Polysialic acid glycomimetics promote myelination and functional recovery after peripheral nerve injury in mice

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α2,8 Polysialic acid (PSA) is a carbohydrate attached to the glycoprotein backbone of the neural cell adhesion molecule (NCAM) and implicated in nervous system development and repair. Here, we investigated whether PSA can improve functional recovery after peripheral nerve lesion in adult mice. We applied a functional PSA mimicking peptide or a control peptide in a polyethylene cuff used to surgically reconnect the severed stumps of the femoral nerve before it bifurcates into the motor and sensory branches. Using video-based motion analysis to monitor motor recovery over a 3 month postoperative period, we observed a better functional outcome in the PSA mimetic-treated than in control mice receiving a control peptide or phosphate buffered saline. Retrograde tracing of regenerated motoneurons and morphometric analyses showed that motoneuron survival, motoneuron soma size and axonal diameters were not affected by treatment with the PSA mimetic. However, remyelination of regenerated axons distal to the injury site was considerably improved by the PSA mimetic indicating that effects on Schwann cells in the denervated nerve may underlie the functional effects seen in motor recovery. In line with this notion was the observation that the PSA mimetic enhanced the elongation of Schwann cell processes and Schwann cell proliferation in vitro, when compared with the control peptide. Moreover, Schwann cell proliferation in vivo was enhanced in both motor and sensory branches of the femoral nerve by application of the PSA mimetic. These effects were likely mediated by NCAM through its interaction with the fibroblast growth factor receptor (FGFR), since they were not observed when the PSA mimetic was applied to NCAM-deficient Schwann cells, and since application of two different FGFR inhibitors reduced process elongation from Schwann cells in vitro. Our results indicate the potential of PSA mimetics as therapeutic agents promoting motor recovery and myelination after peripheral nerve injury.

Keywords: PSA mimetic; functional recovery; myelination; Schwann cells

Abbreviations: DRG = dorsal root ganglia; FBA = foot-base angle; FGFR = fibroblast growth factor receptor; HNK = human natural killer; HTA = heels-tail angle; NCAM = neural cell adhesion molecule; PMR = Preferential motor reinnervation; PSA = α2,8 polysialic acid
Introduction

Peripheral nerves of mammals regenerate after injury and this ability is attributed to the growth-permissive environment provided by Schwann cells and their basal lamina (Fawcett and Keynes, 1990). Despite this regenerative potential, functional recovery after peripheral injury and surgical reconstruction is often poor in both humans and laboratory animals (Fu and Gordon, 1997; Lee and Wolfe, 2000; Lundborg, 2003). Different strategies which aim at improving the outcome of peripheral nerve injury have been tested in animal experiments, for example, electrical stimulation (Al-Majed et al., 2000a; Ahlborn et al., 2007), stem cell transplantation (Tohill and Terenghi, 2004), application of neurotrophic factors (Boy and Gordon, 2003; Piquilloud et al., 2007), application of antibodies against neurotrophic factors (Streppel et al., 2002) or mechanical stimulation of the target musculature (Angelov et al., 2007).

We have been interested in the therapeutic potential of glycans, carbohydrate moieties on protein or lipid molecules, for treatment of nerve injuries. Glycans enable essential functional properties of the carrier proteins and increasing evidence indicates that these carbohydrates are of functional significance during neural repair (Kleine and Schachner, 2004; Eberhardt et al., 2006; Gravvanis et al., 2007; Papastefanaki et al., 2008). For example, the unusual acidic glycan, the human natural killer (HNK) cell glycan (3′ sulphoglucuronyl β1,3 galactoside) known as HNK-1 epitope and expressed on different cell adhesion molecules (Kruse et al., 1984, 1985; ffrench-Constant et al., 1986; Löw et al., 1994; Schachner and Martini, 1995), has been associated with proper targeting of regenerating motor axons (Martini et al., 1992; Brashart, 1993). By applying a peptide that mimics the functional properties of the HNK-1 epitope (Simon-Haldi et al., 2002) to the injured femoral nerve of adult mice, we have shown that a HNK-1 mimetic promotes functional recovery and regrowth of severely axons (Simova et al., 2006). Moreover, this experimental evidence demonstrated for the first time the potential of glycomimetics as therapeutic tools for neural repair.

Another glycan involved in nerve regeneration is α2,8 polysialic acid (PSA), a carbohydrate carried by the membrane-associated and soluble forms of the neural cell adhesion molecule (NCAM) (Bock et al., 1987; Olsen et al., 1993; Hildebrandt et al., 2007; Rutishauser, 2008), as well as by neurophilin-2 (Curreli et al., 2007). Expressed abundantly on Schwann cells and axons in the peripheral nervous system during embryonic development, PSA-NCAM expression decreases in adulthood. After nerve injury in adult rodents, PSA-NCAM is upregulated in axons and skeletal muscle fibres (Covault et al., 1986; Olsen et al., 1995; Carratu et al., 1996). Recent work (Franz et al., 2008) suggested that increased PSA-NCAM expression after nerve injury is necessary for preferential targeting of regenerating motor axons to skeletal muscle as opposed to skin, a phenomenon known as preferential motor reinnervation (PMR) (Brushart, 1988, 1993).

Here we tested the effect of PSA mimicking peptides on peripheral nerve regeneration using an established paradigm of femoral nerve injury in adult mice (Eberhardt et al., 2006; Simova et al., 2006; Ahlborn et al., 2007), allowing simultaneous functional and anatomic assessment of regeneration. Our results indicate that PSA is beneficial for peripheral nerve regeneration. Most remarkably, the positive effect is related to enhancement of remyelination, an observation holding promise for the usefulness of PSA mimetics in different types of nervous system disorders.

