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STUDIES ON ANTEROGRADE TROPHIC INTERACTIONS BASED ON GENERAL MUSCLE PROPERTIES

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INTRODUCTION

One of the main reasons for the interest of neurobiologists in trophic interactions is the importance of their role during development and response to injury of neural tissues. Establishment of neural connections depend not only on a rigidly programmed genetic plan but also on ongoing interactions between partner cells during development which allow some flexibility in the final outcome. These influences are precisely what trophic interactions are all about. Of the huge number of retrograde and anterograde interactions, that is target cell on innervating neuron and vice versa, only the latter are the focus of this presentation. A further limitation is that to the neuromuscular system which is a convenient, relatively simple preparation for studying synaptic physiology in general.

A basic distinction among neurally controlled muscle properties is between the very discrete junctional properties and general properties of myofibres which include the contractile apparatus and the extrajunctional membrane. The importance of this distinction is further appreciated if one considers that 1) not all the properties are different in the two cases, and 2) properties which are represented in both the junctional and the extrajunctional membrane, such as the acetylcholine receptors (AChRs) at various stages of development, may be controlled differently. In this review the emphasis is placed on the motoneuronal regulation of the general structural and functional features of myofibres, particularly of the extrajunctional membrane.

Investigations on the nature of the neural signals which mediate the anterograde trophic regulation of general muscle properties have generated two candidates: 1) nerve-borne substances or 2) electrical activity evoked in muscle (4, 13, 14, for a review). Thus, development after denervation of profound changes of the extrajunctional membrane (diffuse appearance of AChRs and of Na^+ channels insensitive to tetrodotoxin (TTX) block; spontaneous firing of action potentials, called fibrillation) or of contraction-related properties (decrease in muscle mass and force output) have been attributed to loss of muscle regulation (down- or up-regulation for the two groups of properties, respectively) by one or the other factor. What follows is a rapid survey of the contributions of our group to the elucidation of this problem.

I. *Muscle activity vs trophic factors.*

Proponents of the regulation of general muscle properties by nerve-borne substances, generally referred to as "trophic" factors, have based their conviction on the following facts: 1) Cutting the nerve at "short" or "long" distances from muscle is followed by different delays in onset of muscle changes (the shorter the nerve stump, the earlier the onset; see Figure 1 for an example on development of TTX-resistance) (1, 13); 2) Changes by denervation and by pure muscle paralysis are not equivalent, because in the latter condition they develop later and to a lesser extent (example in Figure 2 on rat Soleus and EDL muscle) (8, 9, 17, 24); 3) Blocking anterograde axonal transport in motor nerves by colchicine, induces denervation-like extrajunctional membrane changes while muscle fibres retain a normal innervation (5); 4) Denervated myofibres develop their changes in spite of exhibiting some degree of activity, since fibrillation is one of the consequences of denervation (example in Figure 3).

On the other hand, proponents of the theory that spike activity evoked by motoneurons in myofibres is the factor controlling general properties of muscle, have shown that: 1) chronic electrical stimulation of denervated muscle *in vivo* can normalise the extrajunctional membrane and reverse towards normality to a good

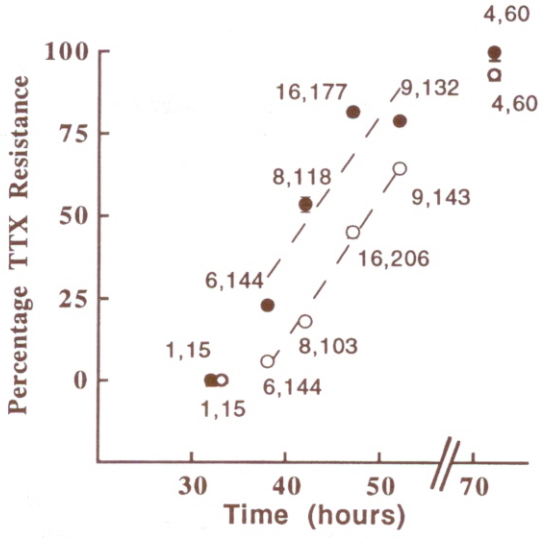

Fig. 1. - *Effect of the length of the nerve stump on the development of TTX-resistant action potentials, after sectioning the original axons, close (filled circles) or far (empty circles) from the Soleus muscle.*

First and second number near symbols indicate muscles and fibres, respectively. Average difference in stump length is 42 mm. A velocity factor (147 mm/day) can be calculated by dividing this value by the time separation between the two lines fitting the two sets of data (fitted by least squares method). Most standard error (S.E.) bars are smaller than symbols. Values of TTX-resistance are expressed as percentage of the maximum value of the distribution, attained by closely denervated muscles at 72 hours and equalling 143.1 V.s-1. Resistance to TTX 10^{-6} M was assayed with a double microelectrode intracellular technique (see Ref. 10 for details).

