1

Dosi-efecte del tractament de l'FK506 sobre la taxa de regeneració axonal

Bimodal dose dependence of FK506 on the rate of axonal regeneration in mouse peripheral nerve.
Udina E, Ceballos D, Verdú E, Gold BG, Navarro X.
Muscle Nerve 2002, 26:348-355.

ABSTRACT: FK506 has been shown to enhance the rate of axonal regeneration after peripheral nerve lesions. However, quite variable doses of FK506 have been used in different animal studies. We examined the dose-dependence of FK506 on the rate of axonal regeneration after crush lesion of the mouse sciatic nerve. Mice received daily subcutaneous injections of FK506 at 0.2, 0.5, 1, 2, 5, or 10 mg/kg for 7 days after lesioning. A control group was injected with saline. The distance that regenerative axons advanced from the crush site was measured by the pinch test at 2, 4, and 7 days. Regenerating axons reached greater mean distances in all FK506-treated groups compared to the control group. The fastest regeneration rate was found at 5 mg/kg (12% increase over controls), although the 0.2 and 2 mg/kg doses achieved similar regeneration rates. In contrast, intermediate doses (0.5 and 1 mg/kg) and a higher dose (10 mg/kg) were not different from controls. Calcitonin gene–related peptide immunohistochemical labeling of regenerating axons yielded similar results to those found with the pinch test. Based on our finding of a double peak in the dose–response for FK506, it is hypothesized that at least two mechanisms of action (perhaps corresponding to distinct functional binding sites) are evoked at different concentrations of the drug to accelerate nerve regeneration. These results have clinical implications for the pharmacological treatment of nerve injuries while avoiding immunosuppressive effects and for the design of related drugs with more specific activities.
 Muscle Nerve **26:** 348–355, 2002

BIMODAL DOSE-DEPENDENCE OF FK506 ON THE RATE OF AXONAL REGENERATION IN MOUSE PERIPHERAL NERVE

ESTHER UDINA, MD,[1] DOLORES CEBALLOS, PhD,[1] ENRIQUE VERDÚ, PhD,[1] BRUCE G. GOLD, PhD,[2] and XAVIER NAVARRO, MD, PhD[1]

[1] Department of Cell Biology, Physiology, and Immunology, Universitat Autònoma de Barcelona, Bellaterra, Spain
[2] Center for Research on Occupational & Environmental Toxicology and Department of Cell & Developmental Biology, Oregon Health & Science University, Portland, Oregon, USA

Accepted 15 April 2002

FK506 (tacrolimus) is an immunosuppressant drug widely used in humans to prevent rejection after solid transplants. FK506 acts through binding to a family of immunophilin receptors (FK506-binding proteins, FKBPs) to form a complex whose geometry determines the activation of specific signaling pathways.[42] In T cells, the FK506–FKBP-12 complex inhibits the activity of calcineurin, a calcium-dependent phosphatase, thereby blocking cell proliferation.[31] Although immunophilins were first related to the immune system, it is now well known that they are plentiful in the nervous system[46] and numerous studies have shown the neuroprotective and neuroregenerative properties of FK506.[19–21]

In our recent study evaluating the effect of FK506 on nerve regeneration after a 6-mm gap repaired by autograft in mice, we found a beneficial effect of FK506 in accelerating the beginning of reinnervation.[38] However, the magnitude of the effect was less that that observed in previous studies in rats.[23,24] Although we used the most effective dose assessed in the rat (i.e., 5 mg/kg per day),[52] we could not discount the possibility that this dose is suboptimal for the mouse, because a dose–response characterization has not been reported in this species. Experimental studies on axonal regeneration after peripheral nerve lesions have involved doses ranging from

Abbreviations: ANOVA, analysis of variance; CGRP, calcitonin gene–related peptide; FKBP, FK506 binding protein; GAP, growth-associated protein

Key words: calcitonin gene–related peptide (CGRP); crush; nerve regeneration; sciatic nerve; tacrolimus

Correspondence to: X. Navarro, Group of Neuroplasticity and Regeneration, Unitat de Fisiologia, Facultat de Medicina, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Spain; e-mail: xavier.navarro@uab.es

Published online 8 July 2002 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/mus.10195

0.3 to 5 mg/kg per day, given as subcutaneous or intraperitoneal injections,[7,13,15,16,23,24,39,38] making comparisons between reports difficult. Wang et al.[52] compared dosages of 1, 2, 5, and 10 mg/kg of FK506 given after crush to the rat sciatic nerve, and found a bell-shaped dose–response curve, with 5 mg/kg resulting in a slightly higher regeneration rate than lower and higher doses. Our results were also consistent with those of other studies in rats that evaluated the effects of FK506 after nerve autograft, where FK506 was administered at lower doses (between 0.6 and 1 mg/kg per day).[13,16] Nevertheless, the dose–response for FK506 in nerve regeneration has not been addressed in the mouse. This issue is clinically relevant, as it is important to know whether nonimmunosuppressant dosages are able to enhance nerve regeneration.

There are indirect clues in humans that FK506, at immunosuppressive doses, offers some benefit to nerve regeneration. In two patients with hand allografts in whom FK506 was used to prevent rejection, a surprisingly fast recovery of sensorimotor functions was observed, which was attributed to an enhancement of axonal regeneration by FK506.[14,27] The ability of FK506 to enhance nerve regeneration, therefore, has potential clinical application.[29] Immunosuppressive therapy usually involves doses of FK506 lower than those used in experimental studies. Blood levels higher than 30 ng/ml imply a risk of toxic effects, whereas levels lower than 10 ng/ml can lead to rejection of the transplant.[44,48] The doses administered in experimental studies are usually higher than those used clinically. For example, a single intravenous administration of 1 mg/kg FK506 results in peak blood levels of about 160 ng/ml in the rat.[6] The aims of this study were to characterize the dose–response effects of FK506 on the rate of axonal regeneration after a crush injury to the sciatic nerve of the mouse by testing a wide range of doses.

MATERIALS AND METHODS

Surgical Procedure. Operations were performed under pentobarbital anesthesia (60 mg/kg intraperitoneally) in seven groups of 3-month-old female OF1 mice (n = 74). The sciatic nerve was exposed at the midthigh and crushed three times (30 s each, in succession, with a Dumont No. 5 forceps) at a constant point, 47 mm from the tip of the third digit.[37] A 10-0 suture stitch was placed at this point through the epineurium of the peroneal branch. Immediately after injury, the distal sciatic nerve was stimulated by light pinching to guarantee that axotomy was complete. The wound was sutured and disinfected with povidone–iodine. In order to avoid autotomy after denervation, animals were treated with amitriptyline.[36] Mice in six of the groups were given daily subcutaneous injections in the back of different doses of FK506 (Fujisawa Pharmaceuticals, Inc., Osaka, Japan) diluted in saline. The first dose was given just after operation. Animals were given daily injections of FK506 at dosages of 0.2 mg/kg (n = 10), 0.5 mg/kg (n = 12), 1 mg/kg (n = 12), 2 mg/kg (n = 10), 5 mg/kg (n = 12), or 10 mg/kg (n = 9). A control group (n = 9) received daily injections of an equivalent volume of saline solution. The experimental procedures were approved by the ethics committee of our institution and were carried out in accordance with the European Community Council Directive (86/609/EEC).

Evaluation. At 2, 4, and 7 days after lesioning, three or four mice from each group were reanesthetized, and the sciatic and its main branch, the tibial nerve, were exposed. The regeneration distance of the leading axons was evaluated by the pinch test. This sensitive test reliably demonstrates the location of the most rapidly growing sensory axons and has frequently been used in studies evaluating the rate of nerve regeneration.[3,4,34] The nerve was successively pinched with a fine forceps at 0.5-mm intervals from distal to proximal until a reflex response (contraction of muscles of the trunk) was elicited; this position along the nerve was labeled with one epineurial 10-0 suture. The positive pinch response results from the activation of the growing tips of regenerating axons, which are far more sensitive to mechanical stimulation than more proximal regions of the regenerated nerve.[34] The distance between the two suture-labeled points was measured with a microruler before and after harvesting the nerve. In order to avoid possible false responses, at 7 days after lesioning the saphenous nerve was cut at the femoral space prior to performing the pinch test. The pinch test was performed in all the animals by the same investigator who was blinded to the treatment group.

Immediately thereafter the sciatic-tibial nerve was removed, pinned in a Sylgard-coated Petri dish, and fixed by immersion in Zamboni's solution. After 24 h at 4°C, the fixative was substituted with 10% sucrose in 0.1 M Sorenson solution and kept at 4°C. Longitudinal sections of 80 μm were cut with a freezing cryotome, and washed free-floating in phosphate-buffered saline (PBS) with 0.3% Triton-X100 (Fluka, Buchs, Switzerland) and 1% normal goat serum (ICN, Costa Mesa, California) for 1 h, then incubated overnight in primary rabbit antiserum to calcitonin gene–related peptide (CGRP, 1:1000; Amersham, Buckinghamshire, UK). After washes, sections

were incubated in secondary antiserum, goat anti-rabbit cyanine 3.18–labeled immunoglobulin G (1:200; Jackson Immunoresearch, West Grove, Pennsylvania) overnight at 4°C. Following additional washes, sections were mounted on gelatin-coated slides, dehydrated in ethanol, and mounted with DPX (Fluka). Samples were viewed with an Olympus BX-40 microscope equipped for epifluorescence. At least three sections from each sample were examined to determine the distance from the crush point to the front of immunolabeled regenerative fibers to confirm the values obtained with the pinch test. CGRP is a neuropeptide present in motoneurons and in a large population of sensory neurons in the peripheral nervous system. Heavy CGRP immunoreactivity has been observed on the growth cones of regenerating neurites after nerve injury, suggesting that CGRP expression may be involved in the interaction between regenerating axons and nonneuronal cells during nerve regeneration.[41]

Data Analysis. All results are presented as mean ± SEM. Statistical comparisons between groups were conducted using a one-way analysis of variance (ANOVA) followed by Fisher's post hoc test. Regression analyses were performed from the regeneration distance (in millimeters) at each data collection period and the number of hours postlesion. A linear regression was made to assess the slope of the regeneration rate from the pinch test measurements in each group. The delay (or latency time), expressed in hours, was determined from the intersection of the regression line with the x-axis. The regeneration rate (during the first week postlesion) was estimated from the regeneration distance for each animal at 7 days after lesioning and the hour interval, minus the mean latency time of the corresponding group. Rate is expressed in millimeters per day.

RESULTS

The front of regenerative axons reached greater mean distances in all the groups treated with FK506 compared to the control group. By 7 days the maximal regenerated distance was found in the groups treated with 5 and 0.2 mg/kg FK506, with these values being significantly higher than in control mice. The rate of axonal regeneration after a crush follows a two-period course,[43,54] with an initial slow growing rate during the first 2–3 days followed by a period in which regeneration follows a linear rate. Comparison of the mean regeneration rate from days 2–7 and from days 4–7 (Fig. 1) shows a bimodal (double peak) dose-dependent relationship. Maximal effect of FK506 on the rate of nerve regeneration was observed at doses of 0.2 and 5 mg/kg, whereas no significant effect was found at the 1 and 10 mg/kg doses. The plots of the regeneration distance versus time were significantly ($P < 0.001$) fitted to a regression line in all the groups (Table 1 and Fig. 2). The slope of the regression line was greatest in the FK506 5 mg/kg group, followed by the FK506 0.2 and 2 mg/kg groups, whereas in the FK506 1 and 10 mg/kg groups the slope was similar to control mice (Table 1). From extrapolation of the best-fit regression line, the initial delay of regeneration was calculated to be 35.6 h in the control group and between 29.8 and 34.8 h in the FK506-treated groups. The estimated rate of regeneration during the entire first week after injury was also significantly greater in the 5 mg/kg (3.09 mm/day) and 0.2 mg/kg (3.03 mm/day) FK506 groups than in control group (2.79 mm/day); 0.5 and 2 mg/kg FK506 groups exhibited intermediate values.

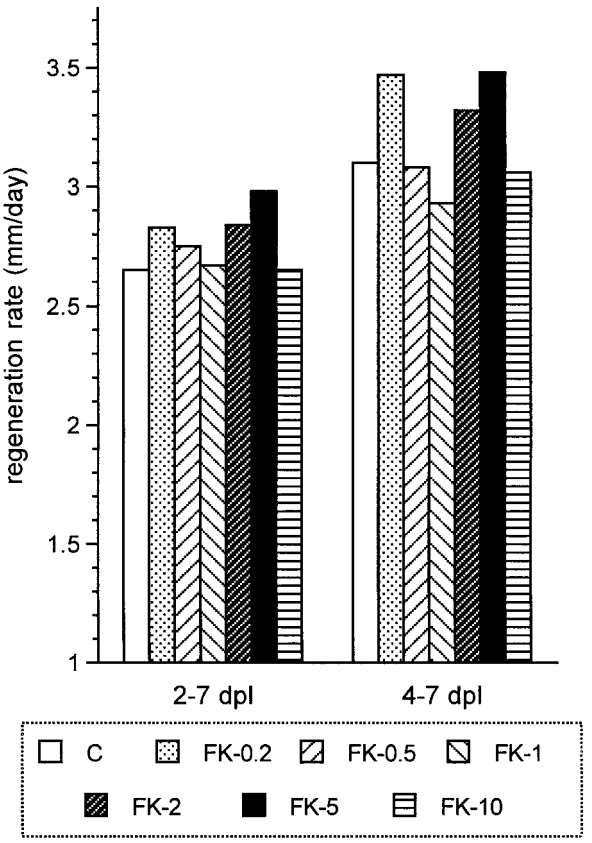

FIGURE 1. Regeneration rate in the interval between 2 and 7 days postlesion (dpl) and between 4 and 7 days postlesion in the control (C) and FK506-treated groups (FK dose expressed in milligrams per kilogram).

