Nervous immunity: A study on the role of complement system in neuronal degeneration and regeneration
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The functional and morphological characteristics of sciatic nerve degeneration and regeneration after crush injury in rats

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Abstract

Background: Peripheral nerve damage induces a sequence of degeneration and regeneration events with a specific time course that leads to (partial) functional recovery. Quantitative electrophysiological analysis of degeneration and recovery over time is essential to understand the process.

New method: The presented ex vivo neurophysiological method evaluates functional recovery of the propagation of the compound action potential after crush injury of the rat sciatic nerve. A 32-channel electrode array was used to monitor compound action potential propagation at time points between 1 hour and 35 days after semi-quantitative crush injury of the rat sciatic nerve.

Results: The compound action potential was characterized by four measures: the latency, the duration, the amplitude and a measure that combined time and location. These four parameters reflected the subsequent steps in early axonal degradation, the transition to rapid degeneration followed by sprouting and the long period of remyelination that accompanied regeneration.

Comparison with existing methods: The neurophysiology measures of the compound action potential were compared with the morphology of the nerve at representative time points and analysis of functional recovery of action potential propagation was compared with a behavioral test: the foot flick test.

Conclusions: Our data suggests that the ex vivo electrophysiological method is complementary to the classical behavioral foot flick test in that it allows a detailed time analysis of the degeneration and early regeneration phases at a high spatial and temporal sensitivity. The results were well-matched with observations made with immunohistochemical and morphological methods.
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Introduction

Peripheral nerve injury is a common trauma and although peripheral nerves have the capacity to regenerate, functional recovery in humans after nerve injury is often poor (Wood et al., 2011). Damage to the peripheral nerve is followed by Wallerian degeneration (WD), a process during which myelin is removed from the axons and axons disintegrate distal to the site of injury (Bradl and Lintingon, 1996; Chen et al., 2007; Koltzenburg and Bendszus, 2004; Misgeld, 2005; Stoll et al., 1989b; Stoll et al., 1989a). In the regeneration phase, the axon stumps located just proximal to the injured site grow sprouts (Verma et al., 2005); that extend distally along the basal lamina and ideally re-innervate their original target (Tomita et al., 2007). At the same time, remyelination engages the new axons in a one-to-one relationship with Schwann cells (Chen et al., 2005; Chen and Strickland, 2003). Regeneration as well as degeneration are studied with a wide range of (semi-quantitative) morphological, behavioral and electrophysiological techniques (Mazzer et al., 2008; Schiaveto de Souza et al., 2004). Morphological parameters such as axon diameter and the presence of myelin and neurofilament can be quantified during all phases of nerve recovery and they distinguish between normal and degrading myelin and axons. Remyelination can be visualized as well (Moore et al., 2012). Functional recovery is most often evaluated using behavioral assays such as the analysis of the walking track or the foot flick test which measures the propagation of sensory information from the periphery (Bain et al., 1989; de Medinaceli et al., 1982).

In animals with autotomy or contracture of the hind limbs, walking track analysis is difficult to perform as it relies on toe spread and print length to calculate the sciatic function index. Functional recovery measurements that rely on behavioral parameters can, in most instances, only provide information after the animal has regained sufficient nerve function in the extremities to perform the motor/sensory task. They provide little information during degeneration and early regeneration. In our behavioral experiments the foot flick test is used prior to and after inflictning the nerve injury but no functional response will be measured until at least a few axons have reached the foot sole (de Koning et al., 1986).

Neuropsychological tools enable to quantify signal propagation in the nerve in vivo as well as ex vivo (Cragg and Thomas, 1957; Cragg and Thomas, 1964; Korte et al., 2011; Krarup et al., 1988; Krarup and Loeb, 1988; Kuypers et al., 1999; Loffredo et al., 2009; Martins et al., 2005; Wood et al., 2011). Continuous neurophysiological in vivo monitoring allows a longitudinal evaluation of the regeneration, but the spatial resolution is poor, since at best three or four electrodes can be implanted (Korte et al., 2011; Oguzhanoglu et al., 2010; Smit, 2006). Another drawback is that many in vivo electrophysiological methods require repeated anesthesia exposing the animal to substantial stress. Ex vivo methods can attain a higher spatial resolution using multi electrodes but are destructive, Jacobson and Guth, were among the first to describe an electrode grid for ex vivo electrophysiology (Jacobson and Guth, 1965).