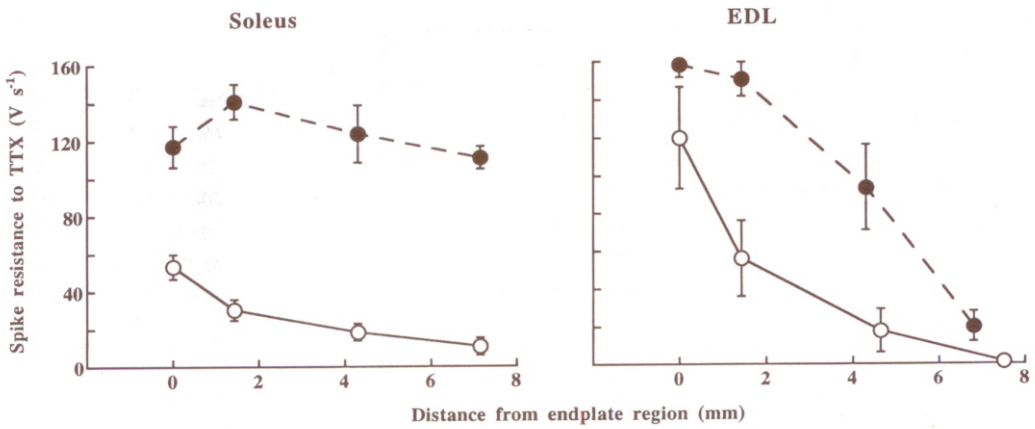

Fig. 2. - *Resistance of action potentials to TTX in fibres of denervated (filled circles) and impulse-blocked (empty circles) rat Soleus and EDL muscles.*

Both muscles examined at 72 hours. In each muscle measurements were made in 5-8 adjacent fibres, at different positions along the distance between endplate region and myotendinous junction. Mean±S.E. are shown. Nerve impulses were chronically blocked in the sciatic nerve with silicone cuffs impregnated with TTX (Ref. 17).

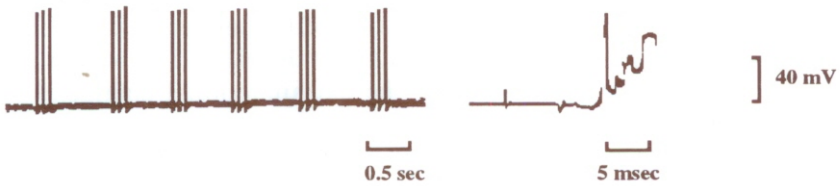

Fig. 3. - *Example of fibrillation potentials recorded intracellularly from an impulse blocked EDL fibre which has retained its normal innervation.*

The fibre exhibits a regular discharge organised in triplets. The record to the right shows the effect of two supramaximal electrical shocks applied in rapid sequence to the nerve, the first proximal and the second distal to the blocking cuff. The fibre is excited only by the distal shock.

extent the contractile properties (see also, 15, 19, 20), 2) pure muscle paralysis can induce denervation-like membrane and contractile changes, as indicated above (8, 9, 17, 19, 24).

A combined interpretation of all the data presented so far, one which is favoured by many authors, is that the general properties of muscle are under the physiological regulation of both muscle evoked activity and putative neural chemical factors. However, while the evidence in favour of the role of activity has been invariably confirmed since its initial presentation, that in support of trophic factors has been challenged by several investigators including our group. Thus, we have shown that after nerve treatment with colchicine extrajunctional membrane changes (development of AChRs and TTX-resistant Na^+ channels) affect both ipsilateral as well as

contralateral muscles. Since no block of axonal transport is detectable contralaterally and the muscle effects are quantitatively comparable on the two sides, we concluded that they are induced by systemic effects of colchicine (5, see also 18). Furthermore, concerning the role of fibrillatory activity, it has been shown that in any given myofibre such activity is not continuous but undergoes cycles in which the active phase lasts in rat muscle about 1 day and the silent phase 2-4 days (25). This phase is thus long enough to allow build up of extrajunctional membrane changes. Also, a cyclical nature of fibrillatory activity is expected if spike activity is the factor controlling, through negative feedback, extrajunctional changes including fibrillation itself.