The longitudinal nerve sections immunolabeled against CGRP showed an increased density of labeled axonal profiles around the crush site from

Table 1. Linear regression analysis of regeneration distance versus time after sciatic nerve crush and estimated regeneration rate in mice of the control group and of those treated with different doses of FK506.

Group	*n*	Regression analysis			Estimated rate at day 7 (mm/day)
		Latency (h)	Slope (mm/24 h)	Coefficient	
Control	9	35.6 ± 4.7	2.68 ± 0.14	0.989	2.79 ± 0.06
FK506 0.2 mg/kg	10	32.6 ± 6.0	2.88 ± 0.16	0.986	3.03 ± 0.08*‡
FK506 0.5 mg/kg	12	32.6 ± 6.1	2.80 ± 0.15	0.981	2.89 ± 0.11
FK506 1 mg/kg	12	33.3 ± 4.8	2.72 ± 0.12	0.989	2.77 ± 0.09
FK506 2 mg/kg	10	31.7 ± 4.9	2.88 ± 0.15	0.989	2.97 ± 0.11
FK506 5 mg/kg	12	34.8 ± 3.4	3.01 ± 0.12	0.992	3.09 ± 0.09*†‡
FK506 10 mg/kg	9	29.8 ± 4.4	2.68 ± 0.12	0.992	2.74 ± 0.05

*$P < 0.05$ vs. control group; †$P < 0.5$ vs. FK506 1 mg/kg; ‡$P < 0.05$ vs. FK506 10 mg/kg.

which an array of fibers elongated through the distal nerve, decreasing progressively in density (Fig. 3). At day 2, CGRP immunoreactivity was very marked just proximal to the crush site.[41] Bundles of labeled fibers emerged distally, but the ending front of regenerative axons was difficult to ascertain at day 2 as CGRP-labeling was still present in the degenerating fibers. At days 4 and 7, the enhanced immunoreactivity against CGRP at the crush was lower than that seen by day 2, and the degenerated distal axons did not show CGRP immunoreactivity at these timepoints. Thus, the regenerated-labeled axons could be traced in continuity to their termination (Fig. 3). This analysis revealed a gradual decrease in density of reactive axons along the distal nerve segment to the front of regeneration, some fibers reaching the suture made to mark the regenerative distance in the pinch test (Fig. 3). The measurements made in the immunohistochemical samples showed regeneration distances that were in agreement with those found with the pinch test, further corroborating the differences between treated and control groups.

DISCUSSION

The main finding of this study is that the ability of FK506 administration to enhance the rate of axonal regeneration following a sciatic nerve crush in the mouse demonstrates a bimodal (double peak) dose–response, maximal activity being found at dosages of 0.2 and 5 mg/kg per day. The best overall regeneration rate was achieved with 5 mg/kg, although doses of 0.2 and 2 mg/kg yielded a very similar regeneration course through the 7 days after lesioning. In contrast, intermediate doses (0.5 and 1 mg/kg) and a higher dose (10 mg/kg) did not significantly increase the regeneration rate from control values.

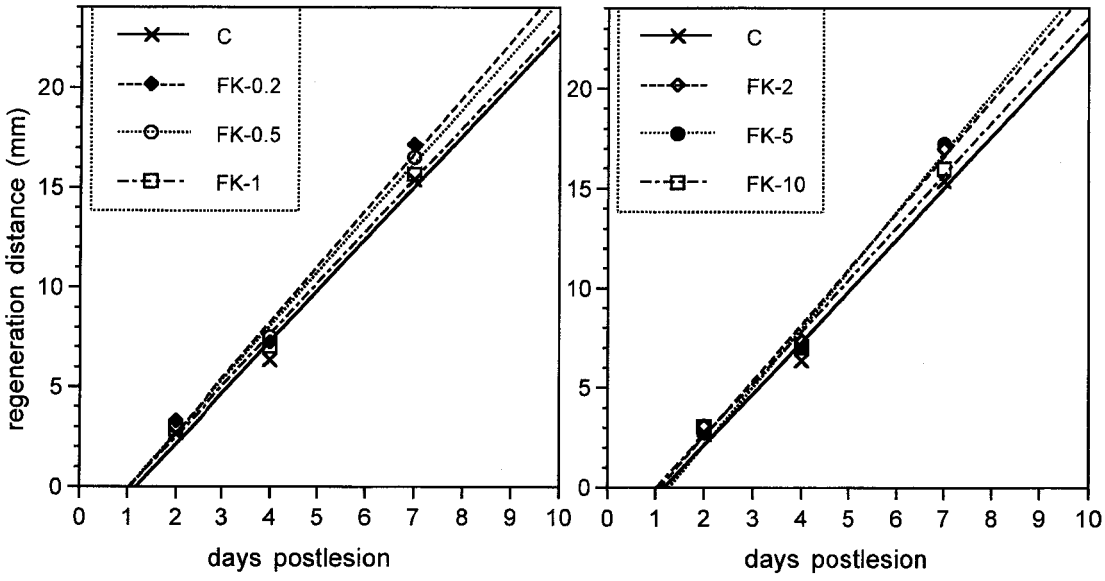

FIGURE 2. Regeneration distances after a crush lesion measured by the pinch reflex test plotted against postlesion time in the control (C) and FK506-treated groups (FK dose expressed in milligrams per kilogram).

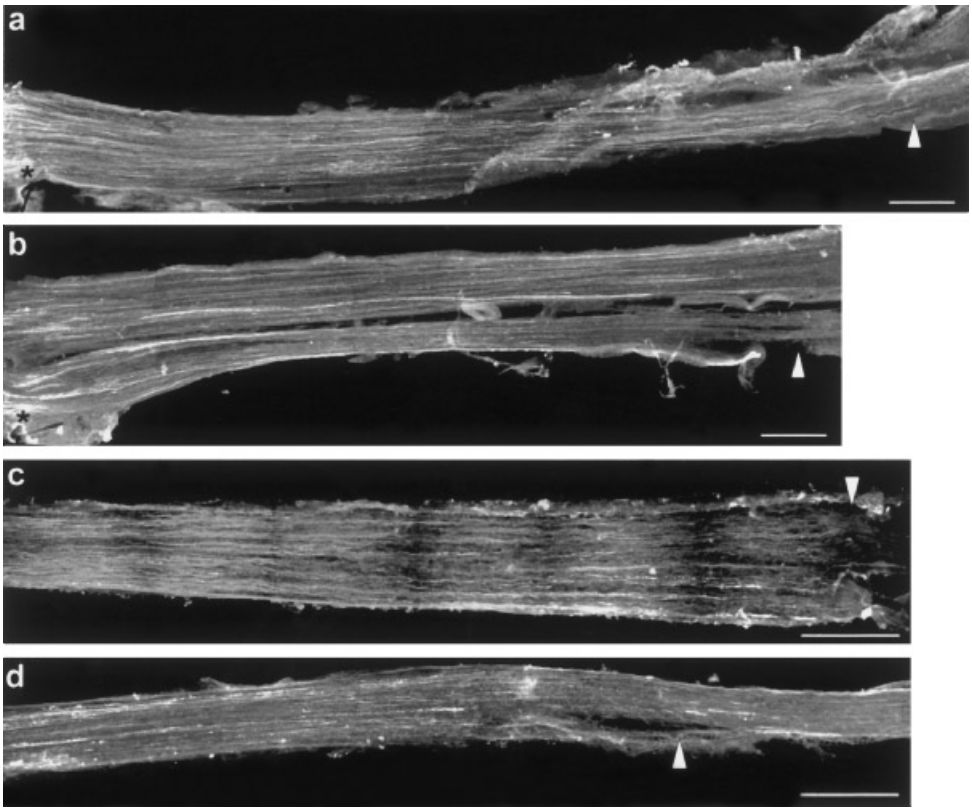

FIGURE 3. Photomontages showing CGRP-labeled axons in the regenerating mouse sciatic nerve at day 4 **(a, b)** and day 7 **(c, d)** from control (b, d) and 5.0 mg/kg FK506-treated (a, c) animals. The crush site is indicated by black asterisks in **(a)** and **(b)** and the regenerative front by white arrows. The samples at 7 days after lesioning correspond to the regenerated tibial nerve from 12.5 mm distal to the crush site to past the regenerative front. Bundles and individual CGRP-immunoreactive fibers are seen with decreasing density from proximal to distal. However, CGRP-labeled axons are seen at greater distances from the crush site (more distally) in the nerves from the FK506-treated animals at both timepoints. Bar = 0.5 mm in all.

This pattern is in general agreement with the dose–response characterization previously described in rats,[52] in which a dose of 5 mg/kg accelerated axonal regeneration more than doses of 1 and of 10 mg/kg. However, in the rat, both the 1 and 10 mg/kg doses were found to increase regeneration rate significantly, albeit not as much as the 5 mg/kg dose.[23,24,52] Accordingly, in previous studies after either sciatic nerve crush[30] or resection with autograft repair,[13,16] FK506 treatment with low doses (0.6–1 mg/kg) expedited walking track recovery and increased the number of regenerating axons at midterm, although there was no difference with respect to untreated animals by the end of the study. This is consistent with an acceleration in the rate of regeneration and further indicates that FK506 speeds regeneration after nerve injuries of different severity. However, differences in the methodology used to evaluate regeneration and functional recovery as well as in pharmacokinetics between species may explain variations in the magnitude of the effect reported between different studies. In normal animals, an estimated regeneration rate of between 2.5 and 4.0 mm/day has been reported in previous studies using the pinch test,[3,34,43] axonal transport labeling,[5,40] or immunohistochemical labeling[50] to measure axonal growth in the sciatic nerve of rats and mice. In this context, the maximally effective dose (5 mg/kg) increased the regeneration rate by only 12% in the mouse, which is lower than the 34% increase reported in the rat.[52] However, such an increase in the rat was found by measurements made between 12 and 15 days after lesioning, an interval when the rate of regeneration is considered faster than at the earlier times than can be tested in the mouse nerve.[54] In addition, as an acceleration in the rate of axonal regeneration will give a greater increase in regenerative distance with time, the net effect is expected to be less apparent in smaller species of animals and along the length of shorter nerves.[34] Thus, an appreciable increase in regeneration rate would be expected to be less significant in the mouse than the rat sciatic nerve.

The mechanisms influencing the rate of axonal

regeneration after axotomy mainly include changes in the neuronal response to the lesion and changes induced in the local environment of the lesion.[18] Several experimental paradigms that alter either the neuronal cell body reaction or the local nerve environment, such as a conditioning lesion[17,34,37,43] and aging,[5,40,49] have been shown to significantly change the rate of axonal regeneration after a nerve lesion. Axonal outgrowth requires delivery of materials to the growth cones mainly following the slow component b of axonal transport.[53] At the level of the growth cone, new microtubules are assembled and stabilized by means of posttranslational modifications of tubulin and microtubule-associated proteins, which are substrates for phosphorylating enzymes, such as growth-associated proteins (GAPs), particularly GAP-43. Finally, the local environment surrounding the regenerative growth cones controls axonal elongation through changes in nonneuronal cells and production of neurotrophic and neurotropic factors.[18,51] In the distal injured nerve, Schwann cells deprived of axonal contact proliferate, help inflammatory infiltrating cells to eliminate debris, and upregulate the synthesis and release of a variety of neurotrophic factors and basal lamina components that create an appropriate microenvironment for regenerating axons. However, it is unclear whether an effect on Schwann cells would elicit a change in rate.

At relatively high concentrations of 100 μM, FK506 has been shown to promote Schwann-cell proliferation in vitro.[16] However, at concentrations of 1 μM or higher, FK506 inhibits neurite extension.[9] Moreover, although proliferation of Schwann cells promoted in vitro by FK506 seems related to calcium ion entrance into the cell,[16] this could be detrimental for axonal regeneration. Although FK506 is a selective suppressor of the cellular immune response, at high doses it may affect indiscriminately other immune responses, inhibiting also granulocyte and macrophage responses. FK506, at concentrations 10- to 100-fold higher than those required to inhibit T cell proliferation, also suppresses synthesis and secretion of some cytokines from macrophages.[1,28] This could also delay the process of wallerian degeneration in which infiltrating macrophages play an important role.[39,47]

Based on our results showing a double peak in the dose response, it is logical to hypothesize that FK506 exerts opposing mechanisms of action on the injured peripheral nervous system that are evoked at different concentrations of the drug and may be counterbalanced depending on the concentration. In this regard, the FK506 0.2 and 2 mg/kg groups followed a very similar regeneration course through 7 days after lesioning, whereas intermediate doses (0.5 and 1 mg/kg) were not effective. The mechanisms by which FK506 accelerates nerve regeneration are not completely known, but it is not unreasonable to propose that different FKBPs (perhaps involving different cell types)[21] mediate different actions and that this is a function of the dose administered. One possibility is that low doses act through binding to FKBP-52, an immunophilin associated with steroid receptors.[19–21] Higher doses, by binding to FKBP-12, could activate a wider range of mechanisms. The possible involvement of these two FKBPs is discussed in what follows.