In this study, we analyzed the relations between electrophysiological, behavioral and morphological parameters of rat sciatic nerves after an induced crush injury. The measurements were performed at relevant time points during degeneration and regeneration, covering the changes that occurred from 1 hour to 35 days after the crush. Each time point was obtained in a separate group of animals. Evoked compound action potentials were recorded ex vivo at 32 points at 1.8 mm intervals as they propagated along a crushed sciatic nerve. This allowed us to localize the crush site and we could determine the efficacy of the crush. We related the electrophysiological measurements to morphological and immuno-histochemical observations.
made in the injured sciatic nerves. The functional recovery of nerve propagation was compared to the outcome of a traditional behavioral test for sensory recovery: the foot flick assay (de Koning et al., 1986; van der Hoop et al., 1988).

**Materials and methods**

*Animals and experimental design*

Twelve-week old male wild type (WT) WT Piebold Virol Glaxo (PVG) rats (Harlan, UK) (www.harlan.com, 2013) of approximately 250 g were used in this study. Care and use of the animals in this study was approved by the Animal Care and Use Committee of the Academic Medical Center (AMC) of the University of Amsterdam and were according to European guidelines. Animals were housed in standard cages under standard conditions and received food and water ad libitum.

*Nerve crush*

For the crush injury, animals were anesthetized (2.5% Vol Isofurane, 2L/min O₂). The left sciatic nerve was exposed and crushed above the splicing between the *m. semimembranosus* and *m. vastus lateralis*, 9.6 mm above the trifurcation. Calibrated force was exerted three times for a period of 10 s each with in-house modified anatomical forceps on which strain gauges were mounted, coupled to a read-out amplifier that provided feed-back to the experimenter. The forceps force was calibrated by applying force on a thin fluid-filled tube connected to a pressure meter; nerves were crushed by about 5.34 N over the 1.5 mm size of the forceps. The right sciatic nerve served as a control and was treated in the same way except for the crush. Crush and control site were marked with non-toxic dye (East India Ink, Talens, NL). After surgery, the wounds were closed and animals received subcutaneous injections of 0.05 mg/kg Temgesic (0.3 mg/ml buprenorfin hydrochlorid; Reckitt Benckiser, RB pharmaceuticals, Slough Berkshire, UK) for three consecutive days after surgery. Animals were tested at eleven time points after the crush injury (early degeneration: 1, 3, 6 hours post injury (h PI); rapid degeneration: 1, 2, and 3 days PI (d PI); regeneration: 7, 14, 21, 28 and 35 d PI. Time points were chosen as in previous studies: at 3 d PI and 7 d PI the degeneration is most intense; earlier time points have not been extensively studied before (Ramaglia et al., 2007; Ramaglia et al., 2008).

*Sensory recovery*

Sensory recovery was assessed *in vivo* with a foot flick test. An electrical stimulus was given at a fixed position on the rat’s paw (de Koning et al., 1986). The threshold stimulus current for paw retraction was determined in the intensity range from 20 to 50 µA. The same foot flick assay was applied to the control leg. Threshold values are expressed as a percentage of normal value in control. To avoid conditioning, animals were tested with and without current, and also the response to the sound of the switch was tested (van der Hoop et al., 1988). These tests prior to the actual measurement were also performed to prevent confounding due to hyperalgesia and allodynia; animals were not tested if hyper responsiveness or hypo responsiveness occurred.
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Figure 1: Schematic representation of the electrode grid and nerve sections. A: Schematic overview of the 32 electrode grid and the placement of the sciatic/tibial nerve (yellow line) on the grid. The crush site was always placed in between electrode 16 and 17 (e-16/17), indicated by the black bar on the nerve. The stimulating electrodes for sciatic stimulation were the two most proximal electrodes on the left hand side and for tibial stimulation the two most distal electrodes on the right hand side were used. B: Schematic overview of the nerve sections studied with immunohistochemistry and quantification of neurofilament and myelin. To study morphology in the regeneration phase, nerves were divided into 5 mm sections, starting from the distal edge of the crush site (dark spot) in distal and proximal direction. 1 = proximal, 2 = crush area, 3 = close distal and 4 = far distal. Direction of sectioning is indicated by the arrows below. 6 µm sections were used for histological analysis. C: Picture of the *ex vivo* recording set up with a nerve placed on the 32 electrode grid.

