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Regeneration and functional recovery in the upper extremity of rats after various types of nerve injuries

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Abstract The aim was to establish an accurate, reproducible, and simple method to evaluate functional recovery after different types of nerve injuries to the brachial plexus of rats. To that end, pawprints, measured as distance between the first and fourth and second and third digits, were used for evaluation of injuries including crush injury, transection/repair, or graft repair of the median, ulnar, and radial nerves. Immunocytochemistry of the C-terminal flanking peptide of neuropeptide Y (CPON) and neurofilaments was used to investigate the cell body response and axonal outgrowth, respectively. Functional recovery was dependent on the severity as well as on the level of the lesion. Neither a single injury to the median nerve nor an injury to the ulnar nerve affected the pawprint, while an injury to both these nerves or a single injury to the radial nerve caused impairment of pawprints. There was a rapid recovery after crush injury to these nerves compared to previous reports of a similar injury to the sciatic nerve. The pattern of axonal outgrowth was related to the severity of the lesion. A conditioning lesion, i.e., an initial lesion of the same nerve preceding a test injury by a few days, of both motor/sensory fibers led to a quicker functional recovery. Surprisingly, conditioning of only sensory fibers had nearly the same effect. The cell body response was dependent on the level of the nerve lesion. The upper extremity of rats might be useful to evaluate the effects of new repair methods after nerve injuries using functional evaluation with pawprints as a simple and accurate method.

Key words: conditioning lesion, CPON, functional recovery, nerve injury, nerve repair

Introduction

Sciatic nerve injury in rats is a dominant model for studies of peripheral nerve regeneration. The majority of human peripheral nerve injuries, however, affect the upper extremity, and for this reason, an experimental model of nerve injury in the upper extremity may prove more useful. In the upper extremity in rats, the distance to the target organs in muscles and skin is short. These organs should be reinnervated rapidly and the time required for studies of functional recovery should

be minimal, because this is of experimental advantage. Furthermore, the complexity of the upper extremity with many nerves and branches offers more experimental possibilities. Thus, selective injuries can be applied to motor or sensory branches, a feat not easily accomplished in the lower extremity. For studies of functional recovery, the integrity of the extremity is crucial. This is a problem in the sciatic nerve model where autotomy and the development of contractures result in the loss of animals available for measurements of walking ability using measures such as the sciatic functional index (SFI) (*De Medinaceli et al., 1982*). In preliminary experiments, we found no indications of articular contractures or automutilations after injuries to nerves from the brachial plexus, suggesting

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that in these respects the upper extremity model could offer advantages as compared with the sciatic nerve model.

There are few reports available describing the rat brachial plexus model (Bertelli et al., 1995). Bertelli and co-workers have described the basic anatomy of the brachial plexus and its terminal branches (Bertelli et al., 1995) and investigated functional recovery after various types of neurotizations and nerve transfers (Bertelli and Mira, 1993; 1995; Bertelli et al., 1995), but a basic description of the rate of reinnervation, outgrowth of nerve fibers, cell body response, etc., is not available.

The present study was aimed at delineating basic regenerative measures like functional recovery, axonal outgrowth, and cell body response, following various injuries to the nerves of the upper extremity in the rat including crush injury, transection and repair or graft interposition, and different types of conditioning lesions (CLs).

Materials and Methods

Animals and surgical procedures

Female Wistar rats, weighing between 180 and 220 g, were used. These studies were approved by the local animal ethics committee at Lund University, Sweden. All animals were kept in plastic cages in a 12 h light and 12 h dark circle.

At surgery, the rats were anesthetized with an intraperitoneal injection of a 1 : 10 solution of pentobarbital (60 mg/ml) and physiological saline. Various injuries to and repair/reconstruction procedures of the brachial plexus branches (Fig. 1) were then performed. The experimental set-up is described in Table 1.

Median and ulnar nerves – crush lesions

The left median and ulnar nerves were exposed above the elbow and a crush injury was induced in either the median (n=5) or the ulnar (n=5), or in both median and ulnar nerves (n=5). A crush lesion was induced twice for 40 s each using a fine forceps. The median nerve was crushed approximately 3 mm

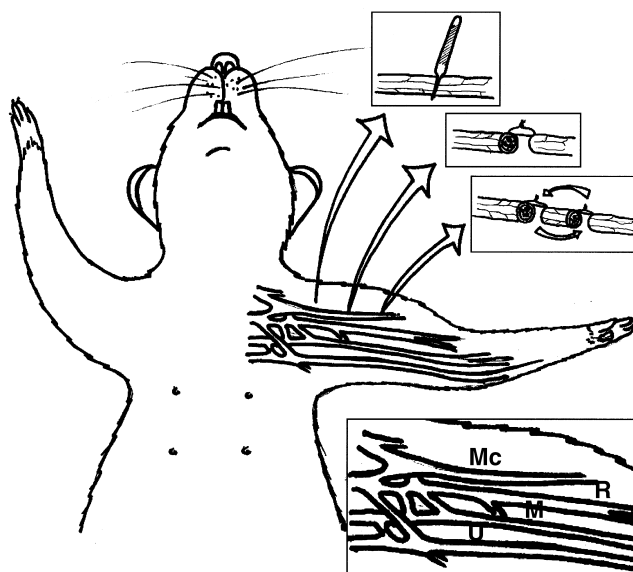


Figure 1. Schematic of the brachial plexus and its branches, and locations of the lesions to the various branches. Mc = musculocutaneous nerve; R = radial nerve; M = median nerve; U = ulnar nerve.

