Adeno-associated virus-mediated L1 adhesion molecule expression ameliorates inhibitory glial scar formation and promotes axonal regeneration and functional recovery after spinal cord injury in adult mice

Jian Chen¹, Junfang Wu¹, Andrey Irintchev², Ivayla Apostolova², Malgorzata Skup³, Sebastian Kügler⁴, and Melitta Schachner¹,²

¹W. M. Keck Center for Collaborative Neuroscience, Department of Cell Biology and Neuroscience, Rutgers the State University of New Jersey, Piscataway, New Jersey 08854, USA
²Zentrum für Molekulare Neurobiologie Hamburg, Universitätsklinikum Hamburg-Eppendorf, Universität Hamburg, 20251 Hamburg, Germany
³Laboratory for Reinnervation Processes, Department of Neuropysiology, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland
⁴DFG Research Center Molecular Physiology of the Brain at Department of Neurology, University of Goettingen, 37073 Goettingen, Germany

Correspondence should be addressed to (schachner@biology.rutgers.edu)
Abstract

Lack of permissive molecules and abundance of inhibitory molecules in the local lesion environment of adult mammals after spinal cord injury prevent axons from successful regeneration and, therefore, contribute to the failure of satisfactory functional recovery. Taking advantage of adeno-associated viral vector (AAV) transduction, we expressed the neural cell adhesion molecule L1 in the spinal cord of adult mice after compression injury. Expression of L1 was observed up to 10 mm rostral and 10 mm caudal to the lesion center 5 weeks after infection, the longest time period studied. L1 expression was not detectable in the fibronectin-positive lesion core. Overexpression of L1 improved motor functional recovery, enhanced serotonergic axonal regeneration, and prevented corticospinal tract axons from degenerative retraction. The expression of the neurite outgrowth inhibitory chondroitin sulfate proteoglycan NG2 was drastically decreased in AAV-L1 treated spinal cords, along with reduction of the reactive astrogial marker GFAP, suggesting that besides acting as a neurite-ougrowth promoting molecule, overexpression of L1 in all neural cell types of the spinal cord promotes axonal regrowth, sparing of severed axons, and functional recovery by reducing inhibitory mechanisms. These beneficial functions may be attributed to intracellular signaling cascades triggered by and in L1 overexpressing cells in the lesioned spinal cord. In vitro experiments confirm that L1 inhibits astrocyte proliferation, migration, process extension, and GFAP expression.

Key words: adeno-associated virus; astrocyte; axonal regeneration; compression; GFAP; L1; locomotor recovery; NG2; spinal cord injury
Introduction

Functional recovery after spinal cord lesion in humans is an important goal in restorative medicine. Strategies used to enhance recovery include neutralization of inhibitory cues in the adult central nervous system of mammals that prevent regeneration (Filbin, 2003; McGee and Strittmatter, 2003; Silver and Miller, 2004; Buchli and Schwab, 2005) or interventions promoting axon regeneration, neuronal survival and synaptic plasticity (Schachner, 1997). The neural recognition molecule L1 favors conducive processes in an inhibitory environment (Castellani et al., 2002; Roonprapunt et al., 2003; Xu et al., 2004; Chen et al., 2005b; Zhang et al., 2005). Inhibition of L1.1 function in the successfully regenerating spinal cord of zebrafish leads to impairment of axonal regrowth and recovery of swimming after injury (Becker et al., 2004). L1 promotes neurite outgrowth in a homophilic (i.e. self-binding) manner and such homophilic interactions not only promote neurite outgrowth and neuronal migration, but also neuronal survival (Lindner et al., 1983; Rathjen and Schachner, 1984; Moos et al., 1988; Lemmon et al., 1989; Appel et al., 1993; Beggs et al., 1997; Kamiguchi and Yoshihara, 2001; Kleene et al., 2001; Dong et al., 2002; 2003). L1 is involved in modification of synaptic efficacy as well as in learning and memory (Luthi et al., 1996; Fransen et al., 1998; Tiunova et al., 1998; Pradel et al., 2000; Law et al., 2003; Venero et al., 2004). L1 also promotes myelination in the central and peripheral nervous systems (Seilheimer et al., 1989; Wood et al., 1990; Haney et al., 1999; Barbin et al., 2004), and thus could promote remyelination after spinal cord injury.

