ESTIMATIONS OF TOPOGRAPHICALLY CORRECT REGENERATION TO NERVE BRANCHES AND SKIN AFTER PERIPHERAL NERVE INJURY AND REPAIR

Anna Puigdellívol-Sánchez [1,2], Alberto Prats-Galino [1], Carl Molander [3]

[1] Department of Human Anatomy and Embryology, Faculty of Medicine, University of Barcelona, 08036 Barcelona, Spain.
[2] CAP Anton de Borja. c/Marconi-cantonada Edison s/n. Consorci Sanitari de Terrassa. 08091 Rubí, Spain.
[3] Department Neuroscience, Rehabilitation Medicine, Uppsala University Hospital, SE-751 85 Uppsala, Sweden.

Number of text pages (including figures and tables): 34
Number of figures: 4
Number of tables: 2

Corresponding Author:
Alberto Prats Galino.
Dept d'Anatomia i Embriologia Humana
Facultat de Medicina (Universitat de Barcelona)
c/ Casanova nº 143
08036 Barcelona
(Spain)
Telephone number: 34-93-4021905
Fax number: 34-93-4035263
aprats@ub.edu

ABSTRACT

Peripheral nerve injury is typically associated with long-term disturbances in sensory localization, despite nerve repair and regeneration. Here we investigate the extent of correct reinnervation by back-labeling neuronal soma with fluorescent tracers applied in the target area before and after sciatic nerve injury and repair in the rat. The subpopulations of sensory or motor neurons that had regenerated their axons to either the tibial branch or the skin of the third hindlimb digit were calculated from the number cell bodies labeled by the first and/or second tracer. Compared to the normal control side, 81% of the sensory and 66% of the motor tibial nerve cells regenerated their axons back to this nerve, while 22% of the afferent cells from the third digit reinnervated this digit. Corresponding percentages based on quantification of the surviving population on the experimental side showed 91%, 87%, and 56%, respectively. The results show that nerve injury followed by nerve repair by epineurial suture results in a high but variable amount of topographically correct regeneration, and that proportionally more neurons regenerate into the correct proximal nerve branch than into the correct innervation territory in the skin.

SECTION: 4. Nervous System Development, Regeneration and Aging.
KEYWORDS. Nerve regeneration; fluorescent dyes; adult rat; peripheral nerve injuries.
ABBREVIATIONS: Footnote [1]

[1] ABBREVIATIONS: DRG: dorsal root ganglion; DRGs: dorsal root ganglia, SC: spinal cord; DY: diamidino yellow; FB: fast blue; FG: fluoro-gold; FBDY: double labeled cells with FB and DY. IR-min: minimal index of regeneration. IR-max: maximal index of regeneration. RLR: Relative lost regeneration. GLR: Global lost regeneration. RTCR: Relative topographically correct regeneration. GTCR: Global topographically correct regeneration. SD: Standard deviation. ctrl: control. exp: experimental.

1. INTRODUCTION

Peripheral nerve injury is followed by degeneration of the axons distal to the lesion and by regenerative growth from the proximal axons still connected to the parental cell body. The number of proximal axons that succeed to regenerate towards their former targets is dependent on several intrinsic and environmental factors. Of particular importance is the survival rate of the injured neurons, the shift from transmission mode to regeneration mode for the injured neurons, and axonal contact to the physical and chemical environment provided by the basal lamina and Schwann cells in the distal nerve stump (for review, see Burnett and Zager, 2004; Fenrich and Gordon, 2004). The reconnection of the regenerating axons with sensory receptors of the correct functional type (Koerber et al., 1989) and in the correct topographical area (Lutz et al., 2001) is also of importance for the functional outcome.

One of the methods to investigate the topographical accuracy of peripheral reinnervation after nerve injury include the study of alteration in the topographical pattern of motoneurons in the spinal cord (Aldskogius et al., 1987; Brown and Hardman, 1987; Shenaq et al., 1989) or the appearance of motoneuron axons in a sensory branch of a nerve (Brushart, 1990; Rath and Green, 1991). Misdirection may also be investigated by applying different tracers to different nerve branches before and after the nerve injury (Molander and Aldskogius, 1992), or to different branches after injury to detect neurons sending collaterals to multiple nerve branches (Valero-Cabré et al., 2001; 2004), or targets, (Henning and Dietrichs, 1994; Ito and Kudo, 1994). A more direct approach is to back-label neurons innervating the peripheral target before and after the injury. The first tracer is injected in the target region before nerve injury to label the original neuronal population and the second tracer after the regeneration period, with the aim to label the population that has regenerated. Double-labeled cell bodies, (with some methodological limitations, see below), represent neurons that have re-innervated the original target. However, the cell counts must be corrected due to at least two types of confounders. First, identification of labeled cells might be compromised by altered uptake of the second tracer, fading of the first tracer and possible cell death (Novikova et al, 1997; Puigdellívol-Sánchez et al., 2000b, 2002). Second, if the first tracer injected in the target remains available in the tissue for "re-uptake", then axons misdirected towards the target of study and that previously projecting to other targets, would take up not only the second tracer but also remaining deposits of the first tracer injected months earlier. This would result in false double

labeling and confuse the identification of the original population and of correctly regenerating cells. The latter problem has been proposed previously (Innocenti et al., 1986; Rende et al., 1991; Puigdellívol-Sánchez et al., 1998b, 2000b; Popratiloff et al., 2001), confirmed by findings in our research group for FB, FG and DY (Puigdellívol-Sánchez et al., 2003) and recently described for DiI (Brushart et al., 2005).

Different combinations of tracers had previously been used: dextran amines/ rhodamine or fluorescein (Fritzsch and Sonntag, 1991), HRP/fluorescent tracer (Wigston and Kennedy, 1987; Brushart, 1990, 1993), FB/FG or DiI (Bodine-Fowler et al., 1997; Popratiloff et al., 2001); FG/DiI (Madison et al., 1996) or fluororuby (Brushart et al., 2005), FB with DY (Hendry et al., 1986; Rende et al., 1991), or DY/FB (Negredo et al, 2004). We have used DY and FB since these fluorescent tracers do not require an axonal injury to allow uptake of the tracer, nor histological processing. They are easily identifiable at the single cell level; DY is found mainly in the nucleus, and FB the cytoplasm, and both are visible through the same ultraviolet or violet microscope filters. They label similar neuronal populations after nerve injection (which minimizes nerve injury) and subcutaneous injection. Long-term accumulation of DY in the cell body does not affect neuronal regeneration or the uptake of FB as second tracer, and there's negligible subcutaneous re-uptake of DY (Puigdellívol-Sánchez et al., 1998b, 2000b, 2002, 2003).

Even though a certain degree of selective reinnervation to peripheral nerve branches and muscular targets has been described, little is known about the extent to which this occurs to distal sensory innervation targets, particularly in relation to the rate of correct axonal growth into a mixed nerve branch from the injured parental main nerve.

We have used the distal phalanges of the hindlimb digits in the rat as a model to study problems related to the topographical accuracy of peripheral regeneration after nerve injury. We have previously shown that the neuronal cell bodies of the afferents belonging to the main hindlimb nerves are somatotopically arranged in dorsal root ganglia (Puigdellívol-Sánchez et al., 1998a). However, the arrangement of neurons innervating the distal phalanges was found to be less organized (Prats-Galino et al., 1999). For this reason, the method of successive application of retrograde tracers is better than the method of detecting somatotopical distortion in DRGs (see above) to study regeneration accuracy in the digits.