II. *Contribution of products of nerve degeneration to the origin of muscle changes induced by denervation.*

The effect of the length of the nerve stump and the lesser efficacy of pure paralysis compared to denervation, which can be considered aspects of essentially the same phenomenon, originated a series of investigations which opened the way to a new interpretation. The neural factor which makes denervation more effective, particularly when closer to muscle, instead of being a physiological one *missing* in denervated muscle, could be a chemical signal which *appears* in the muscle *after denervation.* Products of wallerian degeneration of the intramuscular nerve are an obvious candidate. It is part of the hypothesis that the sign of action on muscle be reversed, degeneration products *inducing* the extrajunctional changes instead of inhibiting them like the putative trophic factors. The initial suggestion, based on development of extrajunctional AChRs under a transplanted piece of nerve (16), was confirmed by the observation that the same occurs around the foreign endplates in a doubly innervated Soleus, by the original and a transplanted nerve (however left connected to the spinal cord), after section of the latter (21). Strong support for the essential role of nerve degeneration, in synergism with muscle inactivity, in causing the powerful effects observed in muscle after denervation, has been given by our laboratory using different approaches which have the following features in common: 1) use of the ordinary process of wallerian degeneration of otherwise normal nerves left *in situ*, 2) use of axons which have not innervated the experimental myofibres before their degeneration, 3) since denervation induces concomitantly muscle inactivity and wallerian degeneration, emphasis is placed on their interaction, 4) no trauma of the muscle surface and consequent inflammatory phenomena are produced which could complicate the interpretation of the muscle effects. Three different approaches are described in the next paragraphs.

1. *Partial denervation.* - Based on the polyradicular innervation of hind limb muscles, sectioning of one radicular nerve (L_4 or L_5 in the rat) produces partial denervation and allows one to test whether, in a given muscle, the *innervated* fibres undergo extrajunctional membrane changes like the denervated fibres. This was

tested in a series of different approaches (6, 7, 10) and gave a clear-cut positive answer. This represents strong support for the products of nerve degeneration hypothesis, since after partial denervation such products are released in the interstitium between the muscle fibres and can equally act on denervated and innervated fibres. Denervation-like changes (such as development of extrajunctional TTX-resistant Na^+ channels and AChRs) although clearly detectable, were actually less pronounced in innervated fibres (6): this should however be expected, since the denervation-like changes are also induced by muscle inactivity and the innervated fibres of the partially denervated muscles are not inactive. This lead us to combine partial denervation and paralysis of the innervated fibres (by a chronic conduction block of their axons), thus studying the interaction between the two key factors that are certainly produced by denervation: nerve breakdown products and paralysis of the myofibres (7, 10). The results of this experiment were striking since the amount of extrajunctional changes in the innervated fibres was found to be as large as that

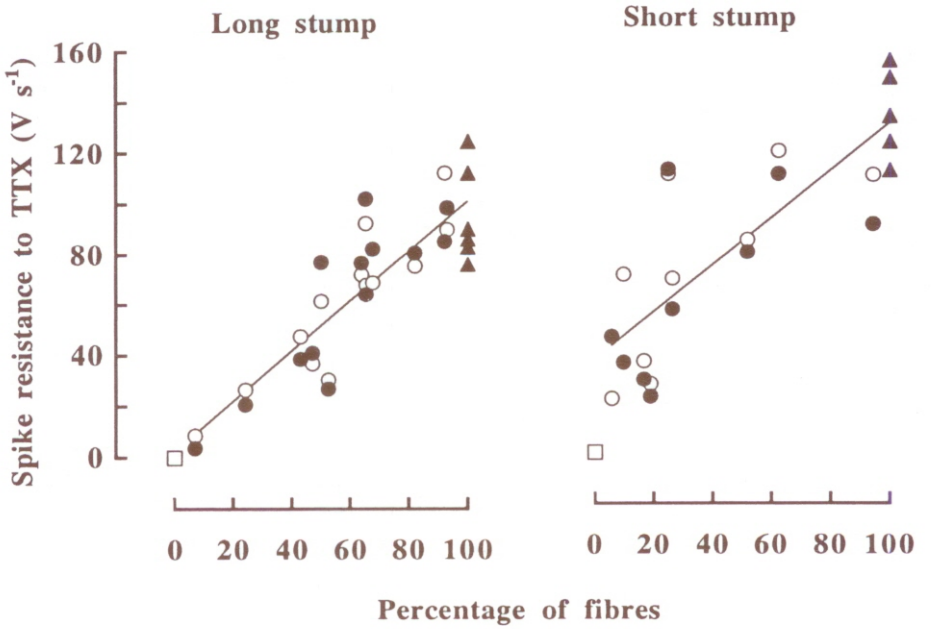

Fig. 4. - *Effects of varying the degree of partial denervation (50 hours) on TTX-resistance of Soleus muscle fibres.*