Application of a fusion protein containing the extracellular domain of L1 and human Fc promotes locomotor recovery in adult rats after contusion spinal cord injury (Roonprapunt et al., 2003). Furthermore, retinal ganglion cell axons regrow in an L1 conducive environment after optic nerve transection (Xu et al., 2004). Embryonic stem cells overexpressing L1 support regrowth of corticospinal tract axons and survive better compared to non-transfected stem cells in the injured spinal cord of adult mice (Chen et al., 2005b). As a next step towards therapeutic application of L1 after spinal cord injury in humans, we expressed full length murine L1 in the lesioned mouse spinal cord and monitored recovery of motor functions. Application of the recombinant virus, which efficiently infects neural cells and has successfully been used in clinical trials (Mandel and Burger, 2004; Mandel et al., 2006), was performed after lesion and thus represents a feasible therapeutic approach. Enhanced functional recovery and regrowth of serotonergic axons, reduced dying-back of corticospinal tract axons, reduced expression
of the neurite outgrowth-inhibitory chondroitin sulfate proteoglycan NG2, and reduced astrogliosis as indicated by glial fibrillary acidic protein expression were observed. Furthermore, L1 mediated changes in signal transduction pathways influencing expression of growth-related molecules in regrowing axons and/or cells residing in the lesioned spinal cord. The combined results encourage further use of L1 in lesion paradigms, not only relating to spinal cord injury, but also in other types of central nervous system trauma.
Materials and methods

Antibodies and reagents

The following antibodies and reagents were used: rat anti-L1 monoclonal antibody 555 (Appel et al., 1995), mouse anti-NeuN, rat anti-MBP, rabbit anti-NG2, rat anti-serotonin (5-HT) (Chemicon, Temecula, CA, USA), rabbit anti-GFAP (DakoCytomation Denmark, Glostrup, Denmark), mouse anti-GFAP (Sigma, Saint Louis, MI, USA), mouse anti-Numb (this antibody developed by Dr. Catherine Saner was obtained from the Developmental Studies Hybridoma Bank), mouse anti-Rac-1 and Rho assay reagent (Upstate, Lake Placid, NY, USA), mouse-anti-RhoA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated and total CREB, ERK, PKA, PI3K (Cell Signaling Technology, Danvers, MA, USA), rat anti-mouse fibroblast growth factor receptor 3 and cyclic AMP kit (R&D Systems, Minneapolis, MN, USA), PD98059, U0126 (Sigma, Saint Louis, MI, USA).

AAV vector production

An adeno-associated virus serotype 5 (AAV-5) was constructed to express L1 in the spinal cord. The genome of the recombinant viral vector consisted of the short version (530 bp) of the murine cytomegalovirus immediately early promoter (mCMV promoter) (Bett et al., 1994), the cDNA for murine L1 NH-terminally tagged with the FLAG-epitope and the bovine growth hormone polyadenylation site. As a control, a respective vector expressing EGFP was constructed. It is noteworthy that the AAV-GFP construct contains a WPRE (woodchuck hepatitis posttranscriptional control element) which stabilizes mRNA, resulting in a 2 - 10 fold higher rate of protein expression. This element is not contained in the AAV-L1 virus due to vector size restrictions. Vectors were propagated in 293 cells using pDP5 as helper plasmid (Grimm et al., 2003), and purified essentially as described (Malik et al., 2005) with the exception that Q-FF anion-exchange columns (Amersham) were used for FPLC. After dialysis, genome titers were determined by quantitative PCR, purity by SDS-gel electrophoresis and infectious titers by transduction of cultured primary brain cells as described (Kügler et al., 2003). Genome particles to transducing units (t.u.) ratio was 25:1 - 35:1.

Animal surgery

All surgical procedures and post-operative care were approved by the local authorities following the guidelines of the European Community and the National
Institutes of Health (USA). Female C57BL/6J mice, 3 months old, were deeply anaesthetized by intraperitoneal injection of Ketanest (20%, Parke-Davis, Berlin, Germany) and Rompun (8%, Bayervital, Leverkusen, Germany), 0.01ml/g body weight. Laminectomy was performed at the T7-T9 level with Mouse Laminectomy Forceps (Fine Science Tools, Heidelberg, Germany). The spinal cord was lesioned using a mouse spinal cord compression device (Curtis et al., 1993), and the viral constructs AAV-L1 or AAV-GFP (3 x10^7 titer, 1 microliter) were injected into the lesion site after compression injury as described previously (Chen et al., 2005b). The skin was then sutured. After the operation, mice were held on a heated cushion before they were returned to their home cages. The animals were subjected to evaluation of motor function at certain time points, and sacrificed for histology or biochemical measurements after the last evaluation for motor function.

