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On regenerative and collateral sprouting to hind limb digits after sciatic nerve injury in the rat

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Abstract. *Purpose:* This study examines the proportions of regenerative and collateral sprouting to the skin after peripheral nerve injury. *Methods:* In the first experimental paradigm, primary afferent neurones were pre-labelled with Diamidino Yellow (DY), injected in digit 3, followed by sciatic nerve section and repair. After three months of regeneration, digit 3 was re-injected with Fast Blue (FB) to label regenerating cells. Fluoro-Gold (FG) was applied to the femoral (FEM) and musculocutaneous (MC) nerves four days later to quantify their contribution to the innervation. In the second experimental paradigm, sciatic nerve was first sectioned and repaired. Three months later, the sciatic was resected, and digit 3 injected with FB. After four more days, FEM and MC were resected and FG injected in all digits. *Results:* Neurones in dorsal root ganglion (DRG) L5 had a higher rate of correct reinnervation of digit 3 (44–72%) than neurones in DRG L4 (14–44%). Like in control cases, only occasional axons were traced from the FEM and MC. In the second experiment, only occasional labelled neurones appeared. *Conclusions:* The results indicate differences in the capacity for correct peripheral sensory reinnervation between segmental levels, and that in this model collateral sprouting was practically non-existent compared to regenerative sprouting.

Keywords: Nerve regeneration, DRG, fluorescent dyes, adult rat

1. Introduction

Peripheral nerve injuries are often followed not only by reduced sensitivity in the central zone of the denervated territory, but also by changes in sensory functions at the boundary which may be attributed to altered functions in neighbouring nerves. The processes that underlie these alterations might be of interest for potential restoration of the sensory loss and for the clinically important phenomena of borderline hypersensitivity [4,12] and neuropathic pain. Theoretically, both central and peripheral mechanisms may be involved in this process.

Centrally, early physiological studies after peripheral nerve injury indicated somatotopical reorganisation of postsynaptic neuronal responsiveness in the spinal cord. After an initial loss of postsynaptic responses to peripheral stimulation in the dorsal horn territory of the injured nerve, neurones were reported to show novel responsiveness to nerve stimulation of neighbour-

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ing uninjured sensory nerves to which they normally would not respond [6,17,19]; see, however [22]. Several controversial mechanisms have been proposed to be associated with these findings, including collateral sprouting from neighbouring uninjured nerves [8,16, 18], or afferents primed to regenerate by a conditioning lesion [20], and unmasking of normally silent contacts from uninjured nerves [3,19,29].

Peripherally, nerves in neighbouring skin territories have been reported to send sprouts into the denervated area. Techniques used were anterograde tracing [13], immunocytochemistry to demonstrate neuronal markers [15,27,28], plasma extravasations of Evans Blue [14,31], or physiological tests [10,14,28]. Briefly, reinnervation of the denervated territory may occur either by collateral sprouting from uninjured axons in adjacent regions, or by regenerative sprouting from either the nerve that was injured in the first place [7], or from neighbouring nerves primed to regenerate by for instance a crush injury "expansive regenerative sprouting" [31]. The main findings reported are that regenerative sprouting from the injured nerve generally results in better restoration than collateral sprouting from neighbouring nerves, at least for coarse calibre fibres [7,15,30,31], and that collateral sprouting from neighbouring nerves primed to regenerate by a conditioning lesion is better than collateral sprouting from uninjured nerves [13,15,30,31]. All those studies provide mainly qualitative descriptions of collateral sprouting.

We have previously proposed the distal phalanges of the toes of the adult rat as an area of special interest to study selective nerve regeneration because of their natural boundaries that allow two tracers to be injected in the same place [24] before and after an injury situation. In this model it would be possible to assess the regeneration of the original and the regenerating population, the contributions of the different hind limb nerves in the process [25], and also the quantification of the regeneration.

The experiments we present here were designed to provide information on changes in innervation patterns as well as quantitative information of collateral sprouting after nerve injury, using the hind limb digits as a model.

2. Material and methods

Nine 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. Anaesthesia was initiated with ether and then continued with chloral hydrate (300 mg/kg) during all surgical procedures and perfusion.

1. Experiment 1. Selective re-innervation of the digits from the injured sciatic nerve and by collateral sprouting from intact neighbouring nerves

Bilateral subcutaneous injections in the tips of the third hind limb digits were performed with 0.5 μl. of Diamidino Yellow (DY, EMS-Polyloy, Gross-Umstadt, Germany) ($n = 6$) 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 introduction 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, realigned, and sutured using nylon 10-0 monofilament.