*Ex vivo electrophysiology*

After crush the animals were allowed to recover for 1 hour to 28 days before they were killed with 300 µl 20% Pentobarbital sodium (Produlab pharma, Raamsdonksveer, The Netherlands) cardially injected under deep isoflurane anesthesia. Both left and right hind leg sciatic and tibial nerves, still connected at the trifurcation, were dissected. Excess fat and connective tissue were removed and the nerves were transferred within 12 minutes after death into a 0.9% saline solution at room temperature. Nerves were kept in saline solution until the start of the recording session on the electrode array.

Compound action potentials (CAPs) were recorded with a custom-made 32-channel silver-wire electrode-array (inter electrode distance 1.8 mm) in an airtight, moisturized holder (Fig. 1A, C). Two electrodes of choice (mostly the electrodes on the most proximal or most distal side of the nerve) were used to apply a biphasic voltage stimulus (Fig. 1A). The orientation of the nerve on the electrode-array was standardized in that the marked crush site was put between electrode 16 and 17 (Fig. 1A).

Electrical stimulation and simultaneous CAP recording was done by custom-made software (MATLAB, The MathWorks, Inc., Natick, MA) that controlled a data acquisition system...
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(NI-6259, National Instruments, Austin, TX) with digital-analog conversion for the stimulation (500 kHz sampling rate) on two electrodes of choice (Fig. 1A) and analog-digital conversion for recording (25 kHz sampling rate) on the remaining 30 channels.

Charged-balanced biphasic rectangular voltage pulses with phase durations of 100 µs and an interphase gap of 100 µs were used for stimulation. Stimuli of different intensities were applied while the CAP was simultaneously recorded from each electrode against a chosen common reference (one of the electrodes) as it propagated over the nerve (Fig. 2A). Stimulus intensity varied between 0 and the intensity required to evoke a CAP of maximal amplitude ($V_{\text{max}}$). We used the following values for $V_{\text{max}}$: injured sciatic: 0.60 ± 0.30 V, uninjured sciatic: 0.71 ± 0.30 V, injured tibial: 0.99 ± 0.53 V and uninjured tibial: 0.73 ± 0.28 V. It took about 30 minutes to complete a full isolated nerve measurement protocol. No decline in response was seen during these measurements (Cappaert et al., 2013).

**CAP data processing**

The analysis of CAP propagation along the nerve was performed with custom-made software, written in MATLAB (Mathworks, Natick, USA). The scaled response to a sub-threshold stimulus was subtracted from each recording to suppress the stimulus artifact. To improve the signal to noise ratio, CAP recordings were averaged over five trials and they were filtered using a 2-dimensional convolution with a rectangular space-time kernel (3.6 mm by 200 µs).

To enhance the detection of the propagating wave front, we estimated the second order spatial derivative of the compound action potential (dCAP, Fig. 2B) at electrode $k$, using three adjacent electrodes ($k-1$, $k$, $k+1$) and assuming an identical inter-electrode distance $\Delta x$:

$$d\text{CAP}_k(t) = \frac{\text{CAP}_{k+1}(t) - 2 \times \text{CAP}_{k}(t) + \text{CAP}_{k-1}(t)}{\Delta x^2}$$

$d\text{CAP}$ is plotted as a function of position and time using a color-coded amplitude scale (yellow/red colors Fig. 2C). The distance between the contour levels is based on the standard deviation in the signal determined in a 300 ms time window before the stimulus pulse (Fig. 2C).

**Tissue processing**

Directly after the CAP recordings nerves were post-fixed in 4% paraformaldehyde and used for paraffin embedding. Nerves harvested at degeneration time points (0, 1, 3, 6 h PI and 1, 2, 3 d PI) were embedded longitudinally to visualize the events around the crush. Nerves harvested at regeneration time points (7, 14, 21, 28 and 35 d PI) were embedded for cross sections. Before embedding of the cross sections, the nerves were divided into 5 mm sections, starting from the crush site, both proximally and distally. Nerve sections were embedded with their distal ends at the cutting plane (Fig. 1B).
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Immunofluorescence

