PERSISTENCE OF TRACER IN THE APPLICATION SITE - A POTENTIAL CONFOUNDING FACTOR IN NERVE REGENERATION STUDIES.

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

[1] Department of Human Anatomy and Embryology, Faculty of Medicine, University of Barcelona, 08036 Barcelona, Spain.

[2] Department of Neuroscience, Karolinska Institutet, S-171 77 Stockholm, Sweden.

[3] Department of Rehabilitation Medicine, Huddinge University Hospital, S-141 86 Flemingsberg, Sweden.

Author for correspondence: Alberto Prats-Galino. Dept. of Human Anatomy and Embryology. University of Barcelona, c/ Casanova nº 143, 08036 Barcelona, Spain. Tel.: 34-93-4021905, Fax.:34-93-4035263, aprats@medicina.ub.es

Key words: fluorescent dyes, nerve regeneration, sciatic nerve, DRG, adult rat.

ABSTRACT

Selective reinnervation of peripheral targets after nerve injury might be assessed by injecting a first tracer in a target before nerve injury to label the original neuronal population, and applying a second tracer after the regeneration period to label the regenerated population. However, altered uptake of tracer, fading, and cell death may interfere with the results. Furthermore, if the first tracer injected remains in the target tissue, available for "re-uptake" by misdirected regenerating axons, which originally innervated another region, then the identification of the original population would be confused. With the aim of studying this problem, the sciatic nerve of adult rats was sectioned and sutured. After three days, to allow the distal axon to degenerate avoiding immediate retrograde transport, one of the dyes: Fast Blue (FB), Fluoro-Gold (FG) or Diamidino Yellow (DY), was injected into the tibial branch of the sciatic nerve, or in the skin of one of the denervated digits. Rats survived 2-3 months. The results showed labelled dorsal root ganglion cells and motoneurones, indicating that late re-uptake of a first tracer occurs. This phenomenon must be considered when the model of sequential labelling is used for studying the accuracy of peripheral reinnervation.

INTRODUCTION

Reinnervation of a peripheral target after nerve injury may be studied by an experimental design in which a first tracer is injected in the target region before nerve injury to label the original axonal population, and a second tracer after the regeneration to label the regenerated population. Double-labelled parental cell bodies could represent axons that have grown to the original target (Hendry et al., 1986; Wigston and Kennedy, 1987; Rende et al., 1991; Madison et al., 1996; Popratiloff et al., 2001). However, the cell counts in this model must be corrected due to at least two types of confounding factors. First, late detection of cell numbers may be compromised by altered uptake, fading and cell death (Puigdellívol et al., 2002). Second, the first tracer injected in the target may remain available in the tissue for "re-uptake". If so, regenerating axons that originally innervated another region and then grow into to the area or nerve of study could absorb both the first and second tracer in the application site. They would represent a non-intended population of double-labelled cells in the model. This possibility has been suggested previously (Innocenti et al., 1986; Rende et al., 1991; Puigdellívol et al., 1998, 2000a; Popratiloff et al., 2001) but not directly demonstrated. We have recently proposed that Fast Blue (FB), Diamidino Yellow (DY) and Fluoro-Gold (FG) can be combined to study the topographic accuracy of reinnervation by using the method of sequential labelling, as they are visible through the same filter combination (Puigdellívol et al., 1998, 2000a). The aim of the present study was to investigate if late “re-uptake” occurs with these tracers.

METHODS

Eighteen adult female Sprague Dawley rats (250-330 g) were used in the present study. All animals were obtained from Harlan Interfauna Ibérica 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. Anaesthesia was initiated with ether and then maintained with chloral hydrate (300 mg/kg) during all surgical procedures and perfusion. Bilateral experiments were performed in some cases in order to reduce the number of animals used.

In all rats, the sciatic nerve was exposed at the level of the thigh and transected using sharp microscissors. The nerve stumps were immediately reapposed by epineurial sutures using 10-0 nylon monofilaments. Upmost care was taken to avoid further damage to the nerve fascicles during the suturing process, but cannot be completely excluded.