Three months after the transection and suture, the same digits were re-injected with 1.5 μl. of Fast Blue (FB, Sigma, St Louis, MO) using a similar technique.

Four days after the FB injection, the musculocutaneous nerve, a proximal branch of the sciatic nerve, was dissected bilaterally from the dorsal side of the thigh. This nerve was given its name because of its similarities with the musculocutaneous nerve of the forelimb, since it innervates the femoral biceps muscle. It contains about 1600 afferent fibres and accounts for about 2% of the sensory innervation of the digits [25]. Once the musculocutaneous nerve was dissected, it was transected immediately distal to where it crosses the caudofemoral muscle, and its proximal cut nerve end exposed to 10% Fluoro-Gold (FG, Fluorochrome.Inc, Denver, CO) in a capsule. Then the femoral nerve was exposed ventrally at the level of the groin, transected and its proximal end exposed 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 removed after 30 minutes, the nerve cleaned, the skin sutured, and the animal allowed to recover (Fig. 1). Animals were perfused after four more days.

2. Experiment 2. Quantification of the reinnervation from other sources than the injured sciatic nerve.

This experiment was designed to quantify the total collateral innervation of the third digit from other nerves than the sciatic nerve, after it had regenerated. We used bilateral experiments to reduce the number of animals.

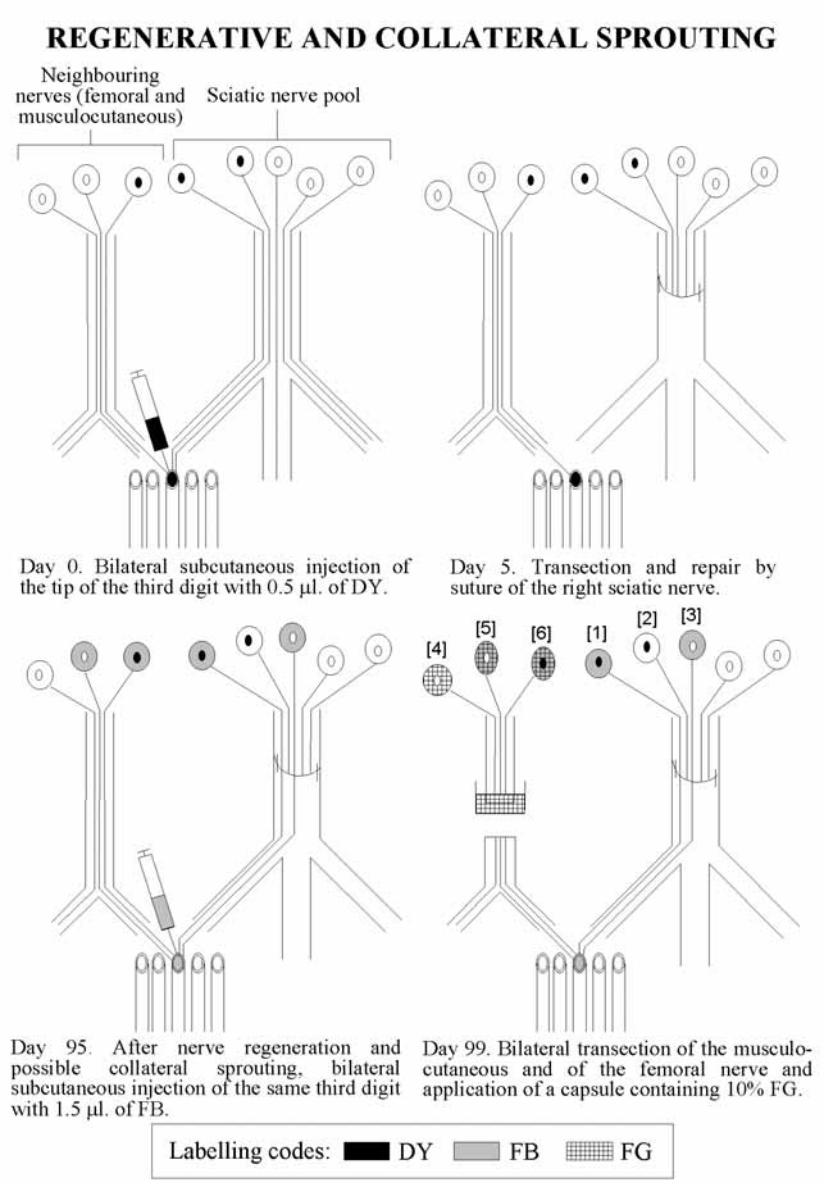

Fig. 1. Experiment 1 designed to study distal selective reinnervation and collateral sprouting to the hind limb digits. Codes of labelled cells
