester R.R. (1980). Sprouting of active nerve terminals in partially inactive muscles of the rat. J. Physiol. 303, 281-297.

Brown M.C. (1984). Sprouting of motor nerves in adult muscles: a recapitulation of ontogeny. Trends Neurosci. 7, 10-14.

Brown M.C., Holland R.L. and Hopkins W.G. (1981). Motor nerve sprouting. Annu. Rev. Neurosci. 4, 17-42.

Cardasis C.A. (1983). Ultrastructural evidence of continued reorganization at the ageing (11-26 months) rat soleus neuromuscular junction. Anat. Rec. 200, 399-415.

Cardasis C.A. and LaFontaine D.M. (1987). Aging rat neuromuscular junctions: a morphometric study of cholinesterase-stained whole mounts and ultrastructure. Muscle Nerve 10, 200-213.

Comella J.X., Molgó J. and Faille L. (1993). Sprouting of mammalian motor nerve terminals induced by in vivo injection of botulinum type-D toxin and the functional recovery of paralysed neuromuscular junctions. Neurosci. Lett. 153, 61-64.

Connor E.A. and Smith M.A. (1994). Retrograde signalling in the formation and maintenance of the neuromuscular junction. J. Neurobiol. 25, 722-739.

Deschenes M.R., Maresh C.M., Crivello J.F., Armstrong L.E., Kraemer W.J. and Covault J. (1993). The effects of exercise training of different intensities on neuromuscular junction morphology. J. Neurocytol. 22, 603-615.

Diamond J., Cooper E., Turner C. and MacIntyre L. (1976). Trophic regulation of nerve sprouting. Science 193, 371-377.

Duchen L.W. and Strich S.J. (1968). The effects of botulinum toxin on the pattern of innervation of skeletal muscle in the mouse. Q. J. Exp. Physiol. 53, 84-89.

Fischback G.D. and Robbins N. (1971). Effect of chronic disuse of rat soleus neuromuscular junctions on postsynaptic membrane. J. Neurophysiol. 43, 562-569.

Funakoshi H., Belluardo N., Arenas E., Yamamoto E., Casabona A., Persson H. and Ibáñez C.F. (1995). Muscle-derived Neurotrophin-4 as an activity-dependent trophic signal for adult motor neurons. Science 268, 1495-1499.

Futerman A.H. and Banker G.A. (1996). The economics of neurite outgrowth - the addition of new membrane to growing axons. Trends Neurosci. 19, 144-149.

Haimann C., Mallart A., Tomas i Ferré J. and Zilber-Gachelin N.F. (1981). Patterns of motor innervation in the pectoral muscle of adult *Xenopus laevis*: evidence for possible synaptic remodelling. J. Physiol. 310, 241-256.

Herrera A.A., Banner L.R. and Nagaya N. (1990). Repeated, in vivo observation of frog neuromuscular junctions: remodelling involves concurrent growth and retraction. J. Neurocytol. 19, 85-99.

Hill R.R., Robbins N. and Fang Z.P. (1991). Plasticity of presynaptic and postsynaptic elements of neuromuscular junctions repeatedly observed in living adult mice. J. Neurocytol. 20, 165-182.

Hoffman H. (1950). Local re-innervation in partially denervated muscle: a histophysiological study. Aust. J. Exp. Biol. Med. Sci. 28, 383-397.

Holland R.L. and Brown M.C. (1980). Postsynaptic transmission block can cause terminal sprouting of a motor nerve. Science 207, 649-651.

Hopkins W.G., Brown M.C. and Keynes R.J. (1981). Nerve growth from nodes of Ranvier in inactive muscle. Brain Res. 222, 125-128.

Jans H., Salzmann R. and Wernig A. (1986). Sprouting and nerve

retraction in frog neuromuscular junction during ontogenesis and environmental changes. Neuroscience 18, 773-781.

Juzans P., Comella J.X., Molgó J., Faille L. and Angaut-Petit D. (1996). Nerve terminal sprouting in botulinum type-A treated mouse Levator auris longus muscle. Neuromuscular Disord. 6, 177-185.

Lanuza M.A., Santafé M., Fenoll M.R. and Tomas J. (1993). α-Bungarotoxin induced motor nerve terminal sprouts can consolidate upon cessation of the growth stimulus. Eur. J. Neurosci. S6, 282.