Furthermore, the distal phalanges of the digits are innervated not only by tibial and peroneal nerve branches, but also by branches of the femoral nerve and by a proximal sciatic branch, which we name the musculocutaneous nerve of the hindlimb (Puigdellívol et al., 2000a). The peripheral innervation from these branches is both separate and overlapping. Thus, it is important to consider possible contribution from collateral sprouting from these nerves after a sciatic nerve injury to avoid confusion with reinnervation from the tibial and peroneal nerves. The detailed results from the collateral sprouting have been presented separately (Puigdellívol-Sánchez et al., 2005).

The aim of the present study has been to use the method of successive labeling with different fluorescent tracers to quantify the rate of accurate regeneration into nerve branches/fascicles just distal to a nerve transection and epineurial repair, and also the rate of topographical correct cutaneous reinnervation. In the longer perspective, the results could be relevant for testing the efficacy of different techniques of nerve repairing after nerve injury.

2. RESULTS

None of the cases showed faint diffuse fluorescent labeling in all dorsal root ganglion (DRG) cells that would have suggested significant vascular spread of tracer from the injection site.

Numbers of FB and DY labeled profiles in DRGs and spinal cord are presented in Tables and Figs. 1 and 2. The FB and DY single labeled neurons and FBDY double labeled neurons found after sciatic lesion and regeneration were used to calculate: a) an index showing the proportion of injured neurons that had regenerated and could be labeled from a site distal to the injury (experimental side/control side), b) the number of neurons that had not regenerated towards the region which they originally innervated, “lost regeneration”, and c) the number of neurons that had regenerated to the region which they previously innervated, “correct regeneration”. The ranges of results obtained varied according to the formulae (described below) that were used to compensate for the technical limitations described in the introduction.

FG labeling alone or with any of the other tracers was used to investigate the possible contribution from collateral sprouting, i.e. ingrowths from an adjacent uninjured nerve into the denervated territory. Detailed results have been presented elsewhere (Puigdellívol-Sánchez et al., 2005). Briefly, the femoral nerve accounted for 1 to 5.7% of the innervation of the injured third digits, and the musculocutaneous nerve

for 1.1 to 4%, without significant differences between the experimental and control hindlimbs.

Estimation of the rate of regeneration

An **index of regeneration** (IR) was defined as the total number of regenerating neurons identified by the second tracer in the right experimental side $(FBDY+FB)_{exp}$, divided by the number of labeled neurons on the control side, identified either by the total number of FB labeled cells $(FBDY+FB)_{ctrl}$ for a maximal index (IR-max), or by the total number of cells (FBDY+FB+DY) for a minimal index (IR-min).

Index of reinnervation= total number of regenerating cells / total number of control cells

$$\text{IR-min} = (FBDY+FB)_{exp} / (FBDY+FB+DY)_{ctrl}$$

$$\text{IR-max} = (FBDY+FB)_{exp} / (FBDY+FB)_{ctrl}$$

Retrograde tracing from the tibial nerve resulted in an IR-min of 87.4 ± 12.2% for DRG cells, and 74.9% ± 16.5% for motoneurons, respectively. There was a significant difference between indexes of DRG cells and motoneurons (p=0.028). Percentages increased to 95.0 ± 13.9% for DRG cells, and 93.4 ± 19.9% for motoneurons when the IR-max was calculated, without significant difference (p=0.60). Retrograde tracing from the digits resulted in an IR-min of 51.9 ± 15.9%, and an IR-max of 59.6 ± 20.6%, respectively. These indexes are significantly lower compared to the indexes calculated after retrograde tracing from the tibial nerve (p=0.004 and p=0.015).

Estimation of the lost regeneration

The **lost regeneration** is defined as the fraction of the original population of neurons from a nerve branch that did not regenerate at all after the nerve injury or that regenerated to other regions than the original region. They correspond to the total number of cells that show only the first tracer (DY_{exp}). This number may be compared: 1) to the original population of the experimental hindlimb $(DY+FBDY)_{exp}$ to obtain an index of the injured neurons on the experimental side that did not regenerate to the same region –relative lost regeneration index (RLR)–, and 2) to the total number of labeled cells showing DY on the control side $(DY+FBDY)_{ctrl}$ –global lost regeneration (GLR)–.

Relative lost regeneration (RLR) =
=Original injured population that did not regenerate to the same region / surviving original population
= DY_{exp} / $(DY+FBDY)_{exp}$

Global lost regeneration (GLR) =
=Original injured population that did not regenerate to the same region / corresponding tibial population on the control side=
=DY_{exp} / $(DY+FBDY)_{ctrl}$

R236 was excluded for the purpose of these calculations because the DY labeling on the control side was unsuccessful.

Retrograde tracing from the tibial nerve resulted in an RLR index of 8.9 ± 4.2% for DRG cells, and 12.6 ± 6.4% for motoneurons. The GLR index was 7.7 ± 4.7% for DRG cells, and 7.6 ± 3.9% for motoneurons. There were no significant differences between DRG cells and motoneurons, using neither the RLR index ($p=0.345$) nor the GLR index ($p=0.753$).

Retrograde tracing from the digits resulted in a GLR index of 15.2 ± 9.5 %, and a RLR index of 43.8 ±12.0%, respectively. The RLR indexes ($p=0.002$), but not the GLR indexes ($p=0.177$), were statistically different when comparing retrograde tracing from the tibial nerve and from the digits.

Estimation of the topographically correct regeneration

Topographically correct regeneration is defined as the fraction of the original population of axons that had regenerated back to the region or nerve branch they innervated before the nerve injury. They would correspond to the number of cells that show both tracers ($FBDY_{exp}$). Like for the lost regeneration (see above), the number of double labeled cells may be compared: 1) to the original population of the experimental hindlimb $(DY+FBDY)_{exp}$ to obtain an index of the total number of injured neurons that had regenerated their axons to the same region –relative topographically correct regeneration (RTCR)–, and 2) to the original population on the control side $(DY+FBDY)_{ctrl}$ –global topographically correct regeneration (GTCR)–. Since 100%

double labeling does not occur even on the control side, and in particular so after retrograde tracing from the digits, the ratio should be related to the maximal double labeling rate obtained in the control hindlimb ($FBDY_{ctrl}$ / $(DY+FBDY)_{ctrl}$).

Relative topographically correct regeneration (RTCR)=
= Topographically correct regenerating cells / surviving original population=
= $FBDY_{exp}$/ $(DY+FBDY)_{exp}$

Global topographically correct regeneration (GTCR)=
= (Topographically correct regenerating cells / corresponding population of an uninjured nerve) / Maximal percentage of double labeling in a control situation=
= ($FBDY_{exp}$ / $(DY+FBDY)_{ctrl}$) / ($FBDY_{ctrl}$ / $(DY+FBDY)_{ctrl}$)=
=$FBDY_{exp}$ / $FBDY_{ctrl}$

Retrograde tracing from the tibial nerve resulted in an RTCR index of 91.1 ± 4.2% for DRG cells, and 87.4 ± 6.4% for motoneurons. These percentages were not significantly different (p=0.345). The GTCR index was 81.9 ± 13.4% for DRG cells, and 66.5 ± 11.8% for motoneurons. This difference between DRG cells and motoneurons was statistically significant (p=0.028). Retrograde tracing from the digits resulted in a RTCR of 56.2 ± 11.9%, and a GTCR of 22.2 ± 3.9%. Both the RTCR index (p=0.002) and the GTCR index (p=0.004) were statistically different when comparing the results from the retrograde tracing from the tibial nerve and from the digits.

3. DISCUSSION

The main results of this investigation are detailed descriptions of the proportion of regenerating sensory and motor neurons that reinnervate a nerve branch or a topographically specified skin territory where they resided before the injury to the sciatic nerve.

The innervation of the digits after the sciatic nerve injury might come from collateral sprouting from uninjured adjacent nerves and/or from regenerative sprouting from the sciatic nerve. We have addressed these two possibilities at the same time by

labeling the sciatic nerve target and the adjacent femoral nerve and musculocutaneous nerves by different tracers.

