Somatosensory and Motor Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713393866

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Online Publication Date: 01 March 2007
To cite this Article: Rupp, Angie, Dornseifer, Ulf, Rodenacker, Karsten, Fichter, Andreas, Jütting, Uta, Gais, Peter, Papadopulos, Nikolaos and Matiasek, Kaspar , (2007) 'Temporal progression and extent of the return of sensation in the foot provided by the saphenous nerve after sciatic nerve transection and repair in the rat - implications for nociceptive assessments', Somatosensory and Motor Research, 24:1, 1 - 13
To link to this article: DOI: 10.1080/08990220601116329
URL: http://dx.doi.org/10.1080/08990220601116329

Somatosensory and Motor Research, March–June 2007; 24(1–2): 1–13

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Temporal progression and extent of the return of sensation in the foot provided by the saphenous nerve after sciatic nerve transection and repair in the rat—implications for nociceptive assessments

ANGIE RUPP[1], ULF DORNSEIFER[2], KARSTEN RODENACKER[3], ANDREAS FICHTER[4], UTA JÜTTING[3], PETER GAIS[5], NIKOLAOS PAPADOPULOS[4], & KASPAR MATIASEK[1]

[1]*Chair of General Pathology & Neuropathology, Institute of Veterinary Pathology, Ludwig-Maximilians University, Munich, Germany,* [2]*Department of Plastic, Reconstructive and Hand Surgery, Academic Hospital Bogenhausen, Munich, Germany,* [3]*Institute of Biometrics and Biomathematics, GSF National Research Centre for Health & Environment, Neuherberg, Germany,* [4]*Department of Plastic & Reconstructive Surgery, Klinikum Rechts der Isar, Technical University, Munich, Germany and* [5]*Institute of Pathology, GSF National Research Centre for Health & Environment, Neuherberg, Germany*

(Received 6 August 2006; revised 8 October 2006; accepted 6 November 2006)

Abstract
Sensory testing, by providing stimuli for nociceptors of the foot, is a popular method of evaluating sensory regeneration after damage to the sciatic nerve in the rat. In the following study, 20 rats were submitted to double transection of the sciatic nerve. The subsequent 14 mm gap was repaired through guidance interponation. In order to evaluate nerve regeneration, sensory testing was performed additionally to other methods, which included motor testing, morphometry, and electron microscopic assessments of nerves. Somatosensory testing revealed that all animals exhibited next to the same amount of sensory reinnervation on their foot regardless of their experimental group. In motor tests, however, two out of the three experimental groups did not improve at all. These groups also failed to show neural regrowth in morphometric and electron microscopic assessments of the associated nerve. Retrograde tracing was able to prove the saphenous nerve as an alternative source of sensory reinnervation in animals with failed sciatic regeneration. This means that results of sensory testing in the rat should be treated with caution, taking into account the areas tested and the likelihood that in these areas saphenous sprouting could have taken place. Furthermore, it is strongly advised that somatosensory testing should be conducted only on toe 5.

Keywords: *Sciatic nerve regeneration, saphenous sprouting, pain response, withdrawal reflex, rat*

Introduction

In sciatic nerve regeneration studies the extent of reinnervation is evaluated by both motor and sensory testing. Generally, sensory assessments can be divided into proprioceptive and exteroceptive testing. To achieve a reliable reaction in the latter, nociceptors have to be stimulated; these are receptors which have a particularly high threshold (Serpell 2005) and which only react to noxes at a level potentially destructive to tissue. The subsequent reaction can take place at two different levels: A subconscious withdrawal reflex or a conscious pain reaction.

The withdrawal reflex is a true reflex which involves the central nervous system on the spinal level. The animal responds to the stimulation of nociceptors simply by withdrawing its leg. For a conscious reaction to pain, however, afferent signals have to reach the thalamus via spinothalamic tracts and the medial lemniscus, and are further projected to the somesthetic cortex via the internal capsule. Conscious pain perception occurs both on the thalamic and on the cortical level, and causes the animal to react with an accurate pain response such as licking its foot or vocalizing its discomfort in

Correspondence: Dr K. Matiasek, Chair of General Pathology & Neuropathology, Institute of Veterinary Pathology, Ludwig-Maximilians University, Veterinärstr. 13, 80539 Munich, Germany. Tel: +49 (0)89 2180 3313. Fax: +49 (0)89 2180 99 3313. E-mail: kaspar.matiasek@neuropathologie.de

ISSN 0899–0220 print/ISSN 1369–1651 online
DOI: 10.1080/08990220601116329

addition to withdrawing its foot from the noxious stimulus (Navarro et al. 1994).

Given that, after experimental nerve trauma, the afferents have been re-established, the question that now arises is whether a rat with residual sciatic dysfunction is still capable of performing a recognizable withdrawal of the foot. This could be a problem if the effector muscles needed for this action are still denervated or incompletely or inappropriately reinnervated. Additionally, the common technique of wrapping the rat in a towel for immobilization and restriction of vision during evaluation (Masters et al. 1993; Hu et al. 1997; Varejão et al. 2004a) might not only hinder the rat from performing a withdrawal reflex, but might also make it more difficult for the examiner to recognize weak responses.

In addition to this aspect, there appears to be little consensus regarding the method adopted to evoke a pain response or withdrawal reflex in the rat. In turn, a number of different noxes have been applied, to date, in different ways and on different areas of the foot (Vogelaar et al. 2004; Nichols et al. 2005). The only generally accepted rule is that the medial aspect of the foot should be avoided, as the saphenous nerve caters for this region (de Lahunta 1977; Devor et al. 1979; de Koning et al. 1986; Varejão et al. 2004b).

Various descriptions of extensive saphenous sprouting following permanent sciatic denervation can be found in the literature (Devor et al. 1979; Markus et al. 1984; Kingery and Vallin 1989). This annexation of what was originally sciatic territory on the foot has been described as occurring as early as within the first 4 days after acute sciatic transection (Devor et al. 1979). Neglecting these observations, recommendations regarding the area to be stimulated for assessment of sciatic function still range from the general area of the sole (Masters et al. 1993; Attal et al. 1994; Hu et al. 1997; Hadlock et al. 1999; Varejão et al. 2004a), the lateral side of the foot, sometimes including toe 5 (de Koning et al. 1986; Chamberlain et al. 2000; den Dunnen and Meek 2001; Meek et al. 2003), or the plantar and sometimes also the dorsal side of the foot including the toes in various places (Devor et al. 1979; Navarro et al. 1994; Rodríguez et al. 2000; Negredo et al. 2004).

