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Thyroid Hormone Enhances Transected Axonal Regeneration and Muscle Reinnervation Following Rat Sciatic Nerve Injury

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Improvement of nerve regeneration and functional recovery following nerve injury is a challenging problem in clinical research. We have already shown that following rat sciatic nerve transection, the local administration of triiodothyronine (T3) significantly increased the number and the myelination of regenerated axons. Functional recovery is a sum of the number of regenerated axons and reinnervation of denervated peripheral targets. In the present study, we investigated whether the increased number of regenerated axons by T3-treatment is linked to improved reinnervation of hind limb muscles. After transection of rat sciatic nerves, silicone or biodegradable nerve guides were implanted and filled with either T3 or phosphate buffer solution (PBS). Neuromuscular junctions (NMJs) were analyzed on gastrocnemius and plantar muscle sections stained with rhodamine α-bungarotoxin and neurofilament antibody. Four weeks after surgery, most end-plates (EPs) of operated limbs were still denervated and no effect of T3 on muscle reinnervation was detected at this stage of nerve repair. In contrast, after 14 weeks of nerve regeneration, T3 clearly enhanced the reinnervation of gastrocnemius and plantar EPs, demonstrated by significantly higher recovery of size and shape complexity of reinnervated EPs and also by increased acetylcholine receptor (AChRs) density on post synaptic membranes compared to PBS-treated EPs. The stimulating effect of T3 on EP reinnervation is confirmed by a higher index of compound muscle action potentials recorded in gastrocnemius muscles. In conclusion, our results provide for the first time strong evidence that T3 enhances the restoration of NMJ structure and improves synaptic transmission.

Key words: Thyroid hormones; peripheral nerve regeneration; muscle reinnervation; neuromuscular junction recovery; compound muscle action potentials

Traumatic injury to peripheral nerves results in considerable motor and sensory disability. Therefore, several research groups have tried to improve the regeneration of traumatized nerves by invention of favourable microsurgery. However, the clinicians soon noticed that despite advancement in microsurgical technique, complete sensory and motor recovery is rarely achieved (Kline and Hudson, 1995; Lundborg, 1988). Therefore, improvement of functional recovery following peripheral nerve injury continues to be an important clinical challenge. Since in some traumatic nerve injuries, nerve loss leaves a significant gap between the cut ends of the nerve, many researchers have been working on techniques to reconstruct nerve gaps by interlocking the severed nerve ends into artificial nerve conduits. Many silicone or other synthetic materials have been used to construct tubular nerve guides. These conduits have the potential to provide an external guidance channel to guide the outgrowing nerve fibers towards the distal target. Several studies have reported success using tubular guides to promote nerve regeneration across a gap. In fact, studies show that when the gap is bridged, the degree of recovery is higher and the beginning of reinnervation occurs sooner, when compared to unrepaired nerve (Danielsen et al., 1988; Buti et al., 1996; Doolabh et al., 1996). The use of nerve guides was even more successful when investigators began to introduce growth factors or cells that may enhance regeneration. Various neurotrophic factors and substrates including extracellular matrix components have been analyzed for regeneration properties (Rich et al., 1989; Dubuisson et al., 1993; Keeley et al., 1993; Laird et al., 1995; Newman et al., 1996; Utley et al., 1996; Lewin et al., 1997; Frostick et al., 1998; Chen et al., 2000; Moir et al., 2000; Itoh et al., 2001; Xu et al., 2003).

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Since Thyroid hormone is one of the most important epigenetic factors regulating the development of the nervous system (Innocenti and Berbel, 1991; Oppenheimer and Schwartz, 1997; Auso et al., 2004), our group studied the effect of local administration of T3 within silicone tubes on sciatic nerve regeneration (Voinesco et al., 1998; Schenker et al., 2002, 2003; Voria et al., 2006). Although silicone and other non biodegradable nerve conduits facilitate peripheral nerve regeneration in rodents, these conduits cannot be used in humans because they induce a chronic inflammatory reaction, (Merle et al., 1989). The design of artificial nerve substitutes has focused on the creation of biodegradable conduits in combination with cells or growth factors (Bini et al., 2004; Nakamura et al., 2004; Yang et al., 2004; Sundback et al., 2005; Ciardelli and Chiono, 2006). Among the several conduits, biodegradable nerve guides composed of an amorphous copolymer of DL-lactide and ε caprolactone [poly (DLLA-ε-CL) was developed by Polyganics (Groningen, the Netherlands) for clinical application. Recently we demonstrated that following rat sciatic nerve transection, the administration of T3 within these nerve guides induced a significant increase in the number and diameter of myelinated regenerated axons (Barakat-Walter et al., 2007). The fact that the ultimate regeneration is the connection of regenerated nerve fibers to a target organ prompted us to investigate whether the increased number of regenerated myelinated axons by T3 local administration is associated with improvement of peripheral target reinnervation. Since the neuromuscular junction is the only synaptic link between a motor neuron and the muscle fibers, we analyzed the gastrocnemius and plantar NMJs in normal and operated hind limbs following sciatic nerve transection and administration either of T3 or phosphate buffer in biodegradable or silicone nerve guides. The NMJs were analyzed at two stages of regeneration: 1) at 4 weeks after surgery, a stage which precedes the penetration of regenerated axons in the distal nerve segment and denervated muscles; 2) and at 14 weeks of surgery, at this later stage all the regenerated axons have already penetrated in the muscles and they reestablished contact with the denervated end-plates.

MATERIALS AND METHODS

Surgical Procedures

All animal procedures were conducted according to local guidelines for care and use of experimental animals. The surgical procedures were carried out under general anaesthesia; efforts were made to minimize suffering of animals during the post-surgery period.

Forty-four adult Wistar rats of 8–10 weeks, weighing 250–300 g, were anaesthetized by intraperitoneal injection of pentobarbital (75 mg/kg). Under aseptic conditions, the skin of the right leg was cut parallel to the femur. The sciatic nerve was exposed by splitting the superficial gluteus muscle. Under a dissecting microscope, the right sciatic nerve was transected close to the vertebral column, and a 6-mm nerve segment removed. The distal and proximal cut ends of the sciatic nerve were secured into the ends of either 10-mm long silicone tubes (an internal diameter of 1.5 mm and an external diameter of 3 mm, Silclear, Medical Grade Tubing, Midland, Michigan) or biodegradable tubes (copolymer of DL-lactide and ε-caprolactone, with an internal diameter of 1.5 mm and wall thickness of 0.3 mm, Polyganics). The transected nerve was held in place with polyamid epineural sutures (Ethicon 8-0, Ethicon, Norderstedt, Germany) at each end, leaving a gap of 8 mm between the nerve stumps. After placement, the tubes (nerve guides) were filled with either a sterile solution of 3,3′,5 triiodo-L-thyronine, (Sigma, St. Louis, MO, 1 mg of T3 was dissolved in 1 ml 0.01N NaOH, then the solution was neutralized with 0.01 N HCl, the final pH is 7.4) or with PBS. Thus four groups of experimental rats were created: two PBS-treated rats (the transected nerve bridged either by silicone or biodegradable guides filled with PBS) and two T3-treated rats (the transected nerve bridged either by silicone or biodegradable guides filled with T3). In each experiment the four experimental rats were taken from the same litter. The overlying muscle and skin of the thigh were sutured with 6-0 and 4-0 polyamid-sutures. The animals were allowed to survive for either 4 or 14 weeks after surgery. According to our previous work, these time points correspond to two specific stages of regeneration which precede or follow the penetration of the regenerated axons in the nerve segment distal to the silicone guide and in the denervated muscles (Voinesco et al., 1998; Schenker et al., 2002). The animals had free access to food and water with 12 hr-light/12 hr-dark cycle. In three additional rats, the right sciatic nerves were transected and the nerve regeneration was prevented by closing the distal part of the nerve conduit.