Sections (6 µm) were deparaffinated using xylene and rehydrated in a decreasing ethanol range, with as final step aquadest. Antigen retrieval in the microwave was performed on all sections, for 3 minutes at 800W and 10 minutes at 440W in 10mM Tris/1mM EDTA (pH 6.5). Aspecific binding of antibodies was reduced using 10% normal serum in Tris buffered saline (TBS) of the secondary antibody host for 30 minutes. Primary antibodies were diluted in 1% bovine serum albumin (BSA) and incubated for 90 minutes (polyclonal rabbit anti-MBP (1:100), DakoCytomation, Glostrup, Denmark); monoclonal mouse anti-SMI31 (1:1000), (Covance, Rotterdam, NL). Primary antibodies were detected with directly conjugated goat-anti-rabbit-FITC or sheep-anti-mouse-Cy3 (Sigma-Aldrich, Saint Louis, MI). As a nuclear counterstain 4.6-diamidine-2-phenylindole, dihydrochloride (DAPI) (blue, 280 nm) was used. Sections were mounted in vectashield (VECTOR, Burlingame, CA). A slide with only the secondary antibodies was used as a control in each staining.

Microscopy and quantification

Immunofluorescence was determined on a fluorescent microscope (Vanox AHBT3, Olympus) using 488 nm excitation for FITC, 568 nm excitation for Cy3 and 350 nm excitation for DAPI. Representative micrographs were taken at 20x and 40x magnification with a digital camera (DP12 camera, Olympus). Percentage of positivity for neurofilament and myelin staining in the cross sections of the regenerated nerves was quantified using the positive/area option in ImagePro 7.0 software (Media Cybernetics, Rockville, MD).
Statistics

All values are given as mean and the standard error of the mean (SEM). Direct comparisons are made with Student t-test unless otherwise mentioned. P < 0.05 is assumed to indicate a significant difference.

Results

Nerve morphology

One hour after the crush (1 h PI) there was acute loss of myelin (MBP) and axons (NF) at the crush site (Fig. 3B), but there was no sign of cell infiltration in the damaged crush area and the distal and proximal parts of the nerve were still intact (Fig. 3A; supplementary Fig. 1A). At 3 h PI a thickening of the nerves was observed distal from the crush site, as well as strong swelling of the crush site (Fig. 3C). This swelling was decreased at 6 h PI (Fig. 3D), while the entire part of the nerve distal from the crush site remained swollen compared to the uninjured nerves. Early signs of myelin and axonal degeneration were found distal from the crush site. At 3 h PI accumulation of phagocytes was observed within the epineurium. Influx of cells was observed from 6 h PI onward (supplementary Fig. 1B).

Figure 3: Neurofilament and myelin changes in the early degeneration phase. Representative photomicrographs of neurofilament (SMI31: red) and myelin basic protein (MBP: green) staining in the early degeneration phase. The nuclei are stained blue. The scale bar in A represents 30 µm: all images are taken at the same magnification. A: Proximal part of the nerve (portion 1 as indicated in Fig. 1B) at 1 h post injury (PI) showed normal morphology; myelin is located around the neurofilament. B: The crush site (portion 2 as indicated in Fig. 1B) at 1 h PI showed slight changes compared to the control situation. Some axons were still surrounded by myelin, some axons showed axonal damage; myelin changes were observed at the crush edge. C: The crush site at 3 h PI. Notice the space in-between the axons, indicating swelling of the nerve. There was loss of normal morphology and presence of myelin ovoids. D: The crush site at 6 h PI: The swelling of the crush site was reduced within the crush area but other changes were similar to changes at 3 h PI (see C).
In the rapid degeneration phase (1–3 d PI) the nerve degenerated at the crush site as well as at the far distal nerve end. In the rapid degeneration phase substantial MBP and NF loss were found around the crush site. The loss of axons and myelin progressed further distal from the crush site compared to the early degeneration phase (Fig. 4) and a large fraction of the nerve was affected at 3 d PI. The changes in myelin and axons were also found just proximal of the crush site, showing ruffled myelin as well as unmyelinated axons. Progression further proximal from the nerve terminal was not observed. The numbers of infiltrated cells increased over time and concentrated within the crush area and further distal along the nerve (supplementary Fig. 1C, D).