For anterograde labeling of the corticospinal tract, 3 animals from each group were re-operated 10 days before sacrifice as described previously (Chen et al., 2005b). In brief, three 1 mm diameter holes were drilled through the cranium overlying the sensorimotor cortex (coordinates: -0.5 mm, -1.75 mm, -3.0 mm Bregma, 2 mm lateral, 2 mm deep). A glass micropipette was used to inject 2 µl 10% Fluoro-ruby (dextran tetramethylrhodamine 10,000 MW, lysine fixable, Molecular Probes Europe BV, Leiden, Netherlands) into the sensorimotor cortex. Ten days after application of Fluoro-ruby, animals were sacrificed with an overdose of sodium pentobarbital and perfused with 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4. Longitudinal spinal cord sections were collected consecutively. To quantify the axon regrowth, the rostral border of the lesion site demarcated by highest levels of GFAP immunostaining (Chen et al., 2005a), was chosen as the reference point for measurement (Fig. 1). Highest levels of GFAP immunoreactivity were seen at the boundary between glial scar and fibronectin immunoreactive core of the lesion. The distance between the rostral border and the furthest detectable axon tips of the corticospinal tract in all consecutive sections was measured. For measurement of regrowth of serotonergic (5-HT) immunoreactive axons from the brainstem consecutive cross-sections of the spinal cord were taken starting 5mm rostral to the GFAP demarcated boundary and ending 5mm caudal to the lesion site.

Evaluation of motor functions
Functional recovery was analyzed in AAV-L1 and AAV-GFP treated animals (N = 15 and 14, respectively). The recovery of ground locomotion was evaluated using a mouse rating scale, the Basso mouse score (BMS) (Engesser-Cesar et al., 2005). In addition motor recovery was analyzed using a novel approach for numerical assessment of different aspects of motor behavior (I. Apostolova, A. Irintchev and M. Schachner, unpublished observations). This method includes evaluation of 4 parameters in three different tests: beam walking, voluntary movements without body weight support and inclined ladder climbing.

Mice were trained to perform a beam-walking test during which they have to walk unforced from one end of a horizontal beam (length 900 mm, width 40 mm) towards their home cage located at the other end of the beam. A left- and a right-side view of each animal during two consecutive walking trials were captured prior to the operation and 1, 2, 3 and 5 weeks after the operation with a high speed video camera of SIMI motion analysis system and analyzed with the affiliated analysis software (SIMI Reality Motion Systems GmbH, Unterschleissheim, Germany). Two parameters, designated foot-stepping angle (FSA) and rump-height index (RHI), were measured. The foot-stepping angle is defined by a line parallel to the dorsal surface of the hind paw and the horizontal line. The angle is measured with respect to the posterior aspect at the beginning of the stance phase in intact mice. In severely disabled mice dragging behind their hind extremities, video frames for analysis were selected from “step cycles” delineated by the forelimbs. In less disabled animals, the angle was measured upon dorsal or ventral placement of the paw on the ground after a swing phase or after a forward sliding of the paw over the beam surface. Three to five measurements per animal, extremity and trial were performed. The values for the left and right leg of individual mice were averaged. The foot-stepping angle is a numerical parameter allowing assessment of the plantar stepping ability. Previous work has shown that results for this parameter are in good agreement with rating scale scores (I. Apostolova, A. Irintchev and M. Schachner, unpublished observations). The second parameter evaluated during beam walking, the rump-height index is defined as height of the rump, i.e. the vertical distance from the dorsal aspect of the animal’s tail base to the beam, normalized to the thickness of the beam measured along the same vertical line. For each animal and trial, at least 3 frames in which the rump height was maximal during different “step cycles”, defined according to the stepping ability of the animal were used for measurements. The rump-height index is a numerical estimate of the ability to support body weight during ground locomotion.
A third parameter, the limb extension-flexion ratio (EFL), was evaluated from video recordings of voluntary movements. In the so called pencil test, an intact mouse, when held by its tail and allowed to grasp a pencil with its fore paws, tries to catch the object with its hind paws and performs cycling flexion-extension movements with the hind limbs. After spinal cord injury, the ability to perform such movements is disabled to varying degrees depending on the lesion severity and recovery period. Left- and right side-view videos were recorded for each animal. Using single video frames, the extension and flexion length of the extremity (distance from the most distal mid-point of the paw to a fixed, well discernible point on the animal’s body, e.g. the tail base) were measured for at least 3 extension-flexion cycles per animal and time-point and used to calculate the so called flexion-extension ratio. Mean values for the two extremities from one animal were averaged. The extension-flexion ratio is a numerical estimate of the animal’s ability to initiate and perform voluntary, non-weight-bearing movements.