FKBP-12 interacts with two calcium-release channels and stabilizes them. A high dose of FK506 is required for breaking this binding and affecting calcium influx. Two elements of the neurite outgrowth process (elongation and growth cone motility) have different calcium requirements.[33] This interaction could play a role in counterbalancing actions underlying the inhibitory effects of FK506 observed at high concentrations of the drug.[9] Interaction of FK506 with FKBP-12 and the calcineurin inhibition pathway was the first mechanism proposed.[24,25,32] Inhibition of calcineurin by FK506 prevents GAP-43 dephosphorylation and promotes its active state. This protein plays an important role in growth cone motility and in the modulation of new synapses.[2] However, the most important form of GAP-43 for elongation of growth cones is the GAP phosphorylated by environmental stimuli,[35] and phosphorylation of GAP-43 by other mechanisms could be ineffective or even disadvantageous in promoting regeneration.

At very low doses, FK506 appears to promote neurite sprouting but no axonal elongation in vitro, whereas at higher doses it enhances elongation and does not influence neurite branching.[10,45] When added to dopamine neurons in culture, immunophilin ligands that inhibit the phosphatase activity of calcineurin (FK506 and cyclosporine A) enhanced elongation of neurites, whereas immunophilin ligands that do not inhibit calcineurin increased branching of neurites and antagonized the elongation effects of FK506.[11] Interestingly, all these drugs showed bell-shaped dose–response curves[11] and, if superimposed, the effects of FK506 on neurite sprouting and on neurite elongation would produce a two-peak curve. However, whereas both FK506 and cyclosporine A inhibit calcineurin and both exert neuroprotective action in vitro and in vivo,[6,12] only FK506 enhances nerve regeneration in vivo.[30,52] Moreover, the demonstration that nonimmunosuppressant derivatives of FK506 do not inhibit

calcineurin yet speed nerve regeneration,[26,45] and that FK506 elicited neurite elongation in hippocampal neurons of FKBP-12 knockout mice,[22] provides strong evidence against the involvement of either calcineurin or FKBP-12 in mediating nerve regeneration. Furthermore, a non–FKBP-12 binder also demonstrates a bimodal dose response for nerve regeneration (B. G. Gold, unpublished observation). This finding seems to exclude the involvement of FKBP-12 in the generation of either peak in nerve regeneration activity. It is of interest that FKBP-52 contains two FK506-binding domains.[8] Thus, it is possible that both activities of FK506 are dependent on its interaction with only one FKBP, namely FKBP-52.[19,21]

This work was supported by grants from the Fondo de Investigación Sanitaria (FIS00-0031-02) and from the Acadèmia de Ciències Mèdiques de Catalunya i Balears, Spain. We thank Fujisawa Pharmaceuticals, Inc., (Osaka, Japan) for the generous gift of FK506.

REFERENCES

1. Andersson J, Nagy S, Groth CG, Andersson U. Effects of FK506 and cyclosporine A on cytokine production. Studies in vitro at a single-cell level. Immunology 1992;75:136–142.
2. Benowitz LI, Routtenberg A. GAP-43: an intrinsic determinant of neuronal development and plasticity. Trends Neurosci 1997;20:84–91.
3. Berenberg RA, Forman DS, Wood DK, DeSilva A, Demaree J. Recovery of peripheral nerve function after axotomy—effect of triiodothyronine. Exp Neurol 1977;57:349–363.
4. Bisby MA. Fast axonal transport of labeled protein in sensory axons during regeneration. Exp Neurol 1978;61:281–300.
5. Black MM, Lasek RY. Slowing of the rate of axonal regeneration during growth and maturation. Exp Neurol 1979;63: 108–119.
6. Butcher SP, Henshall DC, Teramura Y, Iwasaki K, Sharkey J. Neuroprotective actions of FK506 in experimental stroke: in vivo evidence against an excitotoxic mechanism. J Neurosci 1997;17:6939–6946.
7. Büttemeyer R, Rao U, Jones NF. Peripheral nerve allograft transplantation with FK506: functional, histological, and immunological results before and after discontinuation of immunosuppression. Ann Plast Surg 1995;35:396–401.
8. Callebaut I, Renoir J-M, Lebeau M-C, Massol N, Burny A, Baulieu E-E, Mornon J-P. An immunophilin that binds Mr 90,000 heat shock protein: main structural features of a mammalian p59 protein. Proc Natl Acad Sci USA 1992;89: 6270–6274.
9. Chang HY, Takei K, Sydor AM, Born T, Rusnak F, Jay DG. Asymmetric retraction of growth cone filopodia following focal inactivation of calcineurin. Nature 1995;376:686–689.
10. Constantini LC, Chaturvedi P, Armistead DM, McCaffrey PG, Deacon TW, Isacson O. A novel immunophilin ligand: distinct branching effects on dopaminergic neurons in culture and neurotrophic actions after oral administration in an animal model of Parkinson's disease. Neurobiol Dis 1998;5: 97–106.
11. Constantini LC, Isacson O. Immunophilin ligands and GDNF enhance neurite branching or elongation from developing dopamine neurons in culture. Exp Neurol 2000;164:60–70.
12. Dawson TM, Steiner JP, Dawson VL, Dinerman JL, Uhl GR, Snyder SH. Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate neurotoxicity. Proc Natl Acad Sci USA 1993;90:9808–9812.
13. Doolabh VB, Mackinnon SE. FK506 accelerates functional recovery following nerve grafting in a rat model. Plast Reconstr Surg 1999;103:1928–1936.
14. Dubernard J-M, Owen E, Herzberg G, Lanzetta M, Martin X, Kapila H, Dawahra M, Hakim NS Human hand allograft: report on first 6 months. Lancet 1999;353:1315–1320.
15. Fansa H, Keilhoff G, Altmann S, Plogmeier K, Wolf G, Schneider W. The effect of the immunosuppressant FK506 on peripheral nerve regeneration following nerve grafting. J Hand Surg 1999;24B:38–42.
16. Fansa H, Keilhoff G, Horn T, Altmann S, Wolf G, Schneider W. Stimulation of Schwann cell proliferation and axonal regeneration by FK506. Restor Neurol Neurosci 2000;16:77–86.
17. Forman DS, McQuarrie IG, Labore FW, Wood DK, Stone LS, Braddock CH, Fuchs DA. Time course of the conditioning lesion effect on axonal regeneration. Brain Res 1980;182: 180–185.
18. Fu SY, Gordon T. The cellular and molecular basis of peripheral nerve regeneration. Mol Neurobiol 1997;14:67–116.
19. Gold BG. FK506 and the role of the immunophilin FKBP-52 in nerve regeneration. Drug Metab Rev 1999;31:649–663.
20. Gold BG. Therapeutic potential of immunophilin ligands in neurological disorders. Exp Opin Invest Drugs 2000;9: 2331–2342.
21. Gold BG. Neuroimmunophilin ligands and the role of steroid hormone chaperone proteins in nerve regeneration. In: Gold BG, Fischer G, Herdegen T, editors. Immunophilins in the brain. FKBP-ligands: novel strategies for the treatment of neurodegenerative disorders. Barcelona: Prous Science; 2000. p 3–22.
22. Gold BG, Densmore V, Shou W, Matzuk MM, Gordon HS. Immunophilin FK506-binding protein 52 (not FK506-binding protein 12) mediates the neuroprotective action of FK506. J Pharmacol Exp Ther 1999;289:1202–1210.
23. Gold BG, Katoh K, Storm-Dickerson T. The immunosuppressant FK506 increases the rate of axonal regeneration in rat sciatic nerve. J Neurosci 1995;15:7509–7516.
24. Gold BG, Storm-Dickerson T, Austin DR. The immunosuppressant FK506 increases functional recovery and nerve regeneration following peripheral nerve injury. Restor Neurol Neurosci 1994;6:287–296.
25. Gold BG, Yew JY, Zeleny-Pooley M. The immunosuppressant FK506 increases GAP-43 mRNA levels in axotomized sensory neurons. Neurosci Lett 1998;241:25–28.
26. Gold BG, Zeleny-Pooley M, Wang M-S, Chaturvedi P, Armistead DM. A nonimmunosuppressant FKBP-12 ligand increases nerve regeneration. Exp Neurol 1997;147:269–278.
27. Jones JW, Gruber SA, Barker JH, Breidenbach WC. Successful hand transplantation: one year follow-up. N Engl J Med 2000; 343:468–473.
28. Keicho N, Sawado S, Kitamura K, Yotsumoto H, Takaku F. Effects of an immunosuppressor, FK506, on IL-1 production by human macrophages and a macrophage-like cell line, U937. Cell Immunol 1991;132:285–294.
29. Kline DG. Nerve surgery as it is now and as it may be. Neurosurgery 2000;46:1285–1293.
30. Lee M, Doolabh VB, Mackinnon SE, Jost S. FK506 promotes functional recovery in crushed rat sciatic nerve. Muscle Nerve 2000; 23:633–640.
31. Liu J, Farmer JD, Lane SW, Friedman J, Weissman I, Schreiber SL. Calcineurin is a common target of cyclophilin–cyclosporine A and FKBP–FK506 complexes. Cell 1991;66:807–815.
32. Lyons WE, George EB, Dawson TM, Steiner JP, Snyder SH. Immunosuppressant FK506 promotes neurite outgrowth in cultures of PC12 cells and sensory ganglia. Proc Natl Acad Sci USA 1994;91:3191–3195.
33. Mattson MP, Kater SB. Calcium regulation of neurite elongation and growth cone motility. J Neurosci 1987;7:4034–4043.
34. McQuarrie IG, Grafstein B, Gershon MD. Axonal regeneration in the rat sciatic nerve: effect of a conditioning lesion and of dbcAMP. Brain Res 1977;132:443–453.

35. Meiri KF, Bickerstaff LE, Schwob JE. Monoclonal antibodies show that kinase C phosphorylation of GAP43 during axonogenesis is both spatially and temporally restricted in vivo. J Cell Biol 1991;112:991–1005.
36. Navarro X, Butí M, Verdú E. Autotomy prevention by amitriptyline after peripheral nerve section in different strains of mice. Restor Neurol Neurosci 1994;6:151–157.
37. Navarro X, Kennedy WR. The effect of a conditioning lesion on sudomotor axon regeneration. Brain Res 1990;509: 232–236.
38. Navarro X, Udina E, Ceballos D, Gold BG. Effects of FK506 on nerve regeneration and reinnervation after graft or tube repair of long gaps. Muscle Nerve 2001;24:905–915.
39. Perry VH, Brown MC, Lunn ER, Tree P, Gordon S. Evidence that very slow Wallerian degeneration in C57BL/Ola mice is an intrinsic property of the peripheral nerve. Eur J Neurosci 1990;2:802–808.
40. Pestronk A, Drachman DB, Griffin JW. Effects of aging on nerve sprouting and regeneration. Exp Neurol 1980;70: 65–82.
41. Raivich G, Dumoulin FL, Streit WJ, Kreutzberg GW. Calcitonin gene-related peptide (CGRP) in the regenerating rat sciatic nerve. Restor Neurol Neurosci 1992;4:107–115.
42. Schreiber SL. Chemistry and biology of the immunophilins and their immunosuppressive ligands. Science 1991;251: 283–287
43. Sjöberg J, Kanje M. The initial period of peripheral nerve regeneration and the importance of the local environment for the conditioning lesion effect. Brain Res 1990;529:79–84.
44. Spencer CM, Goa KL, Gillis JC. Tacrolimus, an update of its pharmacology and clinical efficacy in the management of organ transplantation. Drug 1997;54:925–975.
45. Steiner JP, Connolly MA, Valentine HL, Hamilton GS, Dawson TM, Hester L, Snyder SH. Neurotrophic actions of nonimmunosuppressive analogues of immunosuppressive drugs FK506, rapamycin and cyclosporin A. Nat Med 1997;3: 421–428.
46. Steiner JP, Dawson TM, Fotuhi M, Eglatt C, Snowman AM, Cohen N, Snyder SH. High brain densities of the immunophilin FKBP colocalized with calcineurin. Nature 1992;358: 584–587.
47. Tanaka K, Zhang QL, Webster HF. Myelinated fiber regeneration after sciatic nerve crush: morphometric observations in young adult and aging mice and the effects of macrophage suppression and conditioning lesions. Exp Neurol 1992;118: 53–61.
48. Undre NA, Stevenson P, Schäfer A. Pharmacokinetics of tacrolimus: clinically relevant aspects. Transplant Proc 1999; 31:21–24.
49. Verdú E, Butí M, Navarro X. The effect of aging on efferent nerve fibers regeneration in mice. Brain Res 1995;696:76–82.
50. Verdú E, Navarro X. Comparison of immunohistochemical and functional reinnervation of skin and muscle after peripheral nerve injury. Exp Neurol 1997;146:187–198.
51. Verdú E, Navarro X. The role of Schwann cell in nerve regeneration. In: Castellano B, González B, Nieto-Sampedro M, editors. Understanding glial cells. Boston: Kluwer; 1998. p 319–359.
52. Wang M-S, Zeleny-Pooley M, Gold BG. Comparative dose dependence study of FK506 and cyclosporin A on the rate of axonal regeneration in the rat sciatic nerve. J Pharmacol Exp Ther 1997;282:1084–1097.
53. Wyjek JR, Lasek RJ. Correlation of axonal regeneration and slow component b in two branches of a single axon. J Neurosci 1983;3:243–251.
54. Wyrwicka W. On the rate of regeneration of the sciatic nerve in white mouse. Acta Biol Exp 1950;15:147–153.