Figure 4: Neurofilament and myelin changes in the rapid degeneration phase. Representative photomicrographs of neurofilament (SMI31; red) and myelin basic protein (MBP; green) staining in the rapid degeneration phase. The nuclei are stained blue. Scale bar in A represents 200µm; all images are taken at the same magnification. The insets are magnification (2.5x) of the panel. A: The proximal part of the nerve (portion 1 as indicated in Fig. 1B) at 1 day post injury (PI) showed normal morphology of axons and myelin. B: Crush site (portion 2 as indicated in Fig. 1B) at 1 day PI showed a lack of neurofilament positivity in the crush site, damaged axons as well as myelin degradation. Notice the large amount of nuclei inside the crush area (blue staining indicated by a plus sign) and some unmyelinated axons (*). C: Crush site at 2 days PI, showed small calibre unmyelinated axons (*) which might represent newly growing axons in the crush area. Myelin debris was not completely cleared yet. At this time point, there was still an increase of nuclei inside the crush area (+). D: Crush site at 3 days PI. The myelin debris was almost entirely cleared and the crush area was negative for myelin. The amount of nuclei inside the crush area remained high (+).

The regeneration phase (7–35 d PI) was characterized by axonal outgrowth observed as early as 7 d PI (Fig. 5A), although myelin debris was still present. Numbers of infiltrated phagocytes remained high until 35 d PI and peaked at 14 d PI (supplementary Fig. 1 E-I). The number of new axons and their diameter increased with time (compare Fig. 5B, C, D-E; new axons indicated by *). Remyelination was first found in the former crush area at 21 d PI (Fig. 5C). A similar sequence of events was observed in the areas distal of the crush site (supplementary Fig. 2), indicating a wave like pattern of degeneration, followed by axonal sprouting, enlarging of the axons and remyelination.
Figure 5: Neurofilament and myelin changes in the regeneration phase. Representative photomicrographs of neurofilament (SMI31: red) and myelin basic protein (MBP; green) staining in the crush area (portion 2 as indicated in Fig. 1B) in the regeneration phase. The nuclei are stained blue. Scale bar in A represents 15.55µm; all images are taken at the same magnification. A: Myelin debris (D) was still found at the crush site at 7 d PI. Some new axons were observed (concentrated red dots, indicated with *) as well as degrading axons (large diffuse stained red structures, indicated with D). B: New axons (*) were found at the crush site at 14 d PI, as well as myelin debris (D) and remyelination (arrow). C: At 21 d PI the remyelination process started (arrows) and the new axons (*) became thicker. There were more new sprouts found in clusters as well. D: Most new axons were myelinated (arrows) at 28 d PI and some clusters of unmyelinated axons were still observed (*). E: Compared to 28 d PI, 35 d PI showed no additional axonal changes, except for the amount and thickness of myelination (arrows) some axons remain unmyelinated (*).

Recovery of sensory function

Recovery of sensory function was monitored weekly using a foot flick test (Fig. 6). The first sensory recovery occurred at 17 d PI, but remained fairly small until 24 d PI. After 24 d PI, a rapid recovery of sensory function was observed in the animals. At 35 d PI animals showed a sensory recovery of 96 ± 4%, which is not distinguishable from control.

Nerve conduction

Stimulation at the proximal end of the nerve results in propagation of the induced CAP from the most proximal to the most distal part of the nerve (Fig. 2). During stimulation (total of 300 µs) the signals in Fig. 2 and 7 are blanked for clarity. Figure 2 also illustrates the relation between the CAP (Fig. 2A) and its second order spatial derivative (here after called dCAP) in which the moving wave front can be more easily detected (Fig. 2B). Stimulation at the distal end of the nerve evoked a similar pattern, which started in the most distal part and traveled into the opposite direction. After crushing the nerve, the propagation pattern changed (Fig. 7A): At 1 – 24 h PI the dCAP propagated along the proximal electrodes after stimulation at the most proximal electrode, but disappeared around the crush site. Stimulation at the most distal electrode evoked a propagating dCAP response only in the distal part of the nerve which did not propagate to the proximal part. We determined for each nerve at which electrode the dCAP disappeared in response to proximal (Fig. 7) and distal stimulation. In this way we could precisely localize the crush gap: the section of the nerve that can no longer propagate activity after the crush (Fig. 6).