Two population of muscles are shown, one with axonal section far (long stump) and the other close (short stump) to the muscle belly. Innervated fibres (empty circles), denervated fibres (filled circles). Partially denervated muscles are, in addition, totally paralysed by chronic conduction block of the sciatic nerve. Control muscles are: totally denervated (filled triangles) and purely paralysed (empty square, representing 4 muscles), also at 50 hours. Each point represents mean±S.E. of values from 4-15 fibres in a given muscle. Regression lines fit both innervated-paralysed and denervated fibres in each of the two populations and indicate that the "short stump" population has higher values. Distant denervation is obtained by cutting L4 or L5 radicular nerve, just outside the vertebral canal. Close denervation is obtained by cutting incompletely the soleus nerve.

of the denervated fibres (Figure 4). We considered the possibility that the effects seen in the innervated fibres are not due to products of nerve degeneration but to a retrograde action through the spinal cord, similar to the effect described by Rotshenker on the contralateral side after cutting nerves on one side (26). This was however discarded because when the partial denervation was placed close to the Soleus muscle, by cutting incompletely the soleus nerve, the effects on the extrajunctional membrane of the innervated fibres became larger (Figure 4, compare short with long stump). This is expected if the effects occur earlier, as it should be if they depend on nerve degeneration. Finally, Figure 4 shows that there is a "dose-dependency" of the effects of products of nerve degeneration since the higher the degree of partial denervation the larger the amount of membrane changes. Furthermore the amount is often comparable in innervated and denervated fibres, whether the degree of denervation is high or low, further indicating that the changes in the two classes of fibres start in synchrony.

2. *Degeneration of a transplanted foreign nerve.* - When a foreign nerve (left connected to the spinal cord) is first transplanted onto the surface of the Soleus muscle, it grows *but does not make synapses,* provided the original nerve is left intact. If later in time the foreign nerve is sectioned, striking development of membrane changes occurs in the region of the degenerating nerve, p*rovided the muscle is paralysed* by chronic nerve conduction block (10). The interpretation of this experiment is the same as that of partial denervation combined with paralysis and speaks in favour of actions of products of nerve degeneration.

3. *Effect of nerve stump.* - The paradigm described in the preceding paragraph has also been used by us to investigate the effect of the length of the nerve stump, by cutting the foreign nerve close or distant from the muscle. In either case the muscle is chronically paralysed. The effect of the stump was clearly detectable (1) as shown in Figure 5 and is quantitatively comparable to that obtained with the original nerve (see Figures 1, 5 and their legends). The significance of this experiment is that a nerve does not have to make synapses with the muscle in order to exhibit the stump length effect, thus fitting nicely the products of nerve degeneration interpretation to this effect as well. In fact it is long known that the closer the nerve is cut to nerve endings, the sooner they start to degenerate (23).

4. *Long term comparison of paralysis to denervation.* - Since products of nerve degeneration are removed from the interstitium a few weeks at most after denervation, the amount of extrajunctional membrane changes should eventually decrease in denervated muscles to a lower level and become equal to that of paralysed-innervated muscles. Several investigators deny the occurrence of this equalisation, thus supporting the continuing action of a trophic inhibitory factor which would keep the paralysed-innervated muscles closer to normal (2, 11, 28). A similar long lasting difference between denervated and purely paralysed muscles has been reported for loss of muscle mass and force output (27), the paralysed muscles being

