ELECTROPHYSIOLOGICAL EVALUATION OF THE EFFECTS OF NAFTIDROFURYL ON SKELETAL MUSCLE REINNERVATION IN THE RAT

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The effects of naftidrofuryl on the reinnervation of the rat gastrocnemius muscle after its denervation by localized freezing of the sciatic nerve were tested with electrophysiological techniques. Daily intraperitoneal injections of 30mg/kg of naftidrofuryl do not increase the rate of axonal regeneration since early signs of reinnervation appeared as in controls around the 10th day after surgery. However, axonal sprouting is markedly increased since the percentage of muscle fibers with polyneuronal innervations was almost twice as high as in controls at the 15 and 21 day postoperative stages. The promoting effects of naftidrofuryl on polyneuronal innervation which gives rise to a redundant innervation during the first period of reinnervation constitutes an improvement of motor function which might be efficient for treatment of nerve injury and neuropathies.

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EVALUATION ELECTROPHYSIOLOGIQUE DES EFFETS DU NAFTIDROFURYL SUR LA REINNERVATION DU MUSCLE SQUELETTIQUE DU RAT.

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Les effets du Naftidrofuryl sur la réinnervation du muscle gastrocnémien du Rat après dénervation par congélation localisée du nerf sciatique ont été étudiés au moyen de techniques électrophysiologiques.

Des injections intrapéritonéales quotidiennes de Naftidrofuryl (30 mg/kg) n'augmentent pas la vitesse de régénération des axones, les premiers signes de réinnervation du muscle n'étant détectés, comme sur les muscles témoins, qu'à partir du 10ème jour post-opératoire. Cependant, chez les rats traités, une augmentation importante du pourcentage de fibres musculaires à innervation multiple et polyneuronale, est observée 15 et 21 jours après l'opération. Cela indique que le Naftidrofuryl favorise le bourgeonnement axonal qui donne naissance à une innervation motrice redondante pendant les premiers stades de la réinnervation.

L'effet sur le bourgeonnement axonal pourrait apporter une amélioration de la fonction motrice dans le traitement des lésions nerveuses périphériques ou de certains types de neuropathies.

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Several years are sometimes necessary for recovery of functional activity of muscles after nerve lesion. Many studies have been undertaken during the last decades to discover drugs that would either facilitate peripheral nerve regeneration and consecutive muscle reinnervation after traumatic injury or protect neurons against hostile microenvironments in neuropathies of various origins. Drugs have been discovered that intervene successfully at crucial metabolic steps in nerve regeneration. They act either by reducing the amount of scar tissue,[1,2] by enhancing protein synthesis in the neuron,[3–9] by accelerating axonal flow,[10–12] by stimulating proliferation of nonneural cells and thereby enhancing release of growth factors,[13–16] or by facilitating axonal sprouting.[4,8,12,17–22] However, their use in human therapy was often limited, since, in most cases, side effects and a low therapeutic coefficient did not allow prolonged treatment.

It was assumed recently that naftidrofuryl,* which is used with success in peripheral and cerebral ischemic accidents, could act as a protective factor for neurons not only by interfering at different levels in blood vessels[23–26] but also by interacting directly with nerve cells. It has been shown that, in vitro, naftidrofuryl at low concentrations activates several enzymes involved in energy transduction in synaptosomes prepared from brain cortex of rats submitted to different types of hypoxia,[27–29] promotes neurite outgrowth of dorsal root ganglionic neurons[30] and facilitates