The fourth parameter was evaluated from video recordings of inclined ladder climbing. The ladder consisted of a 4-mm-thick frame made of a Resopal® plate (Resopal, Groß-Umstadt, Germany, 96 long, 12 cm wide with central incision of 88 x 6 cm) to which 43 round wooden rungs (100 x 2 mm) were glued at equal intervals (2 cm). The ladder was fixed in an inclined position (55°) using a Plexiglas platform. The mice were placed at the bottom rungs of the ladder and climbing was video recorded from a position “below” the ladder, i.e., viewing the ventral aspect of the animals. The video recordings were observed at slow-speed playback and the number of correct steps (correct placing of the hind paw and sustained position until the next forward move) over 36 rungs was counted. Numbers of correct steps made by the two extremities per animal and trial were averaged. This parameter allows numerical assessment of “skilled” locomotion requiring high levels of supraspinal control and proprioceptive control.

Using the original data, we calculated a recovery index for each parameter, animal and time-point according to the formula

$$ RI = \left[ \frac{(X_{7+n} - X_7)}{(X_0 - X_7)} \right] \times 100, $$

where $X_0$, $X_7$ and $X_{7+n}$ are values prior to operation, 7 days after injury, and a time-point $n$ days after the spinal cord injury, respectively. This measure estimates gain of function ($X_{7+n} - X_7$) as a fraction of the functional loss ($X_0 - X_7$) induced by the operation. Overall recovery indices were calculated per animal and time-point as means of the recovery indices for all 5 parameters measured, including the BMS score.
Western blot analysis

Spinal cords from 4 AAV-L1 and 4 AAV-GFP injected mice were used for Western blot analysis. Total protein was extracted by homogenizing and sonicating the tissue in RIPA buffer (Sigma). After centrifugation of the homogenate at 1000g and 4°C the protein concentration in the supernatant was determined by BCA (Pierce Biotechnology, Rockford, IL, USA). The supernatant was denatured by boiling for 5 minutes in SDS sample buffer. Twenty micrograms of total protein was then subjected to 4 - 12% gradient SDS-PAGE. Gels were transferred to poly-vinylidene difluoride membrane and probed with the following antibodies: L1 monoclonal antibody 555 (1:5000 diluted), Numb (1:50), GFAP (1:15000), MBP (1:2000), GAPDH (1:5000), PI3K (1:1000), Rac1 (1:1000), phosphorylated ERK 42/44 (1:500), total ERK 42/44 (1:500), phosphorylated CREB (1:1000), total CREB (1:1000), phosphorylated PKA (1:500), and total PKA (1:500). Secondary mouse, rat, or rabbit antibodies conjugated to horseradish peroxidase with ECL illumination intensification (Pierce Biotechnology) were used for detection.

GTP-RhoA pull-down assay

GTP-RhoA levels in spinal cord homogenates of 4 AAV-L1 and 4 AAV-GFP treated mice were detected using the Rhotekin Rho binding domain reagent according to the manufacturer’s instructions (Upstate, Charlottesville, USA).

Immunohistochemistry and immunocytochemistry

For immunohistochemistry of the spinal cords taken from mice that had been perfused with 4% formaldehyde in 0.1 M phosphate buffer, pH 7.3. Cross-sections (from 3 AAV-L1 and 2 AAV-GFP animals) or longitudinal sections (from 3 animals for each group) were subjected to antigen retrieval with 10mM sodium citrate (pH 9.0, 80°C, 30 minutes). Normal goat serum (5% in phosphate buffered saline, pH 7.3) with 0.2% Triton-X 100 was used for blocking the sections for 1 hour at room temperature. For detection of L1, serotonin, NeuN, MBP and GFAP, monoclonal antibodies to L1 (diluted 1:1000 in the blocking serum solution), serotonin (1:100), NeuN (1:1000), and MBP (1:200), and polyclonal antibody to GFAP (1:500) were applied to the sections overnight at 4°C followed by incubation with Cy2 or Cy3 conjugated anti-mouse, rat or rabbit IgG antibodies. For immunocytochemistry, the coverslips were briefly washed twice with phosphate buffered saline, pH 7.2 (PBS) followed by fixation of the cells with 4%
formaldehyde for 10 minutes at room temperature. After washing with PBS the coverslips were blocked with 5% normal goat serum in PBS containing 0.2% Triton-X 100 for 30 minutes at room temperature followed by incubating the coverslips with primary antibodies at 4°C overnight and then secondary antibodies for 30 minutes at room temperature. All sections and coverslips were finally counterstained with DAPI and, after washing, mounted with Aqua-Poly/mount (Polysciences, Warrington, PA, USA). The slides were then observed by fluorescence (Axiophot2, Zeiss, Jena, Germany) and/or confocal (LSM510mega, Zeiss) microscopy. For quantitative morphological evaluation, the NIH software of ImageJ was used.