During survival, the behaviour and gait of each animal was observed and the return of motor and sensory functions was examined. The rate of regeneration of sensory axons was evaluated by a skin pinch test. After very light anaesthesia, the skin of the foot was mechanically stimulated by gentle pinching with a pair of fine forceps (diameter of the tip = 0.2 mm), beginning distally in the foot and moving proximally in 1-mm intervals until a muscle contraction. The recovery of reflex motor function was observed by a toe spreading test after rapid lowering of the animal toward the ground. The sign of recovery is the abduction of the fifth digit. The unoperated foot served as a control for the success of the sudden lowering. No rats died during the operation or in the follow-up period.

Standard Electrophysiology

After 14 weeks of nerve repair, and before perfusion, the unoperated contralateral nerve and the segment proximal to the regenerated sciatic nerve from each rat was electrically stimulated and the evoked compound muscle action potentials (CMAP area and amplitude) were recorded from the flexor gastrocnemius muscle as described in previous studies (Barakat-Walter et al., 2007). The relevant parameters were extracted from the reports automatically generated by the EMG machine. The data were compiled and mean ± SD

calculated using Microsoft Excel (Microsoft, Downers Grove, IL).

Processing for Microscopic Analysis

Following survival times of 4 or 14 weeks, the operated rats were deeply anaesthetized then transcardially perfused with 0.1-M phosphate-buffered saline (PBS) containing 0.1% heparin and 0.1% procaine, followed by a fixative solution of 4% paraformaldehyde in 0.1-M PBS at pH 7.4. The silicone or biodegradable guides with regenerated nerves were excised, the guides cut and the regenerated nerves removed. The hind limb muscles (gastrocnemius and plantar) were also taken. Moreover, from each animal, the sciatic nerves and muscles from unoperated contralateral limbs were collected.

All specimens were first post-fixed for 2 hr at 4°C in the same fixative. For immunohistochemical studies, the tissues were cryoprotected in 30% sucrose over-night then embedded in Tissue-Tek O.C.T Compound medium (Sakura Finetek Europe B.V., Zoeterwoude, the Netherlands), and rapidly frozen in liquid nitrogen. For semi-thin sections, nerve segments were placed in 2% osmium tetroxide in PBS for 3 hr, followed by dehydration in a series of graded ethanols and embedded in Epon.

Light Microscopy and Morphometric Analysis of Regenerated Sciatic Nerves

Transverse semi-thin sections (1-μm thick) were cut from the midpoint of regenerated nerves and stained with Toluidine blue for light microscopy and morphometric analysis. All microscopic analysis and counting were performed blind to the experimental condition. The number of myelinated axons was estimated on sections from each group of rats using systematic sampling and the StereoInvestigator program (MBF Bioscience, Williston, VT) as described previously by (Barakat-Walter et al., 2007).

Immunocytochemistry for Identification of Myelinated Motor Axons

Cryostat transverse sections 12-μm thick were prepared from the midpoint of regenerated nerve segments taken 14 weeks after surgery. The sections were first incubated for 30 min, at room temperature (RT) in blocking solution (0.1 M phosphate buffer, pH 7.4, containing 15% of normal donkey serum and 0.5% triton X-100), followed by incubation overnight at 4°C with polyclonal primary antibodies; anti-Choline-Acetyltransferase (ChAT, 1:50; AB144P, Millipore, Billerica, MA) and anti-neurofilament (NF 200 kDa 1:200; AB1982, Millipore). Sections were washed 3 times (3 × 10 min) in PBS and incubated for 2 hr at RT with secondary antibody, Alexa Flour 488 Donkey anti-Goat 1:200 (Invitrogen Carlsbad, CA) and Cy3-conjugated Donkey anti-Rabbit (1:500; Jackson Immuno Research, West Grove, PA). Following another wash with PBS, sections were mounted with VectaShield Mounting Media (Vector Laboratories, Burlingame, CA).

Preparation of Hind Limb Muscle Sections for Neuromuscular Junction Analysis

Serial cryostat longitudinal sections were cut (20 μm thick) from gastrocnemius and plantar muscles from operated and unoperated hind limbs from each rat. Sections were incubated for 45 min in 1 μg/ml tetramethyl rhodamine-conjugated α-bungarotoxin (α-BTX, Invitrogen) at room temperature to stain acetylcholine receptors. Sections were washed in PBS and blocked in 0.1% triton, 10% goat serum in PBS for 30 min before incubation overnight, at 4°C, with the primary polyclonal antibody (1:200), directed against the 200-KDa neurofilament protein (AB1982, Millipore). After washing with PBS, the muscle sections were incubated for 2 hr with a 1:200 dilution of the secondary antibody conjugated to Alexa Fluor 488 (Invitrogen). Muscle sections were observed under fluorescence microscopy (Zeiss, Axioplan 2). As a variation can exist in the structure of the end-plates, and to remove bias in interpretation, all end-plates observed in the same microscope field were photographed using an image-acquisition program (Zeiss, AxioVision with Axiocam HRc). For each field, a series of multichannel fluorescent pictures were taken in successive focal planes of the slice to obtain z-stacks. The photographed images were imported into an image-processing program (ImageJ 1.40, NIH, Bethesda, MD) to analyze the end-plates. To avoid counting and measuring the same junction twice, every third section was analyzed. The morphometric parameters of every EP were determined by drawing the highly labelled post-synaptic membrane following the edge of each fold. The shape complexity was estimated using the following formula: $C = P^3/(4\pi^2 \star A \star D_F)$, where P = Perimeter, A = Area and D_F = Ferret Diameter (Panaite et al., 2008).

Quantitative fluorescence imaging: The fluorescence intensity of rhodamine-α-bungarotoxin labelling at the EPs was measured by using a quantitative fluorescence imaging technique. To calculate the density of pixels within the endplate region, the background fluorescence was approximated by selecting a boundary region around the junction and automatically subtracted from the original image, then the fluorescence intensity of the pixels inside the EP was measured. More than 1500 EPs were measured from 5 rats from each experimental condition. The same EP parameters were also measured on unoperated contralateral limb muscle sections to normalize the data and to quantitate the recovery of reinnervated EPs. All analyses and counts were performed blinded to the experimental animal condition.

Statistical Analysis

The statistical analysis was performed using Analyze-it add-in for Excel (Analyse-it Software, Ltd., Leeds, UK). The measured size, shape complexity and the α-BTX labelling intensity of EPs in PBS and T3-treated rats were normalized and compared to unoperated hind limb EP. First, the distribution of values was checked for normality, transformations were applied when necessary, and then comparisons were performed using the hierarchical ANOVA and Fisher's protected t-test; $P < 0.05$ was considered significant. Graphics were composed by using Microsoft Excel based on mean values per animal.