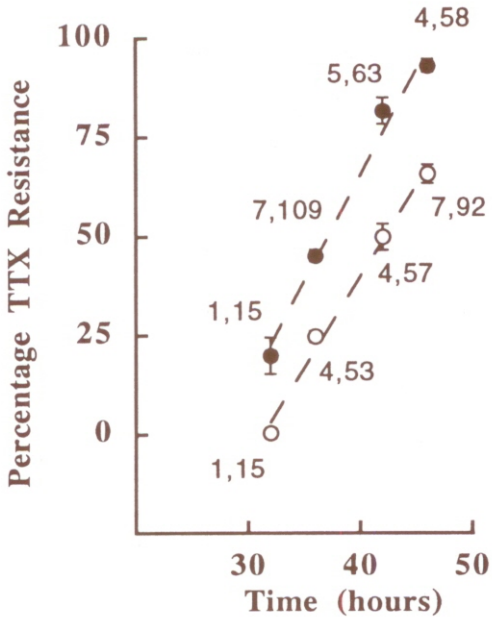

Fig. 5. - *Effect of the length of the nerve stump on the development of TTX-resistance in Soleus muscle fibres, after sectioning the axons of a previously transplanted fibular nerve, close (filled circles) or far (empty circles) from the muscle.*

Transplanted axons did not make synapses before their section and degeneration. Average difference in stump length is 28 mm. A velocity factor (132 mm/day) can be calculated by dividing this value by the time separation between the two lines fitting the two sets of data. All other conditions are as in Fig. 1.

less affected than the denervated ones. Acceptance of this conclusion, however, requires that muscle paralysis remains total over the long period of its duration. This in turn depends on completeness of the chronic conduction block applied to the nerve.

We tested this problem by trying to obtain an optimal conduction block by using adequately high doses of the blocking agent (TTX) and constructing a special perfusion cuff for the rat sciatic nerve which ensures a uniform distribution of the TTX-containing solution (3). For both EDL and Soleus muscles, blocking the sciatic nerve for about one month with different daily doses of TTX, induces extrajunctional membrane changes in the blocked muscles which are closer to their denervated contralateral counterparts when using the higher dosage (Fig. 6). Equalisation between the effects of the two muscle treatments are actually obtained for EDL (not shown) whereas for Soleus some difference still remains even at the highest dose tested. Larger doses could not be used to avoid general toxic effects. Nonetheless, equalisation was also obtained for Soleus, whose axons are contained (unlike the EDL axons) in the large tibial fascicle of the sciatic nerve: the diffusion distance for TTX was reduced by enclosing the dissociated tibial fascicle in a

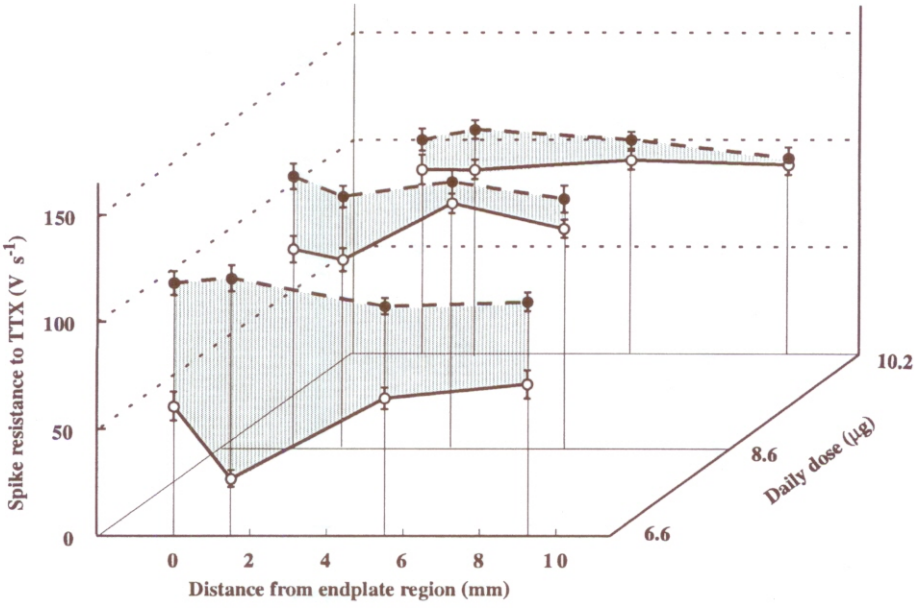

Fig. 6. - *Effects of increasing the daily doses of TTX, perfusing the rat sciatic nerve, on TTX-resistance of Soleus muscles measured along the extrajunctional region between endplates and myotendinous junction.*