The aim of the present study was to evaluate sciatic nerve regeneration in three different surgical groups after double transection and repair of the subsequent 14 mm gap by guidance interponation. Recently developed collagen type IV tubes, both empty and filled with denaturated autologous muscle tissue, were to be assessed with regard to their ability to sustain neural regeneration across a relatively large gap in a peripheral nerve and benchmarked against the gold standard for the bridging of larger gaps, the autologous nerve graft (Bellamkonda 2006; Chalfoun et al. 2006; Keune et al. 2006). The collagen tubes were filled with denaturated muscle as this modification had proved to be successful in promoting neural regeneration in the past (den Dunnen and Meek 2001).

Multiple test methods covering every aspect of regeneration were applied, ranging from assessments concerned with gait (Sciatic Function Index (SFI), Static Sciatic Index, toe spread factor, ankle angles, balancing), nociceptive and proprioceptive testing, and electrophysiologic examinations to histological and morphological assessments of the muscle and nerve. In view of the very diverse information available on the exact procedure to be followed for somatosensory testing, it was decided to test the rats in a greater number of small fields on the foot than previously suggested in the literature to obtain more detailed information about the return of sensation. Only a conscious pain response would be counted as a positive reaction.

Unlike primarily motor-focused evaluations such as the SFI (Walker et al. 1994; Hadlock et al. 1999) and morphometric assessments, the results of nociceptive testing did not differ significantly between the three surgical groups. The nociceptive assessments established a characteristic pattern common to all the groups for the return of sensation in the foot in more detail than previously described in the literature. The primary source of the sensory reinnervation, however, was revealed to be the saphenous nerve.

This study demonstrates how easily somatosensory assessments can be misleading in the evaluation of sciatic nerve regeneration, especially if they are not backed up by morphometric assessments of the associated structures. Largely undetected compensatory abilities of the nervous system, such as extensive saphenous sprouting in this case, are the cause of this phenomenon and can give the researcher the erroneous impression of successful sensory reinnervation of the foot by the sciatic nerve. Morphometric assessments and retrograde tracing studies tell a different story.

As a consequence, caution is advised both in interpreting and executing somatosensory investigations in sciatic nerve regeneration studies.

Material and methods

Surgical procedures

Twenty male Lewis rats (Charles River, Sulzfeld, Germany; 320–440 g) had a 14 mm segment of the sciatic nerve extracted and the defect subsequently repaired by one of the following three methods: Group A ($n = 8$) autograft repair, group B ($n = 6$) repair with empty collagen tubes (length 2 cm), and group C ($n = 6$) repair with collagen tubes filled with

denaturated autologous muscle (Meek et al. 1999). All lesions were set at the same place, with the distal end located 4 mm proximal to the submersion of the tibial branch of the sciatic nerve into the gastrocnemius muscle. Lewis rats were chosen on account of their proven resistance to autotomy after sciatic lesions (Inbal et al. 1980; Panerai et al. 1987; Carr et al. 1992; Chamberlain et al. 2000), their suitable anatomy of sciatic nerve (Rupp et al. 2006), and their friendly nature (Strasberg et al. 1999), as many evaluations would require frequent handling.

Animal health and housing

The rats were weighed weekly and inspected daily as regards grooming, activity levels, signs of autotomy, and infection or inflammation of the foot.

All rats were housed in groups of four on soft bedding in a temperature-controlled room with 12-h light cycles, and had free access to standard rat food and water. Additionally they experienced 4–6 h of "playtime" daily on weekdays in a 45 cm × 55 cm × 120 cm cage in groups of 8–12. Animal studies were approved by the local animal care committee.

Intra vitam evaluations

The rats were accustomed to being handled twice daily for 5 days as from 9 days before the operation (D−9 to D−5). The operation itself took place on D0; 2 days before the operation (D−2) the integrity of sensation on their feet was checked and all reference values were obtained. The first post-op evaluation was on day (D)5, further examinations taking place twice weekly until D56. For nociceptive testing the animals were held firmly in one hand whilst being pinched with a pair of atraumatic forceps at defined points on the lateral, plantar, dorsal, and medial aspects of their right hind feet and then of their left hind feet (Figures 1 and 2A). Definite vocalization of protest at the moment of being pinched—with or without retraction of the foot—was rated as a sign that pain had been consciously registered. Application of pressure was stopped as soon as a reaction in the rat could be provoked; the maximum pressure applied was 0.8 N/mm^2. Results were noted in a diagram and also expressed as a score denoting the number of remaining denervated areas of the foot. For the latter, the scores of the biweekly examinations were averaged to minimize possible errors in reading the signs of the rat's pain response. All tests were carried out by the same investigator to avoid interrater variabilities. The investigator was blinded to group assignment of the different rats.

In addition to nociceptive testing, motor return was evaluated by measuring traits of footprints and determining the SFI. To make the prints, the soles of the rats' hind paws were covered from the tip of their toes to the heel with non-toxic children's paint. The rats were then placed on an elevated 8 cm wide balancing beam, which ended in a darkened shelter

Figure 1. Rat being pinched.

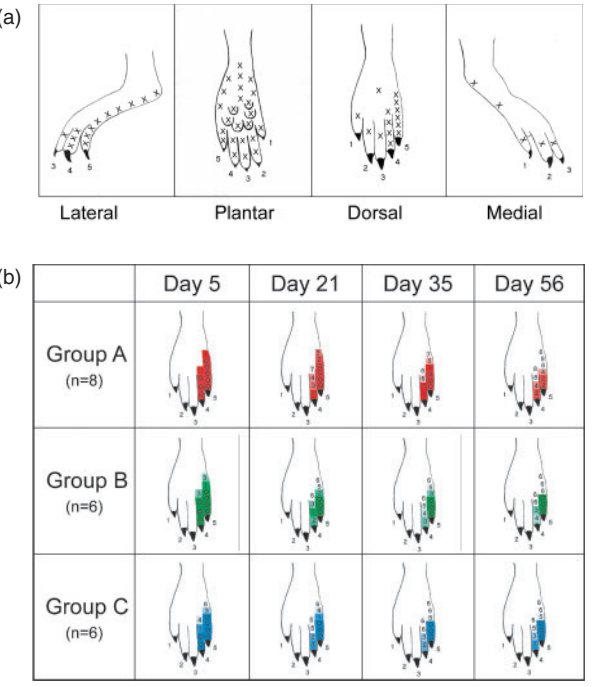

Figure 2A–D. Pinching points and results. (A) Pinching points on different aspects of the foot. On the dorsal aspect the animals were pinched at 14 spots, on the lateral aspect at 13 spots, on the plantar aspect at 24 spots, and on the medial aspect at 5 spots. Return of sensation on the dorsal (B), lateral (C), and plantar (D) aspect of the foot. Numbers show how many rats reacted to being pinched at the spot indicated. The colour code represents the same results: The lighter the shade, the greater the number of rats which reacted to being pinched. In white areas there was always a reaction. (Medial areas are not shown, as here a reaction to being pinched could always be demonstrated.) Note toe 5, where only animals in group A (autograft group) regained sensation.