In the early degeneration (1 – 6 h PI), the dCAP did not propagate at all over the crush site (Fig. 6 and 7A). During the beginning of the rapid degeneration phase (24 h PI) the distal and proximal part (Fig. 6) of the nerve could still initiate and propagate the dCAP. Later on in the rapid degeneration phase (48 and 72 h PI) the dCAP could no longer be evoked in the distal part of the nerve and no recovery of dCAP inducibility by distal stimulation was observed during the regeneration phase (7 – 28 d PI; Fig. 6). However, by stimulation of the proximal part, the dCAP propagated further along the nerve with increasing time PI (Fig. 6 and 7B-D). The mean
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regeneration rate was 0.2 mm/day based on a calculation that assumed a constant increase of the regenerated distance over time.

Figure 6: Comparing ex vivo electrophysiology with foot flick test results. Change of the crush gap (represented in blue; left y-axis) and the normalized foot flick response (represented in red; right y-axis) after the crush. Error bars represent the SEM. The solid blue line shows the distance dCAP reached by sciatic stimulation (at −24 mm); the dotted blue line shows the distance dCAP reached by tibial stimulation (at +24 mm). The crush site is indicated by the dotted black line at distance 0 mm. Area between the two lines shows the crush gap, the section of the nerve that can no longer be activated by distal and proximal stimulation after the crush. A conduction block was observed at the crush site at time points 1–24 h post injury (PI). Restoration of the CAP propagation in the damaged nerves was observed as early as 48 h PI, as the CAP response evoked in the proximal part propagated further along the nerve, crossing the crush site. Foot flick analysis (red line) showed that sensory recovery starts slowly at 7 d PI and only rapidly increased after 24 d PI. At 35 d PI sensory recovery was almost completed.

Figure 7: Color coded space-time representation of dCAP propagation at different stages of degeneration/regeneration. The second order spatial derivative of the compound action potential (dCAP) as a function of space and time illustrates its propagation. The dCAP space-time profile in a crushed nerve recorded at 6 h PI, 3 d PI (B) 14 d PI (C) and 28 d PI (D). Amplitude is color coded as indicated by the bar at the right. The crush site is marked by a black dashed horizontal line. During the stimulus the signal is blanked. The compound action potential now clearly crosses the crush and propagates further into the distal portion of the crushed nerve site indicating recovery.
Properties of the dCAP

During the degeneration and regeneration phases the shape of the dCAP changed. During the early degeneration (1 – 6 h PI; Fig. 8A, D, G, J) in a small proximal and distal region around the crush site, we found an increase in peak latency and a small decrease in dCAP width, dCAP peak amplitude and a related, slightly more stable amplitude measure, area under the dCAP curve (indicated as "surface"). This could indicate axonal loss at both sides of the crush. In the rapid degeneration phase (1 – 3 d PI; Fig. 8B, E, H, K) the peak latency increased even more but the peak amplitude and "surface" around the crush were not different from control. The part of the nerve more proximal to the crush showed an increase in dCAP peak amplitude, "surface" and dCAP width. This could indicate the switch from degeneration to regeneration. In the regeneration phase (Fig. 8C, F, I, L), the latency and the dCAP width are still larger than in controls. The dCAP peak amplitude and "surface" are hardly different from control.

Figure 8: dCAP properties. The latency, peak amplitude, the width and the surface of the dCAP are determined measured with sciatic stimulation: A-C: The latency of the peak amplitude dCAP; D-F: The surface of the dCAP response; G-I: The peak amplitude of the dCAP and J-L: The width of the dCAP response. The left column (A, D, G and J) shows the results in the early degeneration phase (0-6 h post injury (PI)), the middle column (B, E, H and K) shows the results of the rapid degeneration phase (1-3 d PI) and the right column (C, F, I and L) shows the results of the regeneration phase (7-35 d PI). In all graphs the black line represents the values measured on an uninjured control nerve. The error bars represent the SEM.
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Relation between neurophysiological and immunohistochemistry parameter

We quantified the percentage positivity per area for neurofilament and myelin staining in each 5 mm section. dCAP data was collected from all electrodes within the same 5 mm sections and the mean values of maximal dCAP amplitude and the "surface" were determined. As to be expected, higher values for neurofilament and myelin staining were indicative of a higher CAP signal. The variation on both techniques was so large that within a single distal section no clear correlation could be detected. The maximum electrode reached with proximal stimulation slowly increased over recovery time. There was no direct correlation between this time course and the steep increase observed in the foot flick response.