proximal to the elbow joint and the ulnar about 3 mm proximal to the superior ulnar collateral ligament. The skin was sutured and the animals were allowed to recover. To investigate the effects of a more proximal nerve crush, the left median and ulnar nerves, in 12 rats, were exposed at the same level just above the elbow and a crush injury was induced. In another 12 rats, the crush was induced further proximal after their exit from the brachial plexus after any branches.

Median and ulnar nerves – conditioning lesions

The effect of CLs was investigated. Two types of CLs were induced. In some rats, both the left median and ulnar nerves (n=5) were crushed just below the elbow, resulting in a preferential CL of sensory nerve fibers. In other rats, the crush was induced above the elbow, thus inducing lesions of both motor and sensory nerve fibers (n=5). The skin was closed and the animals were allowed to recover. All rats were

Table 1. Experimental set-up.

Nerve lesions	Number of rats				Days (final evaluation)
	Median	Ulnar	Median and ulnar	Radial	
Crush	5	5	5	5	30
Proximal versus distal crush	—	—	24	—	30
Motor versus sensory crush	—	—	10	—	30
Transection/repair	—	—	5	5	30
Graft	5	5	5	5	60

reanesthetized after 3 days and another crush injury (test injury) was induced proximal to the CL where the nerves had branched from the brachial plexus. The skin was closed and the animals were allowed to recover again.

Median and ulnar nerves – transection and repair

The left median and ulnar nerves were exposed proximal to the elbow ($n=5$). Both nerves were transected and then repaired using three epineurial stitches with 10-0 sutures (Ethilon[®], Ethicon).

Median and ulnar nerves – nerve graft

The left median and ulnar nerves were exposed at the same level as above. A 10 mm long nerve segment was resected and inserted as a graft at the same place, just above the elbow, following a 180° rotation. The graft was sutured using Ethilon 10-0 sutures. The graft procedure was performed on the median nerve ($n=5$), the ulnar ($n=5$), or both nerves ($n=5$).

Radial nerve – crush lesion

The left radial nerve was exposed on the side where it circumflexes the humerus. The nerve was crushed with a fine forceps, as described for the median and ulnar nerves ($n=5$).

Radial nerve – transection and repair

The left radial nerve was exposed as above. The nerve was transected and repaired at the same site as in the previous group using three epineurial stitches with 10-0 sutures ($n=5$).

Radial nerve – nerve graft

The radial nerve was exposed as above. A 10 mm nerve segment was resected, rotated 180°, inserted as graft, and secured with 10-0 sutures ($n=5$).

The skin was closed and the animals were allowed to recover. The right contralateral median, ulnar, or radial nerve of the animals was used as a control in all the above-described experiments.

Evaluation

Gait analysis

Functional assessment in all operated rats was carried out using measurement of 'pawprints' on days 0, 3, 6, 14, 21, 30, 45, and 60 (Table 1), but in animals where a CL (median and ulnar nerves) was induced, the gait was evaluated every other day from day 0 until day 14 and then on days 21 and 30. To

obtain records during walking, the forepaw of the rats was marked with ink. The animals were allowed to walk freely across a 'corridor' on a sheet of paper. Toe spread during walking, defined as the distance between the first and the fourth digits and between the second and the third digits, was measured using a caliper. The pawprint measured was the one obtained from a non-interrupted step. The individual values were expressed in percentage of the preoperative (day 0) control value of those specific digits and expressed as mean \pm SE.

Immunocytochemistry and morphology

On the thirtieth or sixtieth day, depending on the group (Table 1), the rats were killed with a lethal dose of pentobarbital, and the nerves that had been previously injured were harvested. The median, ulnar, or radial nerves, including 5 mm proximal and distal to the injury site, were taken out and prepared for immunocytochemical neurofilament staining to visualize regenerating nerve fibers (Brandt et al., 1999). In short, the nerve segments containing the lesion were fixed in Stefanini's fixation (2% paraformaldehyde and 1.9% picric acid in 0.1 M phosphate buffer, pH 7.2) for 2 h and then cryoprotected in 20% sucrose phosphate-buffered saline (PBS). They were then mounted in Tissue Tek (Sakura) and sections 8 μ m thick were prepared. The nerve sections were then incubated in methanol and 3% H₂O₂ for 30 min, washed in PBS for 15 min, and soaked in horse serum for 20 min. The sections were then incubated with primary antibody against neurofilament (NF 70 kDa, DAKO) (1 : 80) for 2 h. After washing in PBS, the sections were then incubated with the secondary biotinylated horse anti-mouse IgG antibodies (1 : 200) for 30 min. They were washed with PBS, soaked in peroxidase conjugated avidin-biotin complex (ABC; Vectastain, Vector Laboratories) and then stained with carbazole. The sections were treated with Mayer's Heamatoxylin (HTX) and washed in tap water and in PBS. At the end, they were mounted in Kaiser's glycerin. The sections were examined by light microscopy.