RESULTS

The behaviour and gait of each operated rat was observed throughout the survival period. During the first 4 weeks post surgery, PBS and T3-treated rats walked

with an abnormal gait on the dorsal surface of the affected toes. Afterwards, improvement in gait and resting posture were observed first in T3-treated rats and later (4–5 days) in PBS-treated rats. Between 12–14 weeks of nerve regeneration, the gait returned more or less to normal and the toes, which were initially in the flexed position at rest, changed gradually to resemble the neutral position. In addition, the rapid lowering of rats' results in reflex spreading of the toes of operated hind limbs and a mechanical stimulation is followed by withdrawal of the operated leg. All our observations showed that PBS-treated rats displayed a slower recovery and clear delay in the return of sensitive and motor functions compared to T3-treated rats.

Morphological and Morphometric Evaluation of the Regenerated Sciatic Nerve

The microscopic examination of semi-thin sections taken from the midpoint of numerous regenerated nerves, at 4 or 14 weeks after surgery confirmed our previous results which showed that T3-regenerated nerves either within silicone or biodegradable guides were more mature than PBS regenerated nerves. In addition, the results of axon counting and statistical analysis confirmed also two findings: the number of regenerated myelinated axons within biodegradable guides is identical to the number of regenerated axons within silicone guides, thus they are grouped together, and the second point is that the number of regenerated myelinated axons in T3-treated nerves (10777 ± 1063) was significantly ($P < 0.001$) higher than that in PBS-treated nerves (6953 ± 1531) (Voinesco et al., 1998; Barakat-Walter et al., 2007).

Identification of Myelinated Motor Axons

The ChAT and NF double immunostaining allows motor axon identification (ChAT positive) among the total myelinated axons (NF positive) and provides information about the organization of motor axons in fascicles (Lago and Navarro, 2006). In intact contralateral nerves, 22.3% of myelinated axons were ChAT positive, which are organized into clear fascicles (Fig. 1A-C). In operated nerves treated with PBS administrated either within silicone or biodegradable guides, and after 14 weeks of regeneration, about 19.5% of myelinated axons displayed ChAT immunostaining. In this experimental condition the regenerated motor axons were scattered and intermingle with the NF positive myelinated axons (Fig. 1D-F). In T3-treated nerves, a larger percentage (25.9%) of myelinated axons were ChAT immunoreactive, most of these motor axons have the tendency to group together (Fig. 1G-I), which indicates that T3 improves functional recovery, because the fascicular organization of axons is related to locomotor function.

Changes in Neuromuscular Junctions Following Denervation and Reinnervation

To investigate whether the increase in the number of regenerated axons by local T3 treatment is associated with an improvement in peripheral target reinnervation, we analyzed the gastrocnemius and plantar neuromuscular junctions (NMJs) stained with rhodamine α-BTX and neurofilament antibody in PBS and T3-treated rats.

Gastrocnemius NMJs. Four weeks after surgery, the histological examination of gastrocnemius muscle sections prepared from unoperated contralateral limbs demonstrated the presence of several end-plates (EPs) and fascicles of axons (Fig. 2A,B). Most EPs displayed typical pretzel-shape with strong fluorescence labelling and direct contact to nerve terminals (Fig. 2C,D). However, gastrocnemius muscle sections prepared from operated limbs treated with either PBS (Fig. 2E-H) or with T3 (Fig. 2I-L) showed mainly the presence of denervated EPs with an atrophic aspect and faint fluorescence labelling. On some sections, a few regenerated axons which reinnervate about 3% ± 1.5 of EPs in PBS and 7% ± 2.5 of EPs in T3-treated limbs were observed (Fig. 2K). The measurement of the size, shape complexity and fluorescence intensity of EPs revealed that in both PBS and T3-treated limbs, all parameters were clearly smaller in operated limbs compared to those in unoperated limbs. In fact, EPs from operated limbs (PBS and T3 considered together) showed a 30% ± 3 decrease in their size, 24% ± 4 in shape complexity and 40% ± 6 in the total fluorescence intensity compared to normal EPs from unoperated limbs. Therefore, at this stage of regeneration, no significant difference could be detected in EP parameters between PBS and T3-treated limbs, and also there is no difference between rats implanted with silicone or biodegradable nerve guides.

After fourteen weeks of nerve repair, the gastrocnemius muscle sections of either PBS (Fig. 3A-D) or of T3 (Fig. 3E-H) treated limbs showed the presence of regenerated axons which reinnervate about 83% ± 1 of EPs in PBS and 88% ± 2 in T3-treated limbs. This reinnervation likely leads to the restoration of the morphological aspect of EPs because most reinnervated EPs exhibited a pretzel-shape with intense fluorescence labelling. The statistical analysis of the morphometric results revealed that the restoration of size and shape complexity of EPs in T3-treated limbs was significantly ($P < 0.01$) higher than in PBS-treated rats; in T3 treated limbs, EPs recover 89% ± 6.5 of their size and 83% ± 7 of shape complexity compared to 75% ± 6 and 70% ± 5 in PBS-treated limbs, respectively. Moreover, the recovery of the total fluorescence intensity by EPs is also significantly ($P < 0.05$) higher in T3-treated limbs (68% ± 6) than in PBS-treated limbs (59% ± 4; Fig. 4). Another important point revealed by the statistical analysis of morphometric results is that no difference in the EP parameters is observed between limbs implanted with biodegradable or silicone guides. These results again confirmed that nerve regeneration within biodegradable guides is equivalent to that within silicone guides. When nerve regeneration is prevented, all EPs remained dener-