The animals were then divided in two groups (Fig.1); one group used to study late re-uptake from the tibial nerve branch distal to the sciatic transection, and a second group used to study late re-uptake from the skin of lateral digits. In the latter group, the denervation of the lateral digits by the sciatic nerve transection was supplemented in the same session by transection also of the musculocutaneous nerve of the lower limb (a proximal branch of the sciatic nerve, see Puigdellívol et al., 2000b) at the level of the caudofemoralis muscle. The femoral nerve does not send axons to the lateral digits and was left intact to avoid autotomy. After nerve transections, the opened skin was sutured and the rats allowed to recover.

Three days later, to allow the distal axons to degenerate and to avoid any immediate retrograde transport of tracers, the animals were re-anesthetised and

subjected to application of tracers. In the first group, the tibial nerve was dissected at the level of the knee joint and a swab was placed below the nerve. Then, 1 µl of 5% FB (Sigma, n=6), or 10% FG (Fluorochrome. Inc, n=6), or 5% DY (EMS-Polyloy, n=8) dissolved in distilled water was injected with a 10 µl Hamilton syringe equipped with a 25 or 26 gauge needle. The needle was introduced at the level of the origin of the gastrocnemius branches and pushed 1-3 mm in the proximal direction before injection. In the second group, the distal phalanges of digits 4 and 5 were injected with 0.5 µl of one of the tracers (n=4 for FG and n=5 for either FB or DY). Animals injected in nerves were perfused after two months and animals injected in digits after three months. Control rats used to check for immediate retrograde transport of dye were subjected to similar nerve lesions (n=4), and tibial nerve or digital injections either immediately or after 2-5 days. Those rats were perfused 5 days after tracer application.

The rats were re-anaesthetised and a thoracotomy was performed. After an intracardiac injection of 1000 IU of heparin/kg b.w., the rats were perfused through the ascending aorta with 100 ml of saline and then with 1000 ml 4% paraformaldehyde and 10% sucrose in phosphate buffer (PB; pH 7,4) for twenty minutes. The ganglia L3-5 and corresponding spinal cord segments were removed, postfixed for 3h in the same perfusion solution, and immersed in 15% sucrose in PB overnight. The dorsal root ganglia (DRGs) L4 and L5 were cut on a cryostat in 10 µm thick longitudinal sections for rats used for nerve injection and 16 µm thick sections for rats used for digital injections. The L3 dorsal root ganglion (DRG) and the spinal cord were cut at 30 µm in longitudinal sections. Sections were mounted on chrom-alum gelatinised (5%) slides and then coverslipped with an antifading solution containing 1% paraphenylendiamine and 10% PBS in glycerol. Slides were examined in a Olympus Vanox fluorescent microscope, using ultraviolet light filters (DM 400 dichroic mirror and UG1 excitation filter, which gives a 365 nm

excitation, 420 nm emission wave length) and violet light filters (DM 455 dichroic mirror and BP 405 excitation filter, which gives a 405 nm excitation, 455 nm emission wave length).

Neuronal profiles with an identifiable nucleus were counted in every tenth consecutive DRG section and in every fourth consecutive spinal cord section (see Fig.2 and Puigdellívol et al., 1998, 2000a,b for more information on the identification of labelled neurones) in the tibial nerve group. No corrections were made for the possibility of counting split cells. The total number of labelled neurones was obtained from the cases injected in the digits. In these rats, most sections contained only 1-2 labelled cells per section. It was therefore easy to identify split cells that appeared in two adjacent sections. When this occured, the cell was counted only the first section in which it appeared.

Mean values are stated ± standard deviation (SD). Statistical significance was calculated using a Kruskal-Wallis test.

RESULTS

No labelling was observed in the L3 DRG in any of the described experiments.

In nerve injured control animals examined five days after FB injection in the digits, 11 neuronal profiles were counted in the DRGs. Similar conditions except that FB was injected in the tibial nerve resulted in 82 neuronal profiles in the DRG, and 27 in the spinal cord. No such neurones were observed in the FG and DY injected animals under otherwise similar conditions.