Dorsal root ganglia (DRGs) from C7 to T1 were removed, depending on the injured nerves, were removed at days 6, 14, 21, and 30 from both the experimental and control rats. The DRGs were prepared for immunocytochemical staining for the C-terminal flanking peptide of neuropeptide Y (CPON) after fixation in Stefanini and cryoprotection in 20% sucrose PBS (Bergmark et al., 2001; Widerberg et al., 2001). After mounting in Tissue Tek (Sakura), the DRGs were cut in 10 μ m thick sections. They were washed in PBS and then exposed to a primary rabbit antibody against CPON (DAKO), diluted 1 : 280 in PBS containing 0.25% Triton-X (Packard, Meridian, MS, USA) and 0.25%

bovine serum albumin (Sigma-Aldrich). The sections were then incubated at 4°C overnight. The sections were washed (3 × 5 min in PBS) and exposed to fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulins (DAKO) at a dilution of 1 : 180 in PBS for 1 h at room temperature and in darkness. The sections were finally washed in PBS and mounted in 50% (v/v) glycerol in PBS for fluorescent microscopy.

A more distal part of each nerve was prepared for conventional histology. These distal nerve segments were immersed and fixed in 2.5% glutaraldehyde and then transferred to 0.1M Na-cacodylate buffer. The nerve pieces were then treated with 2% osmiumtetroxide, soaked again in Na-cacodylate buffer, and dehydrated in serial alcohol solutions. They were then immersed in propyleneoxide, followed by immersion in propyleneoxide agar resin 1 : 1 solution, and then embedded in agar resin. The specimens were polymerized for 3 days at 60°C. Cross-sections measuring 1 µm in thickness were cut with a microtome. The sections were stained with Azure II and methylene blue and examined by light microscopy.

Statistics

All values are expressed as mean ± SE. Repeated analysis of variance (ANOVA), followed by Bonferroni–Dunn post-hoc test, was used to compare the data from the contralateral and ipsilateral site and the time point pattern within an experimental group. A two-factor ANOVA (factors: injury and time) was used to compare the CPON-positive sensory neurons after distal versus proximal injury over time. A significant value was accepted at a *p*-value of <0.05.

Results

All animals survived and neither autotomy nor joint contracture was noticed in any of the animals.

Pawprints

Median and ulnar nerves – crush lesion

In animals subjected to a crush lesion on both the median and the ulnar nerves just above the elbow, the toe spread of the first and fourth and second and third digits decreased to 67 ± 3 and 54 ± 4%, respectively, at 14 days as compared with their preoperative values (*p* < 0.001 compared to control side, repeated ANOVA, Fig. 2A). A full recovery of these distances was observed 21 days after the procedure. Surprisingly, in animals where only one of the nerve trunks had been crushed, i.e., either the median or the ulnar nerve, no effect on the toe spread distances could be observed (data not shown).

Median and ulnar nerves – proximal versus distal crush

When the crush lesion was induced at a more proximal site, just after the nerves had emerged from

the brachial plexus (proximal injury), the distance between the first and fourth and second and third toe decreased to 52 ± 3% at 6 days as compared with 67 ± 3% with a more distal lesion of those two nerves (Fig. 2B; *p* < 0.001 and *p* < 0.002, respectively, compared to control, repeated ANOVA). The difference between a proximal and a distal crush was significant (*p* < 0.02).

Median and ulnar nerves – conditioning lesion

If a CL was induced on the median and ulnar nerves just below the elbow (conditioning of sensory fibers), there was just a slight insignificant decrease of 91 ± 6% in toe spread at day 3, while the corresponding value when both nerves were crushed above the elbow (conditioning of both motor and sensory nerve fibers) was 66 ± 5% (Fig. 2C). Three days after the CL, i.e., 6 days after the test lesion, the corresponding values were 61 ± 3 and 64 ± 5%. Functional recovery after the second lesion (test lesion) performed at day 3 was much more rapid than in nerves subjected to a single lesion (compare Fig. 2C with 2A and 2B). Furthermore, in these conditioned nerves, full recovery was observed after 14 and 11 days, respectively (*p* < 0.05), indicating that there was a difference in time course, i.e., recovery was faster following motor/sensory conditioning than after sensory conditioning alone.