Efectes de l'FK506 sobre la reinnervació mitjançant la regeneració axonal i la ramificació col.lateral

FK506 enhances reinnervation by regeneration and by collateral sprouting of peripheral nerve fibers.
Udina E, Ceballos D, Gold BG, Navarro X.
Exp Neurol 2003, 183: 220-231.

2

ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Experimental Neurology 183 (2003) 220–231

Experimental Neurology

www.elsevier.com/locate/yexnr

FK506 enhances reinnervation by regeneration and by collateral sprouting of peripheral nerve fibers

Esther Udina,[a] Dolores Ceballos,[a] Bruce G. Gold,[b] and Xavier Navarro[a,*]

[a] *Group of Neuroplasticity and Regeneration, Institute of Neurosciences and Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Spain*

[b] *Center for Research on Occupational and Environmental Toxicology and Department of Cell and Developmental Biology, Oregon Health and Science University, Portland, OR 97201, USA*

Received 15 July 2002; revised 17 February 2003; accepted 12 March 2003

Abstract

We examined the effects of FK506 administration on the degree of target reinnervation by regenerating axons (following sciatic nerve crush) and by collateral sprouts of the intact saphenous nerve (after sciatic nerve resection) in the mouse. FK506-treated animals received either 0.2 or 5 mg/kg/day, dosages previously found to maximally increase the rate of axonal regeneration in the mouse. Functional reinnervation of motor, sensory, and sweating activities was assessed by noninvasive methods in the hind paw over a 1-month period following lesion. Morphometric analysis of the regenerated nerves and immunohistochemical labeling of the paw pads were performed at the end of follow-up. In the sciatic nerve crush model, FK506 administration shortened the time until target reinnervation and increased the degree of functional and morphological reinnervation achieved. The recovery achieved by regeneration was greater overall with the 5 mg/kg dose than with the dose of 0.2 mg/kg of FK506. In the collateral sprouting model, reinnervation by nociceptive and sudomotor axons was enhanced by FK506. Here, the field expansion followed a faster course between 4 and 14 days in FK506-treated animals. In regard to dose, while collateral sprouting of nociceptive axons was similarly increased at both dosages (0.2 and 5 mg/kg), sprouting of sympathetic axons was more extensive at the high dose. This suggests that the efficacy of FK506 varies between subtypes of neurons. Taken together, our findings indicate that, in addition to an effect on rate of axonal elongation, FK506 improves functional recovery of denervated targets by increasing both regenerative and collateral reinnervation.

Keywords: Nerve regeneration; Sprouting; Reinnervation; FK506; Sudomotor; Nociception

Introduction

FK506 is an immunosuppressant drug widely used to prevent rejection after solid organ transplantation. FK506 and its nonimmunosuppressant derivatives also exert neuroprotective and neuroregenerative activities (for reviews, see Gold, 1999, 2000a, 2000b; Snyder et al., 1998). Several experimental studies have shown that administration of FK506 increases the rate of axonal regeneration after axotomy induced by nerve crush (Gold et al., 1995, 1994; Lee et al., 2000; Wang et al., 1997) or after nerve transection and suture or graft repair (Büttemeyer et al., 1995; Doolabh and Mackinnon, 1999; Fansa et al., 1999; Navarro et al., 2001). Functional recovery of distal targets, measured by the walking track or by electrophysiological methods, starts earlier in rats and mice treated with FK506, although ultimate levels of recovery are close to those of untreated animals (Doolabh and Mackinnon, 1999; Fansa et al., 1999; Navarro et al., 2001). In patients with a hand allograft where FK506 was used to prevent rejection, recovery of sensorimotor functions was faster than expected, this being attributed to an enhancement of axonal regeneration by FK506 (Dubernard et al., 1999; Jones et al., 2000). However, after severe nerve injuries in larger species, including humans, accelerating target reinnervation may be more important for

* Corresponding author. Group of Neuroplasticity and Regeneration, Facultat de Medicina, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Spain. Fax: +34-935812986.

E-mail address: xavier.navarro@uab.es (X. Navarro).

0014-4886/03/$ – see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0014-4886(03)00173-0

reducing the consequences of denervation (muscle atrophy, loss of sensory receptors, denervation hypersensitivity) (Fu and Gordon, 1995). In this context, the ability of FK506 to increase nerve regeneration following a chronic axotomy (Sulaiman et al., 2002) may be of importance for improving the chances for functional recovery. However, studies on the effects of FK506 on target reinnervation are lacking using any of these models. Furthermore, while FK506 is presently being used in human hand transplantations (Dubernard et al., 1999; Jones et al., 2000) and allograft nerve repairs (Mackinnon et al., 2001), the dose–response needs to be characterized in multiple models to determine its optimal dose for use in clinical practice.

We recently examined the dose dependency for FK506 on the rate of nerve regeneration in the mouse and found a novel bimodal response (Udina et al., 2002). The largest increase in regeneration rate was achieved at a dose of 5 mg/kg, but doses of 0.2 and 2 mg/kg yielded a similar regeneration course through the first week following sciatic nerve crush. In contrast, results with intermediate doses of 0.5 and 1 mg/kg were not different from those of controls. In the rat, the 5 mg/kg dose also maximally accelerates axonal regeneration (Wang et al., 1997), while the 1 mg/kg was less effective; lower dosages were not examined. The efficacy of lower dosages has obvious implications for clinical use for nerve regeneration since low doses (e.g., 0.2 mg/kg) would have less risk of toxicity and would presumably not exhibit immunosuppressant activity (Spencer et al., 1997; Undre et al., 1999).

In the present study, we examined the effects of FK506 on both the speed of axonal elongation and the degree of target reinnervation. Reinnervation of target tissue is achieved by either axonal regeneration from injured nerves or collateral sprouting from intact neurons. In regard to collateral sprouting, one immunophilin ligand (V-10,367) has been shown to increase neurite branching in vitro (Constantini and Isacson, 2000). Therefore, we also investigated the capabilities of FK506 to promote collateral sprouting in vivo. Thus, two models were used to study the effect of FK506 on target reinnervation: nerve regeneration following sciatic nerve crush and collateral sprouting following partial denervation of the mouse hind paw. In both models, we assessed recovery of different targets over a 1-month period in mice receiving FK506 at either 0.2 or 5 mg/kg/day, the two doses previously found as most effective (Udina et al., 2002).

Material and methods

Surgical procedures

Operations were performed under pentobarbital anesthesia (60 mg/kg ip) in 3-month-old female OF1 mice. For nerve regeneration assessment, the sciatic nerve was exposed at the mid thigh and crushed three times in succession with a Dumont No. 5 forceps at a constant point, 45 mm from the tip of the third digit. The saphenous nerve was also cut in the femoral space and a long segment of the distal stump removed to prevent regeneration. The wound was then sutured by layers and disinfected with povidone–iodine. For the study on collateral sprouting and reinnervation, the sciatic nerve was transected and a 10-mm-long segment resected to avoid regeneration. In contrast to the above, the saphenous nerve was left intact. To avoid autotomy after denervation, animals were treated with amitriptyline (Navarro et al., 1994a).

The study consisted of three groups: two treated with FK506 at 0.2 and 5 mg/kg/day and one control untreated for each injury model (regeneration and collateral sprouting). FK506 (Fujisawa Pharmaceuticals, Osaka, Japan) was given as daily subcutaneous injections in the back diluted in saline solution at a dose of 0.2 mg/kg (FK506 0.2 group; $n = 7$ for regeneration and $n = 6$ for sprouting) or 5 mg/kg (FK506 5.0 group; $n = 9$ for regeneration and $n = 6$ for sprouting) beginning on the day of operation until the end of the study. For the control injured group (C, $n = 10$), mice received an equivalent volume of saline solution. For the histological study of regeneration, we also used a group of mice with intact sciatic nerve (Intact, $n = 10$) to obtain the normal counts of myelinated and unmyelinated axons. The experimental procedures were approved by the Ethics Committee of our university and were carried out in accordance with the European Community Council Directive (86/609/EEC).

Functional evaluation of reinnervation by regenerating axons

Target organ reinnervation was serially evaluated after sciatic nerve crush with a battery of functional tests (Navarro et al., 1994b). With animals under anesthesia (pentobarbital, 40 mg/kg ip) the sciatic nerve was stimulated percutaneously through a pair of needle electrodes at the sciatic notch and the compound muscle action potential (CMAP) recorded from plantar muscles with microneedle electrodes. For sensory nerve conduction, the electrodes were inserted on the fourth toe to record the compound nerve action potentials (CNAP) of the digital nerves. Square pulses (0.01 ms in duration) were applied at a voltage that gave a maximal response. The evoked CMAPs and CNAPs were amplified and displayed on a storage oscilloscope at settings appropriate to measure the amplitude and the latency time.

Sweat gland (SG) reinnervation was evaluated by the silicone mold technique. Pilocarpine nitrate (5 mg/kg) was subcutaneously injected and 10 min later a silicone material (Elasticon, Kerr) was spread over the plantar surface of the hind paw. As the material hardens, it retains the impressions made by the sweat droplets emerging from individual SGs. The number of reactive SGs was determined by counting the sweat droplet impressions made in the mold under a dissecting microscope. Recovery of pain sensitivity was

assessed by pricking with a needle at five distinct areas (from the most proximal pawpad to the tip of the second digit on the plantar surface of the denervated paw) under a dissecting microscope by an investigator who was blinded regarding the treatment group. A score to pinprick (PP) was assigned from no response (0), reduced or inconsistent responses (1), to normal reaction (2) in each area tested. Functional tests of reinnervation were performed before operation to obtain baseline control values and at 7, 11, 15, 18, 22, 25, and 32 days postoperation (dpo). For normalization, values obtained in each test after operation were expressed as the percentage of preoperative values for each mouse. For each parameter indicative of the degree of reinnervation (amplitude of CMAP and CNAP, number of SGs, and PP score), we determined the day of the first response after denervation and the percentage of maximal recovery achieved during follow-up. The mean values for each test and an overall mean of the four tests used were derived (see Table 1).

The walking track test was performed at similar intervals postoperatively to assess recovery of locomotor pattern. The plantar surface of the hind paws was painted with acrylic paint and the mice were allowed to walk along a narrow corridor with white paper on its base. The print lengths and toe spread distances (the total toe spread distance between the first and fifth toe prints and the intermediate toe distance between the second and fourth toe prints) were measured with a precision device on footprints of the operated and intact paws. The results of the operated paw were expressed as the percentage of the contralateral paw at each test.

Functional evaluation of collateral reinnervation

Following sciatic nerve resection, the expansion of innervated territories supplied by thin nerve fibers of the intact saphenous nerve was evaluated at 4, 7, 14, and 21 dpo by the pinprick and the silicone mold methods, as described above. Assessment of sensory and SG reinnervation was made at the six paw pads and at the proximal and distal areas of the toes. To confirm the expansion of cutaneous innervation found by pinpricking, at the end of follow-up, a solution of Evans blue (50 mg/kg) was injected into the femoral vein of anesthetized mice (Brenan et al., 1988). Ten minutes later, the exposed saphenous nerve was suspended on a pair of hook wire electrodes and subjected to electrical stimulation for 5 min at 10 Hz pulses (1 ms duration and 12 V); this stimulation excites C fibers in the saphenous nerve inducing neurogenic plasma extravasation, leading to the accumulation of Evans blue-labeled plasma proteins in the skin supplied by the nerve (Brenan et al., 1988; Weisenfeld-Hallin et al., 1989). The extent of blue coloration of the skin of the hind paw was then mapped on scale diagrams of the foot.

Morphological evaluation of nerve regeneration

At the end of follow-up, the mice were anesthetized, the operated area was opened, and the regenerated nerve was harvested and fixed in glutaraldehyde–paraformaldehyde (3%–3%) in 0.1 M cacodylate buffer (pH 7.4, 4 h, 4°C). Samples were postfixed in OsO_4 (2%, 2 h), dehydrated through ethanol series, and embedded in Epon resin. Transverse semithin sections (0.5 μm) of the entire nerve (sampled at 3 mm distal to the crush site) were stained with toluidine blue and examined under light microscopy. Images were obtained using an Olympus DP10 camera to a PowerMacintosh G3 computer and processed to a magnification of 200× (for measurement of the cross-sectional area of the entire regenerated nerve) and to 2600× (for myelinated axon counting). Ultrastructural observations were performed on ultrathin sections (90 nm) collected on Formvar film-coated hole grids, stained with lead citrate, and examined under a Hitachi 7000 electron microscope at 100 KeV. Randomly selected fields covering at least 25% of the tibial branch of the sciatic nerve were used to count the number of unmyelinated and of myelinated axons at 10,000×.

Immunohistochemical evaluation of reinnervation

To visualize nerve profiles in the epidermis and in the sweat glands, we used immunolabeling of protein gene product 9.5 (PGP9.5), a neuron-specific protein that has been recognized as a pan-neuronal cytoplasmic marker (Navarro et al., 1997; Thompson et al., 1983).