Lichtman J.W., Magrassi L. and Purves D. (1987). Visualization of neuromuscular junctions over periods of several months in living mice. J. Neurosci. 7, 1215-1222.

Lichtman J.W., Sunderland W.J. and Wilkinson R.S. (1989). High-resolution imaging of synaptic structure with a simple confocal microscope. New Biol. 1, 75-82.

Molgó J., Comella J.X., Angaut-Petit D., Pecot-Dechavassine M., Tabti N., Faille L., Mallart A. and Thesleff S. (1990). Presynaptic actions of botulinal neurotoxins at vertebrate neuromuscular junctions. J. Physiol. (Paris) 84, 152-166.

Pestronk A. and Drachman D.B. (1978). Motor nerve sprouting and acetylcholine receptors. Science 199, 1223-1225.

Pestronk A. and Drachman D.B. (1988). Motor nerve outgrowth: reduced capacity for sprouting in the terminals of longer axons. Brain Res. 463, 218-222.

Plomp J.J., van Kempen G.T. and Molenaar P.C. (1994). The upregulation of acetylcholine release at endplates of alpha-bungarotoxin-treated rats: its dependency on calcium. J. Physiol. 478, 125-136.

Ramón y Cajal S. (1929). Quelques remarques sur les plaques motrices de la langue des mammifères. Trab. Lab. Invest. Biol. Univ. Madrid, 23. Reprinted in Etudes sur la Neurogénèse de Quelques Vertébrés. Madrid. pp 384-393.

Ravdin P.M. and Berg D.K. (1979). Inhibition of neuronal acetylcholine sensitivity by alpha-toxins from *Bungarus multicinctus* venom. Proc. Natl. Acad. Sci. USA 76, 2072-2076.

Robbins N. (1991). Focal specializations for stability and plasticity at neuromuscular junctions of young and old mice. In: Plasticity of motoneuronal connections. Wernig A. (ed) Elsevier. Amsterdam. pp 71-84.

Robbins N. and Fahim M.A. (1985). Progression of age changes in mature mouse motor nerve terminals and its relation to locomotor activity. J. Neurosci. 14, 1019-1036.

Rosenheimer J.L. (1985). Effects of chronic stress and exercise on age-related changes in end-plate architecture. J. Neurophysiol. 53, 1582-1589.

Tello J.F. (1917). Génesis de las terminaciones nerviosas motoras y sensitivas. Trab. Lab. Invest. Biol. Univ. Madrid. 15, 101-199.

Tomas J., Fenoll M.R., Santafé M., Batlle J. and Mayayo E. (1989). Motor nerve terminal morphologic plasticity induced by small changes in the locomotor activity of the adult rat. Neurosci. Lett. 106, 137-140.

Tomas J., Santafé M., Fenoll R., Mayayo E., Batlle J., Lanuza A. and Piera V. (1992). Pattern of arborization of the motor nerve terminals in the fast and slow mammalian muscles. Biol. Cell 74, 299-305.

Tomas J., Batlle J., Fenoll M.R., Santafé M. and Lanuza M.A. (1993). Activity-dependent plastic changes in the motor nerve terminals of the adult rat. Biol. Cell 79, 133-137.

Tsujimoto T. and Kuno M. (1988). Calcitonin gene-related peptide prevents disuse-induced sprouting of rat motor nerve terminals. J. Neurosci. 8, 3951-3957.

Tuffery A.R. (1971). Growth and degeneration of motor end-plates in normal cat hind limb muscles. J. Anat. 110, 221-247.

Turney S.G., Culican S.M. and Lichtman J.W. (1996). A quantitative fluorescence-imaging technique for studying acetylcholine receptor turnover at neuromuscular junctions in living animals. J. Neurosci. Meth. 64, 199-208.

Velasco M.E. and Pécot-Dechavassine M. (1993). Membrane events related to transmitter release in mouse motor nerve terminals captured by ultrarapid cryofixation. J. Neurocytol. 22, 913-923.

Wernig A. and Herrera A.A. (1986). Sprouting and remodelling at the nerve-muscle junction. Prog. Neurobiol. 27, 251-291.

Wernig A., Salvini T.F. and Irintchev A. (1991). Axonal sprouting and changes in muscle fibre types after running-induced muscle damage. J. Neurocytol. 20, 903-913.

Wigston D. (1990). Repeated in vivo visualization of neuromuscular junctions in adult mouse lateral gastrocnemius. J. Neurosci. 10, 1753-1761.

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