The distal phalanges have the advantage of being comparatively densely innervated by well-defined nerves, and their natural boundaries facilitated reproducible injections of tracers. However, some technical aspects, associated to any sequential double labeling design, must be considered before interpreting the results.

3.1. Technical considerations

As application of a dye in a capsule to a transected nerve seems to label most, if not all, neurons of that nerve (Puigdellívol-Sánchez et al., 2000b) we assume that in the present experiments, labeled cells that contain only tracers applied to a digit or to the tibial branch (DY and FB), without tracer applied to the adjacent nerves (FG), is indeed a result of regenerative sprouting from the sciatic population.

The different mathematical formulae proposed are based on a theoretical model in which the first tracer, DY, should label the original population, and the second dye, FB, the regenerating population. Double FB-DY labeled cells then correspond to neurons that have re-innervated the region they innervated before the injury. However, the number of neurons detected after long survival times depend on differences in the fluorescent intensity between the experimental side and the control side, which may be due to several factors discussed in detail below, in particular differences in tracer uptake and fading.

Tibial nerve

In the control hindlimb, continuous uptake for months of DY results in not only an intense labeling in the nuclei, but also a moderate labeling in the cytoplasm, that might interfere with the visualization of the cytoplasmatic FB.

In the experimental hindlimb, the re-uptake of remaining deposits of DY does not increase the intensity of the labeling of the original population, probably because the damaged axons have lost the contact with DY after the sciatic injury, and because a certain fading of the first tracer might occur (Puigdellívol-Sánchez et al., 2002), resulting in reliable FB cell counts. However, re-uptake affects the identification of the original and regenerated populations: fibers that originally resided in other sciatic branches may be misdirected and regenerate into the tibial and uptake remaining deposits of DY, resulting in false detection as original tibial neurons. Since they will

probably uptake also the second tracer, they would become a false positive fraction of the double-labeled cells that have regenerated correctly (Puigdellívol-Sánchez et al, 2003).

Digits

In the skin, the re-uptake has been found to be negligible for DY (Puigdellívol-Sánchez et al., 2003). Furthermore, the DY labeling on the control hindlimb is not so intense, probably because it was applied by a subcutaneous injection instead of a direct nerve application and because a prolonged survival time might lead to a certain fading. Consequently, the DY would not hide FB, neither on the control side, nor on the experimental side.

3.1.1. Estimation of the regeneration

The regenerating population is labeled with the second tracer, FB, and is compared with the labeled profiles in the control hindlimb.

Tibial nerve

The maximal index of reinnervation, expressing the relation of FB labeling between the hindlimbs, could be a slight overestimation because the total counts of FB cells in the control side might be affected by the intense DY labeling, reducing the denominator of the formula. The minimal index of reinnervation expresses the relation of FB labeling in the regenerating limb with the total number of labeled profiles with any of the two tracers in the control limb. This is, on the other hand, likely to be an underestimation, since it compares the number of labeled profiles with one tracer with the total number of profiles labeled with two tracers. Since the maximal index is likely to be an overestimation and the minimal index an underestimation, we can infer that the real rate of regeneration ranges between these two (87 – 95% for DRG neurons and 75 – 93% for motoneurons).

Digits

The maximal index of regeneration (59%) is likely to be a good estimation because the FB is not likely to be hidden by DY in the control side after skin application. A previous morphological study of sensory regeneration to distal peripheral targets used a combination of axonal counting in the regenerating nerve with

observation of distal plasma extravasation of Evans Blue after C fiber stimulation, and visualization of nerve terminals in the epidermis (Udina et al., 2003). We think that our method is more advantageous because is allows quantification of the neuronal innervation.

3.1.2. Estimation of the lost regeneration

The "lost innervation" represents cells that have not regenerated, or that have been misdirected to nerve branches other than those in which they originally resided. They are identified as cells labeled by only the first tracer, DY. Neurons with faded labeling and dead cells would not be included in this index.

Tibial nerve

The *global index* (about 7% for both DRG and motoneurons) is a likely underestimation since relates the underestimated DY counts (due to a potential DY fading) on the experimental side in the numerator, to the more reliable DY counts on the control side (potential fading compensated by continuous uptake of the tracer), represented in the denominator.

The *relative index* is calculated from DY counts on the experimental side only. These neurons are assumed to show a similar amount of fading even though it can not be excluded that certain subtypes of neurons are more vulnerable than others. On the other hand, the re-uptake of remains of the first dye by regenerating axons once they reach their target might also contribute to increased double labeling, resulting in an underestimation of the true proportion of neurons single labeled with DY. Thus, in theory, the denominator of the formula could be compensated by the previously described quantification of re-uptake for DY (DYr) in nerve branches (Puigdellívol-Sánchez et al., 2003), shown to affect up to a 18% of the tibial nerve population.

Relative lost regeneration, corrected for re-uptake =

$= \text{mean } DY_{exp} / [\text{mean } (DY+FBDY)_{exp} - \text{mean } (DYr)]$

Then, the relative index of the lost regeneration increases from 8,9% to 12.3% for DRG cells, and from 12,6 to 15.3% for motoneurons. Furthermore, this formula implies an over-correction because some DYr include also those that have regenerated to the correct branch. Thus, it is likely that the real rate of lost regeneration is included

in the reduced range between the corrected and uncorrected index. This reduced rate is consistent with the high degree of regeneration reported above.

Digits

Although fading may result in an underestimation of the global index, the relative index is not affected (see above), while re-uptake is negligible in the skin of the digits (Puigdellívol-Sánchez et al., 2003). The estimation of the lost regeneration to 43%, inferred from the formula based on DY labeling, is consistent with the regeneration rate of 59% discussed above, inferred from the FB results.

3.1.3. Estimation of misdirection

In the ideal model, misdirected axons could be studied by quantifying the number of neurons single labeled by the second tracer, FB. However, in the tibial nerve branch approach, the DY injection labeled most, but not all, of the neurons of the nerve (Taylor et al., 1983). The technique of nerve injection is likely to have resulted in very limited injury to the nerve branches during the important application of the first dye, resulting in minimal interference with the quantification of the regenerated parental nerve injured neurons (Puigdellívol-Sánchez et al., 2000b, 2002). The later application of FB in a capsule to the transected nerve exposed most axons to the dye. In digits, we applied a larger amount of FB than DY in order to ensure a maximal labeling of the previously DY-labeled original population. This probably contributed to the higher amount of FB single labeled neurons, compared to DY labeled neurons, on both the control and the experimental side following both proximal and distal dye application. Furthermore, on the experimental side, potential fading of the first tracer might lead to an overestimation of the proportion of FB single labeled neurons. Therefore, the proportion of FB single labeling would not be a good indicator of misdirection with this design and was not calculated. The other indexes presented in this study are based on calculations of the subgroup of DY labeled neurons among the original population and the resulting percentages should therefore not be affected by a potential incomplete digit or nerve branch labeling.

3.1.4. Estimation of the topographically correct regeneration

Ideally, the index of topographically correct regeneration would be calculated by dividing the number of double-labeled neurons after regeneration $(FBDY)_{exp}$ by the

number of all neurons containing the first tracer (FBDY+DY) on the experimental side (relative index) or on the control side (global index).

Tibial nerve

In the control limb, the number of detected double labeled cells in the denominator might be reduced because of the high intensity of the first tracer. In the experimental limb the re-uptake of the first tracer by regenerating axons could increase double labeling while fading of the first tracer would decrease it. Thus, the global index is indicative rather than precise.

The relative index is probably not affected by fading of the first tracer since single- and double DY labeled neurons are likely to be equally affected. Furthermore, it will not interfere with the visualization of the second tracer. Therefore, this index could just be corrected by the mean rate of re-uptake for DY.