The plantar pads of the hind paw were carefully removed and fixed in Zamboni's solution at 4°C overnight. They were then cryoprotected in 0.2 M Sorenson's buffered solution (pH 7.2) with 10% sucrose and stored at 4°C. Cryotome sections (80 μm) were washed free-floating in 0.1 M phosphate-buffered saline (PBS) with 0.3 Triton X-100 (Fluka) and 1% normal donkey serum (ICN Pharmaceuticals) for 1 h, incubated overnight in rabbit antiserum to PGP9.5 (1:800; Ultraclone), washed in PBS–Triton, and incubated with goat antirabbit cyanine-3.18-labeled immunoglobulin G (1:200; Jackson Immunoresearch) overnight at 4°C (Verdú and Navarro, 1997). Sections were placed on agarose-coated slides, dehydrated, mounted in dextropropoxiphene (Fluka), and viewed using an Olympus BX-40 microscope equipped for epifluorescence. For image analysis, three images from three sections for each paw pad were collected using an Olympus DP10 digital camera. Images were analyzed using NIH Image software to quantify the number of intraepidermal nerve fibers and the area of innervation of SGs. A background subtraction procedure was performed (Vilches et al., 2002).

Statistical analysis

All data are expressed as means ± SEM. Statistical comparisons between groups were made by ANOVA fol-

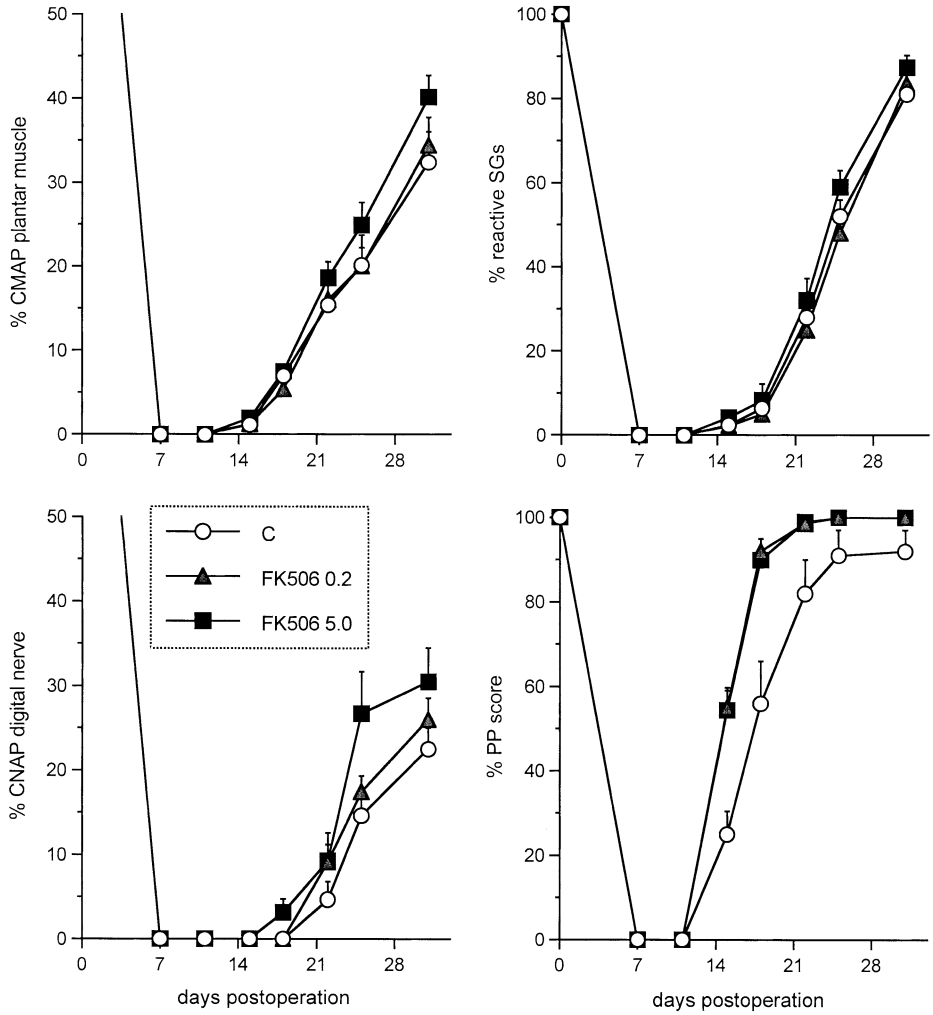

Fig. 1. Percentages (compared to uninjured) for the amplitude of the plantar compound muscle action potential (CMAP), the digital compound nerve action potential (CNAP), the number of reactive sweat glands (SG), and the pinprick (PP) score over time in the groups of mice after sciatic nerve crush.

lowed by a post-hoc Fisher test. Differences were considered significant at $P < 0.05$.

Results

Effects of FK506 on nerve regeneration after sciatic nerve crush

Functional tests performed at 7 dpo demonstrated that all nerve-mediated functions were abolished in the denervated hind paw of all animals. The first CMAPs evoked by sciatic nerve stimulation and recorded from plantar muscles reappeared at 15 dpo in most mice and at 18 dpo in two mice in group C. During the following weeks, the latency of the CMAP shortened to close to normal (data not shown) and the amplitude progressively increased to reach mean final values (relative to preoperative values) of 32, 34, and 40% in the C, FK506 0.2, and FK506 5.0 groups, respectively (Fig. 1; Table 1). Digital CNAPs were recorded earlier in mice treated with FK506 compared to untreated mice. The amplitude of the CNAPs elicited at the end of follow-up averaged 22, 26, and 30% in the C, FK506 0.2, and FK506 5.0 groups, respectively (Table 1). Sudomotor responses returned by 18 dpo in all three groups. The total number of reactive SGs increased with a similar slope, albeit reaching significantly higher maximal recovery in the FK506 5.0 group; 87% vs 81% in the FK506 5.0 and C groups, respectively (Fig. 1; Table 1). On the other hand, nociceptive responses to pinprick were found at the most proximal pad from day 15 in all mice, increasing with a higher slope in both FK506 groups than in the C group (Fig. 1).

To allow direct comparison of the differences between groups for each type of functional nerve fiber assessed, the onset day of reinnervation and the maximal percentage of recovery are shown in Table 1. As found in previous studies (Navarro et al., 1994b; Verdú and Navarro, 1997), functional reinnervation was significantly higher for small nerve fibers (nociceptive and sudomotor) than for large myelinated fibers (sensory and α-motor neurons). FK506 treatment

Table 1
Onset day of reinnervation and percentage of maximal reinnervation for each distal target tested after sciatic nerve crush in the control group ($n = 10$) and in groups treated with FK506 at doses of 0.2 mg/kg ($n = 7$) and 5 mg/kg ($n = 9$)

	Plantar muscle	Digital nerve	Nociception	Sweat glands	Mean
Onset (day)					
Control	15.6 ± 0.4	25.6 ± 1.2	15.0 ± 0.0	18.3 ± 0.5	18.6 ± 0.3
FK506 (0.2 mg/kg)	15.0 ± 0.0	23.7 ± 0.8	15.0 ± 0.0	18.1 ± 0.4	18.0 ± 0.3
FK506 (5.0 mg/kg)	15.0 ± 0.0	21.6 ± 1.0*	15.0 ± 0.0	17.7 ± 0.6	17.4 ± 0.4*
Reinnervation (%)					
Control	32.4 ± 3.6	22.5 ± 2.5	92.0 ± 6.1	81.0 ± 1.7	57.1 ± 2.1
FK506 (0.2 mg/kg)	34.4 ± 7.4	26.0 ± 2.5	100.0 ± 0.0	83.3 ± 1.4	60.9 ± 1.9
FK506 (5.0 mg/kg)	40.1 ± 2.6	30.5 ± 2.8*	100.0 ± 0.0	87.3 ± 2.1*	64.5 ± 1.6*

* $P < 0.05$ vs group C.

produced a dose-dependent, albeit slight, acceleration in the time until reinnervation and an increase of the mean levels of maximal reinnervation. For the CNAP recorded at the digital nerve, the most sensitive parameter to detect differences in this model (see Navarro et al., 2001, 1994b), the onset of recovery was shortened in both FK506-treated groups; values were reduced by 13 and 7.5% in FK506 5.0 and FK506 0.2 groups, respectively, compared to group C. Furthermore, the percentages of recovery were increased by 35 and 15% in FK506 5.0 and FK506 0.2 groups, respectively.

The walking track test showed full functional recovery in all animals during the follow-up. Analysis of the print length factor indicated a rapid return to near-normal values in the three groups by 16 dpo. The total toe spread distance (between the first and fifth digits) and the intermediate toe distance (between the second and fourth digits), which are dependent on reinnervation of intrinsic foot muscles, showed a faster recovery to normal values in the FK506 5.0 group (22 dpo) than in the other two groups (26 dpo) (Fig. 2).

Morphological results after sciatic nerve crush

Light microscopy revealed a well-formed regenerated nerve distal to the crush site in all groups. The regenerated nerves demonstrated a thin epineurium and their original fascicular organization was preserved. The density of myelinated fibers was lower than that in normal nerves and there was more connective tissue between the fibers (Fig. 3). Remnants of degenerating fibers and phagocytic cells were still present. Table 2 shows the average cross-sectional area of the nerves and the number of regenerated myelinated fibers. The number of fibers was significantly increased in the FK506 5.0 group compared to group C (Table 2); values were 71, 79, and 84% of those in the normal (uninjured) sciatic nerve in the C, FK506 0.2, and FK506 5.0 groups, respectively. The numbers of unmyelinated axons were significantly lower in group C compared to those in both FK506-treated groups (Table 2, Fig. 4); for the FK506-treated groups, the counts were similar to that found in the intact nerve (Table 2). The normal ratio of unmyelinated/myelinated axons in the tibial nerve (1.95 ± 0.09) was slightly reduced in group C (1.78 ± 0.10), but it was increased in both treated groups (2.34 ± 0.26). Comparatively the increase in regenerated myelinated axons in FK506-treated groups was about 15–20%, whereas the in-

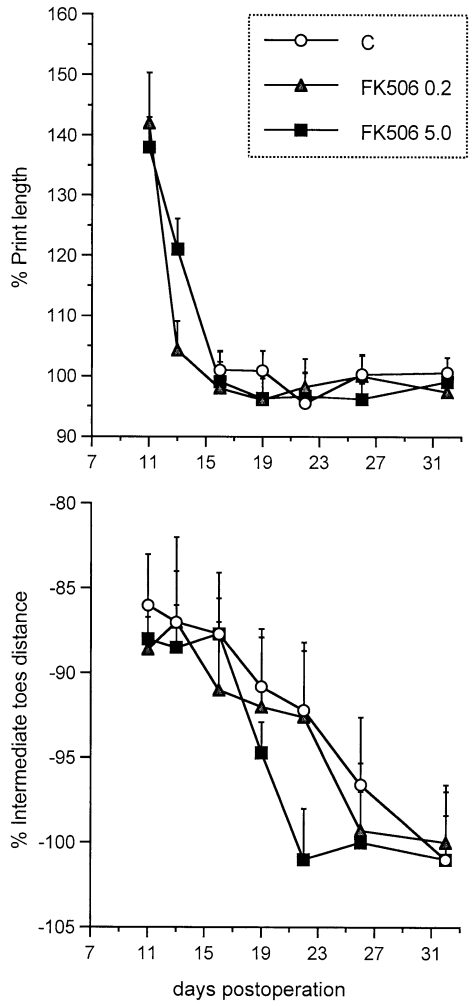

Fig. 2. Changes over time of the print length and the intermediate toe distance of the operated hindlimb, expressed as percentages of the contralateral hindlimb, following crush injury to the sciatic nerve.

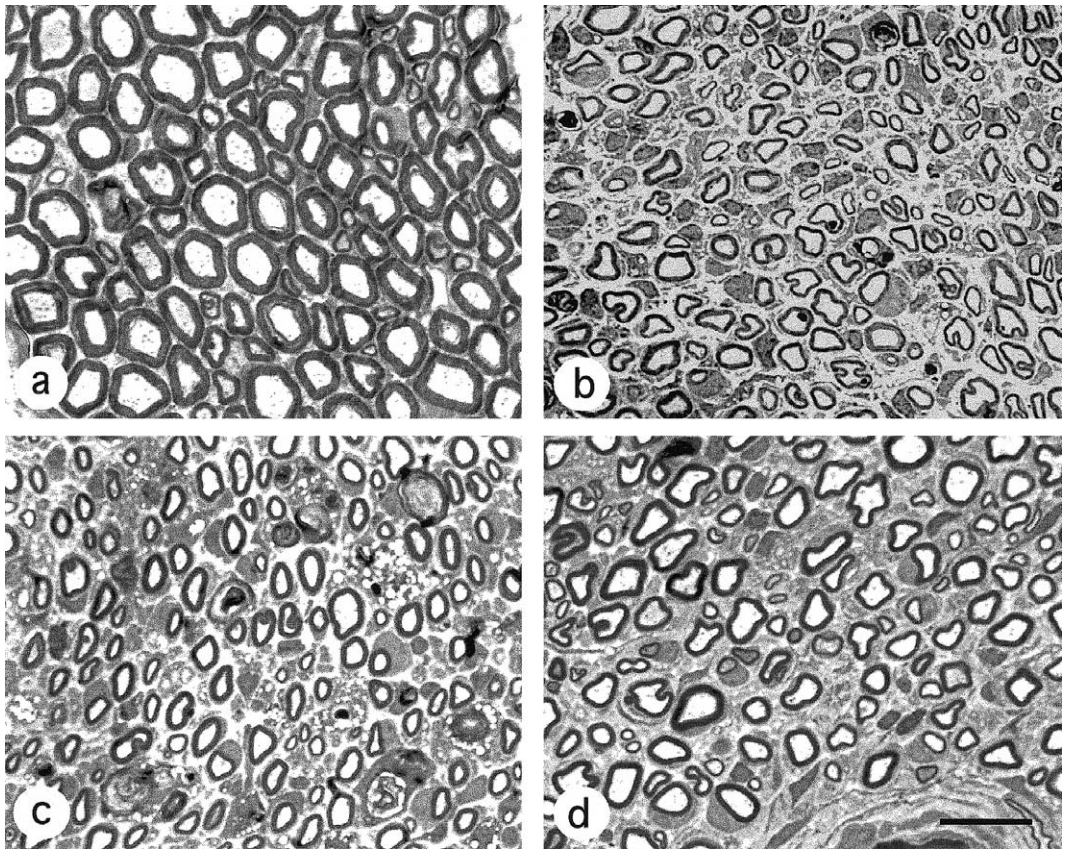

Fig. 3. Semithin transverse sections of sciatic nerves representative of intact mice (a) and regenerated nerves from control (b), FK506 0.2 mg/kg (c), and FK506 5 mg/kg (d) groups. The density and size of the regenerated myelinated fibers in nerves regenerated 1 month after sciatic nerve crush remain lower than those in the normal nerve. In mice treated with FK506 (c and d), there are a higher number of regenerated fibers and a more mature appearance than in the control nerve (b). Bar, 17 μm.

crease for unmyelinated axons was about 60% with respect to the control group.