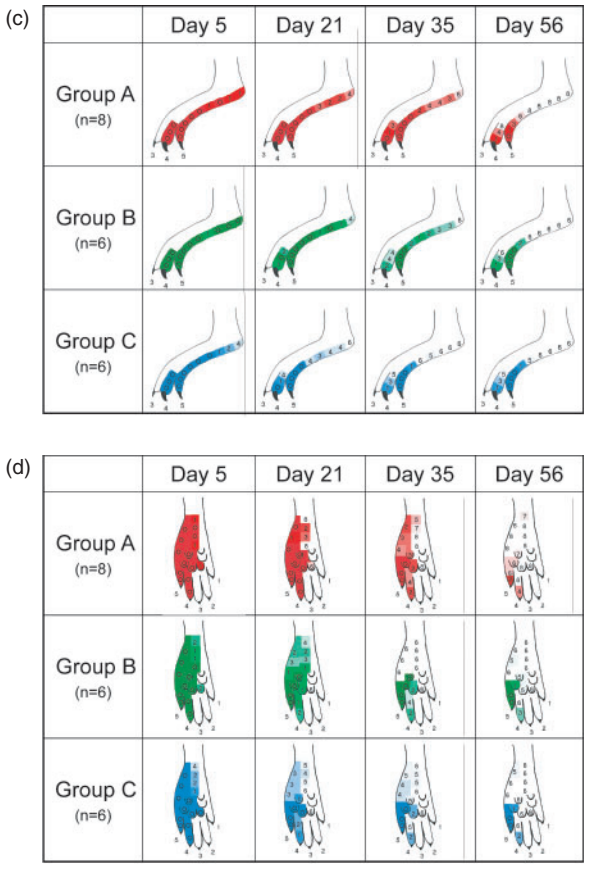

Figure 2B–D. Continued.

and was covered with a strip of paper. This technique produces more usable footprints—especially on the operated side—than those obtained when letting the animals run in a corridor (unpublished observations). As there is no wall, the rats have nothing to lean on, and there is no chance of exorotated foot swings hitting the wall and thus producing no footprints. Also, whilst traversing the beam the rats subconsciously trust their unoperated (right) hind limb and keep to the right, enabling clear footprints to be obtained on the left (operated) side. Having experienced extensive prior training, the rats made their way towards the shelter quickly and confidently. Runs were repeated until at least three well recognizable and distinct prints for each side had been acquired; at the most a rat had to undergo five runs. Three footprints for each hind limb were selected for evaluation and the distances between toes 1 and 5 (toe spread (TS)) and toes 2 and 4 (intermediate toe spread (ITS)) and the print length (PL) were measured as described by Varejão et al. (2004a,b). The average measurements for each animal were determined by two independent researchers, who then compared their values blindly. If the difference did not exceed 3 mm, the average between the two values of the examiners was incorporated into the following formula: $SFI = -38.3 \times PLF + 109.5 \times TSF + 13.3 \times ITF - 8.8$ (Varejão et al. 2004a,b). The PLF, TSF, and ITF were calculated by subtracting measurements taken from the physiological hind limb from those taken from the operated hind limb. The difference was then divided by measurements for the physiological side. SFI scores of approximately −100 indicate total impairment, whereas scores around 0 can only be achieved in rats with complete function of the sciatic nerve (Varejão et al. 2004a,b).

Retrograde tracing

On D47, retrograde tracers were applied to the operated hind limb. One intracutaneous injection, consisting of 1.5 µl of 5% Fast Blue (Polysciences, Eppelheim, Germany), was performed on the lateral side of the foot at the level of the fifth metatarsal bone. On the medial side of the foot three injections amounting to 2.5 µl of 1% Fluorogold (Biotium, Hayward, CA, USA) were conducted at the level of the first metatarsal bone.

Eight weeks after the operation (D56) the rats were killed with a lethal dose of intraperitoneal pentobarbitone. The dorsal root ganglia (DRG) of the first to sixth lumbar segments (L) were extracted and immersed in 4% paraformaldehyde and 10% sucrose in 0.1 M phosphate buffered saline (PBS) at pH 7.4 for 5 h. After fixation the DRG were transferred into 15% sucrose in PBS for at least 15 h and then frozen in liquid nitrogen. Cryostat sections were cut at 16 µm and thaw-mounted on polylysine-coated glass slides. The DRG were then examined by epifluorescence under a Zeiss Axiophot® microscope equipped with a mercury lamp, a 365 nm excitation filter, a 395 nm dichroic beam splitter, and a LP 420 nm barrier filter.

The numbers of blue and yellow labelled cells were semiquantitatively assessed by counting the fluorescent cells on what appeared to be the largest cross section.

Morphometric and electron microscopic assessments

After extraction of the DRG, the sciatic nerve plus its tibial successor were harvested from their most proximal accessible point (near the major trochanter) right down to the middle of the plantar side of the foot in both hind limbs. The nerves were immediately cut into three segments, stretched on a piece of paper, and immersed in 2.5% glutaraldehyde in Soerensen's phosphate buffer (pH 7.4) for 1 h. After fixation, samples were rinsed with Soerensen's phosphate buffer and cut into 2 mm transverse segments, which then underwent post-fixation in 2% OsO_4 for 2 h at room temperature, repeated

buffer rinses and a graded alcohol series before being embedded in epoxy resin. For morphometric evaluations semithin sections (0.5 μm) were mounted on triethoxysilane-coated slides and stained with *p*-phenylene diamine.

Morphometry was performed on cross sections of the tibial nerve 0.5 cm distal to the calcaneus. Photographs were taken of these sections with a Zeiss Axiovert 100® light microscope equipped with a PLANAPO oil immersion objective (100 ×, n.A. 1.25), a CCD camera, and a motorized stage. Pictures were assembled semi-automatically and picture processing was performed by the MT_O_P (Research System Inc., Boulder, CO, USA) software programme.