[1] Double labelled neurones (DYFB): belonging to the original population, have selectively reinnervated its original area.
[2] Neurones single labelled with the first tracer (DY): belonging to the original population, have not regenerated or have been misdirected to another area.
[3] Neurones single labelled with the second tracer (FB): originally belonging to other populations, now misdirected during regeneration to the area of study.
[4] Neurones single labelled with the third tracer (FG): belonging to an adjacent nerve, not innervating the area of study.
[5] Neurones double labelled with FG and FB (FGFB): resulting from collateral sprouting from an adjacent nerve.
[6] Neurones triple labelled with FG, FB and DY (FGFBDY): original population of the area of study belonging to an adjacent uninjured nerve.

The right sciatic nerve was exposed ($n = 3$), and transected at the level of the thigh. The cut ends were realigned and sutured. Three months after this procedure, rats were re-anaesthetised, the sciatic nerves were bilaterally dissected, resected at the level of the suture and subcutaneous injections of 1.5 μl. of FB were performed in the third digit bilaterally. Rats were allowed to recover. After four days, rats were re-anaesthetized and the femoral and musculocutaneous nerves were exposed and resected bilaterally. Then, 1.5 μl. of FG

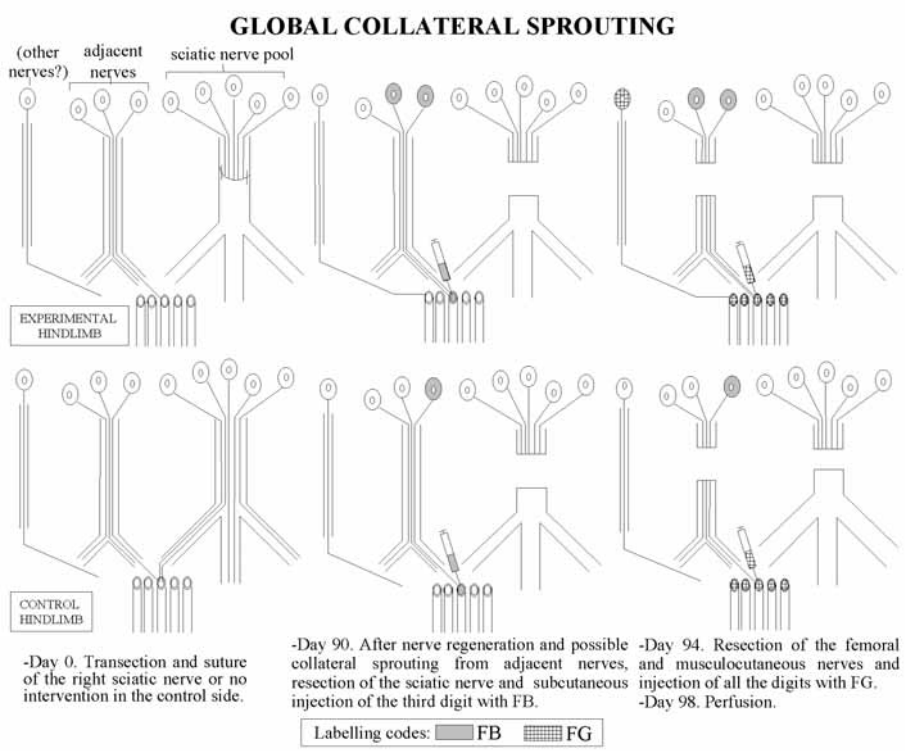

Fig. 2. Experiment 2 designed to study general collateral sprouting to the hind limb digits.

were injected bilaterally in the third digit and 0.5 μl. of the same tracer in the rest of the digits (Fig. 2). Animals were perfused after four days.