The immunohistochemical evaluation of reinnervated plantar pads of the hind paw showed the presence of PGP-immunoreactive fibers in nerve trunks, subepidermal nerve plexus, epidermal layers, and around SGs, but at lower densities than those in contralateral normal (uninjured) samples (Fig. 5). The intraepidermal thin nerve fibers were mainly located at the apex of the pads and less at lateral sides. The number of intraepidermal nerve profiles labeled in the injured groups was about 1/3 of that in normal skin (Table 2). FK506-treated mice demonstrated a higher number of profiles (about 25–30% more) than control mice. The density of PGP-immunoreactive fibers surrounding the SG tubules was moderate, with some SGs remaining denervated or only partially reinnervated. The total areas of SGs occupied by nerve fibers were 34, 72, and 66% of normal values in the C, FK506 0.2, and FK506 5.0 groups, respectively (Table 2). These findings indicate that FK506 increases terminal sprouting and arborization by the regenerating fibers that reached distal cutaneous targets. Whereas the number of regenerated fibers in the sciatic nerve increased mildly (see above), the innervation of targets was notably superior both FK506-treated groups compared to that of untreated mice.

Effects of FK506 on collateral reinnervation by the intact saphenous nerve

Four days after resection of the sciatic nerve, the intact saphenous nerve innervated an average of 21, 11, and 17 SGs (6, 3, and 4% of the normal number of SGs) in the C, FK506 0.2 and FK506 5.0 groups, respectively (Fig. 6). These glands were located in the usual saphenous nerve territory (Kennedy et al., 1984; Navarro and Kennedy, 1988). The number of reactive SGs increased during the second and third weeks to a maximum of 54, 38, and 79 in the C, FK506 0.2, and FK506 5.0 groups, respectively. A few SGs outside of the saphenous territory started to be reactive to pilocarpine in some animals. Because the original saphenous innervated SGs at 4 dpo widely varied in number between mice, the increase over time is expressed as the percentage with respect to the baseline number found at 4 dpo for each animal. The expansion of sudomotor innervation by collateral sprouting of the saphenous nerve followed a higher slope and to larger territory in the FK506

Table 2
Morphological parameters of the regenerated sciatic nerve and immunohistochemical results of the innervation of the paw pads 1 month after crush in the control group and in groups treated with FK506 at doses of 0.2 and 5 mg/kg

Group	*n*	Sciatic nerve	
		Nerve area (mm^2)	No. myelinated fibers
Intact	10	0.210 ± 0.006	4801 ± 207
Control	10	0.155 ± 0.008*	3407 ± 95*
FK506 (0.2 mg/kg)	7	0.192 ± 0.006**	3784 ± 82*
FK506 (5.0 mg/kg)	9	0.166 ± 0.006*	4008 ± 136***
		Tibial nerve	
		No. unmyelinated fibers	No. myelinated fibers
Intact	10	5948 ± 382	3109 ± 189
Control	5	3739 ± 406*	2208 ± 118*
FK506 (0.2 mg/kg)	5	5900 ± 596**	2521 ± 121*
FK506 (5.0 mg/kg)	5	6166 ± 421**	2680 ± 122***
		Cutaneous innervation	
		Epidermal fibers/mm	Percentage of innervated SG area
Intact	10	57.7 ± 2.6	37.8 ± 2.8
Control	10	22.1 ± 1.6*	13.0 ± 1.6*
FK506 (0.2 mg/kg)	7	27.1 ± 1.2*	27.3 ± 1.7***
FK506 (5.0 mg/kg)	9	30.4 ± 1.5***	24.9 ± 1.7***

For comparison the results obtained for mice with normal intact nerves are also shown. Values are expressed as means ± SEM.
$P < 0.05$* vs intact nerves; ** vs group C.

5.0 group, reaching a final increase of 472% compared to 340% in group C; group FK506 0.2 showed an intermediate course, reaching a value of 382% (Fig. 7).

Positive responses to nociceptive pinprick stimulation after resection of the sciatic nerve were found in the normal sensory territory of the saphenous nerve (Brenan et al., 1988; Kinnman and Aldskogius, 1986). This covered the first and second toes in all the mice, being more variable in the medial pads of the hindpaw. From 7 to 21 days, the responses were more consistent in these areas. Moreover, an extension of the response was found to medial pads and to the third toe. The total PP score increased faster during the second week postdenervation in both FK506-treated groups (Fig. 7). The final score for nociceptive responses found at 21 dpo were increased (with respect to baseline values at 4 dpo) by 285, 355, and 378% in the C, FK506 0.2, and FK506 5.0 groups, respectively.

The areas of the plantar skin labeled by extravasation of Evans blue corresponded in all cases to the areas where functional nociceptive responses were previously found, except for the pads, which show little or no extravasation (Weisenfeld-Hallin et al., 1989). However, because the extravasation of the dye gives a lower and less homogeneous stain in the plantar skin than in the dorsal hairy skin, quantitation was not attempted.

Discussion

The present study reveals that FK506 administration enhances the reinnervation of target organs both by regenerating axons following a nerve crush and by collateral sprouts of an intact nerve induced to expand following denervation of its nearby territory. The recovery achieved by regeneration and by collateral sprouting was greater overall with the 5 mg/kg dose than with the dose of 0.2 mg/kg of FK506. Nevertheless, the 0.2 mg/kg dose still produced better results than those found in untreated, control mice.

FK506 enhances nerve regeneration and reinnervation

In agreement with previous reports (Gold et al., 1995, 1994; Lee et al., 2000; Udina et al., 2002; Wang et al., 1997), FK506 administration accelerated the rate of axonal regeneration and shortened the time until target reinnervation after sciatic nerve crush. We have recently reported (Udina et al., 2002) that FK506 exerts a bimodal dose–response enhancement on the rate of axonal regeneration following sciatic nerve crush in the mouse, the best regeneration rate being achieved at 5 mg/kg/day, although doses

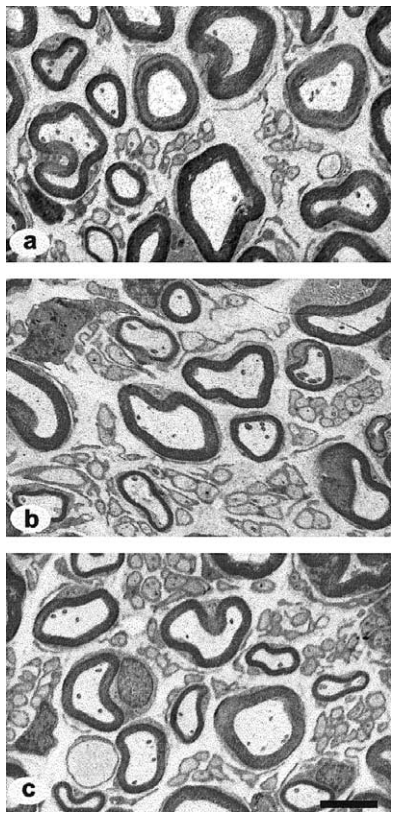

Fig. 4. Ultrathin sections of regenerated nerves 3 mm distal to the crush from (a) untreated control, (b) FK506 0.2 mg/kg and (c) FK506 5 mg/kg mice. Note the increased density of unmyelinated axons in the nerves from both FK506-treated animals. Original magnification, ×10,000.

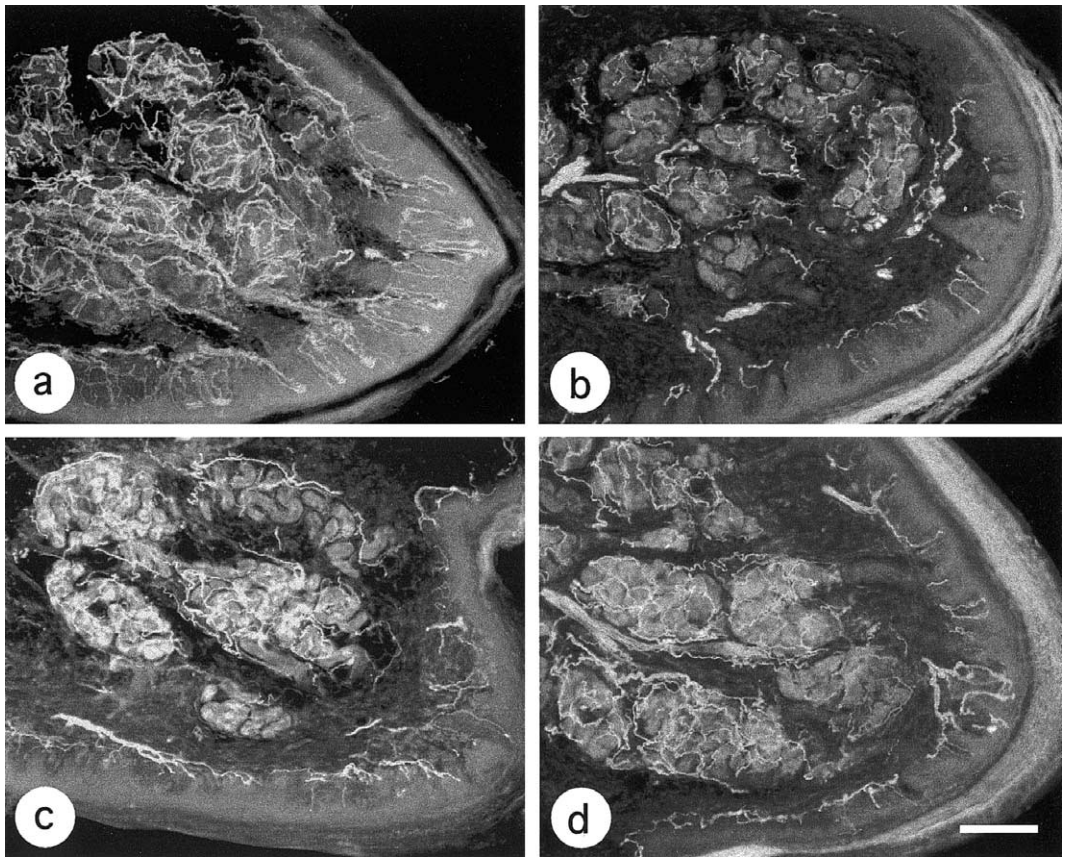

Fig. 5. Immunohistochemical images of PGP 9.5 immunoreactivity in intact (a) and in reinnervated mouse foot pads representative of control (b), FK506 0.2 mg/kg (c), and FK506 5 mg/kg (d) groups 1 month after sciatic nerve crush. (a) Nerves in control footpad form a dense network around SGs. Large trunks extend through the central area to form a subepidermal plexus, from which numerous fibers emerge to innervate the epidermis and Meissner's corpuscles in the tufts of papillary dermis. (b) Immunofluorescence is greatly reduced in the SGs, and few nerve fibers enter the epidermis. (c and d) The density of immunoreactive profiles is considerably increased in the SG area and in the epidermis in mice treated with FK506. Bar, 100 μm.

of 0.2 and 2 mg/kg yielded a very similar regeneration course through the first week postlesion. The more marked effect found in this study with the dose of 5 mg/kg than with the low dose of 0.2 mg/kg arises from two differences from the previous model employed. First, the present model enabled a doubling of the distance examined for regeneration; in the previous study, the regenerative front at 7 days postcrush was always found proximal to the ankle, whereas in the present study regeneration was assessed further distally (at the toes). Second, the present model allowed us to also assess the effect of FK506 on terminal branching and reconnection of regenerating axons with postsynaptic targets. Using immunohistochemical methods, different types of nerve fibers have been found to exhibit similar regeneration rates until they reappeared within corresponding targets (Verdú and Navarro, 1997). In contrast, differences of a few days were found in the time lapse between the reappearance of axonal profiles and recovery of detectable functional responses (Verdú and Navarro, 1997). Peripheral reinnervation by terminal branching and synaptogenesis appears to be dependent on different factors in the regenerating tissue that those regulating axonal elongation (see below).