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axonal growth and microtubule assembly in the tectal plate of mice embryos postimplanted in rotatory cultures.[31] Furthermore, this drug is able to interact with a fraction of neurons present in cultures of chick embryo forebrain, increasing the cyclic AMP levels, glucose transport, and glycolysis rate, and thereby promoting neuronal survival by a specific neuroprotective effect.[32] The suspected beneficial effect of naftidrofuryl on the nervous system has been recently documented by clinical results. An improvement of clinical and electrophysiological signs of diabetic polyneuropathy has been reported in humans.[33] The effect could be related, according to the authors, not only to the vasodilating properties of the drug but also to a specific neurotropic action. Moreover, naftidrofuryl is commonly prescribed for several months or years, both intravenously and orally for the treatment of peripheral arterial disease,[34–37] of acute as well as of chronic cerebrovascular disorders,[38,39] and of diabetic polyneuropathy.[33] Hence, the absence of toxicity in humans, the efficient action on nerve vascularization, its neuroprotective effect and its promoting action on neurite outgrowth, in vitro, suggested that the drug might have a beneficial effect on nerve regeneration and skeletal muscle reinnervation in vivo. To test this possibility, we used an experimental procedure previously developed[40–42] and already applied to a similar study with another drug.[22] Briefly, it consists of denervating a muscle of the limb by locally freezing the sciatic nerve in a defined site of the sciatic fossa. This procedure of denervation has been shown to provide more reproducible results and more complete anatomical and electrophysiologic nerve recovery than has been observed after other types of injury.[43] The basal lamina surrounding each nerve fiber remains intact all along the frozen and the distal segments, axonal sprouts develop in the continuous tubes that are formed and are then guided directly to the periphery. In the present work, two phenomena were evaluated by electrophysiology and compared in control and naftidrofuryl treated-animals: 1) the rate of motor axonal regeneration by detecting the first signs of reinnervation in the medial head of the gastrocnemius muscle, and 2) the degree of intramuscular axonal sprouting by comparing the extent and the time course of polyneuronal innervation which normally follows the first stage of monoinnervation at the beginning of muscle reinnervation.[44–46]

MATERIALS AND METHODS

Experiments were performed on male Wistar albino rats, about 6 weeks old, weighing 200 ± 20 g at the time of operation. The animals were observed closely for at least 3 days before surgery to eliminate those exhibiting abnormal growth or state. Animals were handled according to the French laws governing their use for experimental purposes. Seventy-six animals were used for the evaluations. Before any surgical procedure or dissection of the nerve-muscle preparation, the animals were deeply anesthetized with a 7% intraperitoneal chloral hydrate solution (0.6 ml/100 g body weight). Each animal was weighed at the time of surgery and just before sacrifice. The methods used in the present experiments are identical to those already reported.[22,40–46] They will be described briefly in this paper.

Surgical Procedure

The left sciatic nerve was frozen locally in situ using a liquid nitrogen cryode whose extremity was applied successively at three points on the same circumference of the nerve, just after its emergence into the sciatic fossa. The successive freezing and thawing cycles lasted about 30 seconds. The center of the 2–3 mm long frozen segment was indicated by a non-resorbable Flexilon thread that was inserted into the perineurium before freezing.

Electrophysiological Techniques

The nerve-muscle preparation (sciatic nerve, nerve to the medial head of the gastrocnemius muscle and gastrocnemius muscle) was rapidly removed from the left hindlimb, immediately transferred into a recording chamber and bathed with a continuous flow of oxygenated solution.[22] The nerve was stimulated through a suction electrode.

Each preparation was first examined under an operating microscope to detect muscle fiber contraction evoked by electrical stimulation of the nerve. After adding (+)-tubocurarine ($2–6.10^{-7}$M), the "white" portion of the muscle, which is the thinnest, was preferentially explored with intracellular microelectrodes to record endplate potentials (EPPs) evoked by stimulating the nerve. The intensity of 0.1–0.3 msec pulses delivered at 0.5 Hz was gradually increased in order to recruit an increasing number of axons and thereby to detect polyneuronal innervation. Muscle fibers were considered as polyneuronally innervated when they exhibited multiple EPPs characterized by abrupt changes in their amplitude due to the responses of motor axons with different excitation thresholds or when they were formed of two or more components of different latencies (Fig. 1). The percentage of muscle fibers exhibiting multiple EPPs was determined for each muscle from the responses (simple or multiple) of twenty-five to fifty innervated muscle fibers examined. Mean percentages ± standard error were calculated on the basis of these individual values (n = number of muscles).

After the electrophysiologic recordings, the distance between the approximate center of the frozen area, indicated by the Flexilon thread, and the point of entry of the nerve into the gastrocnemius muscle was measured and each muscle was weighed at day 10 (D_{10}) and day 21 (D_{21}) postoperatively.