Electron microscopic assessments were performed on the segments of the tibial nerve taken from the left, operated, hind limb (0.5 cm distal to the calcaneus). Slices with a thickness of 80 nm were contrasted with uranyl acetate and lead citrate, and then examined under a Zeiss-EM10 (Germany).

Statistical analyses

Scores for denervated areas were subjected to statistical analysis using Sigma Stat Software (SPSS); as the scores originated from counted data, non-parametric tests were chosen for comparing groups. The Kruskal–Wallis test was applied for determining significant differences ($p < 0.05$) between the three experimental groups in the different areas of the foot and at the various stages during the test period; the same test was also used for assessing the progress made by the different experimental groups between weeks (W)5 and W8. The Mann–Whitey test was then applied for more detailed comparison of score differences between only two groups at a given point in time and area of investigation.

Statistical analysis of the SFI values was also carried out with the SPSS. Here, the Welch test was applied for determining significant differences between the three experimental groups at W8, as there was a large disparity between the mean variations in the individual groups. The *t*-test was then applied in combination with the Levene test for more detailed comparison of the SFI scores of only two groups at W8. To determine whether significant progress had been made in groups between W1 and W8 the paired *t*-test was chosen. Morphometric results on the unoperated side were assessed by the SAS system. The ANOVA was used to determine whether significant differences existed between the mean fibre densities on the unoperated side of the different surgical groups. On the operated side no statistics were applied in view of the disparate results, which were in any case obvious.

Results

Nociceptive testing

As a general rule heightened reactions to pain could be noted on the operated side when the rat's foot was pinched in areas where pain sensation still existed or had been regained. This was so despite the fact that the same amount of pressure was applied on both the operated and unoperated sides. When pinched on the operated side the rats responded with shriller tones compared to the healthy side, or even by biting and trying to attack the source of pain, which they soon identified as the forceps. Often the area of pain was also licked in an attempt to alleviate discomfort.

This finding stood in stark contrast to the unoperated side, which was always evaluated first, and where occasionally withdrawal reactions without any vocalization could be elicited. However, when quickly pinching the right (unoperated) side as a control whilst actually testing the left foot, some rats also responded on the contralateral side with shrill squeaks.

On D5 sensory innervation was virtually nil in all the groups both on the *lateral* aspect of the foot and on the lateral sides of toes 4 and 5; however, four of the six rats in group C showed innervation of the heel. A few randomly innervated spots were found which cannot be accounted for. On the *plantar* aspect, all areas apart from the medial edge of the sole, the two medial pads, and toes 1, 2, and 3 appeared to be denervated in all the groups. Sometimes a reaction could be elicited from areas in the midline and the heel (mainly group C). On the *dorsal* side of the foot, pinching of the surfaces of toes 4 and 5 only triggered a signal of discomfort in one animal; about half the rats in groups B and C responded to being pinched at one or more points in a wedge-shaped area extending from these toes towards the ankle. The rest of the dorsal and the *medial* sides of the foot as well as the lateral, medial, plantar, and dorsal sides of toes 1–3 were fully innervated throughout testing from D5 to D56.

Over the next 8 weeks the return of sensation occurred at different rates for individual rats, but following a set pattern (Figure 2B–D).

On the *lateral* and *dorsal* aspects of the foot, innervation reappeared very slowly from proximal to distal, with lateral and dorsal aspects of the toes being reinnervated at the same time or with a slight time lag on the lateral side. By D21 reactions to pinching could be evoked in animals of all groups in areas stretching from the heel to about halfway down the lateral side of the foot and occasionally on the lateral side of toe 4. The lateral and dorsal aspects of toe 5 remained without sensation, whereas frequently the rats showed a pain reaction when pinched on the dorsal aspect of toe 4. The following 5 weeks then

Table I. Numerical results of nociceptive assessments.

Area of foot	Group	D–2	D5	W3	W5	W8
Dorsal	A	0.00	9.71	7.75	6.31	3.69
	B	0.00	8.83	6.67	4.75	4.17
	C	0.00	7.67	6.08	4.92	4.50
	p-value		0.003	0.066	0.039	0.942
Lateral	A	0.00	11.57	10.94	9.38	4.63
	B	0.00	11.83	10.67	8.00	5.58
	C	0.00	10.83	8.50	6.42	5.25
	p-value		0.040	0.005	0.003	0.363
Plantar	A	0.00	14.57	11.50	8.19	2.56
	B	0.00	14.00	10.93	6.08	4.17
	C	0.00	13.50	8.67	6.42	4.00
	p-value		0.152	0.020	0.084	0.309
Total foot	A	0.00	35.86	30.19	23.86	10.89
	B	0.00	34.67	28.25	18.83	13.91
	C	0.00	32.00	23.25	17.75	13.75
	p-value		0.006	0.005	0.010	0.464

Notes: The values represent the number of denervated spots in the different areas of the foot. The maximum score for the dorsal aspect of the foot is 10, for the lateral aspect of the foot 12, and for the plantar aspect of the foot 15, with a grand total of 37 for the entire foot. Significant differences in scores between groups are present when $p < 0.05$ (Kruskal–Wallis test). Until week (W)5 group A clearly lags behind the other two groups (B and C), which are almost identical. After this the autograft rats catch up and finish at W8 with the lowest scores, mostly attributable to the sensory reinnervation of toe 5.

showed distal reinnervation creeping forward at an extremely slow pace. By D56, reinnervation of both the lateral and dorsal aspects had reached the base of toe 4 in all but one animal, and the middle of toe 4 in half the animals of each group. The dorsal side of the tip of toe 4 was reached in just a third of the animals and the lateral side in only one rat of each group. On the dorsal and lateral aspects of toe 5 the situation was even poorer. No animals in groups B and C showed any reaction to being pinched in these areas right up to the end of the study. In the autograft group (group A) the tip of toe 5 was reinnervated by W8 on the dorsal aspect in one-quarter of the animals, on the lateral side, however, in none.

The *sole* of the foot—apart from the area of the heel—appeared to be innervated from medial. By D21 almost all animals under observation had pain sensation around the heel and the pad between toes 2 and 3, approximately half the animals along the midline, and only very few (but half the animals in group C) on the lateral side of the plantar aspect of the foot. The underside of toes 4 and 5 with corresponding pads and the pad proximal to those remained more or less without sensation. In the following 5 weeks reinnervation moved across the sole of the foot to the lateral side—very hesitantly at first in group A, but then with increasing speed—and spread towards and into toes 4 and 5. (In most cases pain sensation in the pads was regained within the same week reinnervation had reached the corresponding toe or in the following 2 weeks.) On D56 almost every rat had achieved complete sensory reinnervation of the soles of its feet and the base of toe 4. The plantar aspect of the tip of toe 4 was reached in nearly half the animals in every group. As with the reinnervation on the lateral and dorsal sides of toe 5, group A was the only group in which a pain response could be evoked when the rats were pinched on the plantar aspect of toe 5.