Discussion

Peripheral nerve injury in humans most often results in severe disability. Understanding and wherever possible facilitating regeneration is therefore of high relevance and animal models of degeneration/regeneration after nerve injury are a valid tool to this aim. The approach presented here focuses on functional aspects of action potential propagation in particular at the early stages of regeneration, around the injury site. The usual behavioral paradigms for regeneration provide little information in that stage since they measure recovery at the end of the extremities and therefore need substantial functional recovery before any response can be detected (Bain et al., 1989; de Koning et al., 1986; de Medinaceli et al., 1982; van der Hoop et al., 1988). Immunohistochemistry on the other hand provides considerable information about ongoing processes, but gives no insight in their relevance for actual functional recovery.

We followed degeneration and regeneration of the sciatic nerve after a well-defined crush injury with a neurophysiological ex vivo method that uses a medium resolution electrode array. Stimulation on two electrodes induced a compound action potential (CAP) in the nerve fiber that was simultaneously monitored on all other 30 electrodes. Although we realize that many factors are involved in the shape of this extracellularly recorded representation of the action potentials, it appeared to be quite reproducible and could be quantified with the following characteristic parameters: the latency, the amplitude, the width and the "surface". We used the second derivative in space (dCAP) instead of the CAP which locates the moving wave front more precisely. The latency (from the moment of stimulation) is dependent on the distance to the stimulus electrode and directly reflects propagation speed. In the control situation, we see slowing of speed at the distal part of the nerve where the fibers taper. Besides the clear effect of nerve fiber diameter on propagation speed, it should also reflect the degree of myelination.

The amplitude of the recorded response at least scales with the number of activated fibers. The width of the response, in particularly when it is substantially wider than a single action potential, originates from the dispersion in the fiber bundle, e.g., the variation in propagation speed; it is expected to increase with distance traveled. In addition to these parameters we also used the "surface", which is the time integral under the curve. This parameter is strongly related to the amplitude, but is less affected by noise and by dispersion.

In the early degeneration (1 – 6 h PI) phase, it was still possible to induce a propagating dCAP in both the distal and the proximal part of the nerve. The properties of the dCAP were different from those in the control situation: smaller amplitude and "surface", smaller width and longer latency. These changes indicate that the number of axons in the nerve section just proximal to the crush is smaller and/or that the axons have less myelin coating. The latter was
confirmed by our morphological data where the crushed nerves showed axonal and myelin degeneration and swelling of the crush location as has been often reported (Koltzenburg and Bendszus, 2004; Lichtman and Fraser, 2001; Misgeld, 2005; Misgeld and Kerschensteiner, 2006; Ramaglia et al., 2007).

During the rapid degeneration phase (1 – 3 d PI) myelin and axonal losses expanded further distally. This is corroborated by the neurophysiological results: CAPs could no longer be evoked in the distal part of the nerve after 2 d PI. dCAP induction from the proximal end was still possible; propagation occurred although the dCAP just proximal to the crush site showed a higher amplitude, width and “surface” than controls. Compared to the early degeneration, the latency of the CAP increased even further. The increased CAP amplitude and “surface” most likely reflect the outgrowth of new axons. The individual axons in the proximal stump initiate approximately 5 sprouts during the recovery (Aitken et al., 1947; Mackinnon et al., 1991; Wolters et al., 2005; Wood et al., 2011). These newly sprouted axons are known to have a smaller diameter than the surviving axons (Cragg and Thomas, 1957; Gutmann and Sanders, 1943) and many are not yet myelinated, which would explain the larger width and longer latency of the CAP at this time point. This increased variation in the diameter of the axons and the variance in myelination, increase the variation in conduction speed and thus the width of the CAP.

The fast degenerative phase is followed by a long-lasting phase of regeneration (7 – 28 d PI) in which immunohistochemical, functional and neurophysiological results all support the slow extension of nerve fibers in distal direction (Li et al., 2008; Mackinnon et al., 1991; Sunderland, 1947). In this phase the CAP propagates further and further across the crush site and the sensory function shows signs of recovery (Ikeda and Oka, 2012; Varejao et al., 2004). The histological data showed outgrowth of axons. The increase in MPB staining indicates gradual remyelination. The propagation velocity was reduced proximal to the crush site in the regeneration phase (Cragg and Thomas, 1961; Kuypers et al., 1999; Walbeeinh et al., 2003), but, by the end of this phase, the latency tends to reach control values. That these values are not completely reached can be understood because the regenerated axons only regain their full diameter once they reach their target (Gordon and Stein, 1982b; Gordon and Stein, 1982a; Wood et al., 2011) and they also still have shorter internode distances (Ikeda and Oka, 2012; Korte et al., 2011; Wood et al., 2011).