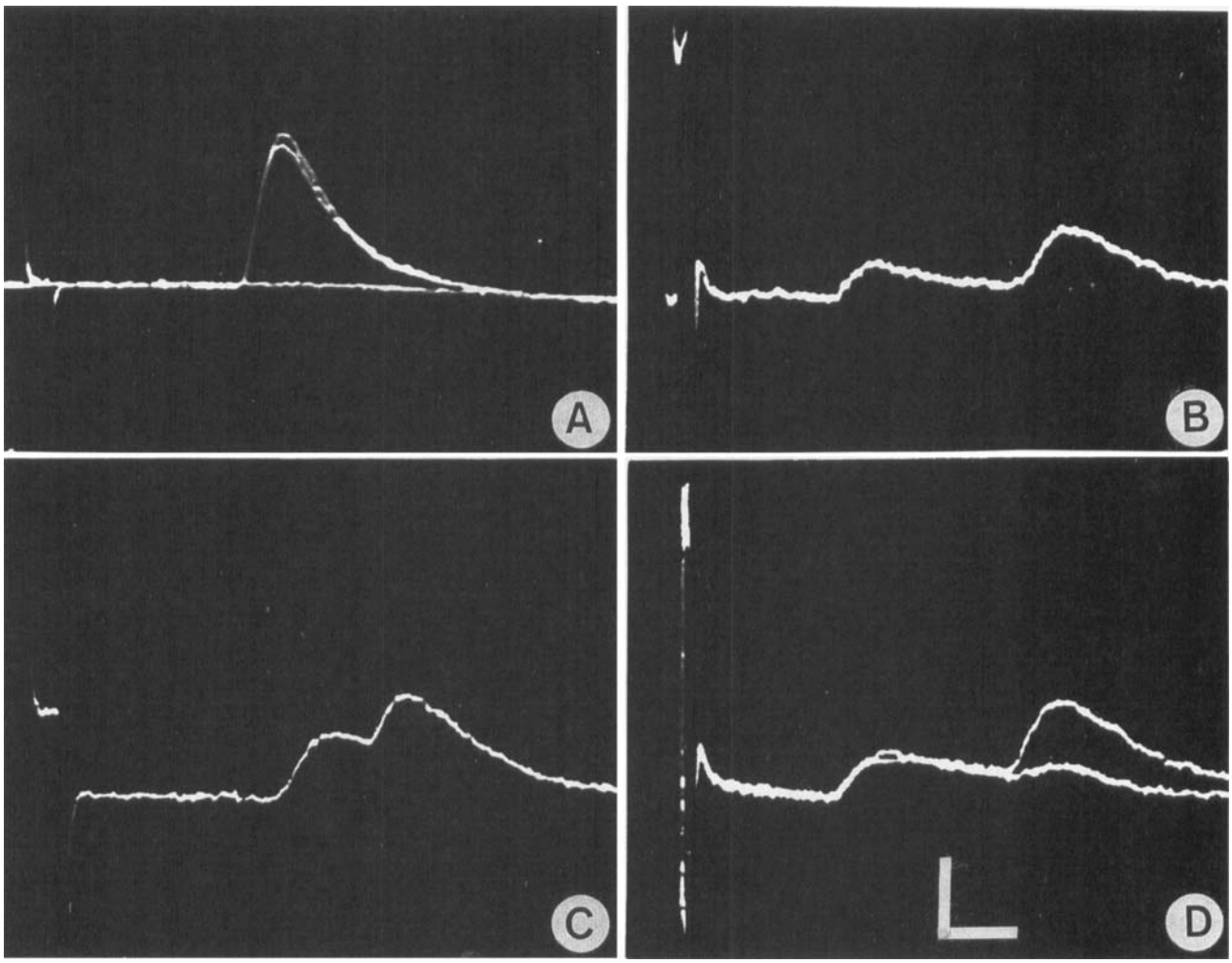

Figure 1. Examples of different types of EPPs recorded intracellularly at regenerating neuromuscular junctions in response to electrical stimuli applied on the nerve. ***A:*** *The response is a single "all-or-none" depolarization to stimuli of varying intensity (monoinnervated muscle fiber recorded at day 21 (D_{21}) in a muscle of the control group).* ***B–D:*** *Compound EPPs from muscle fibers with multiple innervation. In B and C, doubly innervated muscle fibers. Two EPPs of different latencies are obtained in response to a single stimulus, indicating that the axons innervating the same neuromuscular junction have different conduction velocities (recorded at D_{21} in muscles from the placebo group). D: Triply innervated muscle fiber. Nerve stimuli of varying intensity permit detection of three elementary EPPs with different latencies and amplitudes. The axons innervating the same neuromuscular junction have different conduction velocities and different excitation thresholds (for the two last responses) (recorded at D_{21} in a muscle of the treated group). (+)-tubocurarine $1–3.10^{-7}$M. Calibration bars: 2 mV; 2 msec.*