Materials and Methods

Glycomimetic peptides

We used a linear PSA mimicking peptide with the sequence H-NTHTDPYIYPID-OH, and a control peptide with the sequence H-DSPLVFIFDHPC-OH. A scrambled version of the PSA mimetic with the sequence H-TNYDITPHDIYC-OH was used in some experiments as a second control. The PSA mimetic was identified by screening of a linear 12-mer phage display peptide library with the PSA recognizing monoclonal antibody 735 (Frosch et al., 1985) and this peptide was verified as a PSA mimetic using competition ELISA (data not shown). Binding affinity of PSA antibody 735 to the PSA mimetic, scrambled and control peptide was evaluated by surface plasmon resonance (SPR) measurements carried out on a BIAcore 3000 instrument (GE Healthcare Europe GmbH, Freiburg, Germany) with sensor chips maintained at 25°C (Schulze, 2000). The running buffer was phosphate buffered saline (PBS, pH 7.4). Peptides were covalently immobilized to CM5 sensor chips (carboxymethyl dextran; GE Healthcare Europe GmbH) via primary amino groups, using standard coupling protocols (Karlsson et al., 1991). In brief, the sensor surface was activated by a 7-min pulse of 0.2 M N-ethyl-N-(3-dimethylaminopropyl) carbodiimide and 50 mM N-hydroxysuccinimide. The antibody solution (0.04 μg/ml in 10 mM sodium acetate, pH 5.2) was then injected for 5–10 min. Ethanolamine, 1 M, pH 8.5, was used to block remaining activated carboxyl groups. Ligand densities of 100–150 fmol/mm² were reached. Immobilized control peptide was used as a reference surface. Regeneration of the sensor chip was achieved with injection of 10 mM glycine pH 2.5 at 10 μl/min. The SPR signal is displayed as resonance versus time where 1000 RU (resonance units) represent a shift in resonance angle of 0.1° corresponding to a change in surface antibody concentration of ~1 ng/mm². The data were analysed using the BIAevaluation 3.0 software. All sensorgrams were corrected for background and bulk refractive index by subtraction of the reference flow cell signal.

To confirm that the antibody binds specifically to the PSA mimetic, a competition experiment was performed. First, the PSA specific monoclonal antibody 735 (10 nM) was preincubated for 1 h at room temperature with different molar concentrations (0.2, 0.5, 1, 2, 5, 10, 20 and 50 nM) of colomic acid, the bacterial analogue of PSA. Then the antibody/colomic acid solution was injected (5 μl/5 min) to the PSA mimetic-coupled chip. The surface of the sensor chip was then regenerated with 10 mM glycine pH 2.5.

We also used a cyclic PSA mimetic peptide (sequence H-CSSVTAWTTCG-NH₂, Torregrosa et al., 2004) for in vivo and in vitro experiments yielding results similar to those obtained with the linear PSA mimetic.

Animals and surgical procedures

Three-month-old female C57BL/6J mice were obtained from the central animal facility of the Universitätsklinikum Hamburg-Eppendorf. All experiments were conducted in accordance with the
German and European Community laws on the protection of experimental animals. The procedures used were approved by the responsible committee of the State of Hamburg. For surgery, the animals were anaesthetized by intraperitoneal injections of 0.4 mg/kg fentanyl (Fentanyl-Janssen, Janssen-Cilag GmbH, Neuss, Germany), 10 mg/kg droperidol (Dehydrobenzperidol, OTL Pharma, Paris, France) and 5 mg/kg diazepam (Ratiopharm, Ulm, Germany). The right femoral nerve was exposed and nerve transection performed at a distance of ~3 mm proximal to the bifurcation of the nerve into a motor and a sensory branch. A polyethylene tube (3 mm length, 0.58 mm inner diameter; Becton Dickinson, Heidelberg, Germany) was placed between the two nerve stumps and filled with PBS containing scaffold peptide that forms a gel matrix support (0.5% PuraMatrix Peptide Hydrogel, 3D, BD Biosciences, USA), or PBS/scaffold peptide supplemented with either control peptide, linear or cyclic PSA mimicking peptides (200 µg/ml). The cut ends of the nerve were inserted into the tube and fixed with single epineural 11-0 nylon stitches (Ethicon, Norderstedt, Germany) so that a 2 mm gap was present between the proximal and distal nerve stumps. Finally, the skin wound was closed with 6-0 sutures (Ethicon). Ten animals were operated on for each group.

Analysis of motor function

Functional recovery was assessed by single-frame motion analysis (Irintchev et al., 2005). All experiments were performed blindly with regard to type of treatment. To evaluate quadriceps muscle function during ground locomotion prior to operation, mice were accustomed to a classical beam-walking test. In this test, the animal walks unforced from one end of a horizontal beam (length 1000 mm, width 38 mm) towards its home cage located at the other end of the beam. For all mice, a rear view of one walking trial was captured prior to the operation with a high-speed camera (A602fc, Basler, Ahrensburg, Germany) at 100 frames per second and stored on a personal computer in Audio Video Interleaved (AVI) format. The recordings were repeated 1, 4, 8 and 12 weeks after nerve transection. The video sequences were examined with VirtualDub software, a video capture-processing utility written by Avery Lee (free software available at http://www.virtualdub.org). Selected frames in which the animals were seen in defined phases of the step cycle (see below) were used for measurements performed with UTHSCSA ImageTool 2.0 software (University of Texas, San Antonio, TX, USA, http://ddsdx.uthscsa.edu/dig/). Two parameters were measured: the foot-base angle (FBA) and the heels-tail angle (HTA). The FBA, measured at toe-off position, is defined by a line dividing the sole surface into two halves and the horizontal line (Fig. 2A and B). The angle is measured with respect to the medial aspect. The HTA is defined by the lines connecting the heels with the anus and measured when one leg is in the single support phase and the contralateral extremity has maximum swing altitude (Fig. 2C and D). The angle is measured with respect to the dorsal aspect. Both parameters are directly related to the ability of the quadriceps muscle innervated by the motor branch of the femoral nerve to keep the knee joint extended during contralateral swing phases. As a relative measure of functional recovery at different time-points after nerve injury, we calculated the stance recovery index (RI), which is a measure of the RI for the FBA and HTA. The RI for each angle is calculated in per cent as:

$$RI = \left[ \frac{(X_{\text{pre}} - X_{\text{den}})}{(X_{\text{pre}} - X_{\text{den}})} \right] \times 100,$$

where $X_{\text{pre}}$, $X_{\text{den}}$, and $X_{\text{eine}}$ are values prior to operation, during the state of denervation (7 days after injury), and at any given time-point during reinnervation, respectively.

Retrograde labelling and evaluation of motoneuron number and soma size

Following the last video recording, mice were re-operated for retrograde labelling. Under fentanyl/droperidol/diazepam anaesthesia (see above), the two nerve branches were transected ~5 mm distal to the bifurcation, and two fluorescent dyes, Fast Blue (EMS-Chemie, Großumstadt, Germany) and Fluoro-Gold (Fluorochrome, Denver, CO, USA), were applied to the motor and sensory branches, respectively. One week later, the animals were perfused with 4% formaldehyde (Sigma-Aldrich, Steinheim, Germany) in 0.1 M sodium cacodylate buffer, pH 7.3, and spinal cords and femoral nerves were dissected for morphological analyses.

After overnight fixation, the lumbar part of the spinal cord was cut transversely (serial sections of 50 µm thickness) on a Leica vibratome VT1000S (Leica Instruments, Nußloch, Germany). The sections were examined under a fluorescence microscope (Axioskop 2, Zeiss, Germany) with appropriate filter sets. All cell profiles labelled with one of the dyes or with both tracers are distributed within a stack of 35–45 serial cross-sections. Each section, containing typically 2–5 labelled cell profiles, was examined using a 40× objective by focusing through the section thickness starting from the top surface. All profiles except those visible at the top surfaces of sections were counted (Simova et al., 2006). The application of this simple stereological principle prevents double counting of labelled cells and allows an unbiased evaluation of cell numbers, which does not rely on assumptions or requires corrections. The same sections were used for measurements of soma size using Neurolucida software (see below).

Analysis of degree of myelination

After fixation with formaldehyde, femoral nerves were post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.3, for 1 h at room temperature, dehydrated and embedded in resin according to standard protocols. Transverse 1 µm-thick sections from the motor and sensory nerve branches were cut (Ultramicrotome, Leica) at a distance of ~3 mm distal to the bifurcation and stained with 1% toluidine blue/1% borax in distilled water. Total numbers of myelinated axons per nerve cross-section were estimated on an Axioskop microscope (Zeiss) equipped with a motorized stage and Neurolucida software-controlled computer system (MicroBrightField Europe, Magdeburg, Germany) with a 100× oil objective. Axonal and nerve fibre diameters were measured in a random sample from each section. For sampling, a grid with a line spacing of 30 µm was projected into the microscope visual field using the Neurolucida software. Selection of the reference point (zero coordinates) of the grid was random. For all myelinated axons crossed by or attaching the vertical grid lines through the sections, mean orthogonal diameters of the axon (inside the myelin sheath) and of the nerve fibre (including the myelin sheath) were measured. The mean orthogonal diameter is calculated as a mean of the line connecting the two most distal points of the profile (longest axis) and the line passing through the middle of the longest axis at right angle (Irintchev et al., 1990). The degree of myelination was estimated by the ratio axon to fibre diameter (g-ratio).

Schwann cell culture

Mouse Schwann cells were isolated from peripheral nerves (sciatic and femoral nerves) and dorsal root ganglia (DRG) of 7-day-old
C57BL/6J mice and nerve- and DRG-derived Schwann cells were cultured separately. Tissues were dissected, washed once with ice-cooled Ham’s F-12 (PAA Laboratories, Colbe, Germany) and then incubated with 0.25% trypsin and 0.1% collagenase (Sigma) at 37°C for 30 min. After enzymatic digestion, tissues were washed twice with ice-cooled Ham’s F-12 medium and then suspended in 1 ml Ham’s F-12 medium containing 0.01% DNase (Sigma). Mechanical digestion was performed using fire-polished Pasteur pipettes, and cells were suspended in 5 ml Ham’s F-12 medium, added on top of 5 ml 4% bovine serum albumin (BSA, fraction V, PAA Laboratories) cushion and centrifuged for 10 min at 4°C and 500 g. Finally, Schwann cells were suspended in fresh pre-warmed (37°C) medium and plated on coverslips (Nunc, Roskilde, Denmark) coated with poly-γ-lysine (PLL 0.01%, Sigma), or PLL coated coverslips that were additionally covered with different coatings: control peptide (100 μg/ml), PSA mimicking peptide (100 μg/ml) or laminin (10 μg/ml, Sigma). The medium used for Schwann cell culture contained DMEM high glucose/Ham’s F-12 (1:1) (PAA Laboratories), 60 ng/ml progesterone (Sigma), 16 μg/ml putrescine (Sigma), 5 μg/ml insulin (Sigma), 0.4 μg/ml l-thyroxine (Sigma), 160 ng/ml sodium selenite (Sigma), 10.1 ng/ml triiodothyronine (Sigma), 38 ng/ml dexamethasone (Sigma), 100 U/ml penicillin (PAA Laboratories), 100 μg/ml streptomycin (PAA Laboratories) and 2 mM l-glutamine (PAA Laboratories).