Median and ulnar nerves – transection and repair

After transection and repair of both median and ulnar nerves proximal to the elbow, the toe spread between the first and fourth digits decreased to 64 ± 3% at day 14 and then returned to 88 ± 5% of the preoperative value at day 21 (*p* < 0.001 compared to control; Fig. 2D). No further improvement was observed up to 30 days.

Median and ulnar nerves – nerve grafting

After transection and grafting of both median and ulnar nerves, there was an incomplete recovery of the pawprints. The distance between the first and fourth toes decreased to 48 ± 3% at day 6 and then there was a gradual improvement up to 74 ± 5% at day 60, which was significantly different from the contralateral control side (*p* < 0.01; Fig. 2E). A transection and graft repair of only the median or the ulnar nerve had no significant effect on the pawprints, as compared with the contralateral control side (data not shown).

Radial nerve – crush lesion

When the radial nerve was crushed, the pawprints decreased, with a drop in distance between the first and fourth toe to 50 ± 3% at day 6, but then it returned progressively to normal values at day 14 (*p* < 0.01 compared to control; Fig. 3A).

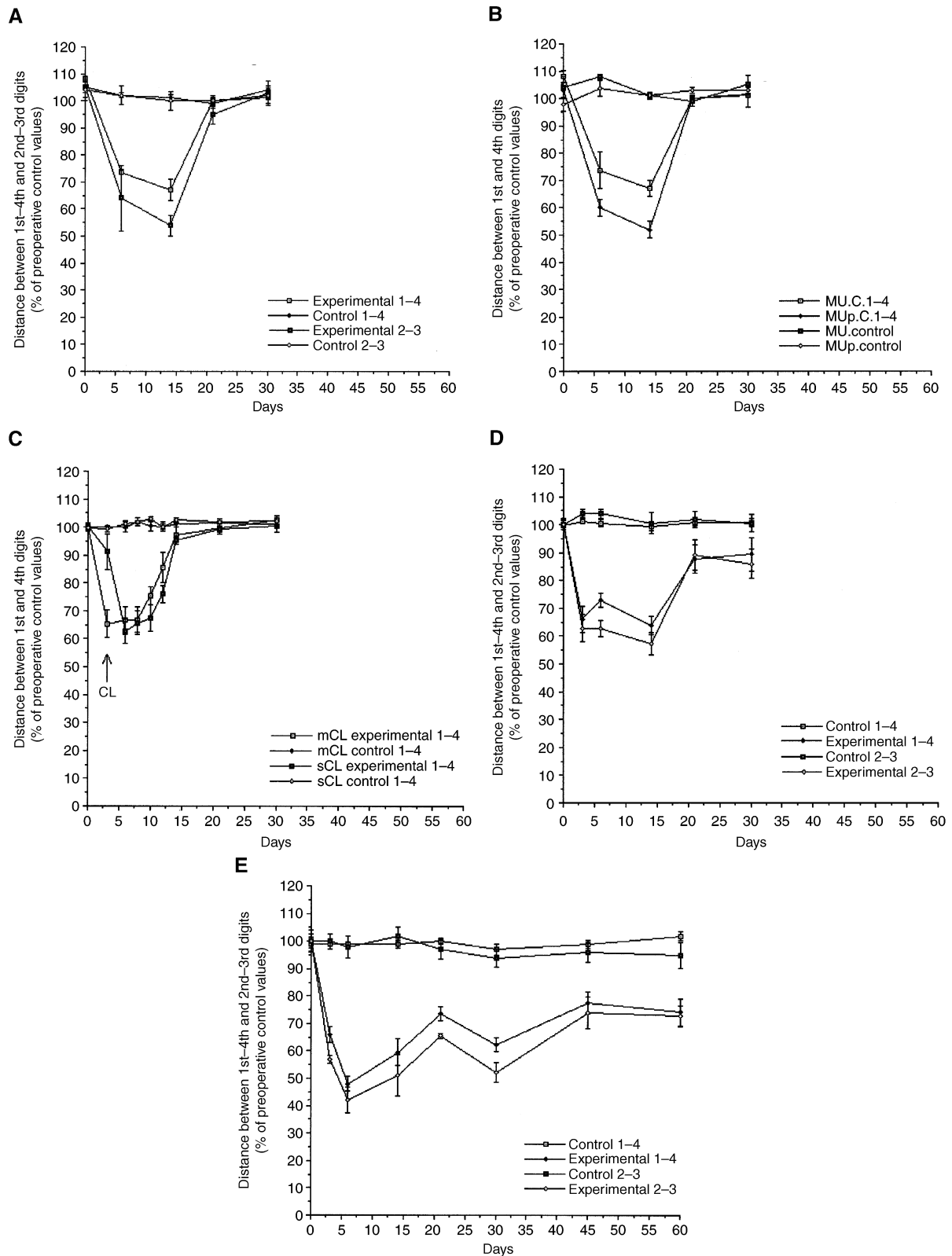


Figure 2. Measurement of pawprints from rat forepaw after various injuries to the median and ulnar nerves. (A) Crush injury above the elbow. (B) Nerves crushed above elbow (distally; MU.C.) or proximally (MUp.C.) in the limb. (C) Conditioning lesion (CL): initial lesion induced at the sensory part of the median and ulnar nerves (sCL) or at the motor/sensory part of those nerves (mCL). (D) Transection and repair. (E) Reconstruction of median and ulnar nerves with nerve graft. Values are mean \pm SE ($n=5$ after each injury) of preoperative measurements of distance between the first and fourth and second and third digits.