3. Fixation, sectioning, microscopic examination

The rats were anaesthetised and given intracardial injections of 1000 UI heparin/kg. body weight followed by perfusion through the ascending aorta with 100 ml. saline and then with 500 ml. 4% paraformaldehyde and 10% sucrose in phosphate buffer (PB, pH=7.40) for 20 minutes. The lumbar dorsal root ganglia (DRG)s L3-6 [9] were removed and post-fixed for three hours in the same fixative + sucrose solution. After that, in experiment 1 the L3-6 DRGs were cut on a cryostat in 16 μm. thick longitudinal sections, while in experiment 2 all ganglia were cut at 30 μm. thick serial longitudinal sections. Sections were thaw-mounted on chrom-alum gelatinised (5%) slides and coverslipped using an antifading 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 wave lengths). Neuronal profiles with an identifiable nucleus were counted (see [24,25], for more information on the identification of labelled neurones). The three tracers can be easily differentiated by the ultraviolet filter; FB is blueish, FG is reddish, and DY is pale yellow. In case of double labelling, FB and FG may hide DY if the ultraviolet filter is used. This difficulty was solved by checking the cells also by the violet filter which clearly facilitated the visualisation of the DY. FG-FB double labelled cells have a special blue-redish appearance. The FB labelling could also be confirmed in by checking the cells through the violet filter, were the FG is less intense.

In experiment 1, the total number of labelled profiles were counted using every fifth section of DRGs L4-5. Total cell counts and percentages are presented in Tables 1 and 2. No corrections were made for the possibility of counting split cells twice in different sections.

In experiment 2, the total number of cells was identified by examining the whole number of consecutive sections obtained. Split cells were identified by exam-

Table 1
Femoral nerve representation in DRG L4 and selective reinnervation (SR) from this DRG

L4 Case	Experimental FBDYFG	FGDY	FGFB	FG	%femoral	FB	DY	FBDY	SR relative	SR total
R236	0	0	0	112	0.0%	15	20	20	50.0%	
R237	0	1	0	192	2.2%	14	4	3	42.9%	8.6%
R238	0	0	3	251	5.7%	28	4	5	55.6%	15.2%
R239	1	0	2	322	3.2%	24	10	8	44.4%	10.6%
R240	0	0	0	142	0.0%	19	19	4	17.4%	19.9%
R273	0	0	1	168	1.0%	18	11	12	52.2%	15.9%
Mean	0.2	0.2	1.0	197.8	2.0%	19.7	11.3	8.7	43.7%	14.1%
SD	0.4	0.4	1.2	77.0	2.2%	5.4	6.9	6.4	13.8%	4.5%
L4 Case	Control FBDYFG	FGDY	FGFB	FG	%femoral	FB	DY	FBDY	FBDY/(FBDY+DY)	
R236	0	0	0	180	0.0%	74	5	10	66.7%	
R237	0	1	7	124	4.9%	55	5	33	86.8%	
R238	0	0	0	61	0.0%	5	9	35	79.5%	
R239	0	0	0	115	0.0%	27	11	70	86.4%	
R240	0	0	0	281	0.0%	28	16	21	56.8%	
R273	0	0	0	264	0.0%	7	12	77	86.5%	
Mean	0.0	0.2	1.2	170.8	0.8%	32.7	9.7	39.3	77.1%	
SD	0.0	0.4	2.9	87.4	2.0%	27.1	4.3	24.9	12.6%	

Number of profiles counted in one of every five DRG consecutive sections (DRG L4).

Table 2
Musculocutaneous nerve representation in DRG L5, and selective reinnervation (SR) from this DRG

L5 Case	Experimental FBDYFG	FGDY	FGFB	FG	%musc-cut.	FB	DY	FBDY	SR relative	SR total
R236	0	0	0	257	0.0%	19	7	10	58.8%	
R237	0	0	0	317	0.0%	17	0	7	100.0%	52.2%
R238	0	0	0	229	0.0%	8	1	4	80.0%	52.3%
R239	0	2	0	301	2.2%	31	7	8	53.3%	47.1%
R240	0	1	0	206	1.1%	23	5	11	68.8%	40.7%
R273	0	1	3	546	4.0%	30	7	17	70.8%	28.6%
Mean	0.0	0.6	0.5	309.3	1.2%	21.3	4.5	9.5	72.0%	44.2%
SD	0.0	0.8	1.2	123.3	1.6%	8.6	3.2	4.4	16.6%	9.9%
L5 Case	Control FBDYFG	FGDY	FGFB	FG	%musc-cut.	FB	DY	FBDY	FBDY/(FBDY+DY)	
R236	2	0	3	93	4.8%	8	0	2	100.0%	
R237	3	0	1	268	2.5%	40	3	14	82.3%	
R238	0	0	0	192	0.0%	1	4	8	66.7%	
R239	0	0	0	106	0.0%	4	2	17	89.5%	
R240	0	0	0	222	0.0%	21	10	31	75.6%	
R273	1	4	0	381	3.0%	4	1	59	98.3%	
Mean	1.0	0.6	0.7	210.3	1.7%	13.0	3.3	21.8	85.4%	
SD	1.3	1.6	1.2	107.3	2.0%	14.9	3.6	20.7	13.0%	

Number of profiles counted in one of every five DRG consecutive sections (DRG L5).

ining the adjacent section once a cell profile was visualized, in order to see if another cell profile appeared in the same position within the adjacent ganglionic tissue. In that case, the second profile, corresponding to the same cell, was excluded from the counting.