Cyclic AMP measurement

Cyclic AMP levels of AAV-L1 and AAV-GFP infected spinal cord homogenates from 4 mice each were quantified using the cyclic AMP ELISA kit according to the manufacturer's instructions (R&D Systems).

Primary culture of astrocytes and in vitro infection

Purified astrocyte cultures were prepared from whole brains of neonatal (P0) C57BL/6J mice. Brains were removed, meninges were peeled off, and the tissue was cut into 1mm³ pieces and dissociated by sequentially passing the pieces through 200μm, 150μm and 30μm filter meshes (VWR GmbH, Darmstadt, Germany). The tissue pieces were then washed three times with pre-warmed DMEM supplemented with 10% fetal calf serum, 0.1mg/ml streptomycin and 10U/ml penicillin (Invitrogen, Carlsbad, CA, USA) and plated at a density of 10⁶ cells/well on poly-L-lysine-coated 12-well-plates. When cells had grown to approximately 50% confluence, AAV-L1 and AAV-GFP were added into the medium (3x10⁷ transducing units (t.u.) /500μl). Ten days after viral transduction, cells were digested with 0.02% trypsin/EDTA (Invitrogen) and subcultured into poly-L-lysine-coated 12-well-plates, 96-well-plates or coverslips for further studies. Transduction efficiency was approximately 50% after one passage.

Astrocyte proliferation assay

Astrocytes transduced with AAV-L1 or AAV-GFP were plated at a density of 20000 cells/well in 200μl DMEM supplemented with 10% fetal calf serum on poly-L-lysine-coated 96-well-plates and grown for 3 days, followed by either changing the medium alone or changing the medium and applying MAPK inhibitors (U0126 10μM; PD98059
10\mu M) or anti-FGFr3 neutralization antibody (10\mu g/ml, R&D Systems). This antibody neutralizes the signal transduction of mouse FGFr3 alpha (IIIb and IIIc) in the presence of all FGF isoforms. Each value was obtained from 6 experiments performed in duplicate. The medium, MAPK inhibitors or FGFr3 antibody were changed three times within a 96-hour interval. Ninety-six hours after the first treatment (which was 24 hours after the last treatment), the cells were subjected to a proliferation assay using the CyQUANT cell proliferation assay kit, according to the manufacturer's instructions (Invitrogen).

**Astrocyte scratch assay**

Astrocytes transduced with AAV-L1 or AAV-GFP were plated in DMEM supplemented with 10% fetal calf serum on poly-L-lysine-coated 24-well-plates and grown to confluence. The medium was then removed, and the monolayer was scratched with a sterile 20-200\mu l plastic pipette tip. The cells were washed twice with pre-warmed medium and maintained for 4 hours and 12 hours of incubation. Cells were fixed with 4% formaldehyde and immunostained for L1 and GFAP, and for DAPI.

**Measurement of GFAP expression in astrocyte cultures**

Four groups of astrocytes were subjected to evaluation of GFAP expression: 1) AAV-L1 transduced astrocytes; 2) AAV-GFP transduced astrocytes; 3) non-transduced astrocytes cultured on L1-Fc; 4) and non-transduced astrocytes cultured on human Fc. AAV-L1 or AAV-GFP transduced astrocytes (1x10^6) were plated into 12-well-plates coated with poly-L-lysine and maintained for 10 days in culture. Astrocytes (1x10^6) were then subcultured into 12-well-plates coated with poly-L-lysine. Non-transduced astrocytes were subcultured into 12-well-plates coated with mouse L1-Fc (10\mu g/ml, Loers et al., 2005) or human Fc (10\mu g/ml, Dianova, Hamburg, Germany). The mouse L1-Fc contains the extracellular domain of mouse L1 in fusion with human Fc. Gel filtration of the L1-Fc fusion protein preparation showed that L1-Fc migrates as a monomer. Cells were cultured in DMEM to confluence, and then lysed and subjected to Western blotting for measurement of GFAP and NG2 expression levels.
Results
Adeno-associated virus mediated expression of target genes in lesioned adult mouse spinal cord