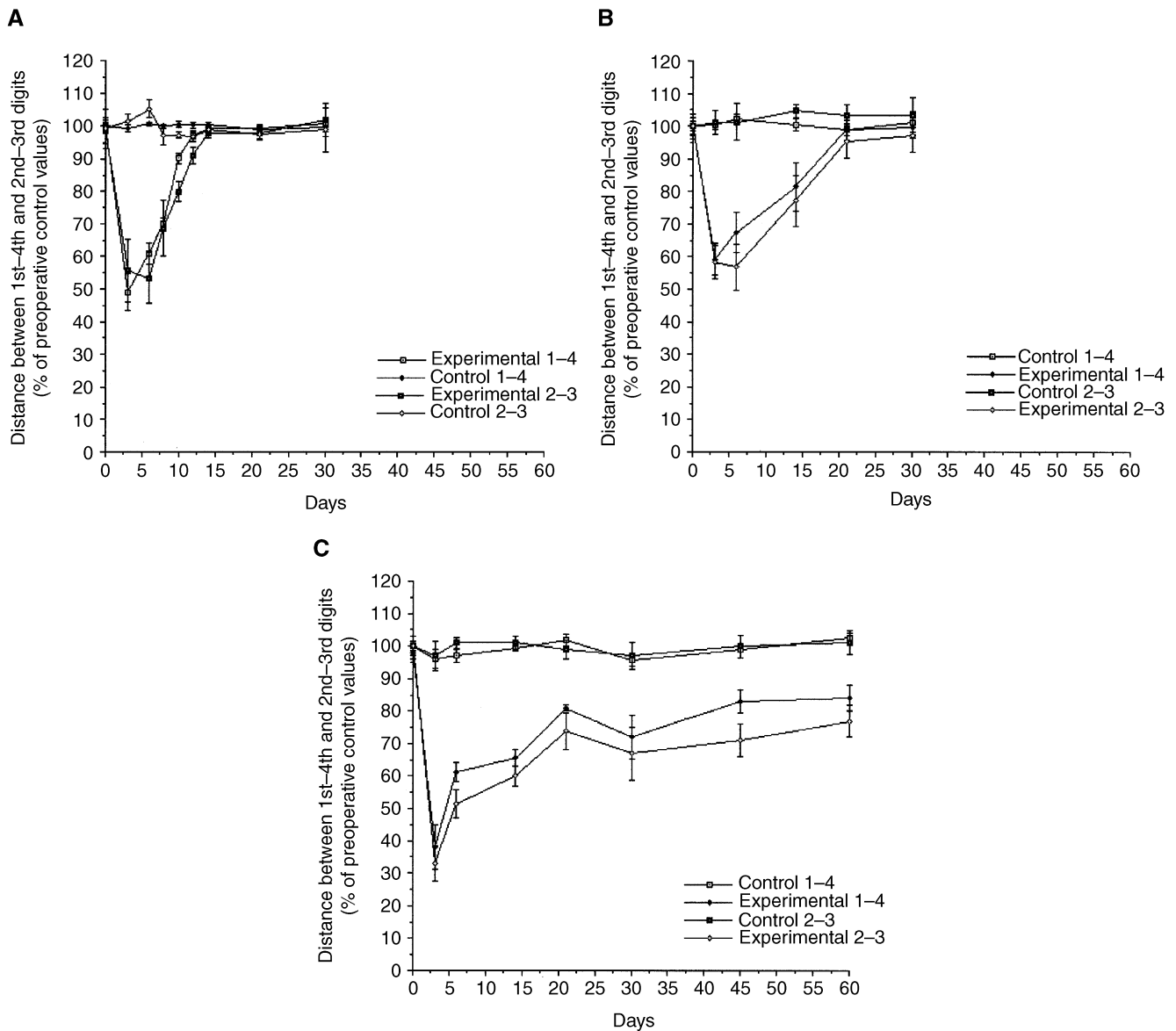


Figure 3. Measurements of pawprints from rat forepaw after various injuries to the radial nerve. (A) Crush injury above the elbow. (B) Transection and repair. (C) Reconstruction of the radial nerve with graft. Values are mean \pm SE ($n=5$ after each injury) of preoperative measurements of distance between the first and fourth and second and third digits.