However, counts of labelled neuronal profiles in DRGs of nerve-injected rats perfused two months after injection showed the following results: 346.2 ± 252.1 for FB, 89.6±124.8 for FG, and 168.4±231.2 for DY. These differences were not significant ($p=0.191$). Corresponding examination of the spinal cord showed the following counts

of labelled motorneuronal profiles: 132.0±114.3 for FB, 8.5±19.86 for FG, and 35.3±67.3 for DY. These numbers were significantly different (p=0.011; table I).

The injection of tracers in the distal phalanges of digits 4 and 5 in previously nerve lesioned animals resulted in the following numbers of labelled neurones in the DRGs: 32.0±16.8 for FB and 2.2±3.3 for DY; these were significantly different, (p=0.005). No labelled cells were found after injection with FG (table II).

DISCUSSION

The absence of diffuse neuronal labelling in the L3 DRG indicated a lack of hematogenous spread of the tracers from the application area and suggests that the labelling observed in other ganglia corresponds to axonal retrograde transport of the dyes.

The application of FB early after the nerve transection and suture, in either the tibial nerve or in the digits, resulted in some DRG and spinal cord FB labelling in rats perfused five days after the procedure. This indicated that axons distal to the suture remain capable of transporting tracers for a short time and that the dye may cross the suture and reach the DRG provided that the distal nerve remains in contact with its proximal stump. This is in accordance with previous reports, which described retrograde transport (Nitta et al., 1999) and excitability (Fu and Gordon, 1997) in the distal part of sectioned axons for a short period after transection. To avoid this “crossing” phenomenon, we waited three days after the nerve lesion and repair before we applied the tracers. The three days period between the injury and the application of tracer allowed the axons in the distal stump to degenerate sufficiently to avoid early retrograde transport (at least of FG and DY) to the DRG and spinal cord. The small number of labelled neurons in the control rats perfused five days after tracer application ensures

that this procedure is for the most part efficient for FG and DY (but not for FB). Similarly, the 78 neurones found in the FB group are far fewer than those found after labelling the intact tibial nerve, suggesting that the "crossing" process, if present, is limited.

The results indicate that FG and DY will label neuronal cell bodies only if their regenerating axons reach the area of the previous dye application, pick up the dye and then transport it retrogradly to the soma. The appearance of labelled neurones in rats perfused two to three months after tracer application indicates that this process does indeed occur to a significant extent for both FG and DY. When comparing these two, FG seems to be less prone to late uptake than DY. Furthermore, the increase of FB labelling, which was up to four times the cell number found in the control situation, also suggests significant re-uptake for this tracer.

Some of the variability between rats in cell numbers in the tibial nerve injection experiments may be due to differences in the rate of proximal diffusion of the tracer to the regenerating axons in the tibial nerve.

To calculate the potential importance of the phenomenon of late uptake of remaining dye in future studies of nerve regeneration, we suggest that the number of cells obtained is compared with the population found after dye injections in normal control animals, using an estimation factor. Our previous experiments (Puigdellívol et al., 2000a) showed that a dye injection in the normal tibial nerve as described above results in about 1000 profiles in the DRGs (cells counted in every tenth section), and about 200 profiles in the spinal cord ventral horn. Similarly, the corrected mean number of neurones innervating a hindlimb digit is 365 (Prats-Galino et al., 1999). Based on these data and the results presented here, we therefore estimate that the re-uptake of remaining dye by regenerating axons labels 35% (FB), 9% (FG) and 17% (DY) of the

DRG cell numbers that would have been labelled after a normal nerve injection, and 66% (FB), 4% (FG) and 18% (DY) of the motoneurones, respectively, provided that the number of regenerating neurones is similar to the original population. The corresponding percentages for the digital skin are estimated to 9.8% (FB), 0% (FG) and 0.6% (DY).