The most striking effect of FK506 observed is its ability to increase target reinnervation and functional recovery by regenerating axons. Previous studies using either a sciatic nerve crush (Gold et al., 1995, 1994; Lee et al., 2000; Wang et al., 1997) or nerve section with autograft repair (Doolabh and Mackinnon, 1999; Fansa et al., 1999) found that FK506 treatment (0.6–10 mg/kg) expedited walking track recovery and increased the number of regenerating axons at early times, but there were no differences with respect to untreated animals by the end of the study. This lack of effect on final outcome may be due to the insensitivity of the methods used. The neurophysiological tests employed in this study to assess reinnervation by different types of nerve fibers allow serial evaluation in the same animal at desired intervals (Navarro et al., 1994b). Together with the immunohistochemical labeling of regenerated fibers into the skin (Navarro et al., 1997; Verdú and Navarro, 1997), these assessments provide more sensitive measurements of recovery than walking track and counts of nerve fibers over only short distances from the lesion.

Differences were observed in the response to FK506 by different neuronal populations. The mean amplitudes of the compound action potentials were increased from controls by 6 and 15% for CMAP and by 15 and 35% for CNAP in

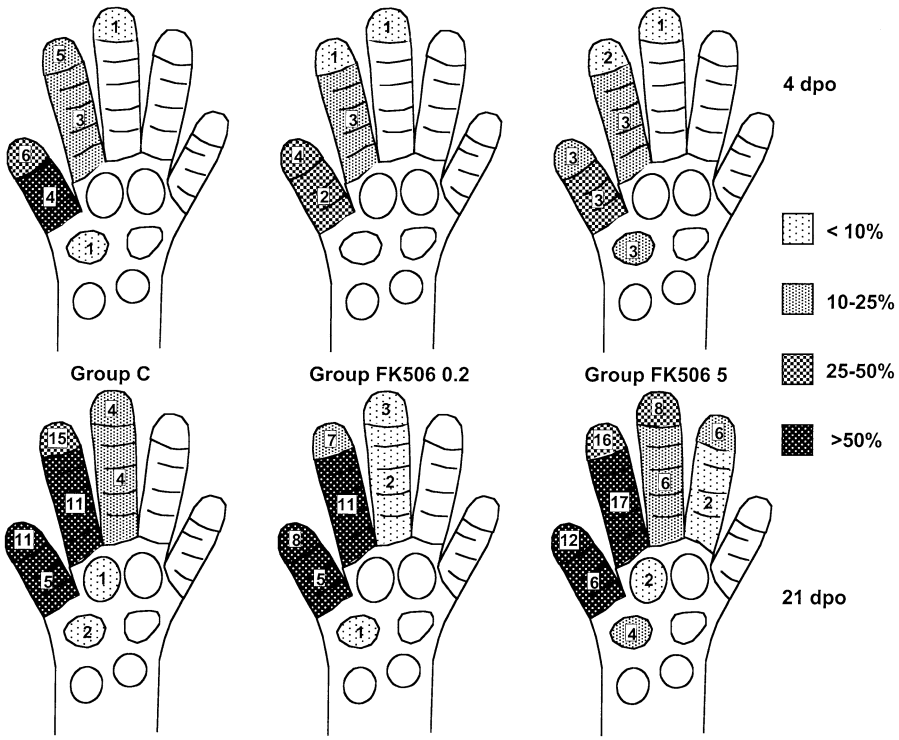

Fig. 6. Distribution and number of sweat glands innervated by the saphenous nerve at 4 days (top panels) and at 21 days (bottom panels) after resection of the sciatic nerve in the three groups of mice. Shadings represent the percentage with respect to the preoperative number of reactive sweat glands found at each area of the paw.

FK506-treated animals given the 0.2 and 5 mg/kg dosages, respectively. In contrast, nociceptive and sudomotor reinnervation increased by less than 10% with FK506 treatment, despite the stronger effect of FK506 on the regeneration of unmyelinated than of myelinated fibers. This difference can be explained by the normally higher percentage of recovery for sweating and pain sensitivity than for muscle action potentials found after nerve lesion and regeneration, thereby minimizing differences between experimental and control groups; the difference is attributable to a greater capacity of unmyelinated axons for terminal sprouting and functional reinnervation compared to large myelinated axons (Navarro et al., 2001, 1994b; Verdú and Navarro, 1997).

The density of reinnervation found by immunohistochemical labeling of target tissues was lower than that in normal (uninjured) nerves, despite the fact that a crush lesion should have provided optimal conditions for regeneration. Although nociceptive and sudomotor functional recovery reached levels similar to preoperative controls, the number and extension of immunolabeled profiles in the epidermis and the SGs remained below normal (see also Navarro et al., 1997; Verdú and Navarro, 1997). Despite this defect in the density of nerve profiles in the epidermis, nociceptive response can be close to normal because free nerve endings reaching deep layers of the epidermis may respond to mechanical stimuli. Regarding the SG reinnervation, a reduced number of sudomotor axons may achieve secretion in a normal number of SGs due to their large capability for collateral sprouting and to the fact that SGs secretion return even if partially reinnervated (Navarro et al., 1997; Verdú and Navarro, 1997). One month following nerve crush, a time when steady levels of cutaneous reinnervation are achieved, the number of axonal profiles in the epidermis was increased about 35% and the density of SG innervation about 100% in FK506-treated mice relative to controls. This can be explained by the fact that FK506 clearly promotes regeneration of unmyelinated axons, achieving values similar to those from intact nerves. In addition, the immunohistochemical observations suggest that FK506 may exert a promoting action on terminal sprouting and target reinnervation.

FK506 enhances reinnervation by collateral sprouting

Following peripheral nerve lesion, reinnervation of denervated targets may occur by means of two different mechanisms: regeneration of injured axons and collateral sprouting of neighboring undamaged axons. These two growth responses have different characteristics. Axonal regeneration after peripheral nerve damage depends on the presence of a suitable pathway for growth (the degenerated nerve) and occurs whether or not appropriate target tissue is available. In contrast, collateral sprouting is a characteristic of normal undamaged nerves in response to elimination of other axons within the same target tissues (Diamond and Foerster, 1992). In adult mammals, large myelinated fibers

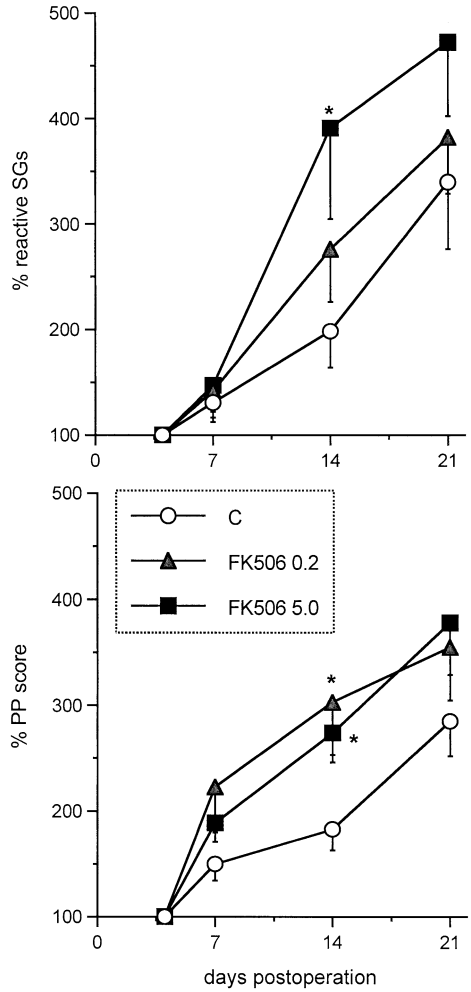

Fig. 7. Plot of the percentage with respect to the baseline values found at 4 days of reactive sweat glands (SGs) and of the pinprick (PP) score achieved by collateral reinnervation from the saphenous nerve during follow-up after resection of the sciatic nerve. Bars represent the SEM. *$P < 0.05$ vs group C.

readily regenerate after axotomy. However, unlike thinly myelinated and unmyelinated axons, uninjured sensory large fibers fail to sprout collateral branches and make functional reinnervation into denervated territories (Devor et al., 1979; Jackson and Diamond, 1984). The extension of the peripheral territory innervated by collateral sprouting of thinly myelinated and unmyelinated sensory (Brenan et al., 1988; Doucette and Diamond, 1987; Kinnman and Aldskogius, 1986; Nixon et al., 1984) and sympathetic nerve fibers (Gloster and Diamond, 1992; Kennedy et al., 1988; Navarro and Kennedy, 1988) does not have the temporal and spatial constraints that affect sprouting of large fibers, either low-threshold sensory (Jackson and Diamond, 1984; Kinnman et al., 1992) or α-motor neurons (Brown et al., 1980; Torigoe, 1985).

In agreement with previous reports (Devor et al., 1979; Navarro and Kennedy, 1988; Wiesenfeld-Hallin et al., 1989), the nociceptive and sudomotor saphenous nerve territories readily expanded after sciatic nerve resection, increasing the responses in areas normally supplied by the saphenous nerve and extending to neighboring sciatic areas of the paw. Since collateral sprouting becomes functionally evident for mechanosensitive nociceptors (Nixon et al., 1984) and for sympathetic sudomotor fibers (Navarro and Kennedy, 1988) at about 7–10 days of denervation, we compared the normal saphenous map to that found in each animal at 4 dpo. This analysis revealed that collateral reinnervation by both types of axons was enhanced by treatment with FK506. The cutaneous field expansion followed a faster course between 4 and 14 days in both FK506-treated groups than in the control animals. However, during the subsequent week the slopes were less different (see Fig. 7). Collateral sprouting of nociceptive axons was similarly increased with both FK506 dosages (0.2 and 5 mg/kg). In contrast, collateral reinnervation of SGs was more extensive with the high dose, suggesting that the effects of FK506 on neural responses to injury may vary between neuronal subtypes.

Possible mechanisms of action and implications

The mechanisms influencing axonal regeneration and collateral sprouting after denervation display also distinct features. In the regenerative growth cone, new microtubules are assembled and stabilized by means of posttranslational modifications of tubulin and microtubule-associated proteins, which are phosphorylated by growth associated proteins (GAPs). The local environment surrounding the regenerative growth cones influences axonal elongation through changes in nonneuronal cells, including infiltrating macrophages and reactive Schwann cells, and production of neurotrophic and neurotropic factors (Fu and Gordon, 1997; Verdú and Navarro, 1998). On another hand, terminal sprouting of regenerative axons seems to have more in common with target-related collateral sprouting of intact axons than with regeneration along peripheral nerve trunks. This is supported by the findings that anti-NGF serum administration does not affect regeneration of injured sensory and sympathetic nerves (Diamond et al., 1992a; Gloster and Diamond, 1992), but does prevent terminal arborization of sympathetic regenerative axons (Bjerre et al., 1974) and collateral sprouting of nociceptive and sympathetic axons into denervated skin (Diamond et al., 1992b; Doubleday and Robinson, 1992; Gloster and Diamond, 1992). Administration of NGF and other neurotrophins in vivo leads to potentiation of collateral sprouting within the denervated skin (Diamond et al., 1992b) and in the spinal cord (Schnell et al., 1994); NGF, by inducing a dynamic reorganization of the cytoskeleton, promotes generation of axon collateral branches (Gallo and Letourneau, 1998). In contrast, application of NGF does not affect the rate of axonal elongation after axotomy but delays the onset of regeneration (Gold, 1997), probably by reducing the neuronal body response to injury (Mohiuddin et al., 1999). Taken

together, the present findings indicate that FK506 increases reinnervation of NGF-independent (nerve regeneration) and NGF-dependent (collateral sprouting) processes.

With respect to the possible mechanisms of action proposed to explain the regeneration-promoting effects of FK506, binding of FK506 to FKBP-12 and subsequent calcineurin inhibition was the first mechanism proposed (Gold et al., 1994, 1998; Lyons et al., 1994). Inhibition of calcineurin by FK506 prevents GAP-43 dephosphorylation and promotes its active state. Axonal elongation during development and regeneration correlates with the expression of GAP-43 (Benowitz and Routtenberg, 1997). Increased expression of GAPs, such as GAP-43 and GAP-23, also promotes spontaneous nerve sprouting at the neuromuscular junction (Caroni, 1997). However, GAP-43 is not increased in motor neurons induced to sprout by muscle inactivity or by partial denervation (Bisby et al., 1996), further indicating that the control of sprouting is largely a target-dependent phenomenon, responsive to increased production of neurotrophic factors in the denervated tissue (Mearow et al., 1993).

The effect of FK506 in accelerating collateral sprouting would, therefore, be inconsistent with a mechanism involving GAP-43 phosphorylation via interaction with FKBP-12. Thus, the present in vivo findings are consistent with the demonstration that FK506 promotes neurite elongation in neuronal cultures from FKBP-12 knockout mice (Gold et al., 1999), indicating that the neurotrophic effects of FK506 are not mediated by FKBP-12. Furthermore, nonimmunosuppressant derivatives of FK506 do not inhibit calcineurin yet speed nerve regeneration (Gold et al., 1997; Steiner et al., 1997). Instead, we suggested that the ability of FK506 to increase both nerve regeneration and collateral sprouting is mediated by FKBP-52 (Gold et al., 1999). Binding to FKBP-52 leads to dissociation of the mature steroid receptor complex, and several downstream effectors may be involved in enhancement of axonal growth, including increased expression of c-jun and GAP-43, stimulation of the MAP kinase (ERK) pathway, and interaction with cytoskeleton components (Gold, 1999, 2000a).