Radial nerve – transection and repair

When the radial nerve was transected and repaired, the distance between the first and fourth toe decreased to $60 \pm 5\%$ of the preoperative value at day 3, and then there was a progressive return of function to near normal level at day 30 ($p < 0.01$ compared to control; Fig. 3B).

Radial nerve – nerve grafting

In the grafted radial nerves, the values of the pawprints between the first and fourth toe fell down to $38 \pm 7\%$ of the preoperative value and then returned to $84 \pm 1\%$ at day 60 ($p < 0.001$ compared to control; Fig. 3C).

Immunocytochemistry and histology

Neurofilaments

The results for the median, ulnar, and radial nerve were essentially the same.

In the crush-injury groups, the neurofilament staining pattern had an appearance similar to that of an uninjured nerve 30 days after the procedure. At this time point, the crush site was difficult to identify (Fig. 4A). In the transection and repair groups, the sections from the repair site showed that many axons had crossed the suture line, but many were misdirected, i.e., they were not parallel to the nerve axis. Axons also grew in the epineurium (Fig. 4B). In

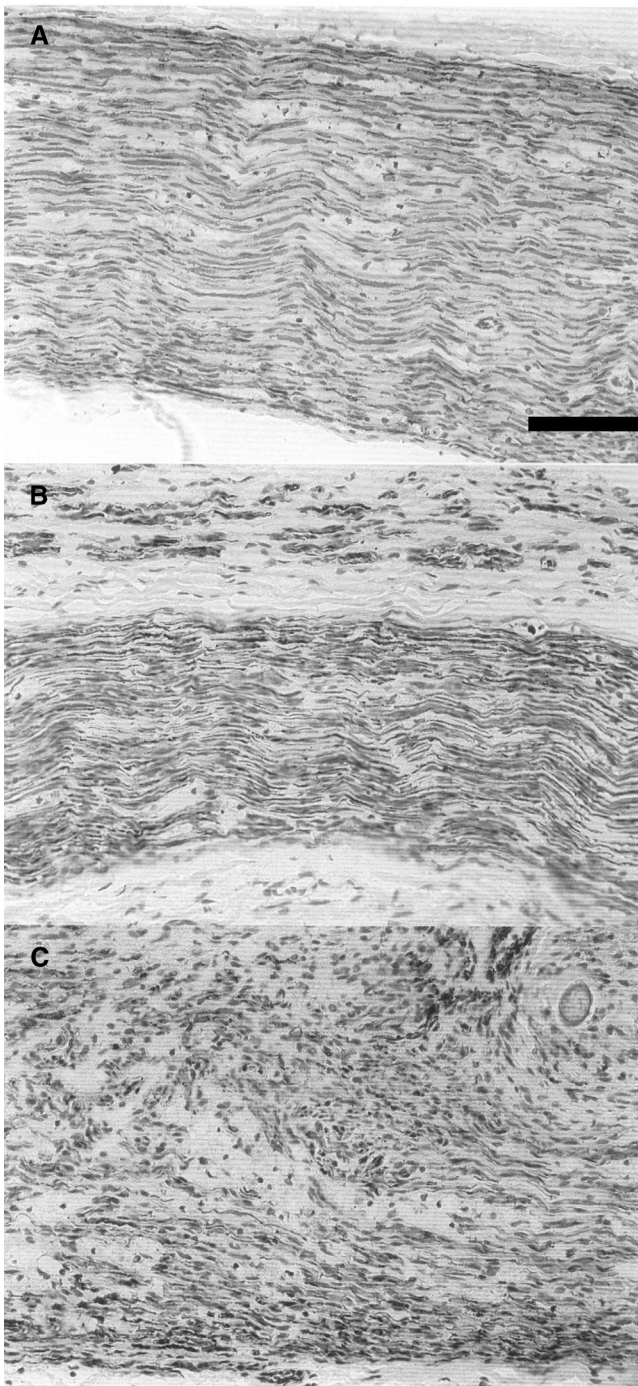


Figure 4. Neurofilament staining of the longitudinal sections of median and ulnar nerve that were crushed (A), transected and repaired (B), and reconstructed with a graft (C). The results were essentially the same for median, ulnar, or radial nerves. Scale bar = 100 μ m.

the graft groups, the neurofilament staining showed a great deal of misdirection at the site of the repair but also inside the graft (Fig. 4C). Fewer axons were noticed in the graft as compared with the nerves that

were crushed or transected and repaired. In the group where both the median and ulnar nerves were grafted at the same level, the fascicular organization was disturbed. The two grafts appeared to have fused, making it difficult to recognize each nerve. In this fused graft structure, many axons had grown outside the main nerve trunk.