A recent study (Popratiloff et al., 2001) showed that injected DiI remains as a "large extracellular deposit" for up to two months after the injection. The authors suggested that the DiI in the deposit is available for uptake by regenerating axons during this period, and that this might be evident also for FB and FG. However, even though the possibility of "re-uptake" has been suggested previously for FB (Innocenti et al., 1986; Rende et al., 1991; Popratiloff et al., 2001), it has not been demonstrated, and no such information seems to be available for DY. The relatively small number of FG labelled cells as compared to the other tracers, found in our material, could be explained by a less significant re-uptake phenomenon combined with fading, which has previously demonstrated for this tracer (Novikova et al., 1997; Puigdellívol et al., 2002).

All three tracers gave rise to a relatively small re-uptake from the digital skin compared to the re-uptake from the nerve. One possibility is that the longer period needed for the axons to reach the distal skin areas allowed for greater degradation of the tracer at the injection site. Secondly, it is also possible that a more pronounced fading occurs in neurones that regenerate first and arrive early to take up the remaining dye. Thirdly, the remaining tracer may be in better condition for axonal uptake in proximal territories to which the regenerating axons are likely to arrive first. Fourthly, if the survival period is long, then axons that have regrown to the injected area may take up the tracer over a longer period of time. We therefore conclude that the re-uptake probably depends on the distance from the tracer application area to the injury site and

less on the survival time, at least when the period is just a few months. Finally, the significance of the re-uptake also depends on the type of tracer used, since differences among them were seen for both proximal and distal targets.

We conclude that the phenomenon of re-uptake shown here needs to be considered but does not invalidate the model of double labelling for assessing selectivity in reinnervation.

In distal areas, in which reduced numbers of cells were observed for FG and DY, the interference of this process is negligible if they are used to label the original population. However, the reported FG fading (Novikova et al., 1997), for up to a 75% of the previously labelled population after three months of follow-up (Puigdellívol et al, 2002), limits its use as a first tracer. Here, DY would appear to be a better option.

After application of tracer close to the site of nerve injury, in our case in the tibial nerve, DY showed slightly less re-uptake than FB. Here, one should modify the interpretation of the results according to the principles presented above when using the model with the assumption that double-labelled cells indicate neurones that have regenerated selectively to their former sites.

The re-uptake phenomenon described in this study indicates that experimental designs in which fluorescent tracers are used to study reinnervation after nerve injury should include tests to check whether re-uptake is significant, for each tracer used. We suggest that the different dyes are tested by applying them in the area of study a few days after the injury and repair of the nerve and that the neuronal populations are checked for labelling after the expected regeneration period.

It is also important to control for the compatibility of the two tracers that have to be used in combination in each area of study (Puigdellívol et al, 1998, 2000a) and for

their efficacy in labelling the population of regenerating neurones studied (Puigdellívol et al, 2002).

The different confounding processes that interfere with the interpretation of the results of double labelling models, such as fading and re-uptake of the first tracer and interferences with the uptake and visualization of the second tracer must be considered together in future experiments designed to assess the accuracy of peripheral reinnervation (manuscript in preparation).

ACKNOWLEDGMENTS

We want to thank Dolors Fuster for efficient technical assistance, Dr. Manuel Llusá for performing some sciatic nerve sutures and Dr Josep Lluís Carrasco for kind statistical assessment. This work was supported by the Secretaría de Estado de Universidades e Investigación, Grant Number PM99/0173.

REFERENCES

Fu SY, Gordon T. The cellular and molecular basis of peripheral nerve regeneration. Mol Neurobiol 1997;14:67-116.

Hendry LA, Hill CE, Watters DJ. Long-term retention of Fast Blue in sympathetic neurones after axotomy and regeneration-demonstration of incorrect reconnections. Brain Res 1986;376:292-98.

Innocenti GM, Clarke S, Kraftsik R. Interchange of callosal and association projections in the developing visual cortex. J Neurosci 1986;6:1384-409.

Madison RD, Archibald SM, Brushart TM. Reinnervation accuracy of the rat femoral nerve by motor and sensory neurons. J Neurosci 1996;16:5698-703.

Nitta A, Ohmiya M, Jin-Nouchi T, Sometani A, Asami T, Kinukawa H, Fukumitsu H, Nomoto H, Fukurawa S. Endogenous neurotrophin-3 is retrogradely transported in the rat sciatic nerve. Neuroscience 1999;88:679-85.