Adeno-associated virus (AAV) has emerged as a powerful transgene delivery vehicle (Flotte, 2004; Shevtsova et al., 2005). AAV-5 vectors expressing transgenes from the murine cytomegalovirus immediate early (mCMV) promoter preferentially transduce glial cells in the brain and in primary cultures and thus should have potential to target the non-permissive environment the neurons have to be faced with in the adult central nervous system (Shevtsova et al., 2005). To investigate whether L1 could thereby be overexpressed in the adult mouse spinal cord, we first looked into whether AAV-5 was capable of stably transducing neural cells in injured spinal cord by introducing AAV-5 encoding green fluorescence protein (GFP) into the lesion site of the spinal cord directly after spinal cord compression injury. Strong GFP signal was found in both rostral and caudal parts of the spinal cord 5 weeks after the operation (Figs. 2, 2A-C). In the core of the lesion site, which is fibronectin immunopositive and GFAP immunonegative (Fig. 1, Chen et al., 2005a), GFP was barely detectable. GFP fluorescence was found not only adjacent to the lesion site in the thoracic spinal cord, but also in the cervical and lumbar spinal cord at distances of at least 10mm away from the center of the lesion site. Thus, the AAV-5 vector is capable of stably transducing neural cells in the injured spinal cord for at least 5 weeks, a time period sufficient to evaluate its effect on spinal cord lesion. It not only diffuses over a considerable distance but also transduces all neural cell types (see below). Expression of GFP was detectable two weeks after infection, peaking at three to four weeks after transduction.

We then investigated whether the full-length L1 cDNA (AAV-L1) could also be introduced into neural cells. We used a highly specific monoclonal L1 antibody 555 which does not recognize the close homologue of L1 (CHL1). Immunoreactivity with this antibody in the non-lesioned adult mouse spinal cord is barely detectable. The transduction of AAV-L1 was comparable to AAV-GFP in terms of extent and longevity of L1 expression 5 weeks after spinal cord injury and viral transduction (Fig. 2D-G), with the knowledge that L1 expression is weaker than GFP expression because AAV-GFP contains the WPRE (woodchuck hepatitis posttranscriptional control element) which stabilizes mRNA, resulting in a 2 - 10 fold higher level of protein expression in comparison to L1 expression.
Previous studies have shown that AAV-5 transduces cell types with different efficiency (Shevtsova et al., 2005). We used different neural cell type-specific markers to detect which cell types were transduced by the AAV vector in the injured spinal cord. We observed transgenic expression in AAV-GFP transduced mice mainly in cells which co-localized with the neuronal marker NeuN (~50% of all transduced cells) and the astrocytic marker GFAP (~30% of all transduced cells). A few transduced cells also co-localized with the oligodendrocyte marker MBP (~10% of all transduced cells). This result differs from that observed in the adult non-lesioned rat brain with the same AAV-5 vector, where mainly oligodendrocytes, but only few neurons or astrocytes were transduced (Shevtsova et al., 2005). These results suggest that successful targeting of the AAV vectors is at least partially dependent on the local context.

**AAV-L1 improves recovery of motor functions**

To evaluate the motor functions after spinal cord injury, we adopted the Basso Mouse Scale (BMS) (Engesser-Cesar et al., 2005), as well as a newly developed motion analysis approach using a set of functional parameters evaluating different types of voluntary movement skills (I. Apostolova, A. Irintchev and M. Schachner, unpublished observations, 2006). Spinal cord compression caused severe disabilities in both AAV-L1 and AAV-GFP treated mice as indicated by the BMS at 1 week after injury (0.3 ± 0.24 and 0.11 ± 0.16, respectively, P > 0.05, t-test for independent). Between 1 and 5 weeks after compression, the mean score values improved more in AAV-L1 treated than in AAV-GFP treated mice to reach values of 5.1 ± 0.83 and 1.7 ± 0.68 in the two groups, respectively, at 5 weeks (P < 0.01). The time course of BMS recovery is shown in Fig. 3A using recovery indices. The recovery index is a relative measure that normalizes, on an individual animal basis, the degree of recovery after the first week post-lesion to the degree of disabling caused by the trauma (difference between the values prior to operation and 7 days thereafter). Analysis of variance for repeated measurements with subsequent Tukey post-hoc tests, both using indices (Fig. 3A) and score values (not shown) revealed better recovery at 3 and 5 in the AAV-L1 group. In addition to the BMS, we analyzed the plantar stepping ability of the animals using a novel parameter, the foot-stepping angle. Since the mean values prior to and at 7 days after injury were similar in the two animal groups, alike those for the BMS score and all other parameters described below, we show all data using recovery indices only. Analysis of the foot-stepping angle also revealed, in agreement with the BMS scores, enhanced recovery in AAV-L1
compared to AAV-GFP treated mice at 3 and 5 weeks (Fig. 3A). These results clearly indicate, based on use of two independent measures, that AAV-L1 application improves the abilities for ground locomotion after spinal cord injury. Good agreement between rating scale scores and the foot-stepping angle has also been observed previously (I. Apostolova, A. Irintchev and M. Schachner, unpublished observations).