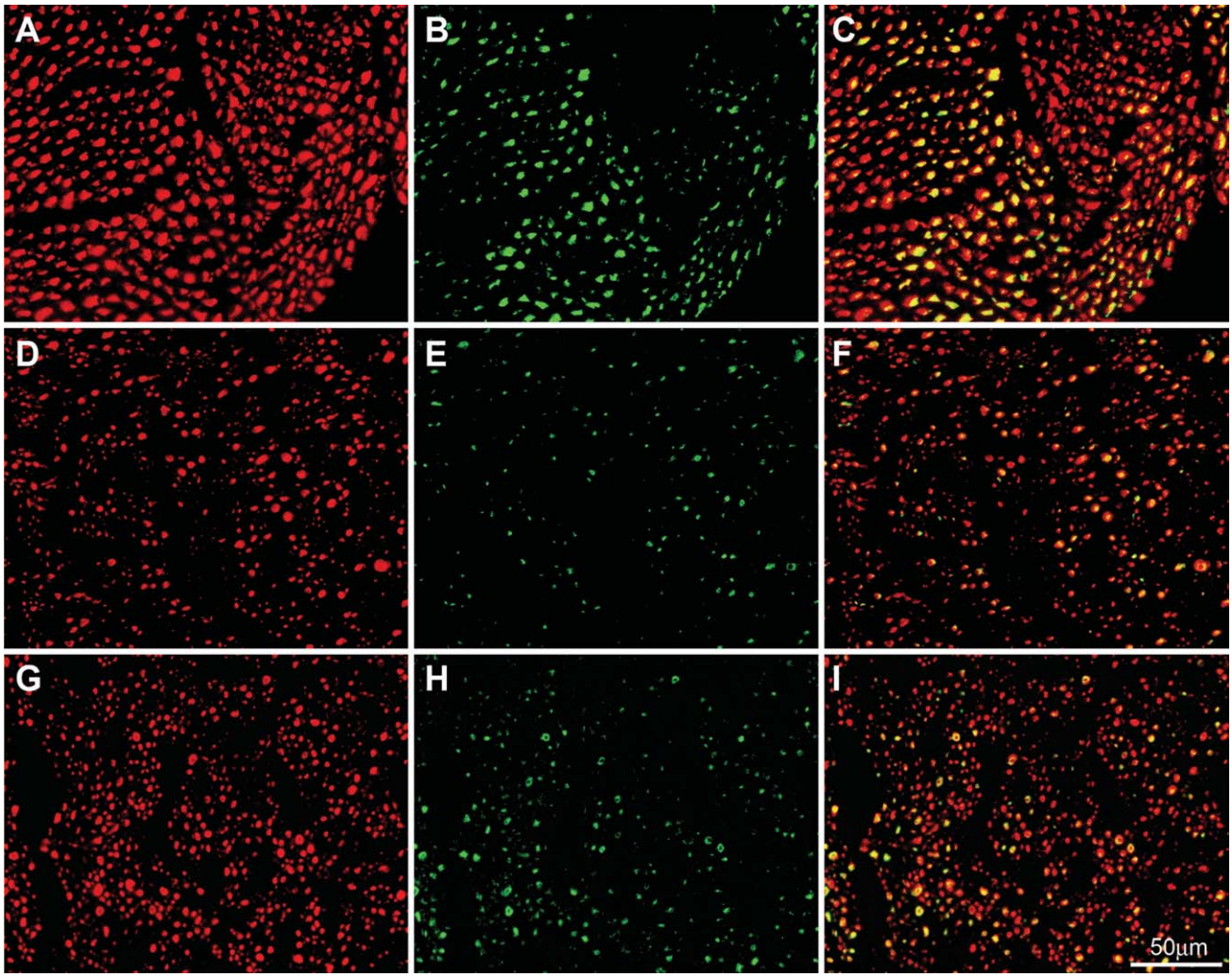

Fig. 1. Cryostat cross sciatic nerve sections double immunolabelled with NF antibody (to stain all myelinated axons - in red) and ChAT antibody (to stain myelinated motor axons - in green). Unoperated contralateral sciatic nerve (**A-C**); PBS-regenerated sciatic nerve (**D-F**) and T3-regenerated sciatic nerve (**G-I**). In contralateral nerve section, a clear fasciculation pattern of ChAT positive axons can be observed. In PBS-treated nerves, the motor axons are thin and scattered among all the myelinated axons; in T3-treated nerve, larger myelinated motor axons have the tendency to group into small fascicles.

vated, characterized by an atrophic, simple and elongated shape without distinguishable folds and weak fluorescence labelling (Fig. 3I-L). The morphometric measurement of long-term denervated EPs revealed that the size, shape complexity and especially the total fluorescence intensity were clearly lower than those of reinnervated EPs.

Plantar NMJs. On unoperated contralateral muscle sections, the normal plantar EPs have a small size but characteristically shaped ''pretzel-like'' pattern (Fig. 5A-D). After 14 weeks of nerve repair, the plantar muscle sections prepared from operated limbs treated with either PBS (Fig. 5E-H) or T3 (Fig. 5I-L) showed the presence of regenerated axons in direct contact with small size EPs. However, T3-treated EPs look more like the normal EPs characterized by a pretzel-shape. At this stage of regeneration, about 82% ± 2.5 of EPs were reinnervated in PBS-treated limbs and 87% ± 3 in T3-treated limbs. Some polyneuronal reinnervated EPs were found in PBS-treated muscle sections especially (Fig. 5H), which is considered normal and a transitory character during development and regeneration (Ijkema-Paassen et al., 2002; Witzemann, 2006). When plantar muscle reinnervation was prevented, the long-term denervated endplates displayed broken and fragmented forms that indicated degeneration (Fig. 5M-P).

Here again the morphometric measurement of EPs and the statistical analysis of the results revealed that T3-treatment significantly enhances the recovery of EPs, compared to PBS-treated EPs (Fig. 6).