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

Popratiloff AS, Neiss WF, Skouras E, Streppel M, Guntinas-Lichius O, Angelov D. Evaluation of muscle re-innervation employing pre- and post-axotomy injections of fluorescent retrograde tracers. Brain Res Bull 2001;54:115-23.

Prats-Galino A, Puigdellívol-Sánchez A, Ruano-Gil D, Molander C. The representations of hindlimb digits in rat dorsal root ganglia. J Comp Neurol 1999;408:137-45.

Puigdellívol-Sánchez, A, Prats-Galino A, Ruano-Gil D, Molander C. 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 1998; 86:7-16.

Puigdellívol-Sánchez A, Prats-Galino A, Ruano-Gil D, Molander C. 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 2000a;95:103-10.

Puigdellívol-Sánchez A, Prats-Galino A, Forcada-Calvet P, Molander C. The relative contribution of femoral and sciatic nerve branches to the sensory innervation of the hindlimb digits in the rat. Anat Rec 2000b;260:180-88.

Puigdellívol-Sánchez A, Valero-Cabré A, Prats-Galino A, Navarro X, Molander C. 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 2002;115:115-27.

Rende M, Granato A, Lo Monaco M, Zelano G, Toesca A. Accuracy of reinnervation by peripheral nerve axons regenerating across a 10-mm gap within an impermeable chamber. Exp Neurol 1991;111:332-39.

Wigston DJ, Kennedy PR. Selective reinnervation of transplanted muscles by their original motoneurons in the axolotl. J Neurosci 1987;7:1857-65.

TABLE I. **Uptake of dye by regenerating axons after nerve injection.** Number of labelled profiles counted in one of every tenth section in L4-L5 DRG and in every fourth in the spinal cord (SC).

FB			FG			DY		
	DRG	SC		DRG	SC		DRG	SC
R274 r	4	4	R277 r	12	0	R225 r	19	1
R274 l	663	309	R277 l	230	2	R226 r	297	20
R275 r	429	118	R278 r	6	0	R227 r	15	2
R275 l	195	26	R278 l	6	0	R258 r	115	27
R276 r	211	125	R279 r	14	0	R259 r	29	7
R276 l	575	210	R279 l	270	49	R260 r	143	20
						R270 r	39	5
						R271 r	690	200
Mean	346.2	132.0		89.7	8.5		168.4	35.3
SD	252.1	114.3		124.9	19.9		231.2	67.3

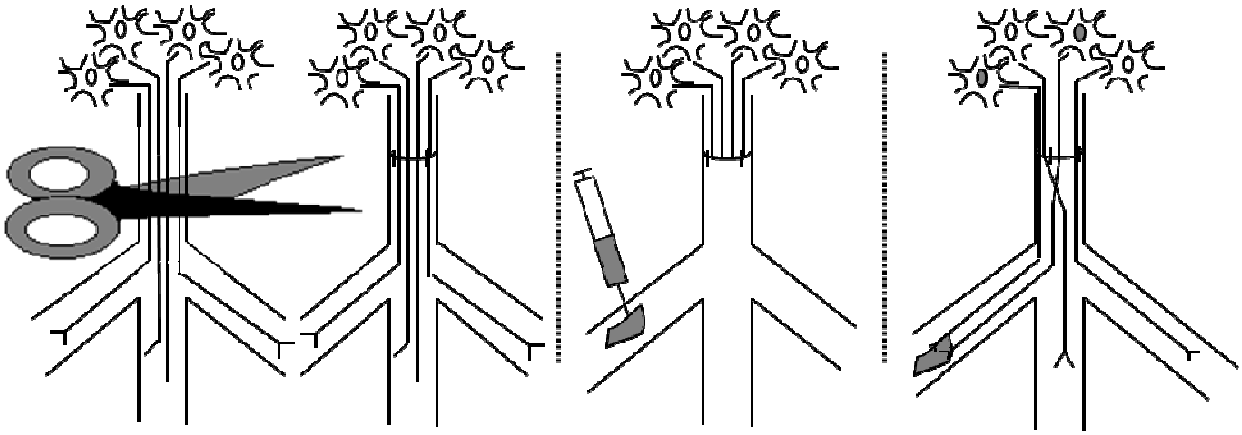
TABLE II. **Uptake of dye by regenerating axons after injection in the lateral digits.**

Total number of labelled neurones in L4-L5 DRG.