The FG labelled neurones belong to nerves other than the sciatic, i.e. the femoral and the musculocutaneous nerves. Depending on the ganglionic level and their localisation within the ganglia [23,25], these neurones were recognised as either femoral or musculocutaneous, respectively. Likewise, neurones containing FG plus any other of the tracers applied in the digits were also considered to belong to primary afferents that reach the digits through other nerves than the sciatic.

The results corresponding to the DY and FB single and double DY-FB labelling will be analysed in more detail separately (manuscript in preparation) and are considered to belong to the selective reinnervation of

the sciatic neurones to the digits. Here, we restrict the presentation to important considerations on differences in the selective regeneration between sensory ganglia.

4. Statistical analysis

Paired Wilcoxon W tests were used to compare percentages between control and experimental hind limbs in the same experiment.

5. Results

Examination of DRGs L3 and L6 showed no general diffuse cellular labelling suggestive of haematogenous spread of the fluorescent tracers.

6. Collateral sprouting to the digits from adjacent uninjured nerves

6.1. Femoral nerve

Sparse FG-neurones were distributed in DRGs L4 and L5. In four of the six experimental cases, DRG L4 neurones labelled with the tracers applied in the digits (FB and/or DY) contained also the FG applied in uninjured nerves. All those neurones were located in the dorsal and rostrolateral part of the L4 ganglion, within the somatotopically arranged neuronal group known to belong to the femoral nerve population [23]. In three of the six cases, FG was found in neurones that contained only the second tracer (FB) but not the first one (DY). The femoral nerve representation was calculated by counting the cell profiles in DRG L4 labelled by both FG and any of the other tracers, divided by the total number of labelled cells in DRGs L4 and L5.

$$\text{Femoral nerve representation} = \frac{(\text{FBDYFG} + \text{FGFB} + \text{FGDY})_{L4}}{= (\text{FBDYFG} + \text{FGFB} + \text{FGDY} + \text{FB} + \text{DY} + \text{FBDY})_{L4+L5}}$$

In total, the femoral nerve accounted for 1 to 5.7% (average 2.0 $\pm$ 2.2%), of the neuronal profiles labelled from the studied third digit in the experimental side (Table 1).

Control cases showed that the femoral nerve normally contribute in average 0.8 $\pm$ 2.0% to the innervation of the same (third) digit. Only one control case showed retrograde labelling from the digits and the nerve, 4.9%. There were no significant differences between the experimental and the control hind limbs ($p = 0.715$), (Table 1).

6.2. Musculocutaneous nerve

Three of the six experimental cases showed L5 neurones that co-localised tracers applied in the digits (FB and DY) and FG applied to the femoral and the musculocutaneous nerves. These three cases all contained neurones that co-localised FG and the first tracer (DY), applied in the digits before the nerve lesion. Only one case showed three neurones labelled with second tracer injected in the digits (FB), without DY. These neurones were assumed to belong to the musculocutaneous nerve since the femoral nerve does not have L5 representation [25]. The musculocutaneous nerve representation was calculated by counting the cell profiles in DRG L5 labelled by both FG and any of the other tracers, divided by the total number of labelled cells in DRGs L4 and L5.

$$\text{Musculocutaneous nerve representation} = \frac{(\text{FBDYFG} + \text{FGFB} + \text{FGDY})_{L5}}{= (\text{FBDYFG} + \text{FGFB} + \text{FGDY} + \text{FB} + \text{DY} + \text{FBDY})_{L4+L5}}$$

The musculocutaneous nerve representation in the experimental side accounted for 1.1% to 4% of the neuronal profiles labelled from the studied digit 3 (average 1.2 $\pm$ 1.6%), (Table 2).

Three of the six control cases showed also retrograde labelling via the musculocutaneous nerve from the digits. In the control cases, the representation of the musculocutaneous, when present, accounted for 2.5 to 4.8% (average 1.7 $\pm$ 2.0%) of the neuronal profiles labelled from the studied digit. Only in one of these cases did the labelling on the control side concur with musculocutaneous nerve labelling on the corresponding experimental side.