Relative index of proximal topographically correct regeneration related to re-uptake = $(FBDY_{exp} - DYr) / (DY+FBDY - DYr)_{exp}$

The resulting rate includes an overcorrection, because the original neuronal population is already labeled by the first tracer, representing a valid estimation of the topographically correct regeneration.

It is reasonable, therefore, that the true rate of relative selective reinnervation is between the reduced range of the over-corrected relative index (87,6% in DRGs and 84.6% for motoneurons), and the corresponding uncorrected indexes (91% and 87%, respectively).

Digits

The double-labelled neurons can be securely identified as those that have regenerated back to their original territory since our previous studies have shown that there is minimal re-uptake of DY by regenerating axons when they reach the skin (Puigdellívol-Sánchez et al., 2003).

The reduced global rate of topographically correct reinnervation to a digit, 22%, is probably an underestimation due to fading of the first tracer, which leads to an underestimation of the total number of double labeled cells. However, the relative rate of topographically correct regeneration might be up to 56% for the detectable surviving

population, provided that the degree of fading is similar for the different populations of regenerating and non-regenerating neurones.

3.1.5. Considerations about quantification

The cell profile counts presented here have not been corrected according to modern stereological methods since ganglia were not sectioned following a random three-dimensional arrangement. One reason is that we aim to make 3-D reconstructions of the DRG (Puigdellívol-Sánchez et al., 1998a; Prats-Galino et al., 1999), and another that the total number of labelled neurons in the digit experiments (there is a maximal number of 500 labeled neurons in two ganglia) was too small to allow for the use stereology. A few studies have attempted to quantify FB labelled sciatic neurons in frozen sectioned material using stereology (Messina et al., 2000, Negredo et al., 2004). Interestingly, the number of neurons found in these studies is much higher than those obtained from complete reconstructions of the neuronal populations labelled with WGA-HRP (Swett et al., 1986, 1991) and from counting of neurons using retrograde tracing with fluorescent without correction for split cells, (Puigdellívol-Sánchez et al., 2000b), which should overestimate the real population. A possible explanation is the variability of section thickness due to the marked and uneven shrinkage of frozen sections, which has been reported to introduce substantial noise in the number estimations, when unbiased stereological methods are used (Negredo et al., 2004). Studies based on confocal microscopy might help to assess the degree of splitting of labelled cells in frozen material. Altogether, the results presented here should be considered as semi-quantitative.

Even though a considerable variability in cell counts was found, partly due to technical factors but also to the true normal anatomical diversity (Puigdellívol-Sánchez et al., 1998; Prats-Galino et al., 1999), clear and significant differences in the rate of topographically correct regeneration between nerve branch and innervated skin could be demonstrated in the present investigation.

3.2. Comments on regeneration

3.2.1. Collateral sprouting

The absence of differences in FG labeling between control and experimental hindlimbs indicate that collateral sprouting is of minor importance, when the injured

parental nerve is allowed to regenerate to its target (see Puigdellívol-Sánchez et al., 2005, for more details), which is consistent with previous findings (Devor et al., 1979).

3.2.2. Regenerative sprouting

The regeneration rates to the tibial nerve branch (between 75-95%) two months after nerve section and suture are in good agreement with previous studies suggesting that most of the neurones have survived and regenerated to the nerve branches after the nerve injury (Fritzsch and Sonntag, 1991; Al-Majed et al., 2000), and also with findings suggesting that sensory neurons regenerate better than motoneurons (Suzuki et al., 1998; Negredo et al., 2004). A smaller proportion of sensory neurones regenerated to the skin of the third digit (59%). The ability of regenerating axons to reinnervate distal targets is likely to be compromised by the progressive collapse of endoneurial tubes after denervation (You et al., 1997). It has been described that the growth-supportive environment of the Schwann cells in the distal nerve stump progressively deteriorates when nerve repair is delayed or if neurones have to regenerate over long distances (Fu and Gordon 1995; Sulaiman et al., 2002). However, some authors report how neurones regenerate their axons better in pre-degenerated distal nerve stumps in studies of delayed nerve repair using cross-anastomosing paradigms (Guntinas-Lichius et al., 2000). A possible degree of progressive long-term cell death after the nerve injury (Tandrup et al., 2000; Jivan et al., 2005), could also explain that decrease. In our experiments, the fading of the first tracer DY on the experimental side prevents quantification of cell death, as compared to the uninjured control side. However, the rate of regenerating cells can be used as an indication of the proportion of surviving neurons, provided that neuronal sprouting to multiple nerve branches is limited after nerve section (Molander and Aldskogius, 1992; Valero-Cabré et al., 2004) and the rate of regeneration is uniform among targets. A certain percentage of surviving non-regenerating neurones is also expected to be present. Our results would be comparable to a previous report showing a survival of about a 60% of the sensory afferents of the saphenous nerve (Baranowsky et al., 1994) and of the sciatic nerve (Puigdellívol-Sánchez et al., 2002), three months after injury and repair.

Studies based on multiple labeling of different nerve branches (see Introduction) reported a small number of double-labeled cells, suggesting that misdirection at the first bifurcation of the sciatic nerve is limited. This is in well agreement with the described preference of peripheral nerve axons to regenerate into the nerve branches in which they

originally resided (Politis, 1985), both for motor axons, which seem to prefer muscle branches (Brushart, 1990, 1993) and for sensory axons (Madison et al., 1996). The problem of re-uptake by regenerating axons was not accounted for in these studies. This problem was avoided, however, in a recent study (Brushart et al., 2005) using Fluoro-gold and Fluoro-ruby to quantify regeneration to nerve branches: the pre-labeled area was replaced by an unlabeled nerve graft from the contra-lateral side at the time of the main nerve injury to avoid re-uptake of the first tracer. Only 40% correct reinnervation from sensory axons was achieved when using the femoral muscle branch as a paradigm. In our study, the larger diameter of the tibial nerve, the comparatively smaller damage of the target during the nerve injection of the first tracer, and the absence of contralateral injury, which might influence the regeneration (Yamaguchi et al., 1999; Pachter and Eberstein, 1991; Koltzenburg et al., 1999), could explain the different rates of quantified reinnervation to a nerve branch.

The rate of topographically correct regeneration to the skin is smaller than the corresponding rate into the tibial nerve. It is possible that the successive bifurcations of the nerve results in increasing difficulties for the growing axons to find the correct pathway, or that an erroneous pathway among the fascicles of the correct nerve branch is taken already at the sciatic cut level. The fascicles could be partly mingled (Lago and Navarro, 2006) and once the wrong path was entered, it would be followed all the way to the target. Previous investigators have reported that neurons reinnervate their previous peripheral targets with a rate of about 52% to the anterior chamber of the eye (Hendry et al., 1986) and of 14%, 30% or 69% to muscles of the hindlimb (Bodine-Fowler et al., 1997; Wigston and Kennedy, 1987; Rende et al., 1991; respectively). Furthermore, others have described the progressive disappearance of misdirected neurons in favor of those with correct connections in the goldfish visual system (Becker and Cook, 1988), in facial nerve motoneurons (Ito and Kudo, 1994), and for the sciatic nerve motoneurons of the rat (Henning and Dietrichs, 1994). However, the eye and muscular models are difficult to compare with the skin model used in the present study because of anatomically different conditions and because of the possible differences in regeneration ability for sensory and motor cells discussed previously. There are no previous quantitative morphological reports on cutaneous re-innervation in the hindlimbs. Nevertheless, experiments with long survival times would be needed to verify if the degree of topographically correctness in sensory regeneration increases

after a more prolonged survival period after injury, as might be inferred from these studies.