In statistical evaluations of the scores for denervated areas on the left hind feet (Table I) there was no significant difference between any of the three experimental groups for any of the aspects of the foot in W8 ($p = 0.942$ for the dorsal aspect of the foot, $p = 0.363$ for the lateral aspect, $p = 0.309$ for the plantar aspect, and $p = 0.464$ for the sum of the three areas). The figures for W5 stand in stark contrast to this, with significant differences being noted between all groups in all areas except the plantar area of the foot. However, there were no significant differences at all between groups B and C ($p = 0.818$ (dorsal aspect), $p = 0.6500$ (lateral aspect), $p = 0.818$ (plantar aspect), $p = 0.589$ (sum of the three aspects)). Analysis of the scores for the return of sensation to the various areas of the foot between W5 and W8, revealed the most striking differences to be in group A (p between 0.001 and 0.005 for all tested aspects of the foot); group B exhibited significant differences for the scores on the lateral ($p = 0.005$), plantar ($p = 0.004$), and combined ($p = 0.024$) areas of the foot, whereas group C only made significant progress on the plantar aspect ($p = 0.004$).

On dissection of one of the rats in group B on D56 no collagen tube could be found, the end of the proximal stump having formed a neuroma. In nociceptive testing this rat had, however, been completely indistinguishable from the other rats in its group.

SFI scores

Analysis of the SFI scores reveals that rats in group A were the only rats to make progress. The return of motor function however did not start until W5 (Figure 4).

At the end of W8, group A, in stark contrast to groups B and C, had made significant progress when scores were compared with W1 (group A: $p = 0.013$, group B: $p = 0.600$, group C: $p = 0.905$). A statistically significant difference between scores in W8 could also be noted between the autograft group and the collagen tube groups, regardless of whether they were empty or filled (A and B: $p = 0.024$; A and C: $p = 0.017$). No significant difference was found between groups B and C ($p = 0.773$).

Retrograde tracing

Fluorescence microscopy revealed yellow (Fluorogold) and blue (Fast Blue) labelled neuronal cell bodies in DRG belonging to the spinal cord segments L2, L3, and L4. No labelled neurons were evident in the DRG corresponding to the spinal cord segments L1 and L6, and only three rats exhibited one to four cells with blue fluorescence in the DRG of L5.

The numbers of labelled ganglion cells, both yellow and blue, were the lowest in the DRG corresponding to the spinal cord segment L2. Here at the most four labelled cells of each colour could be noted, and this not in every animal. The DRG of L3 showed both yellow and blue fluorescence in all animals examined and at least one ganglion cell was double labelled in every animal (Figure 3). The maximum of double labelled cells was four. The largest number of cells exhibiting yellow fluorescence in the DRG of L3 amounted to 52 ganglion cells in one cross section, whereas at the most five cells were labelled with Fast Blue (blue). The DRG of L4 exhibited cells with blue fluorescence in all but two of the rats examined and the blue ganglion cells reached cell counts of up to 15 cells per cross section. Fluorogold-labelled cells (yellow) were visible in all DRG of L4 and amounted to between seven and 27 cells per cross section. In about half the DRG of L4 both blue and yellow (double-labelled) fluorescent cells could be seen.

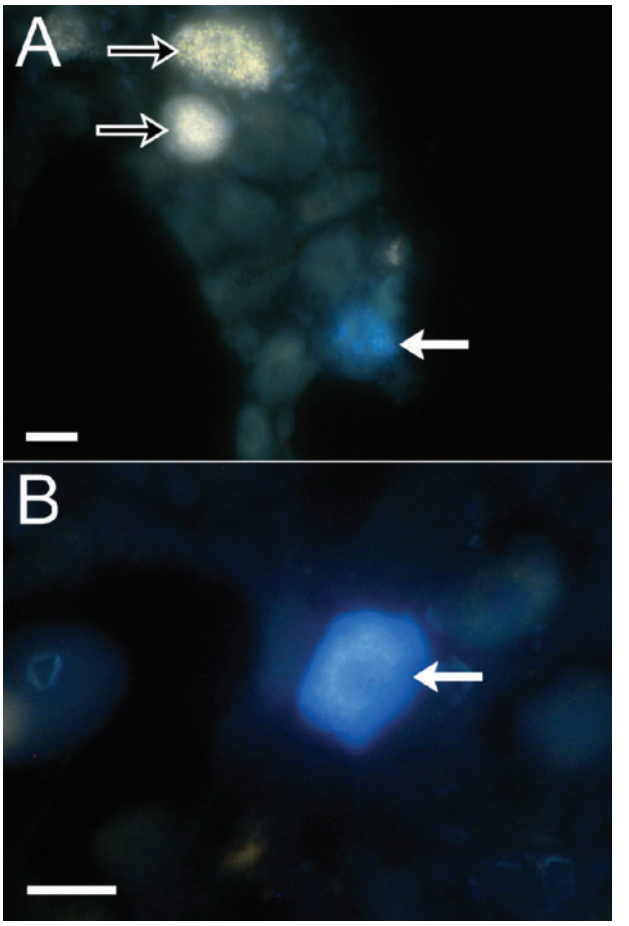

Figure 3A,B. Tracing studies. Yellow (open arrows) and blue (white arrow) labelled neuronal cell bodies in the DRG of the spinal cord segment of L2 (A) and L3 (B). Scale bar is 20 μm (A, B).

Table II. Mean fibre densities [myelinated fibres/mm^2] in plantar extensions of the tibial nerves.

Group	Right hind limb	Left hind limb
A	1181.8	386.1
B	1317.2	0
C	1319.5	0

Note: The values represent the mean fibre densities of myelinated fibres in the three experimental groups A (autograft), B (empty collagen tubes), and C (collagen tubes filled with denaturated muscle) in plantar extensions of the tibial nerves 0.5 cm distal to the calcaneus. When comparing the mean fibre densities in the unoperated right hind limb, no significant difference can be noted between the rats of the different groups ($p = 0.093$). In the operated left hind limb the differences in fibre densities between autograft and interponate groups are striking.