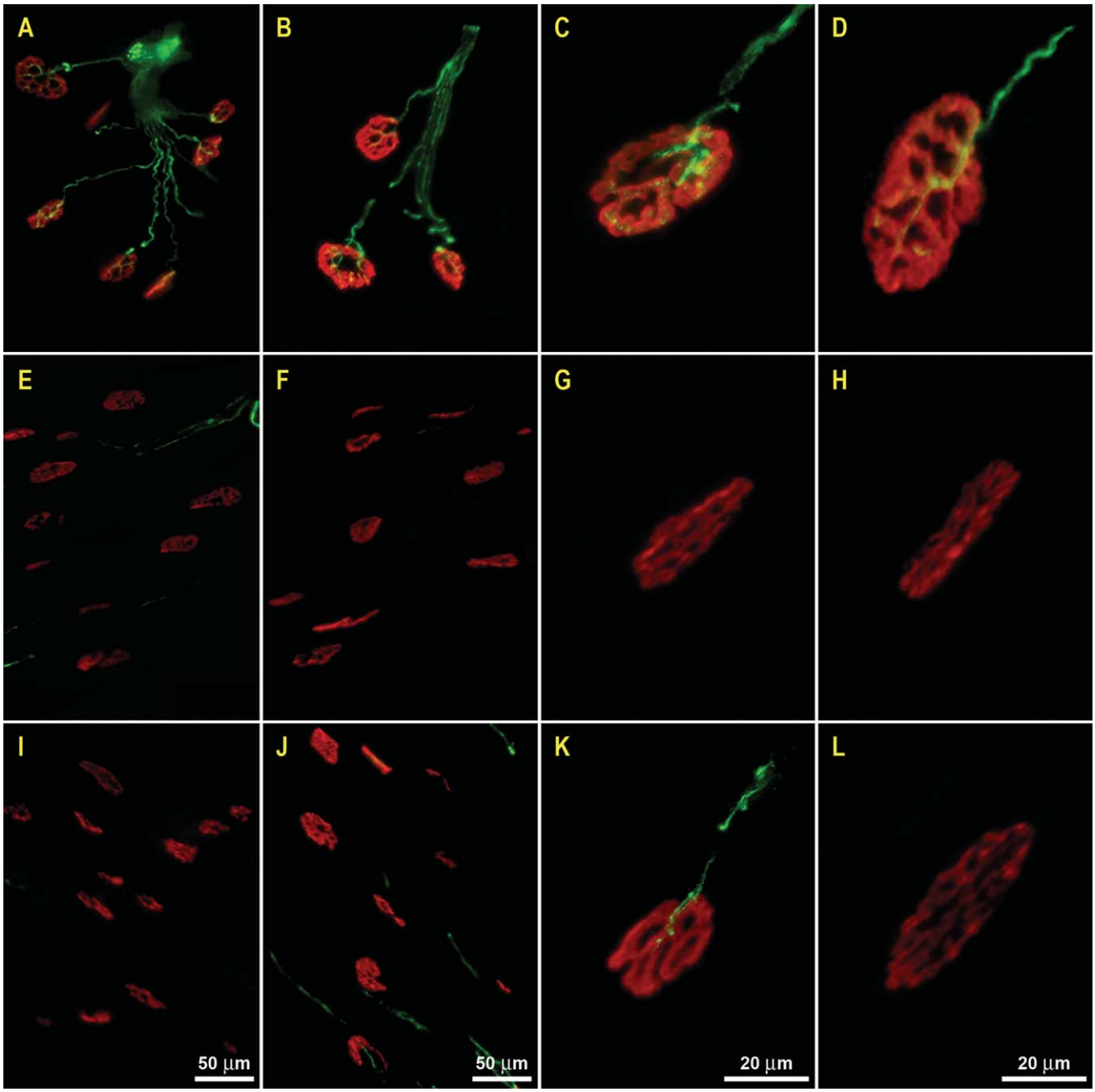

Fig. 2. Representative micrographs of gastrocnemius muscle cryostat sections taken 4 weeks following surgery from: contralateral unoperated limbs (**A-D**), PBS-treated limbs (**E-H**), and T3-treated limbs (**I-L**). Sections were stained with rhodamine conjugated α-bungarotoxin (red) and neurofilament antibody (green). Contralateral unoperated limb sections show the presence of intramuscular fascicles of axons that innervate typical pretzel-shape EPs. Sections from PBS- or T3-treated limbs show mainly the presence of denervated EPs, free from axon contact and displaying lengthened forms with faint fluorescence labelling and less distinct folds. A few regenerated axons reinnervating rare EPs can be observed in T3-treated limbs (K).

Electromyographical Evaluation of the Regenerated Nerve

The beneficial effect of local T3 administration on target muscle reinnervation was confirmed by recording CMAP in gastrocnemius muscles. At 14 weeks of regeneration, the evoked responses in PBS or in T3-treated limbs consisted of typical patterns of CMAPs recorded in unoperated limbs (Fig. 7). However, in T3-treated limbs, the mean recovery index of CMAP area and amplitude (area: 59.12 ± 10.22; amplitude: 19.87 ± 4.07) were significantly ($P < 0.05$) higher than in PBS-treated limbs (area: 47.15 ± 5.44, amplitude: 14.87 ± 2.29). This electromyographical evaluation confirmed that regenerated axons could form functional synapses with

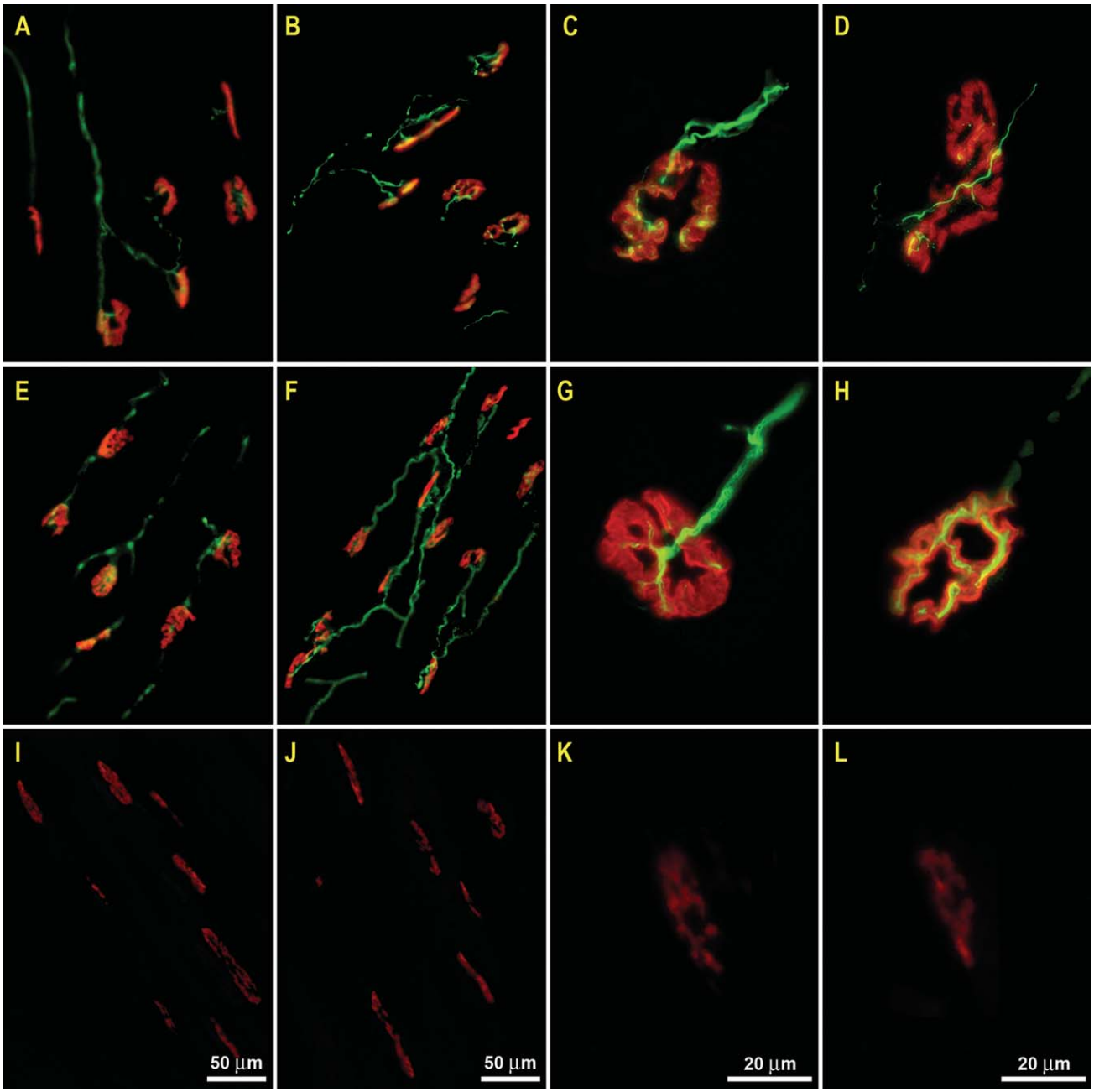

Fig. 3. Representative micrographs of gastrocnemius muscle cryostat sections taken 14 weeks following surgery from: PBS-treated limbs (**A-D**); T3-treated limbs (**E-H**); long-term denervated limbs (**I-L**). At low magnification, on both PBS and T3-treated muscle sections, regenerated axons reinnervating most of the EPs can be observed. Note that most reinnervated EPs exhibit more or less a typical form with postsynaptic folds overlaid by nerve terminals. In contrast, the long-term denervated EPs exhibit thin lengthened shapes, faint labelling and postsynaptic membranes shrunk and deformed.

distal target muscles. Nevertheless, it is worth emphasizing that at this stage of regeneration the CMAPs in both PBS or T3-treated nerve remained significantly lower than CMAPs recorded in unoperated limbs (area: 77.93 ± 6.08. $P < 0.001$; amplitude: 31.75 ± 3.23. $P < 0.01$).