We also evaluated more complex, compared to plantar stepping, motor functions using novel parameters. We analyzed the rump-height index, a measure of the ability to support body weight during ground locomotion, ability requiring activity and coordination of muscles working at different joints in both extremities. In AAV-GFP treated mice the degree of recovery at 5 weeks was close to zero, despite improved values seen at 2 and 3 weeks (Fig. 3C). Most likely, the transient increase at 2 – 3 weeks is due to spasticity rather than to functional improvement (see also I. Apostolova, A. Irintchev and M. Schachner, unpublished observations, for effects of spasticity of the rump-height index). In contrast to the control group, the index values in AAV-L1 treated mice recovered significantly, compared both to zero and to the AAV-GFP group, within the 5-week observation period (Fig. 3C). The animals’ ability to perform voluntary movements without body weight support, estimated by the extension-flexion ratio, was not significantly affected by the kind of treatment, AAV-L1 or AAV-GFP application (Fig. 3D). The same conclusion was reached for numbers of correct steps made by the animals during inclined ladder climbing (Fig. 3E). The ladder-climbing test allows estimation of the ability to perform precise movements requiring highest degree of supraspinal control as compared to the types of movement assessed by the other tests. From the values of the parameters shown in Fig. 3A-E we calculated, on an individual animal basis, overall recovery indices for each animal (Fig. 3G) and group mean values (Fig. 3F). This analysis revealed an overall better outcome in mice treated with AAV-L1 compared to AAV-GFP application.

**AAV-L1 promotes serotonergic fiber regrowth and prevents corticospinal tract axons from degenerative retraction 5 weeks after injury**

To investigate the morphological basis and possible mechanisms which may contribute to the motor recovery, we first studied the serotonergic descending axons in the injured spinal cord. The serotonergic (5-HT) fibers originate in the medullary raphe nuclei and the reticular formation (Shapiro, 1997). The 5-HT system provides tonic
stimulation of the pattern generators which can mediate complex motor functions such as walking (Foster et al., 1989). We stained 5-HT fibers by immunohistochemistry and demarcated the lesion site by GFAP immunostaining (Fig. 4). In all three mice having received AAV-L1 we found robust 5-HT fibers growing into the lesion site. In series of longitudinal sections, fibers had reached the caudal border of the lesion site, extended beyond the border and entered the caudal part of the spinal cord. In contrast, in mice having received AAV-GFP, the 5-HT fibers could only occasionally be observed in the lesion site and caudal to the lesion site. This result was confirmed in consecutive cross-sections of the caudal part of the spinal cords. Six hundred micrometers caudal to the lesion site, robust 5-HT positive fiber bundles were found in white and grey matter in the AAV-L1 treated spinal cords (Fig. 4I, K). In contrast, only very few fibers were observed in either white or gray matter at the same level in the AAV-GFP treated mice (Fig. 4J, M).

We also anterogradely labeled corticospinal tract axons with rhodamine-conjugated dextran Fluoro-ruby (Fig. 5). After injury the proximal segments of corticospinal tract axons had retracted about 500 μm back from the lesion site (Houle and Jin, 2001). This “dying back” allows detection of both positive and negative treatment-related effects such as enhanced axonal regrowth towards the lesion site or reduced retraction. The distance between the tips of the Fluoro-ruby labeled axons and the rostral border of the lesion site demarcated by GFAP immunostaining was measured and used as an indicator of the extent of retrograde axonal degeneration. This distance was significantly shorter in AAV-L1 infected spinal cords (228 ± 21 μm) compared to AAV-GFP infected spinal cords (1207 ± 183 μm, P < 0.01), indicating that overexpression of L1 protects the corticospinal tract axons from retraction in a hostile environment.