Morphometric and electron microscopic assessments (Table II)

Morphometric assessments of the tibial segment 0.5 cm distal to the calcaneus revealed that animals in group A experienced some regeneration in this area. The mean fibre density in this group reached

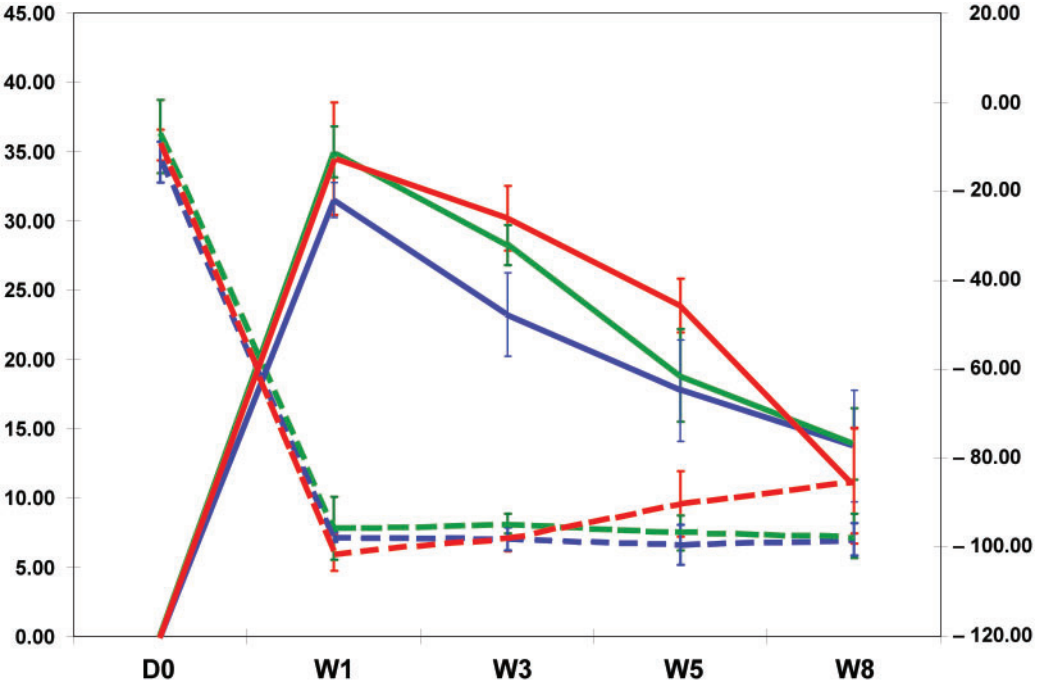

Figure 4. Comparison of results of somatosensory and motor assessments. Somatosensory results (combined scores for nociceptive testing on the whole foot; see Table I for explanations) are shown by continuous lines, the motor results (SFI) by broken lines. Group A (autograft) is red, group B (empty collagen tube) is green, and group C (muscle filled collagen tube) blue. The left ordinate represents the somatosensory scores, the right ordinate the motor (SFI) scores.

about one-third of the reference values which had been calculated from the contralateral unoperated side. Amongst rats in groups B and C, only one animal possessed a total of 23 countable regenerated myelinated fibres on its combined fascicular area of more than 920,000 μm^2. All the others had none. These findings were confirmed by electron microscopy. Here small myelinated and unmyelinated axons could be seen in all animals in group A. The myelinated axons were either set in regenerative groups or clusters, or could be found individually. In contrast to this, only one out of the 12 animals in groups B and C displayed some (but very few) myelinated axons, another displayed one single small myelinated axon, and the rest showed none at all. Unmyelinated axons with decreased diameters and in low numbers were discernible in eight out of the 12 animals. In all segments examined, ovoids, Buengner bands, and collagen pockets could be seen, the rats of groups B and C exhibiting increased signs of myelin destruction and also more denervated Schwann cells when compared to animals of group A.

General health condition

Health and behaviour of the rats was normal throughout the study. All animals exhibited a slight gain in weight, groomed themselves well, and had moderate to high levels of activity. None of the rats displayed any signs of autotomy and all of them placed their feet appropriately when moving. In daily adspectory assessments no evidence of inflammation of the foot could be detected in any rat except for one animal in group B, which developed a chronic ulceration of its heel in W2. This did not regress throughout testing, but also did not provide any hindrance to activity. The affected rat still placed its foot with the plantar side downwards when walking and also exhibited no decrease in weight-bearing or excessive licking. In somatosensory evaluations this rat had not been distinguishable from the other rats in its group either with regard to progress or with regard to final results for sensory reinnervation. Clinical examinations also revealed that this rat was normal.

In none of the rats could any signs of chronic inflammation of the tissue be found upon post-mortem examinations.

Discussion

Sensory testing

In the end, nociceptive testing revealed very little divergence between the autograft (A) and interponate groups (B and C), the major difference being at toe 5, where only animals from group A were able to regain

sensation. Interestingly, groups B and C, which finished the study with more or less the same result as group A, as regards the return of pain sensation, both made faster progress than group A until D35 and then for the most part showed little improvement right up to the end of the study. In contrast, the autograft group maintained reinnervation at a steady pace right up to D56 and, as already mentioned, was the only group to have achieved any pain sensation on the lateral, dorsal, or plantar aspects of toe 5.

A similar timeframe for rats regaining sensation on the lateral aspect of their feet after various injuries to the sciatic nerve and their repair is described by den Dunnen and Meek (2001). In their study the animals reacted to electro-stimulation at the most proximal stimulation point (lateral to the heel) about 3–4 weeks after surgery, and at the most distal stimulation point, just proximal to toe 5, approximately 2 weeks later.

Correlation with other methods of evaluation

Results gained from tests primarily concerned with motor function such as footprint analysis (Walker et al. 1994; Hadlock et al. 1999) follow the same pattern as the return of sensory innervation for animals in groups A (autograft group). In these rats a steady improvement could be seen, accelerating as from W5. In contrast to this, neither of the interponate groups (groups B and C) improved at all in motor tests (SFI). This finding does not correlate at all with their readily detectable progress in sensory assessments (Figure 4). Morphometric examinations of the plantar tibial extensions (5 mm distal to the calcaneus) of the rats in groups B and C revealed only very few myelinated fibres in one of the animals, in the others none at all. In the same segment of most rats in group A moderate amounts of regenerating myelinated axons could be observed.