DISCUSSION

Following a severe injury of peripheral nerves, the loss of motor function greatly affects the life of patients. Therefore, it is necessary to accelerate and improve as quickly as possible the regeneration of transected axons, the reinnervation of denervated peripheral muscles and

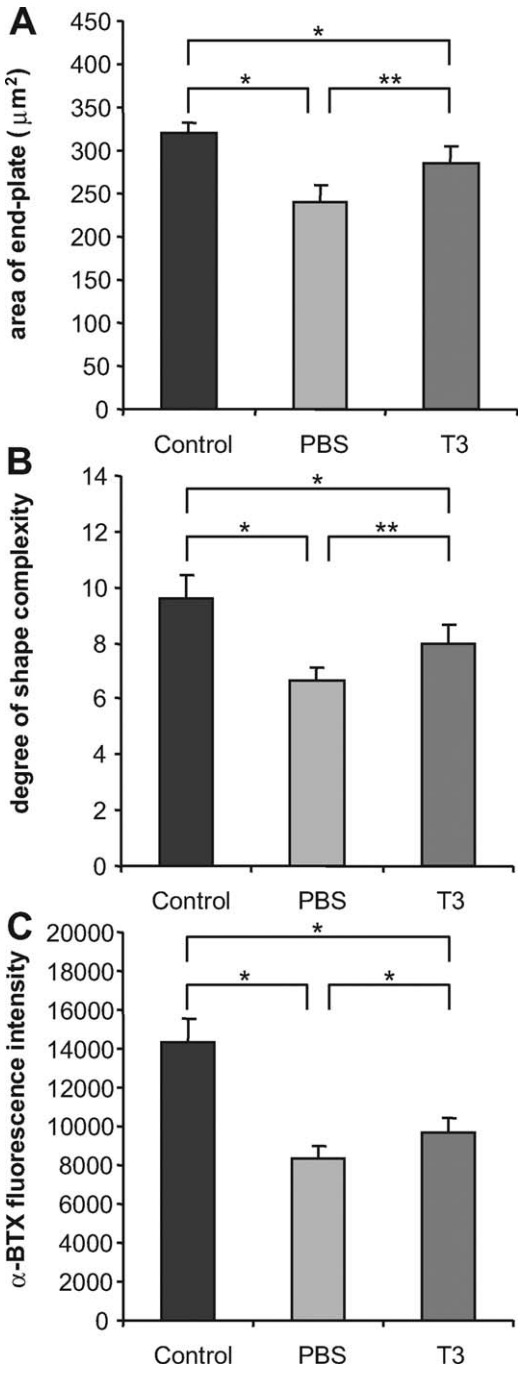

Fig. 4. Histogram of the mean ± SD of size (**A**), shape complexity (**B**) and total α-BTX fluorescence intensity per EP (**C**), measured on longitudinal gastrocnemius muscle sections stained with NF and α-BTX. After 14 weeks of nerve repair, more than 1500 EPs (n = 5) were measured on contralateral unoperated limb sections (Control), PBS-treated limb sections (PBS), and T3-treated limb sections (T3). Since nerve regeneration in biodegradable guides is similar to that in silicone guides, data were pooled. The statistical analysis revealed a significant difference (*$P < 0.01$; **$P < 0.05$) between PBS and T3 treatment.

the recovery of motor and sensory functions. Since in the animals the functional recovery of injured peripheral nerve is difficult to assess by one technique (de Medinaceli et al., 1982), in the present study, we combined morphological and morphometric analysis of regenerated motor axons and hind limb NMJs with electromyography recording. The results of these techniques provide, for the first time, strong evidence that the local administration of T3 at the level of the transected sciatic nerve enhances hind limb muscle reinnervation and functional recovery. Counting ChAT positive axons on PBS and T3 treated nerve sections showed that T3 enhances the number of regenerated motor axons. Also, the morphological and morphometric analysis of gastrocnemius and plantar NMJs revealed that T3 significantly increases the recovery of the size, shape complexity and AChRs concentration of reinnervated EPs. Moreover, T3 raises the index of CMAPs recorded in hind limb gastrocnemius muscles, which indicates an augmentation in the number of formed functional synapses. Together, our results clearly demonstrate that the increase in number of regenerated myelinated axons by T3 treatment described in the present and previous studies (Voinesco et al., 1998; Barakat-Walter et al., 2007) is linked to an improvement in muscle reinnervation, synaptic transmission and motor functional recovery. These findings suggest that consideration should be given to the possibility of using exogenous T3 in the clinical therapy of human peripheral nerve injuries.

To promote axonal growth over a gap produced in a severely injured peripheral nerve, various microsurgical techniques have been used and several trophic factors or molecules known to stimulate axonal growth tested. Thyroid hormones are one of the most important physiological regulators of mammalian nervous system development and maturation (Innocenti and Berbel, 1991; Oppenheimer and Schwartz, 1997; Auso et al., 2004), and therefore we suspected that this factor would play an important role in nerve regeneration. For several years our group has been interested in the effect of T3 on sciatic nerve regeneration. We have already shown that following adult rat sciatic nerve transection, the administration of T3 within nerve guides significantly increased the number and the myelination of regenerated axons; T3 also rescued a significant number of axotomized sensory neurons from death (Voinesco et al., 1998; Barakat-Walter, 1999; Schenker et al., 2002, 2003; Voria et al., 2006; Barakat-Walter et al., 2007). Since it is reported that the greater the number of axons that regenerate through the distal nerve, the greater the extent of neurological recovery (Fawcett and Keynes, 1990), we hypothesize that T3 treatment which increases the number of regenerated axons would also improve functional recovery. However, some recent studies reported that stimulation of axonal regeneration does not systematically produce an enhancement of functional outcome, as some surgical techniques or localized drug delivery increased the number of regenerated axons but did not improve target reinnervation (Lago and Navarro, 2006; Piquilloud et al., 2007). Therefore, it is important to verify whether in our experimental conditions the