There were no significant differences between the control and experimental hind limbs ($p = 0.225$), (Table 2).

6.3. Global collateral sprouting

Application of FB to the third digit after the proximal transection and resection of the regenerated sciatic nerve resulted in no labelled neurones in DRG L4 on either the experimental or the control side. One case showed four neurones in DRG L3, two cases showed two and four labelled neurones, respectively, in DRG L5, and one case showed one neurone in DRG L6. Control cases showed FB labelled neurones in DRG L5 (one neurone per case) and in DRG L6 (three and two

Table 3
Neurones labelled from collateral sprouts

Case	FB	FG	FBFG		FB	FG	FBFG
L3 D				**L3 I**			
R267 D	0	0	0	R267 I	0	0	0
R268 D	4	0	0	R268 I	0	0	0
R269 D	0	0	0	R269 I	0	0	0
Mean	**1.3**	**0**	**0**		**0**	**0**	**0**
L4 D				**L4 I**			
R267 D	0	0	0	R267 I	0	0	0
R268 D	0	0	0	R268 I	0	0	0
R269 D	0	0	0	R269 I	0	0	0
Mean	**0**	**0**	**0**		**0**	**0**	**0**
L5 D				**L5 I**			
R267 D	0	0	0	R267 I	1	0	0
R268 D	2	0	0	R268 I	1	0	0
R269 D	4	0	0	R269 I	1	0	0
Mean	**2**	**0**	**0**		**1**	**0**	**0**
L6 D				**L6 I**			
R267 D	1	0	0	R267 I	3	0	0
R268 D	0	0	2	R268 I	2	0	0
R269 D	0	0	0	R269 I	0	0	0
Mean	**0.3**	**0**	**0.7**		**1.7**	**0**	**0**

Total number of neurones showing FB (most likely from the femoral and musculocutaneous nerves) and total number of neurones with FG (from other sources or innervation) after checking all sections.

neurones, respectively). Only one of the cases with additional resection of the femoral and musculocutaneous nerves showed two labelled neurones in DRG L6 after application of FG (Table 3).

7. Estimation of the selective reinnervation

The selective reinnervation was defined as the number of neurones which were found to reinnervate the same target, identified by quantifying the number of double-labelled cells in the injured hind limb with DY and FB ($\mathrm{FBDY}_{\mathrm{exp}}$). To obtain an index relative to the injury, this number may be compared with the total number of cells corresponding to the original population in the injured hind limb from that area, that is, with the total number of cells that contain the first tracer $(\mathrm{FBDY{+}DY})_{\mathrm{exp}}$. The number of double-labelled cells in the injured limb could also be compared with the original population of the control limb $(\mathrm{FBDY{+}DY})_{\mathrm{ctrol}}$ and be related to the maximal double labelling rate obtained in the control hind limb ($\mathrm{FBDY}_{\mathrm{ctrol}}$ / $(\mathrm{FBDY{+}DY})_{\mathrm{ctrol}}$), with the aim to calculate a global index.

$$\text{Relative selective reinnervation} = \mathrm{FBDY}_{\mathrm{exp}}/(\mathrm{DY}+\mathrm{FBDY})_{\mathrm{exp}}$$

$$\text{Global selective reinnervation} = \frac{(\mathrm{FBDY}_{\mathrm{exp}}/(\mathrm{FBDY}+\mathrm{DY})_{\mathrm{ctrol}})}{(\mathrm{FBDY}_{\mathrm{ctrol}}/(\mathrm{FBDY}+\mathrm{DY})_{\mathrm{ctrol}}) =} = \mathrm{FBDY}_{\mathrm{exp}}/\mathrm{FBDY}_{\mathrm{ctrol}}$$

In the process of counting, we noted a tendency of a higher number of double-labelled neurones in DRG L5 compared to other DRGs. We therefore compared the rate of double labelling in DRGsL4 and L5 with the control hind limb. Percentages of double labelling in the control limb (FBDY) / (FBDY+DY) varied from 77.1 ± 12.6% in DRG L4 to 85.4 ± 13.0% in DRG L5. In the experimental hind limb, the index of double labelling in DRG L4 varied from 14.1 ± 4.5% to 43.7 ± 13.8%, depending on whether the global or the relative index was used, while the rate of double labelling in DRG L5 ranged from 44.2 ± 9.9% to 72.0 ± 16.6% (Tables 1 and 2). Differences between the rate of double labelling in DRG L4 and L5 were significant both for the global index ($p = 0.043$) and the relative index ($p = 0.028$).