The topographically incorrect regrowth described above is likely to contribute to the incomplete functional recovery seen after nerve section and repair (Diamond and Foerster, 1992; Johnson et al., 2005). This impairment is in accordance with the reduced amplitude of the sensory potentials from the digits described after sciatic nerve injury and repair (Puigdellívol-Sánchez et al., 2002; Negredo et al., 2004). The extent to which also sensory axons which have grown to the wrong target become functional will also depend on plasticity of the brain and its ability to re-interpret signals from wrong places as something meaningful (Lundborg and Rosen, 2004) although it is difficult to assess this in rodents.

3.3. Conclusions

Most of the axons in the injured and repaired sciatic nerve that originally resided in the tibial branch of the sciatic, regenerate back to the tibial, and a smaller but substantial number of the sensory population also correctly reinnervate the skin of the same digit.

Double labeling with DY and FB could be used to quantify collateral and regenerative sprouting to nerve branches and to topographically defined cutaneous areas, provided that known confounders are taken into account, and could therefore be an experimental tool of interest to assess techniques of nerve repair.

The proposed formulae may be used also for other dyes to find an ideal combination of tracers: the first tracer should permanently label the whole original population with minimal injury during the application, leaving minimal remains in the deposit area to reduce re-uptake by regenerating axons, with little negative influence on the regenerating ability of the labeled neurons; the second tracer should efficiently label the regenerating population and should easily be visible among structures (such as neuronal cell bodies) labeled by the first tracer.

4. EXPERIMENTAL PROCEDURES

Twelve adult female Sprague Dawley rats (270-380 g) were used in the present study. All animals were obtained from Harlan Interfauna Iberica S.A., maintained in the Animal Care Service, Faculty of Medicine, University of Barcelona, and treated in compliance with the ethical guidelines of this center. Anesthesia was initiated with ether

and then continued with chloral hydrate (300 mg/kg) during all surgical procedures and perfusion.

Experiment 1. Regeneration to correct nerve branch

The tibial nerve (n= 6) was dissected bilaterally at the level of the knee joint and a swab was placed below the nerve to absorb any spread of tracers. The tip of a 25 gauge needle attached to a 10 µl Hamilton syringe was introduced into the tibial nerve at the level of the origin of the branches to the gastrocnemius muscle, pushed 1-3 mm in proximal direction followed by injection of 1 µl of 5% DY (EMS-Polyloy, Groβ-Umstadt, Germany[1]). The skin was sutured and the rats were allowed to recover from the anesthesia.

Five days after this procedure, the right sciatic nerve was exposed at the level of the thigh and transected by means of sharp micro scissors, re-apposed and sutured to realign the epineurium in the proximal and distal stumps, using nylon monofilament 10-0 suture. The skin was sutured and the rats were allowed to recover.

Two months after the section and suture, the previously injected tibial nerves were re-exposed bilaterally, a swab was placed below the nerve, nerve transection was performed just proximal to the site of the previous DY injection and a capsule containing 5% FB (Sigma) was applied at the cut nerve end, kept in this position for 30 minutes and then removed (Fig. 3). Finally, the proximal cut end was cleaned, the skin was sutured and the rat was allowed to recover. Animals were perfused after five more days.

Experiment 2. Reinnervation of correct digit (including collateral sprouting)

Bilateral subcutaneous injections of 0.5 µl of DY (n=6) in the tip of the third digit was performed by means of a 10 µl Hamilton Syringe attached to a 25S gauge needle. An operating microscope was used for optimal control of the needle and to ensure tracer injection in the central plantar part of the distal phalanx of the digit.

Five days after the digit injection, the right sciatic nerve was exposed at the level of the thigh, transected and repaired by epineurial sutures as described above.

Three months after the nerve transection and repair the third digit was again bilaterally injected, this time with 1.5 µl. of FB.

[1] If not longer available from this supplier, we suggest Dr. Illing, GmBH, GrossUmstadt, Germany (see also Puigdellívol-Sánchez et al., 2000b, pg.109)

Four days after the FB injection, the musculocutaneous branch of the sciatic nerve (a proximal branch of the sciatic nerve that innervates the biceps muscle of the thigh and sends some sensory axons that innervate the digits; Puigdellívol-Sánchez et al., 2000a), was dissected bilaterally from the dorsal side of the thigh, transected immediately distal to where it crosses the caudofemoral muscle, and its proximal cut end exposed to 10% Fluoro-Gold (FG) in a capsule for 30 minutes. Then the femoral nerve was exposed and transected ventrally at the level of the groin, and its proximal end exposed for 30 minutes to 10% FG. Frequent inspection ensured that the cut ends of the nerves remained covered with dye during the exposure time. The tracers were then removed, the nerves cleaned, the skin sutured, and the animals allowed to recover (Fig. 4). Animals were perfused after four more days.

Fixation, sectioning, microscopic examination.

Perfusions were performed under anesthesia as described above. After thoracotomy and an intracardial injection of 1000 UI of heparin/kg body weight, rats were perfused through the ascending aorta with 100 ml saline followed by 500 ml 4% paraformaldehyde and 10% sucrose in phosphate buffer (PB, pH=7.40) for 20 minutes. The lumbar dorsal root ganglia L3-6 and corresponding spinal cord segments were removed and post fixed for three hours in the same fixative + 10% sucrose solution. L4-5 DRGs were cut on a cryostat in 10 µm thick longitudinal sections in rats used for experiment 1 (see above). The spinal cord and the L3 and L6 DRGs were cut in 30 µm thick serial longitudinal sections in experiment 1. Ganglia were cut in 16µm thick sections in rats used for experiment 2. Sections were all thaw-mounted on chrome-alum gelatinized (5%) slides and coverslipped using an anti-fading solution containing 1% paraphenylenediamine and 10% phosphate buffered saline in glycerol.

The sections were examined in an Olympus Vanox fluorescence microscope using appropriate filter combinations (ultraviolet light filters: DM 400 dichroic mirror and UG1 excitation filter, which gives 365 nm excitation and 420 nm emission wave lengths; and violet light filters: DM 455 dichroic mirror and BP 405 exciter filter which gives 405 nm excitation and 455 nm emission wavelengths). Neuronal profiles with an identifiable nucleus were counted in every tenth DRG section and in every fourth spinal cord section in experiment 1, and in every fifth DRG section in experiment 2 (see

Puigdellívol-Sánchez et al., 1998b, 2000b, for more information on the identification of labeled neurons). The ultraviolet filter can easily differentiate the three tracers; FB is bluish, FG is reddish, and DY is pale yellow. In case of double labeling, FB or FG may hide DY from view if the ultraviolet filter is used. Checking the cells also by the violet filter, which clearly facilitated the visualization of the DY, solved this difficulty. The total number of FB and DY labeled profiles counted as well as means and standard deviations from total cell profile counts are presented in Tables I and II and Figs. 1 and 2. No corrections were made for the possibility of counting split cells twice in different sections (see discussion). The detailed number of FG containing cells has been presented elsewhere (Puigdellívol-Sánchez et al., 2005).

Statistical analysis

Paired Wilcoxon W tests were used to compare mean double labeling percentages between DRGs and spinal cord, and also percentages between control and experimental hindlimbs in the same experiment. Mann-Whitney U tests were used to compare percentages between cell numbers obtained from experiment 1 and 2.

ACKNOWLEDGEMENTS

We wish to thank Dolors Fuster for excellent technical assistance and the kind collaboration of Olga Fuentes.

This work was supported by the Secretaría de Estado de Universidades e Investigación. Grant number: PM99/0173.

LITERATURE REFERENCES

Aldskogius, A., Molander, C., Persson, J., Thomander, L., 1987. Specific and non-specific regeneration of motor axons after sciatic nerve injury and repair in the rat. J. Neurol. Sci. 80, 249-257.