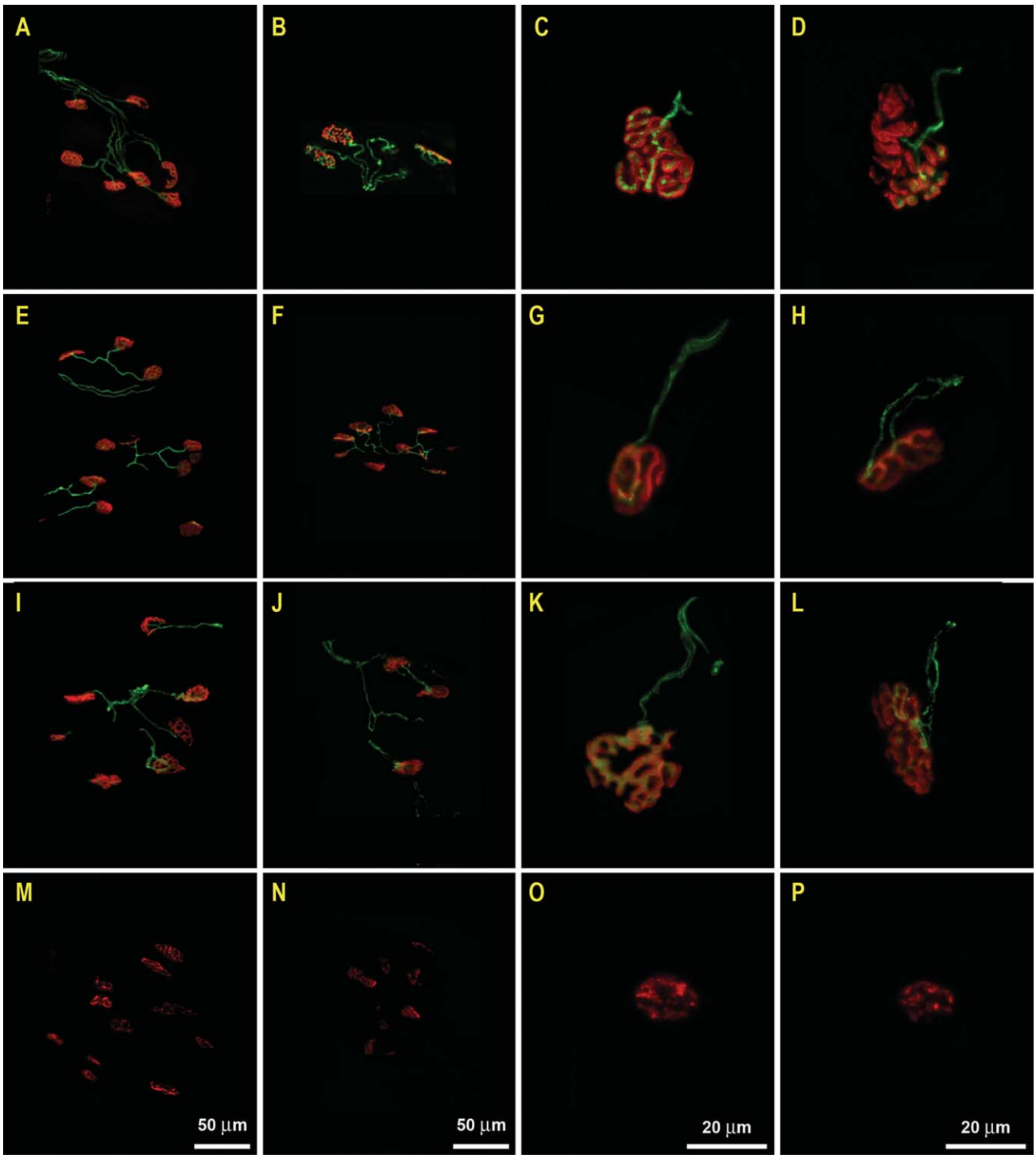

Fig. 5. Representative micrographs of plantar muscle cryostat sections taken either from: contralateral unoperated limb (**A-D**) or 14 weeks after surgery: PBS-treated limbs (**E-H**), T3-treated limbs (**I-L**) and long-term denervated limbs (**M-P**). On unoperated limb sections, intramuscular fascicles of axons innervating small EPs can be observed. On both PBS and T3-treated muscle sections, several small EPs reinnervated by regenerated axons can be seen. At this stage of regeneration, some EPs with polyneuronal reinnervation can be found. Whereas the long-term denervated muscle sections show only the presence of small, shrunken and distorted EPs, the pattern of postsynaptic folds is not distinct.

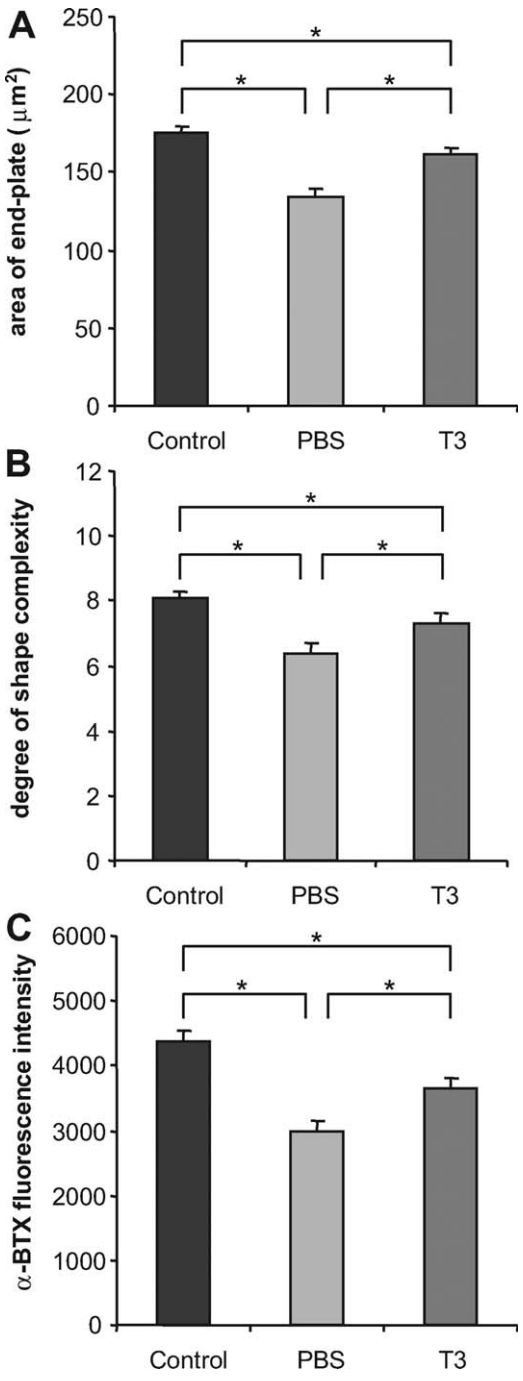

Fig. 6. Histogram of the mean ± SD of size (**A**), shape complexity (**B**) and total α-BTX fluorescence intensity per EP (**C**), measured on longitudinal plantar muscle sections stained with NF and α-BTX. After 14 weeks of nerve repair, more than 1500 EPs (n = 5) were measured on contralateral unoperated limb sections (Control), PBS-treated limb sections (PBS), and T3-treated limb section (T3). Since the nerve regeneration in biodegradable guides is similar to that in silicone guides, data were pooled. The hierarchic analysis revealed a significant difference ($^{\star}P < 0.01$) between PBS and T3 treatment.

increased number of regenerated myelinated axons by local T3 administration is linked to an improvement in peripheral target reinnervation. For this purpose we carefully analyzed the NMJs in the operated hind limbs of rats treated with either PBS or T3, and compared the results to normal NMJs from unoperated limbs. Our morphological and morphometric analysis revealed that at 4 weeks postsurgery more than 93% of gastrocnemius EPs and all plantar EPs are still denervated and free from axonal contact in all operated limb conditions. Therefore, at this stage of regeneration, there is no detectable effect of T3 on muscle reinnervation. The morphological denervation of EPs observed in our study is associated with a clear decrease in the size and shape complexity of EPs, and also with a fall in the density of AChRs on postsynaptic membrane. In the literature, similar changes in the architecture and parameters of denervated EPs are described. The injury of adult rat peripheral nerves induces a complete disappearance of nerve terminals by 24 hr (Tachikawa and Clementi, 1979; Kumai et al., 2005). Muscle denervation also provoked significant ultrastructural alterations at the surface of the postsynaptic part of NMJs (Sakakima et al., 2000; Ijkema-Paassen et al., 2002; Kumai et al., 2005; Deschenes et al., 2006; Nishizawa et al., 2006). In addition to the morphological changes, studies also reported that the density of AChRs on postsynaptic membranes fell significantly after muscle denervation (Andreose et al., 1995; Connor et al., 2002; Kumai et al., 2005; Miyamaru et al., 2008; McMullen and Andrade, 2009). These changes in the denervated EP structure agree with our data and confirm the denervation of hind limb muscles 4 weeks after surgery. Therefore, we could infer that in our experimental conditions and at this stage of nerve repair, most of the regenerated myelinated axons have not yet reached the limb muscles to reinnervate the EPs.