Acknowledgments

This work was supported by grants from the Fondo de Investigación Sanitaria (FIS00-0031-02), the Ministerio de Ciencia y Tecnología (SAF2002-04016), and the Acadèmia de Ciències Mèdiques de Catalunya i Balears, Spain. We thank Fujisawa Pharmaceuticals Inc. (Osaka, Japan) for its generous gift of FK506 and the Kerr Company (Romulus, MI) for providing Elasticon. The technical help of Jessica Jaramillo is greatly acknowledged.

References

Benowitz, L.I., Routtenberg, A., 1997. GAP-43: an intrinsic determinant of neuronal development and plasticity. Trends Neurosci. 20, 84–91.

Bisby, M.A., Tetzlaff, W., Brown, M.C., 1996. GAP-43 mRNA in mouse motoneurons undergoing axonal sprouting in response to muscle paralysis or partial denervation. Eur. J. Neurosci. 8, 1240–1248.

Bjerre, B., Bjorklund, A., Edwards, D.C., 1974. Axonal regeneration of peripheral adrenergic neurons: effects of anti-serum to nerve growth factor in mouse. Cell Tissue Res. 148, 441–476.

Brenan, A., Jones, L., Owain, N.R., 1988. The demonstration of the cutaneous distribution of saphenous C-fibres using a plasma extravasation technique in the normal rat and following nerve injury. J. Anat. 157, 57–66.

Brown, M.C., Holland, R.L., Ironton, R., 1980. Nodal and terminal sprouting from motor nerves in fast and slow muscles in the mouse. J. Physiol. 306, 493–510.

Büttemeyer, R., Rao, U., Jones, N.F., 1995. Peripheral nerve allograft transplantation with FK506: functional, histological, and immunological results before and after discontinuation of immunosuppression. Ann. Plast. Surg. 35, 396–401.

Caroni, P., 1997. Intrinsic neuronal determinants that promote axonal sprouting and elongation. Bioessays 19, 767–775.

Constantini, L.C., Isacson, O., 2000. Immunophilin ligands and GDNF enhance neurite branching or elongation from developing dopamine neurons in culture. Exp. Neurol. 164, 60–70.

Devor, M., Schonfeld, D., Seltzer, Z., Wall, P.D., 1979. Two modes of cutaneous reinnervation following periperal nerve injury. J. Comp. Neurol. 185, 211–220.

Diamond, J., Foerster, A., 1992. Recovery of sensory function in skin deprived of its innervation by lesion of the peripheral nerve. Exp. Neurol. 115, 100–103.

Diamond, J., Foerster, A., Holmes, M., Coughlin, M., 1992a. Sensory nerves in adult rats regenerate and restore function to the skin independently of endogenous NGF. J. Neurosci. 12, 1467–1476.

Diamond, J., Holmes, M., Coughlin, M., 1992b. Endogenous NGF and nerve impulses regulate the collateral sprouting of sensory axons in the skin of the adult rat. J. Neurosci. 12, 1454–1466.

Doolabh, V.B., Mackinnon, S.E., 1999. FK506 accelerates functional recovery following nerve grafting in a rat model. Plastic Reconstr. Surg. 103, 1928–1936.

Doubleday, B., Robinson, P.P., 1992. The role of nerve growth factor in collateral reinnervation by cutaneous C-fibres in the rat. Brain Res. 593, 179–184.

Doucette, R., Diamond, J., 1987. Normal and precocious sprouting of heat nociceptors in the skin of adult rats. J. Comp. Neurol. 261, 592–603.

Dubernard, J-M., Owen, E., Herzberg, G., Lanzetta, M., Martin, X., Kapila, H., Dawahra, M., Hakim, N.S., 1999. Human hand allograft: report on first 6 months. Lancet 353, 1315–1320.

Fansa, H., Keilhoff, G., Altmann, S., Plogmeier, K., Wolf, G., Schneider, W., 1999. The effect of the immunosupressant FK506 on peripheral nerve regeneration following nerve grafting. J. Hand Surg. 24B, 38–42.

Fu, S.Y., Gordon, T., 1995. Contributing factors to poor functional recovery after delayed nerve repair: prolonged axotomy. J. Neurosci. 15, 3876–3885.

Fu, S.Y., Gordon, T., 1997. The cellular and molecular basis of peripheral nerve regeneration. Mol. Neurobiol. 14, 67–116.

Gallo, G., Letourneau, P.C., 1998. Localized sources of neurotrophins initiate axon collateral sprouting. J. Neurosci. 18, 5403–5414.

Gloster, A., Diamond, J., 1992. Sympathetic nerves in adult rats regenerate normally and restore pilomotor function during an anti-NGF treatment that prevents their collateral sprouting. J. Comp. Neurol. 326, 363–374.

Gold, B.G., 1997. Axonal regeneration of sensory nerves is delayed by continuous intrathecal infusion of nerve growth factor. Neuroscience 76, 1153–1158.

Gold, B.G., 1999. FK506 and the role of the immunophilin FKBP-52 in nerve regeneration. Drug Metab. Rev. 31, 649–663.

Gold, B.G., 2000a. Neuroimmunophilin ligands and the role of steroid hormone chaperone proteins in nerve regeneration, in: Gold, B.G., Fischer, G., Herdegen, T. (Eds.), Immunophilins in the Brain. FKBP-Ligands: Novel Strategies for the Treatment of Neurodegenerative Disorders, Prous Science, Barcelona, Spain, pp. 3–22.

Gold, B.G., 2000b. Therapeutic potential of immunophilin ligands in neurological disorders. Exp. Op. Invest. Drugs 9, 2331–2342.

Gold, B.G., Densmore, V., Shou, W., Matzuk, M.M., Gordon, H.S., 1999. Immunophilin FK506-binding protein 52 (not FK506-binding protein 12) mediates the neuroprotective action of FK506. J. Pharmacol. Exp. Ther. 289, 1202–1210.

Gold, B.G., Katoh, K., Storm-Dickerson, T., 1995. The immunosuppressant FK506 increases the rate of axonal regeneration in rat sciatic nerve. J. Neurosci. 15, 7509–7516.

Gold, B.G., Storm-Dickerson, T., Austin, D.R., 1994. The immunosuppressant FK506 increases functional recovery and nerve regeneration following peripheral nerve injury. Restor. Neurol. Neurosci. 6, 287–296.

Gold, B.G., Yew, J.Y., Zeleny-Pooley, M., 1998. The immunosuppressant FK506 increases GAP-43 mRNA levels in axotomized sensory neurons. Neurosci. Lett. 241, 25–28.

Gold, B.G., Zeleny-Pooley, M., Wang, M-S., Chaturvedi, P., Armistead, D.M., 1997. A nonimmunosuppressant FKBP-12 ligand increases nerve regeneration. Exp. Neurol. 147, 269–278.

Jackson, P.C., Diamond, J., 1984. Temporal and spatial constraints on the collateral sprouting of low-threshold mechanosensory nerves in the skin of rats. J. Comp. Neurol. 226, 336–345.

Jones, J.W., Gruber, S.A., Barker, J.H., Breidenbach, W.C., 2000. Successful hand transplantation: one year follow-up. N. Engl. J. Med. 343, 468–473.

Kennedy, W.R., Navarro, X., Kamei, H., 1988. Reinnervation of sweat glands in the mouse: axonal regeneration versus collateral sprouting. Muscle Nerve 11, 603–609.

Kennedy, W.R., Sakuta, M., Quick, D.C., 1984. Rodent eccrine sweat glands: a case of multiple efferent innervation. Neuroscience 11, 741–749.

Kinnman, E., Aldskogius, H., 1986. Collateral sprouting of sensory axons in the glabrous skin of the hindpaw after chronic sciatic nerve lesion in adult and neonatal rats: a morphological study. Brain Res. 377, 73–82.

Kinnman, E., Aldskogius, H., Johansson, O., Wiesenfeld-Hallin, Z., 1992. Collateral reinnervation and expansive regenerative reinnervation by sensory axons into “foreign” denervated skin: an immunohistochemical study in the rat. Exp. Brain Res. 91, 61–72.

Lee, M., Doolabh, V.B., Mackinnon, S.E., Jost, S., 2000. FK506 promotes functional recovery in crushed rat ciatic nerve. Muscle Nerve 23, 633–640.

Lyons, W.E., George, E.B., Dawson, T.M., Steiner, J.P., Snyder, S.H., 1994. Immunosuppressant FK506 promotes neurite outgrowth in cultures of PC12 cells and sensory ganglia. Proc. Natl. Acad. Sci. USA 91, 3191–3195.

Mackinnon, S.E., Doolabh, V.B., Novak, C.B., Trulock, E.P., 2001. Clinical outcome following nerve allograft transplantation. Plastic Reconstr. Surg. 107, 1419–1429.

Mearow, K.M., Kril, Y., Diamond, J., 1993. Increased NGF mRNA expression in denervated rat skin. Neuroreport 4, 351–354.

Mohiuddin, L., Delcroix, J.D., Fernyhough, P., Tomlinson, D.R., 1999. Focally administered nerve growth factor suppresses molecular regenerative responses of axotomized peripheral afferents in rats. Neuroscience 91, 265–271.

Navarro, X., Butí, M., Verdú, E., 1994a. Autotomy prevention by amitriptyline after peripheral nerve section in different strains of mice. Restor. Neurol. Neurosci. 6, 151–157.

Navarro, X., Kennedy, W.R., 1988. Effect of age on collateral reinnervation of sweat glands in the mouse. Brain Res. 463, 174–181.

Navarro, X., Udina, E., Ceballos, D., Gold, B.G., 2001. Effects of FK506 on nerve regeneration and reinnervation after graft or tube repair of long gaps. Muscle Nerve 24, 905–915.

Navarro, X., Verdú, E., Butí, M., 1994b. Comparison of the regenerating and reinnervating capabilities of different functional types of nerve fibers. Exp. Neurol. 129, 217–224.

Navarro, X., Verdú, E., Wendelschafer-Crabb, G., Kennedy, W.R., 1997. Immunohistochemical study of reinnervation by regenerative axons. J. Comp. Neurol. 380, 164–174.

Nixon, B.J., Doucette, R., Jackson, P.C., Diamond, J., 1984. Impulse activity evokes precocious sprouting of nociceptive nerves into denervated skin. Somatosens. Res. 2, 97–126.

Schnell, L., Schneider, R., Kolbeck, R., Barde, Y., Schwab, M.E., 1994. Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. Nature 367, 170–173.

Snyder, S.H., Lai, M.M., Burnett, P.E., 1998. Immunophilins in the nervous system. Neuron 21, 283–294.

Spencer, C.M., Goa, K.L., Gillis, J.C., 1997. Tacrolimus, an update of its pharmacology and clinical efficacy in the management of organ transplantation. Drug 54, 925–975.

Steiner, J.P., Connolly, M.A., Valentine, H.L., Hamilton, G.S., Dawson, T.M., Hester, L., Snyder, S.H., 1997. Neurotrophic actions of nonimmunosuppressive analogues of immunosuppressive drugs FK506, rapamycin and cyclosporin A. Nature Med. 3, 421–428.

Sulaiman, O.A.R., Voda, J., Gold, B.G., Gordon, T., 2002. FK506 increases peripheral nerve regeneration after chronic axotomy but not after chronic Schwann cell denervation. Exp. Neurol. 175, 127–137.

Torigoe, K., 1985. Distribution of motor nerve sproutings in the mouse gastroenemius muscle after partial denervation. Brain Res. 330, 273–282.

Thompson, R.J., Doran, J.F., Jackson, P., Dhillon, A.P., Rode, J., 1983. PGP 9.5—a new marker for vertebrate neurons and neuroendocrine cells. Brain Res. 278, 224–228.

Udina, E., Ceballos, D., Verdú, E., Gold, B.G., Navarro, X., 2002. Bimodal dose-dependence of FK506 on the rate of axonal regeneration in mouse peripheral nerve. Muscle Nerve 26, 348–355.

Undre, N.A., Stevenson, P., Schäfer, A., 1999. Pharmacokinetics of Tacrolimus: clinically relevant aspects. Transplant. Proc. 31, 21–24.

Verdú, E., Navarro, X., 1997. Comparison of immunohistochemical and functional reinnervation of skin and muscle after peripheral nerve injury. Exp. Neurol. 146, 187–198.

Verdú, E., Navarro, X., 1998. The role of Schwann cell in nerve regeneration, in: Castellano, B., González, B., Nieto-Sampedro, M. (Eds.), Understanding Glial Cells, Kluwer Academic, Boston, pp. 319–359.

Vilches, J.J., Verdú, E., Ceballos, D., Navarro, X., 2002. Changes in mouse sudomotor function and sweat gland innervation with aging. Auton. Neurosci. 95, 80–87.

Wang, M-S., Zeleny-Pooley, M., Gold, B.G., 1997. Comparative dose dependence study of FK506 and cyclosporin A on the rate of axonal regeneration in the rat sciatic nerve. J. Pharmacol. Exp. Ther. 282, 1084–1097.

Wiesenfeld-Hallin, Z., Kinnman, E., Aldskogius, H., 1989. Expansion of innervation territory by afferents involved in plasma extravasation after nerve regeneration in adult and neonatal rats. Exp. Brain Res. 76, 88–96.