Al-Majed, A.A., Neumann, C.M., Brushart, T.M., Gordon, T., 2000. Brief electrical stimulation promotes the speed and accuracy of motor axonal regeneration. J. Neurosci. 20, 2602-2608.

Baranowsky, A.P., Priestley, J.V., McMahon, S.B., 1994. The consequence of delayed versus immediate nerve repair on the properties of regenerating sensory nerve fibres in the adult rat. Neurosci. Lett. 168, 197-200.

Becker, D.L., Cook, J.E., 1988. Divergent axon collaterals in the regenerating goldfish optid tract: a fluorescence double label study. Development, 104, 317-320.

Bodine-Fowler, S.C., Meyers, R.S., Moskovitz, A., Abrams, R., Botte, M.J., 1997. Inaccurate projection of rat soleus motoneurons: a comparison of nerve repair techniques. Muscle Nerve, 20, 29-37.

Brown, M.C., Hardman, V.J., 1987. A reassessment of the accuracy of reinnervation by motoneurons following crushing or freezing of the sciatic of lumbar spinal nerves of rats. Brain, 110, 695-705.

Brushart, T.M., 1990. Preferential motor reinnervation: a sequential double-labeling study. Restor. Neurol. Neurosci. 1, 281-287.

Brushart, T.M., 1993. Motor axons preferentially reinnervate motor pathways. J. Neurosci, 13, 2730-2738.

Brushart, T.M., Jari, R., Verge, V., Rohde, C., Gordon, T., 2005. Electrical stimulation restores the specificity of sensory axon regeneration. Exp. Neurol. 194, 221-229.

Burnett, G.M., Zager, E.L., 2004. Pathophysiology of peripheral nerve injury: a brief review. Neurosurg. Focus, 16, 1-7.

Devor, M., Schonfeld, D., Seltzer, Z., Wall, P.D., 1979. Two models of cutaneous reinnervation following peripheral nerve injury. J. Comp. Neurol. 185, 211-20.

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.

Fenrich, K., Gordon, T., 2004. Axonal regeneration in the peripheral and central nervous systems – current issues and advances. Can. J. Neurol. Sci. 31, 142-156.

Fritzsch, B., Sonntag, R., 1991. Sequential double labelling with different fluorescent dyes coupled to dextran amines as a tool to estimate the accuracy of tracer application and of regeneration. J. Neurosci. Methods. 39, 9-17.

Fu, S.Y., Gordon, T., 1995. Contributing factors to poor functional recovery after delayed nerve repair: prolonged denervation. J. Neurosci. 15, 3886-3895.

Guntinas-Lichius, O., Effenberger, K., Angelov, D.N., Klein, J., Streppel, M., Stennert, E., Neiss, W.F., 2000. Delayed rat facial nerve repair leads to accelerated and enhanced muscle reinnervation with reduced collateral axonal sprouting during a definitive denervation period using a cross-anastomosis paradigm. Exp. Neurol. 162, 98-111.

Hendry, L.A., Hill, C.E., Watters, D.J., 1986. Long-term retention of Fast Blue in simpathetic neurones after axotomy and regeneration-demonstration of incorrect reconnections. Brain Res. 376, 292-298.

Henning, R., Dietrichs, E., 1994. Transient reinnervation of antagonistic muscles by the same motoneuron. Exp. Neurol. 130, 331-336.

Ito, M., Kudo, M., 1994. Reinnervation by axon collaterals from single facial motoneurons to multiple muscle targets following axotomy in the adult guinea pig. Acta Anat. 151, 124-30.

Innocenti, G.M., Clarke, S., Kraftsik, R., 1986. Interchange of callosal and association projections in the developing visual cortex. J. Neurosci. 6, 1384-1409.

Jivan, S., Novikova, L.N., Wiberg, M., Novikov, L.N., 2005. The effects of delayed nerve repair on neuronal survival and axonal regeneration after seventh cervical spinal nerve axotomy in adult rats. Exp. Brain Res. 19, 1-10.

Johnson, E.O., Zoubos, A.B., Soucacos, P.N., 2005. Regeneration and repair of peripheral nerves. Injury. 36S, S24-S29.

Koerber, H.R., Seymour, A.W., Mendell, L.M., 1989. Mismatches between peripheral receptor type and central projections after peripheral nerve regeneration. Neurosci. Lett. 99, 67-72.

Koltzenburg, M., Wall, P.D., McMahon, S.B., 1999. Does the right side know what the left is doing? Trends Neurosci. 22, 122-127.

Lago, N., Navarro, X., 2006. Correlation between target reinnervation and distribution of motor axons in the injured rat sciatic nerve. J.Neurotrauma. 23, 227-240.

Lundborg, G., Rosen, B., 2004. The two-point discrimination test – time for a re-appraisal? J. Hand Surg. [Br]. 29, 418-422.

Lutz, B.S., Ma, S.F., Chuang, D.C., Chan, K.H., Wei, F.C., 2001. Interposition of a pedicle fat flap significantly improves specificity of reinnervation and motor recovery after repair of transected nerves in adjacency in rats. Plast. Reconstr. Surg. 107, 116-23.

Madison, R.D., Archibald, S.M., Brushart, T.M., 1996. Reinnervation accuracy of the rat femoral nerve by motor and sensory nerurons. J. Neurosci. 16, 5698-703.

Messina, A., Sangster, C.L., Morrison, W.A., Galea, M.P., 2000. Requirements for obtaining unbiased estimates of neuronal numbers in frozen sections. J. Neurosci. Methods. 97, 133-137.

Molander, C., Alskogius, H., 1992. Directional specificity of regenerating neurons after peripheral nerve crush or transection and epineural suture. A sequential double-labelling study in the rat. J. Restor. Neurol. Neurosci. 4, 339-344.

Negredo, P., Castro, J., Lago, N., Navarro, X., Avendaño, A., 2004. Differential growth of axons from sensory and motor neurons through a regenerative electrode: A stereological, retrograde tracer, and functional study in the rat. Neuroscience, 128, 605-615.

Novikova, L., Novikov, L., Kellerth, J.O., 1997. Persistent neuronal labeling by retrograde fluorescent tracers: a comparison between Fast Blue, Fluoro-Gold and various dextran conjugates. J. Neurosci. Methods. 74, 9-15.

Pachter, B.R., Eberstein, A., 1991. Nerve sprouting and endplate growth induced in normal muscle by contralateral partial denervation of rat plantaris. Brain Res. 560, 311-314.

Politis, M.J., 1985. Specificity in mammalian peripheral nerve regeneration at the level of the nerve trunk. Brain Res. 328, 271-276.

Popratiloff, A.S., Neiss, W.F., Skouras, E., Streppel, M., Guntinas-Lichius, O., Angelov, D., 2001. Evaluation of muscle re-innervation emplying pre- and post-axotomy injections of fluorescent retrograde tracers. Brain Res. Bull. 54, 115-123.

Prats-Galino, A., Puigdellívol-Sánchez, A., Ruano-Gil, D., Molander, C., 1999. The representations of hindlimb digits in rat dorsal root ganglia. J. Comp. Neurol. 408, 137-145.

Puigdellívol-Sánchez, A., Prats-Galino, A., Ruano-Gil, D., Molander, C., 1998a. Sciatic and femoral nerve sensory neurones occupy different regions of the L4 dorsal root ganglion in the adult rat. Neurosci. Lett. 251,169-72.

Puigdellívol-Sánchez, A., Prats-Galino, A., Ruano-Gil, D., Molander, C., 1998b. Efficacy of the fluorescent dyes Fast Blue, Fluoro-Gold and Diamidino Yellow for retrograde tracing to dorsal root ganglia after subcutaneous injection. J. Neuroci. Methods. 86, 7-16.