Groups of Rats

Operated animals were randomized to one of three groups:

1. In the first group (control group, 24 rats), the operated animals did not receive any injection. They were fed and cared for as were the animals of the two other series.
2. In the second group (placebo group, 24 rats), the animals received a daily intraperitoneal (IP) injection of sterile distilled water used in the third group to dissolve naftidrofuryl. The volume injected was 1 ml/100 g of body weight.
3. In the third group (naftidrofuryl group, 28 rats) the animals received a daily IP injection of naftidrofuryl (30 mg/kg as indicated by Oberval Laboratory) dissolved in sterile distilled water so that the injected volume was 1 ml/100 g of body weight as in the placebo group. A new solution was made up each week.

The injections in the placebo and treated groups started the day after the nerve injury and ended the day before the tests.

Stages of the Electrophysiological Tests

The electrophysiologic tests were made at three different stages: at 10 (D_{10}), 15 (D_{15}) and 21 days (D_{21}) after

Table 1. Body Weights (g ± S.E.) Before Surgery and at the Test Time.

Post-operative stages	Control group (n = 24)			Placebo group (n = 24)			Naftidropfuryl group (n = 28)		
	Before surgery	Test time	Increase (%)	Before surgery	Test time	Increase (%)	Before surgery	Test time	Increase (%)
D 10	204.7 ± 8.9 (n = 7)	266.3 ± 13.5	30.1	201.3 ± 6.0 (n = 8)	266.0 ± 15.5	32.1	203.1 ± 5.8 (n = 11)	232.9 ± 7.2*	14.7
D 15	204.7 ± 8.1 (n = 7)	320.7 ± 13.9	56.6	193.4 ± 4.0 (n = 7)	293.9 ± 10.1	51.9	203.3 ± 12.1 (n = 8)	263.9 ± 55.5**	29.8
D 21	197.6 ± 24.5 (n = 10)	320.8 ± 44.6	62.3	179.7 ± 22.9 (n = 9)	296.4 ± 28.6	65.0	192.7 ± 23.3 (n = 9)	306.9 ± 46.5***	59.2

*Significantly different ($P < 0.001$) from both the control and placebo groups.
**Significantly different ($P < 0.02$) from only the control group.
***Not significantly different from both the control and placebo groups.

nerve injury. These postoperative times correspond to three important stages of the curve of reinnervation already described in control rats[22], D_{10} corresponding to the beginning of reinnervation, D_{21} to the period when multi-innervation reaches its highest value and D_{15} to the period when the multi-innervation is increasing.

All the animals of the two first stages (D_{10} and D_{15}) were housed, treated, and tested in the laboratory. Those of the third stage (D_{21}) were operated and tested in the laboratory but they were housed and injected by the Société Trisa (Canly, France). Batches of naftidrofuryl used were 7005 (analysis 12817M) for stages D_{10} and D_{15} and 7005 (analysis 21623M) for stage D_{21}.

Statistical Analysis

Normally distributed data (animal weights and muscle weights) were compared with Student's t-test.

The Mann-Whitney U-test, non-parametric statistical method, was used for comparing the percentages of muscle fibers with polyneuronal innervation.

RESULTS

It is worth recalling that elimination of polyneuronal innervation occurs soon after birth in mammals so that all the motor endplates are singly innervated in adults.[47] Adult muscle fibers exhibit a single "all or none" EPP at the endplate in response to stimuli of varying intensity (Fig. 1A). This characteristic has also been observed previously in the unoperated medial head of the gastrocnemius muscle.[22,45,46]

Control Group

In this group the animals that were operated on but did not receive any injection exhibited normal growth. The increase in body weight was 30%, 57%, and 62% at D_{10}, D_{15}, and D_{21}, respectively (Table 1). In parallel, the mean distance (d) between the center of the frozen area to the point of nerve entry into the gastrocnemius muscle gradually increased with postoperative time from 22.4 ± 0.8 mm at D_{10} to 25.0 ± 1.2 mm at D_{21}; that is to say, an increase of 11.6% (Table 2). No significant change in the weight of muscles was observed during this period (Table 3).