In this study we extrapolated a regeneration rate of 0.2 – 0.8 mm/day, which is lower than the rates reported by other studies: 1-3 mm/day (Gutmann et al., 1942; Sunderland, 1947; Wood et al., 2011). However, this is the recovery rate based on the findings on the ex vivo physiology, the restoration of the ability to propagate a dCAP. It reflects full functional recovery of the nerve bundle. The measurements used to calculate recovery speed in other studies reflect findings on behavioral tests. Additionally, it is known that the recovery time depends on the inflicted amount and type of nerve damage (Mazzer et al., 2008; Sarikcioglu et al., 2007), for example the regeneration time in a transection suture injury model is found to be 0.2-1 mm a day (Al-Majed et al., 2000). This could indicate that the damage inflicted on the nerve is quite severe in our experiment.

It is tempting to correlate the neurophysiological assay with the outcome of the foot flick test. Until now, it was hard to obtain significant correlations between these parameters in the same study (Kanaya et al., 1996; Martins et al., 2005; Munro et al., 1998; Oliveira et al., 2001; Wolthers et al., 2005). Other studies did find correlations, but generally only between the same
class of evaluation methods (Kanaya et al., 1996; Martins et al., 2006; Munro et al., 1998; Oliveira et al., 2001; Wolthers et al., 2005). The explanation most likely lies in the fact that both types of evaluation have a highly non-linear relation to the time course of regeneration. The neurophysiological method is quite sensitive in early regeneration but not very distinctive in the late phase, where signals are large. The opposite is true for the behavioral test, which has hardly any sensitivity in the early phase, but is quite sensitive in the late phase, once neural connectivity has been re-established. Therefore we conclude that these two methods to evaluate degeneration and regeneration are complementary and should each be used in their optimal time window. In all instances (semi-quantitative) morphological data provides additional information, but needs the various modalities of functional evaluation in order to determine relevance. The ex vivo electrode grid method provides detailed information on functional recovery in the early stages of degeneration and regeneration around the site of injury and along the entire length of the nerve, which makes it a useful tool to study the effects of modifiers for neurodegeneration and regeneration on the functionality of the nerve.

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Supplementary figure 1: Phagocyte infiltration in the injured nerves over time (1h – 35 d PI). A-B representative photomicrographs of nerves at 3 and 6 h PI, phagocytes (CD68 positive cells; brown) are found in the perineurium, insert in A, but not inside the nerve at 3 h PI. Cells invade the nerve area at 6 h PI (B). C-I: representative photomicrographs of myelin (MBP: green) and phagocytes (CD68: red) at 1 – 35 d PI, nuclei are stained blue. Phagocytes accumulate inside the nerve and numbers increase over time (C, D, E, F) and peak at 14 d PI. Phagocytes show signs of myelin phagocytosis shown by colocalization of the CD68 with MBP (yellow). Phagocyte numbers decrease a little, but are still present in the injured nerves up to the end of the experiment at 35 d PI (G, I).
Supplementary figure 2: Morphological changes in different nerve portions at 14 and 28 d PI. Representative photomicrographs of neurofilament (SMI31: red) and myelin basic protein (MBP: green) staining in the crush area (portion numbers as indicated in Fig. 1B) in the regeneration phase. The nuclei are stained blue. Scale bar in A represents 15.55µm; all images are taken at the same magnification. A) Nerve at 14 days post injury (d PI). Normal axonal and myelin morphology is shown proximal of the crush (1). The crush area (2) and distal nerve portions (3, 4) showed unmyelinated axons organized in groups (*) and the initiation of remyelination of new axons at the crush area (2; 14 d PI), indicated with an (arrow). B) Nerve at 28 d PI. Normal axonal and myelin morphology was found proximal of the crush (1). Remyelination was observed in the crush area (2) and all distal portions of the nerve (arrows) (3, 4). Some clusters of unmyelinated fibres were still found in the regenerated nerve (3: 28 d PI indicated by *).