Electron microscopic assessments of the tibial plantar extensions distal to the calcaneus were then carried out to rule out the possibility of having overlooked large numbers of regenerating unmyelinating axons which are virtually invisible in light microscope histology. However, only scattered regenerating unmyelinated axons with decreased diameters compared to normal axons could be seen in eight of the 12 animals in groups B and C, and in only one of the animals could myelinated minifascicles be detected. As a consequence of these results the source of sensory reinnervation in groups B and C has to be reconsidered.

Source of sensory reinnervation

Assuming a distance of approximately 53 mm from the proximal end of the lesion to the ankle and a further 38 mm from the heel to the tip of the third toe (Rupp et al. 2006), regenerating axons would have to travel about 91 mm from the proximal stump to completely reinnervate the foot right up to the tip of the toes, if they took the direct route. Given a rate of regeneration of about 1.4–2 mm per day (Gutmann et al. 1944; Navarro et al. 1994), or even faster (de Koning et al. 1986; Varejão et al. 2004a), it is possible that sensory reinnervation of the foot could have occurred as a result of regrowth of the tibial and peroneal nerves. However, if the results of footprint evaluations and morphological studies are taken into account, it seems far more plausible that sensory reinnervation of the foot resulted from extensive sprouting of an uninjured neighbouring nerve. This could be either the saphenous nerve or the musculocutaneous nerve of the hind limb, a branch of the sciatic nerve which also seems to innervate digits of the rat's foot (Puigdellívol-Sánchez et al. 2000). The proximal edge of the operation site, especially in animals with collagen implants, however, lay so close to the point where the sciatic nerve emerges from beneath the medial gluteal muscle, that the chances of the musculocutaneous nerve not being injured during experimental procedures were very slim. Also, the distribution of denervated areas after the insult, together with the distinct reinnervation pattern (from proximal to distal on the lateral and dorsal aspects of the foot, and from medial, then reaching distal, on the sole, with sensory reinnervation always taking place earlier on toe 4 than toe 5), strongly indicate that the source of reinnervation must be located somewhere in the medial region of the ankle, and this is exactly where the saphenous nerve is located (Green 1955; Hebel and Stromberg 1976). Further substantial support is lent to this theory by the findings of the retrograde tracing studies. Injections of a retrograde tracer into the skin on the medial side of the foot resulted in neuronal cell bodies in the DRG of L2, L3, and L4 being labelled. This observation correlates exactly with expectations, since the saphenous nerve has been reported to be responsible for sensory innervation of this area (Green 1955; de Lahunta 1977; Devor et al. 1979; de Koning et al. 1986; Varejão et al. 2004b) and the central distribution of the saphenous nerve lies in spinal cord segments L2–L4 and their respective DRG (Green 1955; Seltzer and Devor 1984). Carl Molander and colleagues state in their retrograde tracing study (Puigdellívol-Sánchez et al. 2000) that the femoral nerve contributes mainly via DRG of L3 and L4 to the sensory innervation of the foot, and this statement corresponds directly with our findings. Application of a different retrograde tracer to the skin on the lateral aspect of the foot, which physiologically is innervated by a branch of the sciatic nerve (Green 1955; Hebel and Stromberg 1976) also resulted in

labelling of neuronal cell bodies in the DRG of segments L2, L3, and L4. At least one double-labelled cell could be found in the L3 DRG of every rat and this finding, coupled with the fact that the central origin of the sciatic nerve normally lies further caudal than that of the saphenous nerve (Markus et al. 1984), namely, in spinal cord segments L4, L5, and L6 (Green 1955), leads to the conclusion that sensory reinnervation on the lateral side of the foot must primarily be attributable to the saphenous nerve. This is definitely the case for the rats in the interponate groups (groups B and C), which did not exhibit any regrowth of the plantar tibial extensions of the sciatic nerve at all. With group A rats, however, one could argue that some regenerating sciatic/tibial fibres (extending to the DRG of L4) were also traced.

Concerning the three rats (one rat in every group) which exhibited up to four blue-labelled cells in the DRG of L5, one could surmise that in these rats the integrity of the very proximal branch of the sciatic nerve, the musculocutaneus nerve, might not have been completely compromised during the operation. Central distribution of this nerve has been reported to lie mainly in the DRG of L5 (Puigdellívol-Sánchez et al. 2000). After a further study, however, on ''regenerative and collateral sprouting to the hind limb digits after sciatic nerve injury in the rat'' the authors reached the conclusion that this nerve only seems to have limited capacity for compensatory innervation of the foot by collateral sprouting after a sciatic injury (Puigdellívol-Sánchez et al. 2005).

Extensive collateral sprouting of the saphenous nerve

The phenomenon of extensive collateral sprouting of the saphenous nerve after sciatic nerve damage has also been described by Devor et al. (1979), who examined distributions of tibial, peroneal, and saphenous nerves in the foot after sciatic nerve crush both by behavioural testing (pinching) and electrophysiological recordings of single unit potentials. The distribution of denervated areas after sciatic damage and also the period of time and the consistent pattern in which sprouting of the saphenous nerve takes place (mostly completed by D35), corresponds with the results recorded in the present study. In Devor et al.'s study, however, the saphenous nerve never progressed into toes 4 and 5, and only into parts of toe 3, even in cases where sciatic regrowth was prevented. This finding lies in direct contrast to the result established for the rats in groups B and C of the present study, where toes 3 and 4 are clearly reinnervated by what must be the saphenous nerve—at least on the sole of the foot, as there are virtually no regenerative myelinated axons of plantar extensions of the tibial nerve to be seen. Kingery and Vallin (1989) support the present findings when they described the average cutaneous nociceptive response to pinch on the dorsum of the toes to take place at 11 weeks after chronic sciatic section in 3.8 ± 0.8 of five test areas. This must mean that toe 3 was reinnervated in all and toe 4 in some animals, assuming the critical areas for reinnervation lie on the lateral side. Pictures in the results indicate that the same seems to apply for the plantar side of the toes. Increase of the saphenous receptive field, however, took longer than in the present study continuing until W11. Markus et al. (1984) described similar results after sciatic denervation of the skin of the foot. Recordings in the medial dorsal horn of the spinal cord indicate that after acute sciatic denervation, stimulation of toes 1, 2, and 3 and the corresponding metatarsal areas—on the plantar side only of toe 1, but on the dorsal aspect of the foot in all three toes—causes a reaction in the normal somatotopic areas of the saphenous nerve. Chronic sciatic denervation for 21 days resulted in receptive fields of the saphenous nerve expanding into the area dorsal to the fourth metatarsal bone and also towards lateral on the plantar aspect of the foot. These results correspond exactly with those established for D21 in the present study.