Total counts of DY labelled neurones in the control limb of R136 were much reduced and were not used for estimation of the global selective reinnervation index. The number of rats was not increased since the obtained results convincingly demonstrated very limited collateral sprouting.

8. Discussion

We have previously shown that only a very limited number (<45) of primary afferents from the distal digital phalanges of the normal hind limb belong to the musculocutaneous nerve [25]. Furthermore, the femoral (saphenous) afferents have been reported to be restricted to digits 1–2, and the proximal phalange of digit 3 [31], which indicates that the sensory innervation of the third digit is mainly sciatic in origin with a minor contribution from the femoral nerve and perhaps the musculocutaneous nerve. Thus, we hypothesized that this digit would be particularly useful for studying compensatory sprouting from other nerves after sciatic injury.

9. Collateral sprouting

The same tracer, FG, was used to label either the femoral or the musculocutaneous nerve. The FG neurones in L4 that appear in the rostro-lateral part of the

ganglion that contain also labelling from the toes (FB and/or DY) have been assumed to belong to the femoral nerve because of the somatotopical arrangement of the femoral neurones in that part of L4 DRG [23] and for the disappearance of the primary afferent cells from the digits in L4 if a resection of the femoral nerve is added to a sciatic nerve resection. Furthermore, no musculocutaneous nerve afferents from L4 innervate the toes in control animals [25].

The FG neurones in DRG L5 that also appear labelled with FB and DY have been assumed to belong to the musculocutaneous nerve because there are no femoral afferents from that ganglion and because the DRG L5 afferents from the digits also disappear when a musculocutaneous nerve resection is added to a sciatic nerve resection in control animals [25]. Since the digit representation in DRG L6 is less than 17 neurones, considering all digits together, we found it reasonable to disregard DRG L6 in this study.

9.1. Femoral nerve

Limited collateral sprouting in adult animals from uninjured saphenous afferents to the adjacent denervated territories that belong to the sciatic nerve has previously been described [13,15,28,31]. In the present experiments, femoral neurones that can be assumed to have sent collateral sprouts would be those that show FB labelling (the new population) and also FG (nerves other than the sciatic) and without DY labelling (original population) in DRG L4. The results show, however, that such neurones, judged by their DRG distribution to be femoral in origin, were found in only three of six cases. Furthermore, even in those three cases, the maximal femoral representation was 5.7% of the total innervation of the digit, which was not significantly different from the control. Thus, from results presented here, our interpretation is that the uninjured femoral nerve, known to innervate the proximal phalange of digit 3 [31], does not efficiently compensate a sciatic denervation of the distal part of the digit.

9.2. Musculocutaneous nerve

The results indicate that the musculocutaneous nerve participated in the innervation of the digits only in three of the six experimental cases. In all these cases the neurones from the musculocutaneous, labelled with FG in L5, were also labelled with the first tracer, DY, indicating that those neurones were supplying the digits before the sciatic nerve injury and therefore participated in their original innervation, in addition to the sciatic. Only one case showed three neurones labelled by the second tracer and the tracer applied in the nerve (FB-FG), but not by the first tracer (DY), indicating possible collateral sprouting from the musculocutaneous nerve.

Less than half of the cases in this study showed retrograde labelling from the third digit that could be accounted for by the musculocutaneous nerve even though we previously showed [25] that this route of innervation from the digits is a consistent finding. This discrepancy may be explained by the fact that in the former study, lateral and medial digits were examined together, while and this nerve may less consistently innervate the third digit examined in the present study. Nevertheless, the maximal participation from the musculocutaneous nerve to the total innervation of the digit was found to be 4%. Thus, its role in compensating innervation by collateral sprouting after sciatic injury is likely to be limited.

9.3. Global collateral sprouting

The quantitatively limited collateral sprouting shown in the first experiment involving FG nerve labelling and application of FB and DY in the digits could theoretically be due to a potential difficulties to observe the double or triple labelled neurones for technical reasons such as dye interactions or problems with microscopic visualization. In theory, a high FB labelling could conceal faint FG labelling, since both are detected through the same ultraviolet excitation filter and there is no filter combination that show FG labelling without showing the FB labelling. Such theoretical limitations could lead to an underestimation of the collateral sprouting. In order to reduce this possibility, we added an experimental design where we performed successive nerve resections with the aim to quantify the entire residual innervation by injecting the digits after the removal of first the regenerated sciatic nerve, and then the contributions from the femoral and musculocutaneous nerves. Injection of FB in the third digit after resection of the regenerated sciatic nerve showed 1–3 FB labelled neurones in DRGs L5–L6 that probably belonged to the musculocutaneous nerve since no femoral nerve afferents reside in these ganglia. Furthermore, additional injections of FG in all digits after resection of also the musculocutaneous and femoral nerves resulted in no FG labelling in the DRGs (excepting two neurones in L6), suggesting practically complete denervation. We therefore conclude that other sources of potential sprouting are unlikely to exist.