Analysis of process elongation

Schwann cells were plated at a density of 50,000 cells/ml. Inhibitors of the fibroblast growth factor receptor (FGFR) were added to the culture 1 h after seeding. Two inhibitors were tested, SU5402 (VWR International, Darmstadt, Germany) and PD173 074 (Parke-Davis, Ann Arbor, MI, USA) at a final concentration of 50 μM and 500 nM, respectively. PD173 074 exhibits a high degree of specificity towards the growth factor receptor in the nanomolar range (Mohammadi et al., 1998; Niethammer et al., 2002). SU5402 also inhibits the growth factor receptor tyrosine kinase, but is less specific, because it weakly affects the PDGF receptor (Mohammadi et al., 1997). L1Fc (10 μg/ml) was used in one experiment as a positive control, as L1 is known to stimulate axonal growth via FGFR (Doherty and Walsh, 1996). Twenty-four hours after seeding and incubation at 37°C, cells were fixed with 2.5% glutaraldehyde (Agar Scientific, Stansted, UK) and stained with 1% methylene blue/toluidine blue (Sigma) in 1% borax. The length of processes was measured by using the Axiovert microscope and the AxioVision imaging system 4.6 (Zeiss). The length of all processes per cell was averaged. At least 150 cells per treatment were analysed.

Analysis of Schwann cell proliferation in vitro

Schwann cells were plated at a density of 250,000 cells/ml onto different substrates. The cells were cultured in the presence of neuregulin (12 ng/ml, ImmunoTools, Friesoythe, Germany). Four hours after seeding, 20 μM BrdU (Sigma) was added to the culture. Cells were cultured for 48 h and then fixed for immunostaining with 4% formaldehyde in 0.1 M phosphate buffer, pH 7.3. After incubation for 30 min with 2 N HCl at 37°C, cells were washed, blocked with normal goat serum and incubated overnight with mouse primary antibody G3G4 (anti-BrdU; 1:100; Developmental Studies Hybridoma Bank, Iowa City, IA, USA). The secondary antibody, goat anti-mouse (1:200, Santa Cruz, Heidelberg, Germany), was applied for 1 h at room temperature. The coverslips were finally washed and mounted with Fluoromount-G (SouthernBiotech, Birmingham, UK).

Analysis of process elongation

To estimate numbers of proliferating cells, 10 photographs per treatment were taken from different areas of the coverslip using an Axioptoph 2 microscope (Zeiss) and a 20× objective. Each area was photographed using phase contrast and epifluorescence. The two digital images were then overlaid using the Image J software (http://rsbweb.nih.gov/ij/download.html) and Image Tool 2.0 software (University of Texas, San Antonio, TX, USA, http://ddsdx.uthscsa.edu/dig/) was used to count proliferating (BrdU-positive) Schwann cells and total number of Schwann cells. Schwann cells in culture have a long spindle-shaped cell body and two processes in opposite directions (Fig. 8A), which makes them easily distinguishable from other contaminating cells (DRG neurons and fibroblasts). We counted ~1000 cells for each experimental value.

Staining of live Schwann cells

Schwann cells were cultured on coverslips coated with PLL (Sigma). Twenty-four hours after seeding, cells were washed three times with the culture medium and incubated on ice for 20 min with the primary antibodies: mouse monoclonal antibody 735 (provided by R. Gerardy-Schahn) and rat monoclonal antibody H28 against NCAM (provided by V. Sytnyk), both diluted in medium containing 5% fetal bovine serum (PAA laboratories). After staining with primary antibodies, cells were washed and incubated at room temperature for 25 min with secondary antibodies goat anti-mouse and goat anti-rat diluted (1:200 and 1:100, respectively; Jackson ImmunoResearch, West Grove, PA, USA) in culture medium containing 5% fetal bovine serum. After washing with PBS, cells were fixed with 4% formaldehyde for 20 min at room temperature, washed again three times with PBS, and then blocked with PBS containing 5% normal goat serum and 0.2% Triton X-100 for 30 min at room temperature. Primary rabbit polyclonal antibody against S-100 (Schwann cell marker; Shearman and Franks, 1987) (diluted 1:500 in PBS; Dako, Glostrup, Denmark) was applied at 4°C overnight. Cells were then washed and secondary goat anti-rabbit antibody (1:100; Jackson ImmunoResearch) was applied for 1 h at room temperature. Finally, cells were washed with PBS, mounted with Fluoromount-G (SouthernBiotech), and images were taken with an Olympus Fluoview 1000 microscope (Olympus, Hamburg, Germany).