C-Terminal flanking peptide of neuropeptide Y

Immunocytochemistry was used to demonstrate the CPON expression of the sensory neurons in the DRGs after proximal versus distal crush of the median and ulnar nerves (Fig. 5). The results are summarized in Table 2. Values from day 30 were not possible to evaluate due to staining problems. On the uninjured control side, only occasional CPON-positive cells were observed in sections from the DRGs C7-T1. In response to a crush injury of both the median and the ulnar nerves proximally, there was a dramatic increase

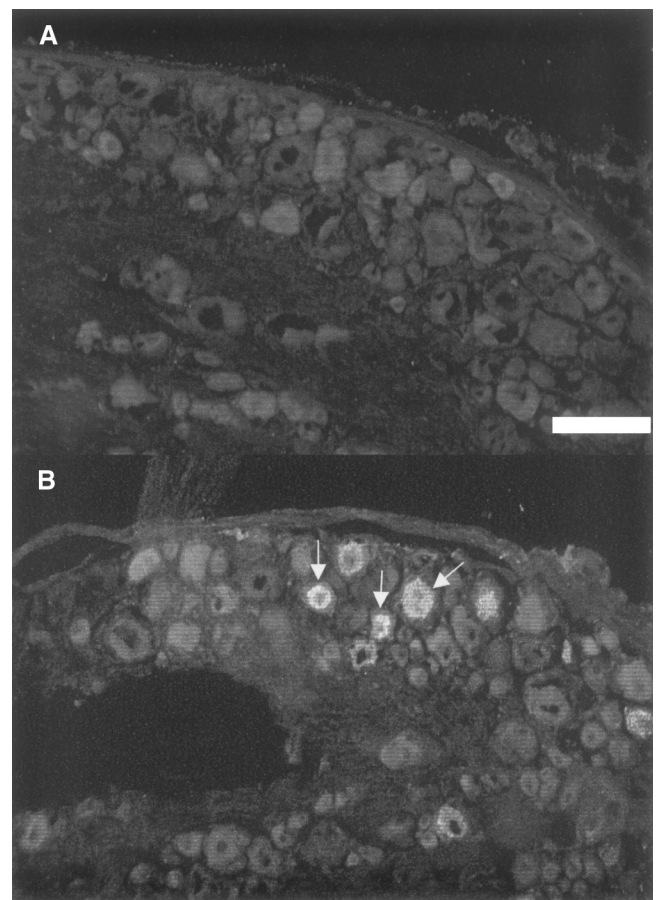


Figure 5. Immunocytochemical staining for C-terminal flanking peptide of neuropeptide Y (CPON) of the sensory neurons of dorsal root ganglion (DRG) (C7) from an uninjured site (A) and from a DRG (C7) where the median and ulnar nerves were crushed (B). The arrows show CPON-positive sensory neurons. Scale bar = 100 μ m.

Table 2. Number of sensory neurons (expressed in %) in dorsal root ganglia (DRGs) C7–T1 expressing C-terminal flanking peptide of neuropeptide Y (CPON) after a proximal or distal crush to the median and ulnar nerves

Days after nerve crush	Proximal nerve crush			Distal nerve crush		
	C7	C8	T1	C7	C8	T1
6	5 ± 2	4 ± 2	2 ± 0	7 ± 1	5 ± 2	9 ± 1
14	23 ± 3	10 ± 2	8 ± 1	8 ± 2	6 ± 0	4 ± 2
21	18 ± 6	19 ± 1	6 ± 2	2 ± 1	9 ± 0	6 ± 2

The number of CPON-positive sensory neurons are expressed in percentage of the total number of cells with a clear nucleus. Values are mean ± SE. At least three sections were examined from each DRG ($n = 3$ at each time point).

in the number of CPON-positive cell bodies, reaching up to $23 \pm 3\%$ of the total number of sensory neurons in C7 at day 14. A lesion induced more distally to both nerves also increased CPON expression. Pooling of the data from DRGs C7–T1 at each time point showed a significant difference (ANOVA; factors: injury and time; $p = 0.04$) in the number of CPON-positive sensory neurons after a distal injury ($6 \pm 2\%$ at day 14) and a proximal lesion of the same nerves (corresponding value $14 \pm 2\%$). In the two-factor ANOVA test, however, each factor (injury and time) was also significant ($p < 0.001$ and $p < 0.002$).

Conventional histology

The results for the median, ulnar, and radial nerve were essentially the same.

The transverse sections of the most distal parts of the nerves in the crush groups revealed no difference from the normal nerves, in number or size of the nerve fibers. In the sections from the most distal part of the transected and repaired nerves, regularly shaped axons and myelin sheaths with various degrees of thickness were noticed. In the distal parts of the grafted nerves, the perineurium was thickened, mostly in the center of the nerves and the number of axons was less than in other groups.

Discussion

The main aims of the present study were, (1) to evaluate the basic regenerative properties of various nerves in the upper extremity of the rat, and (2) to evaluate the possible use of this system as an experimental model for studies of brachial plexus nerve injuries.