At D_{10} no EPP could be recorded in any muscle from the superficial layer of muscle fibers. However, slight contractions evoked by stimulating the nerve were detected in the deep layers of muscle fibers in 1 of the 7 muscles tested at that postoperative time. These results, similar to previous ones, confirm the reproducibility of the model and show that reinnervation actually begins soon after D_{10}. At D_{15}, one third to one half of the white portion of the muscles was reinnervated. The mean percentage of muscle fibers exhibiting EPPs with two components was 19.7 ± 1.2% (n = 7) from a total of 36 out of 183 reinnervated muscle fibers examined. The mean percentage of polyneuronally innervated muscle fibers increased slightly to 22.4 ± 4.4% (n = 10) at D_{21} (Fig. 2), 58 of them being doubly and one triply innervated.

Placebo Group

The animals of this group exhibited a body growth similar to that of animals of the control group. At D_{21}, the percentage increase in body weight relative to the initial weight was 65.0% (Table 1). In parallel, the distance d increased 11.6% between D_{10} and D_{21} (Table 2). At D_{10} no sign of contraction of muscle fibers in response to stimulation of the nerve could be detected under the microscope in the 8 muscles examined. The mean percentages of polyinnervated muscle fibres at D_{15} (20.5 ± 2.1%) (n = 7) and D_{21} (23.4 ± 4.5%) (n = 9) (Fig. 2) were equivalent to those obtained at the same stages in the control group, both showing similarly a slight increase between the two stages. Hence the injections per se did not appear to interfere with the process of reinnervation. In the placebo group, 2 out of a total of 60 polyinnervated muscle fibers tested at D_{21} were triply innervated, a proportion equivalent to that of the control group.

Table 2. Distance (mm ± S.E.) Between the Site of Freezing on the Sciatic Nerve and the Gastrocnemius Muscle.

Control group (n = 24)			Placebo group (n = 24)			Naftidrofuryl group (n = 28)		
D 10	D 15	D 21	D 10	D 15	D 21	D 10	D 15	D 21
n = 7	n = 7	n = 10	n = 8	n = 7	n = 9	n = 11	n = 8	n = 9
22.4 ± 0.8	23.4 ± 0.8	25.0 ± 1.5	22.4 ± 0.5	22.9 ± 0.7	25.0 ± 1.0	22.2 ± 0.8*	22.8 ± 0.9*	25.3 ± 1.2*

**Values not significantly different from the corresponding control and placebo groups.*

Table 3. Muscle Weights at Varying Postoperative Stages.

	Control group (n = 17)		Placebo group (n = 17)		Naftidrofuryl group (n = 20)	
	D 10	D 21	D 10	D 21	D 10	D 21
n	7	10	8	9	11	9
Muscle weight (mg ± S.E.)	430.1 ± 35.2	399.2 ± 63.3	381.3 ± 44.5	388.4 ± 30.7	386.8 ± 42.2	369.4 ± 64.6

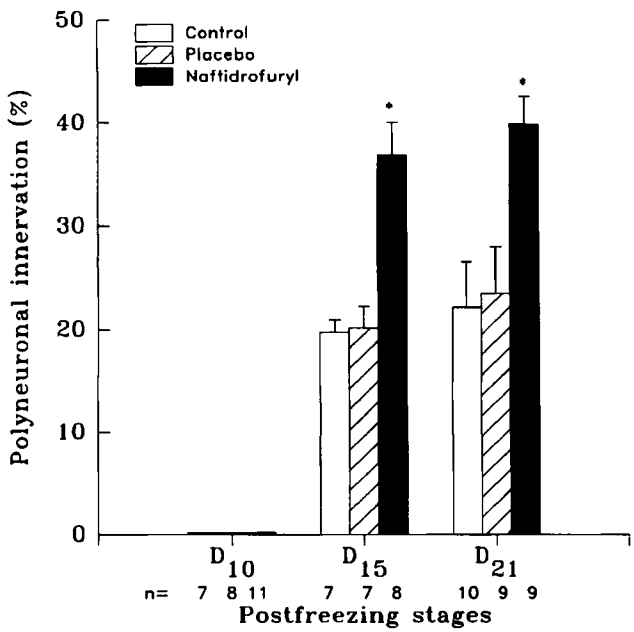

Figure 2. Incidence of polyneuronal innervation in the three groups of animals at three postfreezing stages (D_{10}, D_{15}, and D_{21}). Asterisks indicate that the corresponding values of the treated group are statistically different ($P < 0.001$) from the control values (control and placebo groups, whose values are equivalent). Mann-Whitney U-test. Bars: S.E.