A very plausible reason why in our study toe 3 displayed sensation right from the first day of testing (D5) is given by Puigdellívol-Sanchez et al. (2005), who mention in their work that the saphenous nerve has been reported to be physiologically responsible for toes 1 and 2 and the proximal phalanx of toe 3. Since toe 3 was only tested at one spot on the medial, lateral, and dorsal sides and at two spots on the plantar side, it is highly possible that areas either originally innervated by the saphenous nerve or affected by vast and rapid sprouting of this same nerve were pinched when the animals where evaluated for pain responses.

A vast and very rapid reorganization of sensory somatotopic areas after sciatic transection and ligation has also been described for the spinal cord and the S-I area of the cortex, 85% of which is predominately activated by the sciatic nerve and 15% by the saphenous nerve. Cusick et al. (1990) showed that within 1–3 days after injury to the sciatic nerve, the area activated by the saphenous nerve annexes an additional 23–26% of the total hind paw cortex. Seven to eight months later the area represented by the saphenous nerve does not differ significantly from the normal total hind paw representation. On the spinal cord level, the saphenous nerve already seems to have spread into the representational area of the sciatic nerve and even further on the medial side of the dorsal horn by D21 after sciatic transection and ligation (Markus et al. 1984).

Mechanical hyperalgesia

Mechanical hyperalgesia developed in the present study on the operated side in areas still innervated or reinnervated. However, it should be pointed out that this observation is subjective on the part of the examiner (albeit having conducted more than 320 examinations in all). Increased reactions such as shriller squeaks, increased guarding, or attacking the source of pain could be observed when the rats were pinched with a force not exceeding $0.8\,N/mm^2$ on the foot of the operated hind limb. On the contralateral side normal reactions to pinching were registered. This phenomenon of hyperalgesia on the operated hind limb also has been noted by Markus et al. (1984), Kingery and Vallin (1989), and Kingery et al. (1994). Kingery and Vallin described in 1989 that the adjacent neuropathic hyperalgesia mediated by the saphenous nerve lasts at least 12 weeks after sciatic transection with excision of the distal segment. In a later study Kingery et al. (1994) found that after sciatic crush injuries medial areas innervated by the saphenous nerve exhibited lower thresholds to both pressure and heat until sciatic reinnervation of the foot occurred. Hypersensitivity to pinch is attributed to collateral sprouting of high-threshold mechanoreceptors in the saphenous distribution (Kingery and Vallin 1989; Attal et al. 1994). A reduction in pain-inhibitory control in the central nervous system, together with ectopic excitability, central sensitization after A-fibre sprouting in the dorsal horn (Vogelaar et al. 2004), primary sensory degeneration, and a phenotypic switch are said to contribute to the causation of neuropathic pain. The exact aetiology, however, still remains largely unknown (Abrams and Widenfalk 2005).

In contradiction to all these findings of hyperalgesia, the study by Devor et al. (1979) states that ''no convincing instance of hyperactivity or hyperresponsiveness'' could be noted. On the contrary, some rats showed only sluggish responses. A practical explanation for this large difference in reaction might be the way in which examinations were conducted in the present study. As only ''reaction'' or ''no reaction'' were distinguished, and nothing in between, it could be that subconsciously more pressure was applied when pinching the animals on the operated side. Therefore, whilst evaluating the injured side, internal controls were undertaken by intermittently pinching the uninjured (contralateral) side, and then this side also exhibited hyperalgesia or in this case even allodynia. This, however, is most likely the result of focused attention on stimuli, which can lead to an enhanced perception of pain or stimuli in general (Miltner et al. 1989). In future studies this could be remedied by using an analgesimeter or Frey filaments to exert pressure, as these permit the application of quantified stimuli.

Contribution to mechanical hyperalgesia by chronic tissue inflammation due to repeated nociceptive stimuli, however, can be excluded. In no rat, except one, could any evidence for inflammation be noted in daily inspections or post-mortem examinations of the feet. No behavioural changes regarding the use of the affected foot were evident in any of the rats. Both progress and result of sensory reinnervation of the rat affected with chronic ulceration of the heel were identical with that of the other rats in its group. Therefore, one can surmise that the chronic inflammation did not seem to provide any hindrance to the compensatory sprouting of the saphenous nerve even though a certain spatial closeness between both activities was given.

Source of sensory reinnervation on toe 5

The source of sensation on toe 5 of animals in group A can only be surmised. As reinnervation ceased to change after D35 in animals where hardly any sciatic regeneration could be observed (groups B and C), but exhibited a slow and constant progression in animals with proven regrowth of the sciatic nerve, one can presume that the source of sensation in toe 5 must be the plantar or sural extensions of the tibial or peroneal nerve. The results published by Devor et al. (1979) and Kingery and Vallin (1989) also point in this direction, making it clear that the saphenous nerve never had the capacity to achieve full innervation of the hind foot in a rat. An elegant solution to this question could be provided by further retrograde tracing studies, with the tracer being injected intracutaneously on toe 5.

Conclusions

As a consequence of the results of the present study and the findings published by Devor et al. (1979) and Kingery and Vallin (1989), it must be concluded that results of both withdrawal reflex test and pain response test should be treated with caution. The areas tested, and the likelihood that collateral sprouting of the saphenous nerve could have taken place must be taken into account.

When conducting somatosensory evaluation, care should be taken to stimulate the rat only in areas definitely not affected by sprouting of the saphenous nerve, that is, toe 5. Depending on how much the rat is handicapped in performing a recognizable withdrawal reflex (towel, denervated muscles required), pain response testing might provide more reliable results than withdrawal reflex testing.

Additionally, when assessing neural regeneration by functional tests, nerve samples for histological

assessment should be harvested in sites close to the end organs examined, in order to determine whether reinnervation has actually taken place and minimize misinterpretation of functional results.

Acknowledgements

Many thanks to Marlies Glöde (TU Munich) for her excellent post-operative care of the animals and to Dr Julia Henke, Department for Preclinical Research, TU Munich, for her invaluable information and practical support with regard to the care and analgesia of rats during and after surgery. Also the patience of Christine E. Rupp for proofreading this manuscript more than once and the generous help with the statistics by Wolfgang Lederer and Andrea Ossig, Statistical Consultant Unit, Department of Statistics (LMU Munich) are both greatly appreciated. Furthermore, we would like to thank Professor Stangassinger, Institute for Animal Physiology, LMU Munich, for kindly providing housing and associated equipment for the rats.

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