In the first set of experiments, the effects of different types of lesions and lesion locations on functional recovery were tested. As anticipated, recovery

of function assayed by pawprints was much faster and better after crush injuries than after transection/repair or nerve grafting. The latter repair/reconstruction methods resulted in an incomplete functional recovery. The degree of misdirected axonal outgrowth of regenerating nerve fibers by neurofilament immunocytochemistry correlated with the recovery of function of the pawprints. Thus, no misdirected fibers were found after a crush lesion, while many fibers had misdirected growth after transection/repair and even more after nerve grafting. These results are in accordance with those achieved using the sciatic nerve model (Bodine-Fowler et al., 1997; Lundborg, 2000; Valero-Cabre and Navarro, 2001). The advantage of the upper extremity model, however, is that the recovery is much faster and the time required for reinnervation is shorter. In addition, no experimental animals were discarded due to articular contractures or autotomy, a common condition when the sciatic nerve model is used.

We used pawprints as a measure to evaluate return of function. Pawprints are simple and do not require extensive training of animals (Gonzalez et al., 1986; Montoya et al., 1991; Saling et al., 1992). The pawprints have previously been used by Hassegawa (1978) and by Bernar (2000), and the method seems to be advantageous in comparison with conventional walking track analysis, i.e., SFI (De Medinaceli et al., 1982; Clarke, 1995; Lin et al., 1996; Van Meeteren et al., 1997; Hadlock et al., 1999; Urbanek et al., 1999; Dijkstra et al., 2000; Gillis and Biewener, 2001; Varejao et al., 2001). In contrast to Bertelli and Mira, who found the walking track analysis to assess neurologic impairment after brachial plexus lesion not to be a sufficient method to demonstrate whether a nerve lesion had a specific effect on the walking pattern, we believe that the toe spread index is a more reliable method. Injury to either the ulnar or the median nerves had little or no effect on the pawprints. In this respect, the rat is different from humans, in that in humans the ulnar nerve is known to contribute extensively to finger spreading. However, injury to both the median and the ulnar nerves resulted in the loss of toe spreading, suggesting the presence of compensatory mechanisms for toe spreading in the rat. Injury to the radial nerve resulted in a profound decrease in pawprints, due to the impairment of wrist and finger extension. The pawprints, however, were still useful to evaluate recovery of radial nerve function.

Injuries to the nerves of the upper extremity exhibited the classical CL effect, i.e., an increased regenerative capability following a test crush lesion if the nerve had been injured previously, as seen in the sciatic nerve model (McQuarrie and Grafstein, 1973; McQuarrie et al., 1977; Sjoberg and Kanje, 1990a; 1990b; Dahlin and Thambert, 1993). A CL decreased recovery time

by around 50% after injury to the median and the ulnar nerves. To test whether conditioning of sensory nerve fibers had any effect on the reinnervation of muscles, we compared a CL induced mainly in sensory nerve trunks with such a lesion of motor fibers as well. Surprisingly, both CL procedures resulted in a faster recovery compared to a single crush injury (compare Figs. 2A and 2C). We have no reasonable explanation for the effect of the sensory CL. One possibility is that substances released from the conditioned sensory fibers affect regeneration of motor fibers. This influence could be evoked at all levels of the nerve or, less likely, at the level of the spinal cord. Furthermore, recovery of function was also faster if the CL was induced in the motor/sensory fibers compared to the sensory fibers alone. The mechanisms behind the CL effect are based on changes both at the level of the nerve trunk and at the cell body. Only in the experiments with the CL of the motor/sensory fibers is there a profound cell body reaction evoked in the motor neurons. This may account for the different time taken for recovery seen after the CL induced in the motor/sensory and the sensory fibers, respectively.

CPON was used as a marker of neuronal injury in DRGs C7–T1 (*Ljungberg and Johansson, 1993; Hokfelt et al., 1994; Mille-Hamard et al., 1999; Bergmark et al., 2001; Widerberg et al., 2001*). We found a massive increase in the number of CPON-positive sensory neurons in DRGs C7–T1 after a nerve crush lesion, roughly corresponding to the contribution of nerve fibers of these DRGs to the inflicted nerves. Thus, the number of CPON-immunoreactive sensory neurons was higher in C7 and C8 compared to T1. The CPON reaction was also dependent on the distance of the lesion to the cell bodies. Such a distance-dependent induction of neuropeptides and cell death has also been observed by other authors (*Ygge, 1989; Shi et al., 2001*) and stressed by Fu and Gordon (*1997*). Our results are similar to those of authors who have stated that functional recovery is particularly poor in injuries that sever the nerve far from the target (*Fu and Gordon, 1997*) or that neurons subjected to injuries far from cell bodies are less susceptible than injuries close to cell bodies (*Fu and Gordon, 1997*).

In conclusion, the brachial plexus with its terminal branches offers an excellent experimental model for the study of nerve injuries and repair methods. Functional recovery is rapid and can be evaluated within a few weeks using pawprints and within days with respect to cell body response. With respect to the proximity of the nerve branches in the upper extremity, the model should also prove valuable as a system to study end-to-side nerve repair. Such studies are currently being performed in our laboratory at Malmö University Hospital.

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