Schwann cell proliferation in vivo

Three-month-old female C57BL/6J mice underwent femoral nerve transaction and surgical reconstruction followed by application of either control peptide (n = 5) or PSA mimic peptide (n = 5) as described above. In addition, all mice received intraperitoneal injections of BrdU (Sigma, 200 mg/kg body weight) at Days 2 and 5 after the operation. At the sixth day after injury, mice were perfused with 4% formaldehyde in cacodylate buffer, and the distal nerve stumps were removed, post-fixed overnight at 4°C and cryoprotected by overnight infiltration with sucrose (Sigma, 15% solution in cacodylate buffer, 4°C). Nerves were then cut on a cryostat (Leica) and transverse sections of 10 μm thickness were collected immediately distal from the level of bifurcation of the motor and sensory branch. For immunostaining, sections were incubated in 0.1 M HCl at 60°C for 20 min and then rinsed three times with 50 mM glycine (Merck, Germany) for 10 min each. After 1 h blocking with 5% normal goat serum in PBS at room temperature, sections were incubated with a mixture of rat anti-BrdU (1:200, Abcam, Cambridge, MA, USA) and rabbit anti-S-100.
(1:15000, Dako, Denmark) for 2 days at 4°C. After washing, goat anti-rat and goat anti-rabbit IgG diluted 1:200 (Jackson ImmunoResearch) were applied for 2 h at room temperature. Sections were washed in PBS, stained with bis-benzimide (nuclear staining), washed again and mounted with Fluoromount-G (SouthernBiotech). Images of the femoral nerve were taken on a Olympus Fluoview 1000 microscope with a 20x objective. Analysis of proliferating cells was performed using the Neurolucida system and a 100x oil objective. Cells that were double-labelled with S-100 and BrdU were considered as proliferating Schwann cells.

Results

Similarity between the linear PSA mimicking peptide and the PSA glycan

Analysis of the affinity of PSA mimicking peptide to the antibody against PSA was performed by SPR. Biacore measurements showed a strong signal for the injection of the PSA mimicking peptide (120 resonance units) to 20 nM of PSA antibody 735, while the binding of the scrambled and control peptide was negligible (20 and 0 resonance units, respectively) (Fig. 1A).

Figure 1B shows that the PSA mimicking peptide and colominic acid compete for binding to the 735 antibody. With increasing colominic acid concentration, the binding between the antibody and the PSA mimicking peptide decreased. Additionally, in vitro functional assays, such as neurite outgrowth of DRG and motor neurons from embryonic mice were performed and confirmed that addition of the PSA mimetic to the culture medium induces the same stimulatory effects as colominic acid (data not shown).

PSA mimetics promote functional recovery after femoral nerve injury in adult mice

Damage of the femoral nerve in mice induces changes in gait which can be precisely evaluated by two parameters, the HTAs and the FBAs, measured on single frames of video sequences recorded during beam walking (Fig. 2A–D). These alterations are caused by impaired extensor function of the quadriceps muscle leading to abnormal external rotation of the ankle and high-heel position at defined gate cycle phases shown in Fig. 2A–D. We used these parameters to evaluate the effect of PSA mimetics on locomotor recovery. One week after injury, the degree of functional impairment, as evaluated by the increase of the FBA (Fig. 3A) and decrease of the HTA (Fig. 3B) compared with the preoperative values, was similar in three groups of mice treated with a linear PSA mimicking peptide, control peptide or vehicle (PBS). After the first week, the angles in all three groups of mice gradually returned to the preoperative values, but recovery was incomplete even after 12 weeks (Fig. 3A and B). However, improvement in PSA mimetic-treated mice was better compared with the control groups at 8 and 12 weeks after injury (Fig. 3A and B). As estimated by the stance RI, a measure of the individual degree of recovery calculated for both angles, the outcome of femoral nerve repair at 3 months after injury was significantly better in PSA mimetic-treated mice than in mice receiving control peptide or vehicle ($P<0.05$, ANOVA with Tukey’s post hoc test, Fig. 3C).

To further verify that the positive functional effects of the novel linear peptide are related to its PSA mimicking properties, we also analysed locomotor recovery in a group of mice which received a previously characterized cyclic PSA mimetic peptide (peptide p1 in Torregrossa et al., 2004). As demonstrated by the time course and final degree of recovery, peptide p1 had a similar positive effect to the linear PSA mimetic (Fig. 3D). Thus, the overall results show that PSA glycomimetic treatment leads to a superior functional outcome.

The PSA mimetic does not influence precision of motor reinnervation and motoneuron survival

In search of structural correlates of the superior functional outcome after application of PSA mimetics, we performed retrograde labelling of regenerated motoneurons in the animals analysed.
functionally over a 3-month recovery period. The number of motoneurons back-labelled through the motor (quadriceps) or the sensory (saphenous) branch only, or through both branches of the femoral nerve, as well as the total number of labelled neurons (sum of the three previous categories) was similar in PSA mimetic-, control peptide- or vehicle only-treated mice (Fig. 4). Since numbers of retrogradely labelled motoneurons reflect the extent of motoneuron survival (de la Cruz et al., 1994; Waters et al., 1998; Asahara et al., 1999), we can conclude that treatment with the PSA mimetic did not reduce motoneuron death, a characteristic feature of the femoral nerve injury paradigm in mice (Simova et al., 2006). Also, the numbers of motoneurons projecting aberrantly to only the sensory branch were similar to those projecting correctly to the motor branch of the femoral nerve, irrespective of the treatment of the animals (Fig. 4). Therefore, in all experimental groups of mice there was no PMR, i.e. higher numbers of motoneurons regenerated into the appropriate motor nerve branch (Brushart, 1988). PMR is a characteristic feature of the femoral nerve injury paradigm in rats (Brushart, 1988; Al Majed et al., 2000a). In mice, this phenomenon has also been reported (Franz et al., 2005), but it has also not been observed, in particular in inbred C57BL/6j mice that were used in this study (Robinson and Madison, 2003; Simova et al., 2006; Ahlborn et al., 2007). In conclusion, our results show that better functional recovery in PSA mimetic-treated mice cannot be attributed to better motoneuron regeneration, since neither motoneuron loss was reduced nor precision of motor reinnervation was improved as compared with control mice.