DRG	FB	FG	DY
R220 D	10	R220 I 0	R222 I 2
R221 D	20	R221 I 0	R223 D 1
R222 D	45	R222 I 0	R223 I 0
R223 D	50	R223 I 0	R224 D 8
R224 D	35		R224 I 0
Mean	32.0	0	2.2
SD	16.8	0	3.3

LEGENDS

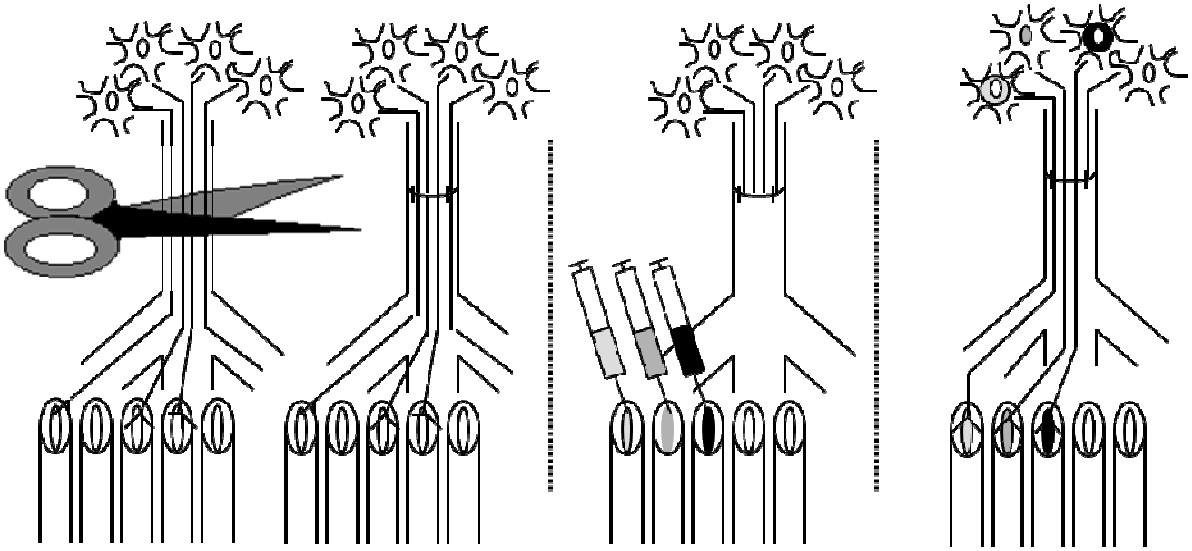
Figure 1. Experimental design. Tracers are injected in either the tibial nerve or a digit at two to five days after sciatic nerve transection and repair. At this time point, regeneration of the axons distal to the injury is assumed to have reached a phase at which retrograde transport has ceased. Any remaining tracer will be available for uptake when approached by regenerating axons.



Day 0: Transection+suture of the sciatic nerve

Day 3: Injection of a tracer in the tibial nerve undergoing wallerian degeneration

Day 3-60: Regeneration (some misdirected), uptake of tracer and subsequent retrograde transport



Day 0: Transection+suture of the sciatic nerve

Day 2-5: Injection of one of several tracers in the denervated digits

Day 3-90: Regeneration (some misdirected), uptake of tracer and subsequent retrograde transport

Figure 2. A, motoneurones labelled with Fluoro-Gold, showing cytoplasmatic labelling. B, dorsal root ganglion neuron showing cytoplasmatic distribution of Fast Blue. C, dorsal root ganglion neurons showing nuclear labelling with Diamidino Yellow. Some cells show pale labelling (thick arrows), but most of them are intensely labelled (thin arrows).

Scale bar: 50 µm.