Fourteen weeks after surgery, we detected a clear stimulating effect of T3 on the regeneration of myelinated motor axons and the reinnervation of peripheral muscles. The morphometric analysis of regenerated nerve sections immunostained with ChAT and NF antibodies showed that T3 increases 1.3 fold the number of regenerated motor myelinated axons (ChAT positive) in T3-treated nerves compared to PBS treated nerves. Also, the total number of myelinated axons was increased 1.56 fold by T3 treatment. Therefore these results clearly indicate that T3 stimulates the regeneration of both motor and sensory myelinated axons. The stimulating effect of thyroid hormones (THs) on peripheral nerve regeneration has already been shown in several studies. Treatment with exogenous THs was shown to accelerate the process of axonal regeneration in the rat sciatic and motor facial nerves (McIsaac and Kiernan, 1975; Cook and Kiernan, 1976; Stelmack and Kiernan, 1977; Talman, 1979; Yu and Srinivasan, 1981; Kontoleon-Vakalopoulou et al., 1985; Oble et al., 2004). Positive clinical effects of TH, such as an acceleration of nerve fiber outgrowth and recovery of sensory conduction in patients with a transected ulnar nerve was also reported

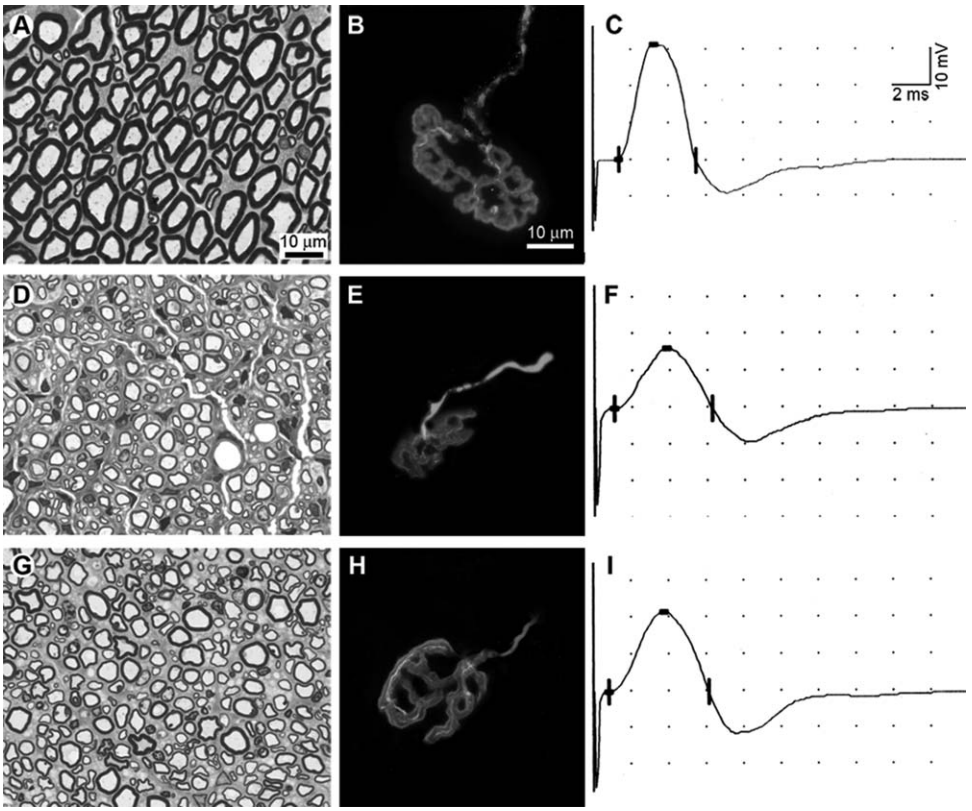

Fig. 7. Representative recordings of compound muscle action potentials in gastrocnemius muscle and photomicrographs of sciatic nerves and EPs. Unoperated contralateral leg (**A-C**), PBS-treated leg (**D-F**) and T3-treated leg (**G-I**) after 14 weeks of nerve repair.

(McQuarrie, 1975). The use of the intubulation technique with the local administration of THs, also enhances outgrowth and myelination of regenerated axons (Danielsen et al., 1988; Voinesco et al., 1998; Barakat-Walter et al., 2007; Papakostas et al., 2009).

Our morphological and morphometric analysis of gastrocnemius and plantar reinnervated EPs in both PBS and T3-treated operated limbs shows that T3 led to normal morphological restoration and significantly increased the recovery of the size, shape complexity and AChR density of reinnervated EPs. A recent study demonstrated that among the factors which affect the efficiency of transmission at NMJs are the size of the junction as measured by the summed area of AChRs aggregates, and the number of postjunctional AChRs (Lomo, 2003). Since T3 increases the restoration of NMJ structure, we believe that T3 improves synaptic transmission. The higher CMAP index recorded in gastrocnemius muscle of T3-treated limbs compared to PBS-treated limbs provides evidence that T3 increases synaptic transmission. The role of T3 in the development and regeneration of synapse function has been demonstrated by others. In newborn rats, the abnormal TH level seriously impairs neural transmission. The lack or excess of THs delays or accelerates the elimination of excess axons in polyneuronal innervation and also the increase in frequency of miniature end-plate potentials, respectively (Kawa and Obata, 1982). In adult rats, the acceleration of axonal regeneration by THs correlates with increased reinnervation of motor end-plates in denervated muscles. The functional recovery of the muscles acting at the ankle joint, occurs faster in rats treated with T3 than in controls (McIsaac and Kiernan, 1975). In hypothyroid rats, expression of the acetylcholinesterase and cholineacetyltransferase genes in the brain was significantly reduced, probably reflecting the reduced number of cholinergic synapses (Ahmed et al., 1993). Moreover, reinnervation of extensor digitorum longus muscles in hypothyroid rats following sciatic nerve crush, indicates that the stabilization of motor innervation of reinnervated muscle is influenced by THs (Cuppini and Ambrogini, 1996). A recent, interesting study, demonstrated that inhibition of TH action in xenopus laevis tadpoles, leads to a defect in spinal cord neurogenesis, and a decreased generation of lumbar motoneurons. This defect in neurogenesis is followed by a reduction in hind limb innervation and a deficiency in functional connections between nerves and muscles within hind limbs (Marsh-Armstrong et al., 2004). Taking into consideration all the observations reported above which demonstrate the direct or indirect

role of T3 in the development and regeneration of NMJs, therefore, it is not surprising that in our experimental conditions the local T3 administration enhances significantly the muscle reinnervation.

It is worth emphasizing that the importance of the use of THs in nerve regeneration comes from the capacity of THs to directly act on both axotomized neurons and Schwann cells. This is supported by the fact that both sensory and motor neurons and also Schwann cells, posses TH nuclear receptors during nerve regeneration (Barakat-Walter et al., 1992; Barakat-Walter and Droz, 1995; Glauser and Barakat-Walter, 1997). Therefore, in our experimental conditions, it is conceivable that T3 acts directly on axotomized sensory and motor neurons to rescue them from the cell death (Schenker et al., 2003). At the same time, T3 acts on Schwann cells to upregulates synthesis and secretion of some trophic factors and other molecules which are necessary to increase and to improve the growth and myelination of new axons, which in turn make a rapid and appropriate contact with denervated muscle end-plates to re-establish functional NMJs.

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