Puigdellívol-Sánchez, A., Prats-Galino, A., Forcada-Calvet, P., Molander, C., 2000a. The relative contribution of femoral and sciatic nerve branches to the sensory innervation of the hindlimb digits in the rat. Anat. Rec. 260, 180-188.

Puigdellívol-Sánchez, A., Prats-Galino, A., Ruano-Gil, D., Molander, C., 2000b. Fast Blue and Diamidino Yellow as retrograde tracers in peripheral nerves: efficacy of combined nerve injection and capsule application to transected nerves in the adult rat. J. Neuroci. Methods, 95, 103-110.

Puigdellívol-Sánchez, A., Valero-Cabré, A., Prats-Galino, A., Navarro, X., Molander, C., 2002. On the use of fast blue, fluoro-gold and diamidino yellow for retrograde tracing after peripheral nerve injury: uptake, fading, dye interactions and toxicity. J. Neurosci. Methods, 115, 115-127.

Puigdellívol-Sánchez, A., Prats-Galino, A., Ruano-Gil, D., Molander, C., 2003. Persistence of tracer in the application site-a potential confounding factor in nerve regeneration studies. J. Neurosci. Methods, 127, 105-110.

Puigdellívol-Sánchez, A., Prats-Galino, A., Molander, C., 2005. On regenerative and collateral sprouting to hind limb digits after sciatic nerve injury in the rat. Restor. Neurol. Neurosci. 23, 97-107.

Rath, S., Green, C.S., 1991. Selectivity of distal reinnervation of regenerating mixed motor and sensory nerve fibres across muscle grafts in rats. Br. J. Plast. Surg. 44, 215-218.

Rende, M., Granato, A., Lo Monaco, M., Zelano, G., Toesca, A., 1991. Accuracy of reinnervation by peripheral nerve axons regenerating across a 10-mm gap within an impermeable chamber. Exp. Neurol. 111, 332-339.

Shenaq, S.M., Mendelow, M., Shenaq, H.M., Spira, M., 1989. Evaluation of axonal specificity of the regenerating rat sciatic nerve using a fluorescent labeling technique. Microsurgery, 10, 210-213.

Sulaiman, O.A.R., Midha, R., Munro, C.A., Matsuyama, T., Al-Majed, A., Gordon, T., 2002. Chronic Schwann cell denervation and the presence of a sensory nerve reduce motor axonal regeneration. Exp. Neurol. 176, 342-354.

Swett, J.E, Wikholm, R.P., Blanks, R.H.I., Swett, A.L., Conley, L.C., 1986. Motoneurons of the rat sciatic nerve. Exp. Neurol. 93, 227-252.

Swett, J.E., Torigoe, Y., Elie, V.R., Bourassa, C.M., Miller, P.G., 1991. Sensory neurons of the rat sciatic nerve. Exp. Neurol. 114, 82-103.

Suzuki, G., Ochi, M., Shu, N., Uchio, Y., Matsuura, Y. 1998. Sensory neurons regenerate more dominantly than motoneurons during the initial stage of the regenerating process after peripheral axotomy. Neuroreport, 9, 3487-3492.

Taylor, D.C.M., Pierau, F.K., Schmid, H., 1983. The use of fluorescent tracers in the peripheral sensory nervous system. J. Neurosci. Meth. 8, 211-224.

Tandrup T., Woolf, C.J., Coggeshall, R.E., 2000. Delayed loss of small dorsal root ganglion cells after transaction of the rat sciatic nerve. J. Comp. Neurol. 422, 172-180.

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

Valero-Cabré, A., Tsinoris, K., Skouras, E., Perego, G., Navarro, X., Neiss, S.F., 2001. Superior muscle reinnervation after autologous nerve graft of poly-L-lactide-epsilon caprolactone (PLC) tube implantation in comparison to silicon tube repair. J. Neurosci. Res. 63, 214-23.

Valero-Cabré, A., Tsinoris, K., Skouras, E., Navarro, X., Neiss, W.F., 2004. Peripheral and spinal motor reorganization after nerve injury and repair. J. Neurotrauma, 21, 95-108.

Wigston, D.J., Kennedy, P.R., 1987. Selective reinnervation of transplanted muscles by their original motoneurons in the axolotl. J. Neurosci. 7, 1857-1865.

Yamaguchi, H., Ochi, M., Mori, R., Ryoke, K., Yamamoto, S., Iwata, A., Uchio, Y., 1999. Unilateral sciatic nerve injury stimulates contralateral nerve regeneration. Neuroreport, 10, 1359-1362.

You, S., Petrov, T., Chung, P.H., Gordon, T., 1997. The expression of the low affinity nerve growth factor receptor in long-term denervated Schwann cells. Glia, 20, 87-100.

FIGURE LEGENDS

Fig. 1. Regeneration in the tibial nerve (Experiment 1). Mean number of counted neuronal profiles in one of every tenth section in DRGs and in every fourth spinal cord section. (DRGs: dorsal root ganglia; SC: spinal cord; FB: fast blue; DY: diamidino yellow; FBDY: double labeled cells with FB and DY).

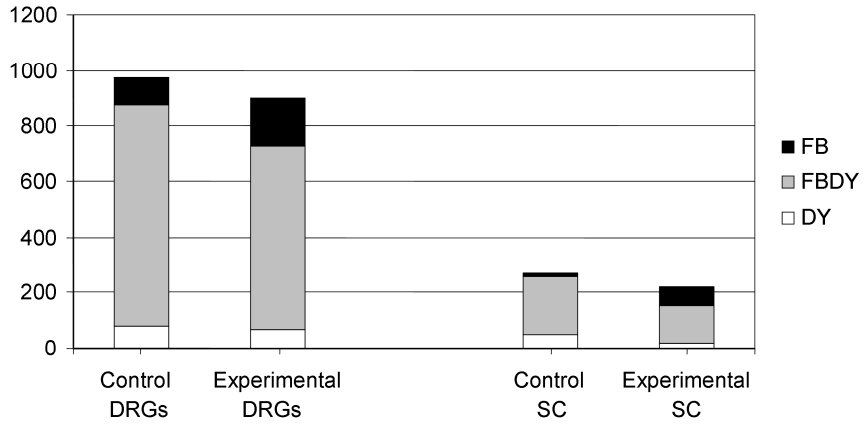


Fig 2. Regeneration in the digits (Experiment 2). Mean number of labeled DRG profiles counted in one of every fifth section. (FB: fast blue; DY: diamidino yellow; FBDY: double labeled cells with FB and DY).