We have previously found significant correlations between RI and motoneuron size at 3 months after femoral nerve injury (Simova et al., 2006; Ahlborn et al., 2007). Therefore, we measured soma size of motoneurons regrowing their axons correctly and found no differences among the groups treated with PSA mimetic, control peptide or PBS (Fig. 5). Similarly, no differences between experimental groups were found for soma size of motoneurons abnormally projecting to the sensory branch of the femoral nerve (Fig. 5). Therefore, we also cannot attribute better functional recovery in PSA mimetic-treated mice to larger motoneuron size, an indicator of a better functional state of regenerated motoneurons (Simova et al., 2006; Ahlborn et al., 2007).

The PSA mimetic improves the quality of axonal regeneration

We next analysed regenerated nerves morphometrically to assess axonal numbers, axonal diameters and degree of myelination. In both the motor and sensory branches of the femoral nerve, we found similar numbers of myelinated axons in PSA mimetic-treated mice and control mice 3 months after nerve injury (data not shown). Also, no differences among the experimental groups were found for axonal diameters (data not shown). However, a remarkable effect was found on the relative degree of myelination, evaluated by the g-ratio (axon- to fibre-diameter ratio) (Fig. 6A and B). The frequency distributions of the g-ratio in both the motor and sensory branches of PSA mimetic-treated nerves were significantly shifted towards lower values, indicating thicker myelin sheaths of the regenerated axons, compared with control mice. Importantly, as compared with intact nerves, the g-ratios in the PSA mimetic-treated motor nerve branch were normal, and even better than normal in the sensory nerve branch (Fig. 6A and B). Thus, considering all morphological results, the major effect of the PSA treatment was enhanced remyelination.

The PSA mimetic influences shape and proliferation of Schwann cells in vitro

The fact that the PSA mimetic improved myelination in vivo suggested an effect on Schwann cells. To test this hypothesis, we separately cultured Schwann cells from two sources: DRG and sciatic/femoral nerves of early postnatal C57BL/6j mice. Morphometric analysis revealed that Schwann cells had longer processes when cultured on substrate-coated PSA mimetic compared with cultures grown on PLL or control peptide (Fig. 7B). We also checked the effect of the PSA mimetic on Schwann cell proliferation by analysis of BrdU incorporation and found that Schwann cell proliferation was enhanced by 26% in the presence of the PSA mimetic compared to the control peptide (Fig. 8A and B). This result is in good agreement with previous
observations that colominic acid enhances proliferation of Schwann cells in vitro (Bruns et al., 2007; Haile et al., 2007). Both effects of PSA reported here, i.e. enhanced process elongation and cell proliferation, were observed regardless from which source Schwann cells were isolated, DRG or peripheral nerves.

To obtain insights into the cell recognition mechanism that underlies the PSA effects on cultured Schwann cells, we first tested whether these effects were dependent on the expression of NCAM, the glycoprotein carrier of PSA on the Schwann cell surface. We isolated Schwann cells from NCAM-deficient mice and tested their response to the PSA mimetic. In contrast to the wild-type cells, NCAM-deficient Schwann cells were not stimulated by the PSA mimetic. Neither process elongation (Fig. 7C) nor cell proliferation (Fig. 8C) was stimulated in NCAM-deficient Schwann cells. To rule out that process elongation of NCAM-deficient Schwann cells was generally impaired,

Figure 3 Time course of motor recovery after femoral nerve lesion. (A–B) Shown are mean values ±SEM of FBAs (A) and HTAs (B) at different time-points after femoral nerve injury and application of linear PSA mimicking peptide (PSA), control peptide (CON) or vehicle (PBS). Preoperative values are plotted at Day 0. Numbers of animals studied per group are indicated in B. (C) Individual animal values (circles) and group mean values (horizontal lines) of the stance RI calculated for the HTAs and the FBAs at 3 months after injury. A RI of 100% indicates complete recovery. Asterisks indicate significant differences (P<0.05, one-way ANOVA with Tukey’s post hoc test) between the PSA mimetic-treated group and the control groups at the given time points. n indicates the number of animals operated per group. (D) Comparison of motor recovery in mice treated with two different PSA mimicking peptides. Shown are mean values ±SEM of stance recovery indices at different time points after femoral nerve transection and application of linear PSA mimicking peptide (PSA), cyclic PSA mimicking peptide (cPSA) and control peptide (CON) or vehicle (PBS). Asterisk and cross-hatches indicate significant differences (P<0.05, one-way ANOVA with Tukey’s post hoc test) between the PSA-treated groups and the control groups at the given time points.
cells were grown on laminin, which is known to strongly stimulate process formation. NCAM-deficient and wild-type Schwann cells showed similar process elongation (Fig. 7B and C), demonstrating that NCAM-deficient Schwann cells are generally responsive to stimulatory agents. As Schwann cells express both PSA and NCAM at the cell surface and on processes (Fig. 9), the PSA mimic acts on NCAM present at the Schwann cell surface or disrupts a PSA-dependent interaction of NCAM with itself or PSA-NCAM interaction partners. To further understand the influences of the PSA mimetic on Schwann cell growth, we used inhibitors of the FGFR, a receptor involved in NCAM-dependent neuritogenesis (Doherty and Walsh, 1996). Application of two different FGFR inhibitors (PD173 074 and SU5402) abolished the stimulatory effect of PSA on process elongation in wild-type Schwann cells (Fig. 10A and B). Moreover, SU5402 inhibited the stimulatory effect also of L1 (Fig. 10B), which is known to interact

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**Figure 4** Analysis of regrowth of motoneuron axons after injury. Mean numbers of motoneurons (+SEM) labelled through the motor branch (correctly projecting ‘CP’), through the sensory branch (incorrectly projecting ‘IP’), through both branches (double labelled ‘DL’) and the sum of neurons in the first three categories (total number ‘Total’) 3 months after femoral nerve injury and application of linear PSA mimicking peptide (PSA), control peptide (CON) or vehicle (PBS). No significant differences between group mean values were found ($P > 0.05$, one-way ANOVA with Tukey’s post hoc test). Nine animals were studied per group.