Comparison is made with the contralateral denervated companions. Paralysed muscles (empty circles), denervated muscles (filled circles). The higher the dose, the smaller the difference between paralysed and denervated muscles. Each point represents mean±S.E. of 6-10 fibres from 3-5 muscles. Average block duration: 23 days (15-32). Experiments of this kind have been done, with similar results, both in purely paralysed and in reinnervated-paralysed muscles: in the latter, a nerve crush of the soleus nerve is placed close to the muscle and followed by quick reinnervation (~6-10 days for the superficial myofibres which are the ones electrophysiologically investigated). The paralysed muscles shown in the figure belong to the reinnervation paradigm.

narrower cuff, an experimental blocking condition which led to equalisation of paralysed and denervated Soleus muscles, when tested at one month distance (not shown). Finally the large difference existing at early times (about first 2 weeks; example in Fig. 2) is a genuine one, since it persists also when using the improved blocking conditions (not shown).

We performed entirely similar experiments for the contractile properties and observed that over a one month period an optimal conduction block induces in rat hind limb muscles (Soleus, EDL, Gastrocnemius medial and lateral, Plantaris, Tibialis anterior) equal atrophy and loss of tetanic force output.

III. *Nicotinic influences.*

The investigations just presented unequivocally show that the quantitative difference in effects on the extrajunctional membrane of paralysis and denervation is

only transient and thus well explained by the action of products of nerve degeneration. Activity appears sufficient to account for the physiological control, inhibitory in nature, of motoneurons on extrajunctional as well as contractile properties. Participation of nerve-borne chemical substances does not need to be called into play. Two points should however be made. First, the neural control of the junctional properties certainly involves chemical factors. Second, even for the general properties (extrajunctional or contraction-related), mention should be made of investigations that suggest a contribution to their neural regulation of the neurotransmitter acetylcholine or of factors released together with it.

Concerning the first point, during synapse formation motor terminals release on the myofibre membrane one or more signals for accumulation of junctional molecules such as AChRs and AChEsterase. Candidates include basal lamina derived protein agrin, brain derived glycoprotein *ARIA* and peptide *CGRP* (14, for a review). Both the accumulation and the induction of specific aspects of their structure and function are controlled by nerve-derived substances, in part in collaboration with evoked muscle activity (see 12). This area is presently under active investigation and is beyond the scope of the present review.

Concerning the second point, use has been made of poisons that interfere with cholinergic transmission, α-bungarotoxin (α BTx) (blocking AChRs) and botulinum toxin (blocking ACh release), to observe the effects on the extrajunctional myofibre membrane (11, 22). In one of these studies changes as large as after denervation have been observed, *even in the short term* (11), suggesting a role for acetylcholine as a trophic regulatory substance for the extrajunctional membrane, quite distinct from its primary function of transmitting the action potential. One problem with the interpretation of this result is that trauma made to the muscle during the initial (by injection) and continuing (by catheter) application of α BTx may produce by itself extrajunctional membrane changes. This is particularly true for chronically paralysed muscles which are much more reactive than normally active muscles to various stimuli (10), a possibility which has not been investigated. More recently, botulinum toxin has been used to see the effects on mRNAs for AChRs (28) and TTX-resistant sodium channels (29). In the first study the toxin was shown to have, at early times, an augmenting effect equal to that of denervation, again suggesting the participation of ACh (or of a factor released together with it) in the control of the extrajunctional membrane. In the study of Yang *et al.* the effect of botulinum toxin was striking since it was much larger than that of denervation (sevenfold) on mRNA encoding the TTX-resistant channel. This seems to indicate a novel, direct action of botulinum toxin on muscle fibres and makes the interpretation of the effects of the poison uncertain. In any event, spontaneous release of acetylcholine is not impaired in our long term muscles paralysed by TTX and is completely ineffective in preventing changes of the extrajunctional membrane as large as those following denervation. Thus the contribution of acetylcholine or other nerve-derived substances to the control of the *extrajunctional* membrane and of other general properties of muscle appears unnecessary.

SUMMARY

General properties of rat skeletal muscle (extrajunctional membrane and contractile properties) are subjected to tight physiological neural regulation, as indicated by their striking alterations (up- or down-regulation) following denervation. The main contributions of the literature concerning the nature of the neural signals which mediate this regulation, are reviewed. The physiological regulation of these general properties appears to be operated by the action potential activity evoked by motoneurons in the muscle fibres. No need to postulate the participation of nerve-borne chemical substances, acetylcholine or unidentified "trophic factors", arises from the main experimental evidence. The stronger response to denervation of extrajunctional membrane properties with respect to pure paralysis is best explained by actions of factors released during wallerian degeneration of the transected nerves.

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