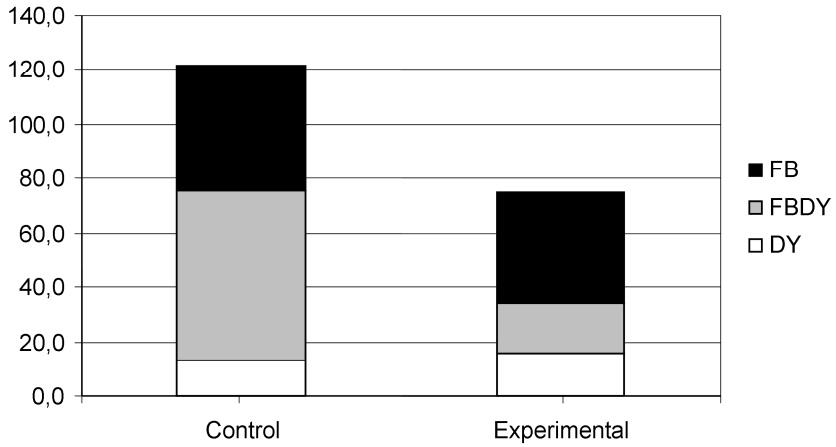


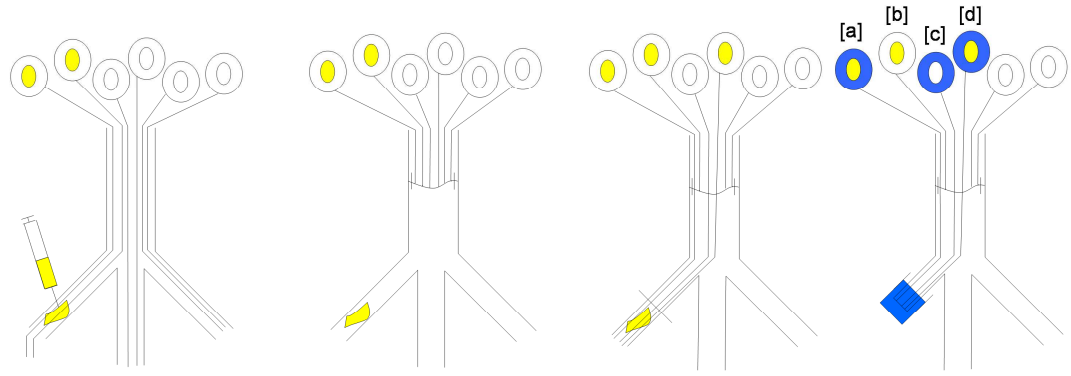
Fig 3. Experimental design for assessing proximal selective reinnervation. Labeling codes:

[a]: Double labeled cells with DY and FB- belonging to the original population, have selectively reinnervated its corresponding nerve branch.

[b]: Single DY labeled cells- belonging to the original population, have not regenerated or have been misdirected.

[c]: Single FB labeled cells- originally belonging to other populations, now misdirected after the regeneration towards the studied nerve branch.

[d]: With a comparable appearance than [a]: belonging to other populations, have now misdirected after the regeneration towards the previously labeled nerve branch, have uptake some remains of the first tracer and also the second, resulting also double labeled with DY and FB.

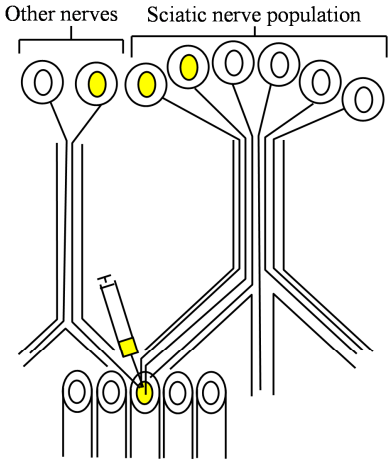


Day 0
Bilateral injection of DY in the tibial nerve.

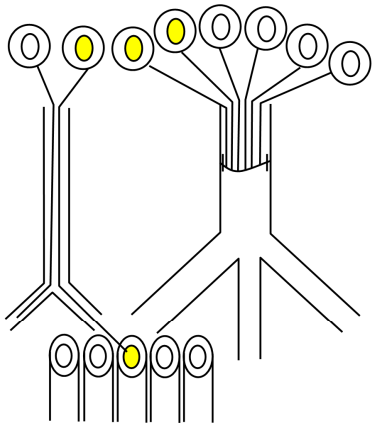
Day 5
Transection and suture of the right sciatic nerve.

Day 65
Bilateral transection of the tibial nerve immediately proximal to the dye injection.
Application of a capsule containing FB at the cut nerve end.

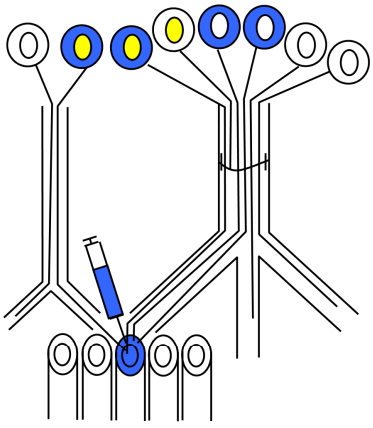
Fig 4. Experimental design for assessing distal selective reinnervation. Labeling codes:
[a]: Double labeled cells with DY and FB- belonging to the original population, have selectively reinnervated its corresponding digit.
[b]: Single DY labeled cells- belonging to the original population, have not regenerated or have been misdirected.
[c]: Single FB labeled cell- belonging to the original population of the digit, was not labeled with the first restricted injection of dye but has been labeled by the second.
[d]: Single FB labeled cell- with similar appearance than [c], originally belonging to other populations, now misdirected after the regeneration towards the studied nerve branch.
[e]: Neurons labeled with FG, with or without other tracers- not belonging to the sciatic nerve population.



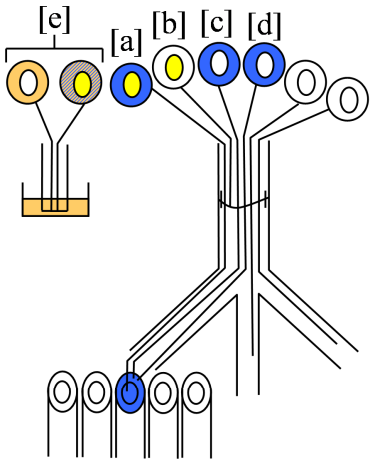
Day 0. Subcutaneous injection of DY.



Day 5. Transection and repair of the right sciatic nerve.



Day 95. After regeneration, subcutaneous injection of the same digit with 1.5 µl. of FB.



Day 99. Transection of femoral and musculocutaneous nerves and application of a capsule containing 10% FG.

Table 1. Labeled neuronal profiles from the tibial nerve (Experiment 1).

Case	**Experimental**			**Control**		
DRG	**FB**	**DY**	**FBDY**	**FB**	**DY**	**FBDY**
R250	276	61	458	52	63	594
R251	147	11	533	57	77	680
R253	291	40	487	63	69	646
R254	162	57	654	58	77	998
R255	55	139	824	101	24	894
R257	87	107	1002	252	155	972
Mean	169.7	69.2	659.7	97.2	77.5	797.3
SD	96.5	46.4	214.8	77.9	42.8	177.8

Case	**Experimental**			**Control**		
SC	**FB**	**DY**	**FBDY**	**FB**	**DY**	**FBDY**
R250	127	27	86	25	46	145
R251	25	14	152	3	40	205
R253	153	22	133	41	37	235
R254	32	11	127	1	44	240
R255	34	14	188	0	76	255
R257	23	21	127	7	69	154
Mean	65.7	18.2	135.5	12.8	52.0	205.7
SD	58.3	6.1	33.6	16.6	16.3	46.5

DY was used to label the tibial nerve before the injury and FB after the regeneration period. Counted profiles in every tenth section in DRGs L4-L5 and in every fourth section of the lumbar spinal cord (SC) ventral horn (motoneurons) without correction for split cells.

Table 2. Experiment 2. Labeled neuronal profiles with FB and DY from the hindlimb digits.

	Experimental			**Control**		
Case	**DY**	**FB**	**DYFB**	**DY**	**FB**	**DYFB**
R236	27	34	30	5	82	12
R237	4	31	10	8	95	47
R238	5	36	9	13	6	43
R239	17	55	16	13	31	87
R240	24	42	15	26	49	52
R273	18	48	29	13	11	136
Mean	15.8	41.0	18.2	13.0	45.7	62.8
SD	9.5	9.2	9.2	7.2	36.8	43.1

DY was used to label the digits before the injury and FB after the regeneration period. Counted profiles in every fifth section in DRGs without correction for split cells. Number of profiles labeled with FG, the tracer applied in the femoral nerve and in the musculocutaneous nerve are presented elsewhere (Puigdellívol-Sánchez et al., 2005).