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**Figure 5** Analysis of soma size of regenerated motoneurons. Shown are mean values of soma area (+SEM) of correctly and incorrectly projecting motoneurons after application of linear PSA mimicking peptide (PSA), control peptide (CON) or vehicle (PBS). No significant differences between group mean values of regrown axons for a given type of projecting neurons were found ($P > 0.05$, one-way ANOVA with Tukey’s post hoc test). However, within each animal group, correctly projecting motoneurons have significantly larger soma sizes than incorrectly projecting motoneurons (**$P < 0.01$**). Six animals were studied per group.

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**Figure 6** Analysis of myelinated nerve fibres in regenerated and intact nerves. Shown are normalized frequency distributions of g-ratios (axon/fibre diameter) in the motor (A) and sensory (B) nerve branches of the femoral nerve of intact mice (‘Intact’) and mice treated with linear PSA mimicking peptide (PSA), control peptide (CON) or vehicle (PBS) after femoral nerve injury. Regenerated nerves were studied 3 months after injury. The number of axons analysed per nerve and nerves analysed per animal group ranged between 60–75 and 5–6, respectively. The distribution for the PSA mimicking peptide-treated nerves is significantly different from that of the CON and PBS groups in A and from the other groups in B ($P < 0.05$, Kolmogorov–Smirnov test). Six nerves per group were used for measurements.
with the FGFR to stimulate neuritogenesis (Doherty and Walsh, 1996).

The PSA mimetic enhances Schwann cell proliferation in vivo

After determining the effect of the PSA mimetic on myelination in vivo and on Schwann cells in vitro, we examined its effect on cell proliferation in vivo. We estimated Schwann cell proliferation using S-100 and BrdU immunostaining of sections from the motor and sensory branch 6 days after nerve transection and application of control peptide or PSA mimetic. The analysis was performed using sections collected immediately distal to the bifurcation of the femoral nerve, i.e. at ~3–4 mm distal to the cuff containing PSA or control peptide. In both the motor and the sensory branch, we found higher numbers of S-100 cells that have incorporated BrdU,
applied at Days 2 and 5 after operation, in PSA-treated mice than in control treated animals (Fig. 11). This finding indicates that the PSA mimetic enhances Schwann cell proliferation during the period of Wallerian degeneration and that this effect is not restricted to the immediate vicinity of the nerve cuff in which the mimetic has been applied, most likely by diffusion out of the cuff, where the mimetic is not covalently bound to the scaffold peptide.

**Discussion**

This study provides first evidence that PSA mimicking peptides are beneficial agents for treatment of peripheral nerve injuries. After a single application of the mimetics performed during surgical reconstruction of the injured femoral nerve of adult C57BL/6J mice, we observed strongly improved axonal remyelination and enhanced functional outcome as measured by recovery of locomotor behaviour. As indicated by results of cell culture experiments, the likely mechanism underlying these effects is NCAM-mediated stimulation of Schwann cell process elongation and proliferation.

### Improvement of motor function by PSA mimic

We attribute the observed effects to the structural similarity of the mimicking peptides with the PSA glycan itself and thus to PSA-related functional effects. Lack of effects of a control peptide with undetectable binding to the monoclonal PSA antibody 735 indicates PSA-induced specificity. More importantly, both a linear and a cyclic PSA mimicking peptide of different amino acid sequences, but high affinities for the PSA antibodies 735 (linear peptide) and MenB (cyclic peptide), were effective. The evidence for better recovery of function was obtained using an objective
video-based analysis of parameters directly related to the quadriceps muscle function that has proved to be reliable and sensitive (Irintchev et al., 2005; Eberhardt et al., 2006; Simova et al., 2006; Ahlborn et al., 2007). Therefore, better recovery of functional parameters after glycomimetic treatment, observed in two independent experiments using different PSA mimetics, shows that functional improvement is related to the treatment. We have observed similar positive effects on recovery after femoral nerve lesion in mice using HNK-1 mimicking peptides (Simova et al., 2006). The time course of improvement is similar...
for the HNK-1 and PSA mimetics, with positive effects becoming apparent later, i.e. at 2–3 months after injury and treatment. Based on results of our morphological analyses, we previously attributed the HNK-1 effects to trophic support of the regenerating motoneurons indicated by reduced motoneuron death and increased motoneuron soma size and diameters of regenerated axons. Other positive HNK-1 effects were increased precision of target motor reinnervation and enhanced axonal remyelination. Here, such trophic effects were not observed with the PSA mimetics. The only marked structural effect of the PSA mimetic treatment was better remyelination, and this effect was more pronounced than the one achieved by HNK-1 mimetic application. Therefore, the question arises whether better remyelination alone can explain enhanced motor recovery. We believe that this explanation is plausible since reduced remyelination has been considered as a major factor limiting recovery after peripheral nerve lesions (Smith and Hall, 1980; Chen et al., 2007). Thus, our observations not only on improved locomotor recovery, but also improved remyelination after injury are of interest from a therapeutic point of view.