Naftidrofuryl Group

The animals of this group showed reduced increase in growth, especially at the beginning of treatment, the percentage increases being 14.7 and 29.8 at D_{10} and D_{15} respectively instead of values averaging 30 and 55% at the corresponding times in the control and placebo groups. However, at D_{21} the level of normal growth was almost recovered since the percentage reached 59% versus 62.3 and 65.0% for the control and placebo groups, respectively (Table 1). The increase in the distance d of the nerve (14%) (Table 2) was similar to that of the other groups. A total of 88 out of 239 muscle fibers examined at D_{15} in 8 muscles were polyinnervated (mean percentage = 36.8 ± 3.2%), one of them exhibiting a triple innervation. The mean proportion of polyinnervated muscle fibers was a little higher at D_{21} (39.8 ± 2.7%) (n = 9) and corresponded to 90 doubly innervated and 5 triply innervated muscle fibers in a total of 239 muscle fibers examined. The percentages of polyinnervation were statistically different at the 0.001 level of significance from those obtained at the corresponding times for the control and placebo groups (Fig. 2).

DISCUSSION

The results provide further evidence of the reproducibility and reliability of localized freezing, to test the effects of drugs on muscle reinnervation. Control values were similar to those obtained in a previous study.[22] Moreover, the different parameters evaluated permitted not only determination of the rate of motor axonal regeneration and the intensity of axonal sprouting but also enabled the changes in these parameters to be related to the growth of the animals.

In spite of reduced increase in body growth observed at the beginning of the treatment with naftidrofuryl, no repercussions on nerve growth were detected at any postoperative stage. Naftidrofuryl does not appear to accelerate muscle reinnervation since, as in the control group, early signs of reinnervation were detected deep in the muscle at D_{10} in 1 of the 11 animals examined. However, naftidrofuryl markedly influences axonal sprouting, preterminal or terminal, which is the beginning of the increase in the level of polyneuronal innervation. The proportion of polyneuronally innervated muscle fibers was higher than in controls as early as D_{15} and the difference with controls, which was maintained and somewhat increased at D_{21}, was highly significant. At the later stage, the propensity of axons to sprout in treated animals was further documented by the rather high proportion of triply innervated muscle fibers recorded, 5.3% (5/95) versus 1.1% (1/88) at D_{15}. The lower value of the level of polyinnervation observed in the present work in

both the control and placebo groups is not due to a delay in the appearance of the phenomenon since, as shown previously,[22] D_{21} corresponds to the period when multiinnervation reaches its highest value in similar control animals. The redundancy of innervation in treated animals can be related to the results of a quantitative evaluation made by another team[48] which shows that 3 weeks after section of the rat sciatic nerve the number of axons in the distal stump of the nerve is markedly increased (about 200%) in naftidrofuryl-treated animals compared to controls. These observations support the view that the increase in the level of polyinnervation observed at D_{21} results, at least partially, from the increase in the preterminal sprouting which normally occurs after any injury.[41,42,43] A correlative increase in motor function has also been demonstrated by electromyography[48] in naftidrofuryl-treated animals. However, it is noteworthy that the transitory appearance of polyneuronal innervation that occurs during muscle reinnervation corresponds, in mammals, to the reexpression of embryonal features of the innervated muscle, that is to say to features of immature muscle fibers. It could be deduced that the increase in the extent of polyinnervation after treatment with naftidrofuryl is able to counterbalance, at a higher level than in controls, the immaturity of the neuromuscular junctions during muscle reinnervation. The mechanism by which naftidrofuryl promotes motor axonal sprouting remains unknown. However, one can postulate that this action does not result (at least totally) from its known action on blood vessels but from a direct action on the metabolism of the motoneurons, as has been demonstrated for other neurons in culture (see introduction). This view is also supported by data which show that, in contrast to naftidrofuryl, no facilitating action on motor function has been detected with two other vasodilator drugs.[48]

In conclusion, naftidrofuryl does not accelerate the rate of nerve regeneration and consequently of muscle reinnervation after a single localized freezing of the nerve. However its promoting effect on polyneuronal innervation, which gives rise to a redundancy of innervation during the first period of reinnervation, might constitute an improvement in the recovery of motor function. This specific action on muscle reinnervation as well as the absence of toxicity in humans make naftidrofuryl a possible candidate for treatment of traumatic nerve injury and neuropathies.

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