Altogether, the results show that significant collateral sprouting from non-sciatic origins towards the denervated sciatic nerve territory could be discarded. Future studies using a similar approach to analyze the tibial and peroneal nerves, which innervate different aspects of the digits, might show if there is collateral sprouting between territories belonging to branches of the sciatic nerve itself.

Human studies have indicated limited recovery of the sensitivity of denervated territories that can be explained by collateral sprouting from intact adjacent nerves [1,2,10,27] even though sprouting may be pronounced near the border to the denervated territories. In adult rats, limited amounts of collateral sprouting into denervated territories have previously been reported to occur in hind limb foot including the digits [13,15]. Thin calibre fibres seem to send out collateral sprouts better than thick calibre fibres [7,13,15]. At least for the TrkA expressing afferents, the amount of collateral sprouting is likely to be dependent on the amount available nerve growth factor, whereas the regenerative sprouting is not [7]. The results of this quantitative study shows that the amount of such collateral sprouting is limited indeed, at least in the present paradigm, where the original sciatic nerve had been allowed to regenerate for such a long period as three months, and as far as the distal phalanges are concerned. It is possible that the limited collateral sprouting found in this study can partly be explained by a positive selection in favour of the regenerating afferents from the original sciatic nerve at late time points after injury. Previous findings in both animals [5,21] and man [27] regarding sensory reinnervation support this view. Similar findings have been made also for motor axons [11]. Another factor likely to be important is that the femoral and musculocutaneous afferents were uninjured, except for the final experimental steps, when also these nerves were injured in order to label their afferents or to exclude them as connectors between the digits and the DRGs. Previous studies have shown that the sprouting capacity is considerably larger if the neighbouring nerves are primed to regenerate by a conditioning injury such as a crush [13,15,30,31]. Whether this would be the case also in the model used here remains to be studied.

10. Selective reinnervation

Neurones double-labelled with DY and FB, but without FG were considered to represent selectively regenerating axons belonging to the sciatic nerve population. A detailed analysis of the topographical selectivity in reinnervation, including differences between proximal and distal re-growth, is now in preparation. No detailed assessment of changes after injury in the participation of the different sciatic nerve branches involved in the innervation of the digits has been performed here. One of the findings so far is that the regeneration after sciatic nerve injury to the original target appears to be more selective for afferents belonging to DRG L5 (44–71%) compared to L4 (14–43%).

One of the possible explanations for this finding is contribution from DRG L5 to the sensory innervation of the digits from other sources than the sciatic nerve, and that afferents in these nerves would send collateral sprouts to the denervated regions shortly after sciatic transection [28]. The sprouts would then take up remaining deposits of the first tracer [26], as well as the second tracer after the regeneration period, resulting in an overestimation of the selective regeneration from the L5 DRG. The results of the present study, however, indicate that this mechanism is unlikely to contribute significantly to the differences between DRGs L4 and L5 as described above.

Other possible mechanisms may be related with unintentional differences in the re-alignment of the fascicles when repairing the nerve, in a way that could favour the selective reinnervation from the L5 DRG. The different rates of correct reinnervation between ganglia presented here prompted us to study the total afferent population from a specific peripheral region, in this case the third digit.

11. Conclusions

The experimental setting presented offers a technique for quantitative studies of collateral sprouting as well as the topographical accuracy of regenerative sprouting, which are the two modes of peripheral reinnervation after nerve injury. The main conclusion is that collateral sprouting from neighbouring intact nerves play a limited role in the reinnervation of the denervated territory, at least in the model used in this study with examination at late time points after injury, and when the injured nerve is repaired and allowed to regenerate. Furthermore, the rate of topographically correct sensory regeneration may differ between neurones at different segmental levels innervating the same territory.

Further studies with this model are needed for a more accurate estimation of the rate of topographically cor-

rect reinnervation by regenerative sprouting after peripheral nerve injury, and to investigate if the amount of collateral sprouting becomes increased if regeneration of the injured nerve is prevented or if the neighbouring nerves are primed to regenerate by a conditioning lesion.

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