**Mode of PSA mimetic action**

Another issue is how a single intra-operative application of a peptide can produce late-appearing and long-lasting effects. Although a fibrin coat is formed around a nerve-guide channel after 24–48 h preventing leakage of the guide’s contents (Hekimian et al., 1995) and a gel-forming matrix was used to immobilize the PSA mimetic, its local availability is most likely limited to hours or at best days, because of degradation by peptidases originating from the damaged tissue and infiltrating proteolytically active immune cells. We favour the explanation that the PSA mimetic causes priming of the injured femoral nerve, both at and around the lesion site, in the sense that early cellular and molecular responses to injury are modulated so that the subsequent regeneration process is favourably influenced over weeks. Considering our *in vitro* and *in vivo* data on the enhanced proliferation and process elongation by Schwann cells, we propose that the PSA mimetic stimulates the early response of these cells to injury *in vivo*. In support of this notion is our recent observation that enhanced Schwann cell proliferation during the first week after femoral nerve injury in L1-deficient mice leads to better functional recovery in these mice compared with wild-type littermates (Guseva et al., in press). The idea of priming the nerve at and around the lesion site is supported by previous observations of long-lasting positive effects after a single intra-operative application of an HNK-1 mimicking peptide to the severed femoral nerve of adult mice (Simova et al., 2006). In this case, we proposed that priming is achieved by activation of the RAGE (receptor for advanced glycation end-products) signalling pathway (Chou et al., 2004). Another example for strikingly long-lasting effects of a short-term postoperative treatment is brief low-frequency electrical stimulation (1 h, 20 Hz) of the proximal nerve stump of the femoral nerve immediately after nerve transection and surgical repair. This treatment significantly shortens the period of asynchronous—and staggered over weeks—axonal regrowth after femoral nerve lesion in rats (Al-Majed et al., 2000a; Brushart et al., 2002) and accelerated functional recovery after femoral nerve lesion in mice (Ahlborn et al., 2007). These beneficial effects are associated with an accelerated and enhanced up-regulation in expression of brain-derived neurotrophic factor (BDNF) and its tyrosine kinase B (TrkB) receptor in motoneurons which results from the depolarization of motoneuron cell bodies during the 1 h stimulation period (Al-Majed et al., 2000b, 2004).

**Possible molecular mechanisms underlying the glycomimetic effects on myelination**

What are the mechanisms by which priming by PSA mimetics function? PSA is associated with NCAM (Bock, 1987; Olsen et al., 1993) and was first considered, because of its negative charges, to be anti-adhesive resulting in inhibition of NCAM functions (Rutishauser et al., 1988). PSA is also a positive modulator of NCAM function, since specific removal of PSA from NCAM by endoneuraminidase N is associated with an inhibition of NCAM function as for instance inhibition of long-term potentiation and reduction of axonal growth (Doherty et al., 1990; Lüthi et al., 1994; Muller et al., 1996). Based on the interpretation of Walsh and Doherty (1997), PSA modulates NCAM function by inhibiting the *cis*-interactions of NCAM in the plane of the plasma membrane, thus preventing the formation of stable, signal-transducing NCAM clusters at the cell surface. This would make more NCAM available for *trans*-interactions (for instance with NCAM, L1 and proteoglycans), which would promote axonal growth and synaptic plasticity. Considering these properties of PSA, we propose that the observed effects of PSA mimetics *in vivo* and *in vitro* are due to PSA interactions with PSA-NCAM on Schwann cells or to disruption of inhibitory PSA-NCAM/receptor interactions. This notion is supported by the fact that Schwann cells of NCAM−/− mice did not respond, in contrast to wild-type cells, to PSA mimetic stimulation *in vitro*. An interaction of the PSA mimetic with PSA-NCAM could lead to the activation of NCAM signalling pathways, which involve a tyrosine kinase receptor, FGFR (Doherty and Walsh, 1996; Walsh and Doherty, 1997; Kolkova et al., 2000; Kiselyov et al., 2003). FGFRs are likely mediators of the effects of PSA mimetics as they are activated by NCAM, expressed on Schwann cells (Grothe et al., 2001) and involved in myelination through the phosphatidylinositol-3-kinase (PI3K) pathways (Ogata et al., 2004). Activation of PI3K-Akt pathways in Schwann cells increases myelination in Schwann cell/DRG neuron co-cultures and allogenic nerve graft experiments *in vivo* (Ogata et al., 2004). Moreover, expression of FGFRs by Schwann cells is increased after sciatic nerve injury (Grothe et al., 2001), and FGFR-3-deficient mice display reduced myelin thickness compared to wild-type controls (Jungnickel et al., 2004). Our finding that application of two different inhibitors of the FGFRs lead to reduction of process outgrowth from Schwann cells cultured in presence of the PSA mimetic strongly supports the idea of a NCAM/FRGR-mediated mechanism of PSA mimetic action.

Our results show that PSA is involved in processes that go beyond its classical role as an anti-adhesive epitope and reveal a promising therapeutic potential of PSA mimetics for peripheral...
nerve injury. Previous studies have shown that PSA and NCAM mimicking peptides diffuse well in the adult central nervous system and facilitate learning and memory (Cambon et al., 2004; Florian et al., 2006). Furthermore, promotion of remyelination has been suggested as a promising therapeutic strategy to treat patients with demyelinating diseases (Fan and Gelman, 1992). Thus, PSA mimetics useful in peripheral nervous system regeneration are likely to be also beneficial for central nervous system regeneration. This notion is currently under investigation.

Acknowledgements

We are grateful to Emanuela Szpotowicz for excellent technical assistance, Geneviève Rougon (CNRS 6156, Marseille, France) for the cyclic PSA mimicking peptide, Rita Gerardy-Schahn (Medizinische Hochschule Hannover, Germany) for the 735 antibody and Vladimir Sytnyk (Zentrum für Molekulare Neurobiologie, Hamburg, Germany) for the H28 antibody. Melitta Schachner is New Jersey Professor of Spinal Cord Research.

Funding

Deutsche Forschungsgemeinschaft.

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