AAV-L1 alters the cellular components in spinal cords 5 weeks after injury

The injured spinal cord undergoes reactive astrogliosis and progressively forms a glial scar around the lesion site, which is widely considered as an important obstacle to the regenerating axons (Fig. 1). GFAP immunoreactive astrocytes highly express chondroitin sulfate proteoglycans which are considered mainly as neurite outgrowth inhibitory molecules (Fitch and Silver, 1997). NG2, a main inhibitory member of this family, is highly expressed in reactive astrocytes (Fidler et al., 1999), oligodendrocyte progenitors, and macrophages (Jones et al., 2002). To evaluate the influence of AAV-L1 on reactive astrogliosis and NG2 expression, homogenates obtained from tissue pieces
with a length of 500µm spinal cord, which contained the lesion site in the middle, were subjected to Western blotting using antibodies against GFAP and NG2. We observed a marked decrease in GFAP and NG2 expression levels (Fig. 6). Normalized to GAPDH, GFAP expression in the AAV-L1 treated spinal cord was 0.45 ± 0.03, while in the AAV-GFP treated spinal cord it was 1.28 ± 0.07 (P < 0.01). NG2 expression level in the AAV-L1 treated spinal cord was 0.42 ± 0.02, and NG2 in the AAV-GFP treated spinal cord it was 0.76 ± 0.03 (P < 0.01). This suggests that L1 overexpression is capable of limiting astrogliosis and of downregulating an inhibitory astrocytic component.

We found one of the MBP isoforms, the larger 21.5 kD isomer, significantly more upregulated in the AAV-L1 group (0.50 ± 0.06) than in the AAV-GFP group (0.30 ± 0.02, P < 0.05, Fig. 6). The other MBP isomers (18.5 kD, 17.0 kD and 14.0 kD) showed no significant difference between the two groups. Since oligodendrocytes are not the major cell type to be infected by the AAV constructs, upregulation of the 21.5 kD isoform of MBP was unexpected. Since the 21.5 kD isoform is the earliest to become expressed during myelination and is re-expressed in remyelinating lesions in multiple sclerosis (Capello et al., 1997), upregulation of this 21.5 kD isoform may reflect the ability of L1 to promote remyelination after spinal cord injury.

Numb is a cell fate determinant which regulates neurogenesis by antagonizing the activity of the Notch receptor. We previously reported that Numb mRNA levels were upregulated after spinal cord injury in adult mice (Chen et al., 2005a). Numb was found to improve neurite outgrowth by promoting L1 endocytosis at the growth cones (Nishimura et al., 2003). This observation suggests that the L1 and Numb signaling pathways may affect each other in regulating neurite outgrowth. To investigate whether L1 overexpression alters Numb expression, we measured levels of Numb expression in AAV-L1 and AAV-GFP transduced spinal cords (Fig. 6). We indeed found that the Numb expression was higher in AAV-L1 (0.32 ± 0.03) than in AAV-GFP transduced spinal cords (0.17 ± 0.02, P < 0.01).

**AAV-L1 activates MAPK/ERK, CREB and Rac1, and inhibits RhoA activation**

To investigate the mechanisms underlying the reduced astroglial reaction, we studied the pathways which had been implicated in signal transduction mediated by L1 (Fig. 7). In neurons, L1 activates mitogen-activated protein kinases (MAPKs), PI3K and PKA (Loers et al., 2005). One of the pathways via which L1 promotes neurite outgrowth is activation of MAPK (Schmid et al., 2000). To study whether L1 overexpression would
activate endogenously expressed MAPK, PI3K and PKA pathways *in vivo*, transduced spinal cord homogenates were analyzed by Western blotting with antibodies recognizing the phosphorylated/activated and non-phosphorylated/total forms of the extracellular-regulated kinases ERK1 and ERK2, and PI3K and PKA. We found that AAV-L1 enhanced phosphorylation of ERK1/2 (phosphorylated/total value: 0.41 ± 0.02) when compared with the AAV-GFP group (phosphorylated/total value: 0.31 ± 0.01, P < 0.05), while the total form of ERK1/2 showed no significant difference between the AAV-L1 and AAV-GFP groups (7.6 ± 0.98 vs. 6.88 ± 0.34, P > 0.05). In contrast, phosphorylation of PKA was not different in the two groups. However, the total level of PKA expression was elevated in the AAV-L1 group compared with the AAV-GFP group, causing a decrease in the ratio of the phosphorylated/total value in the AAV-L1 group (1.14 ± 0.04 vs. 1.59 ± 0.15, P < 0.05). Total PI3K levels were also elevated in the AAV-L1 group (0.28±0.01) compared with the AAV-GFP group (0.19±0.02, P < 0.05). We did not detect phosphorylated PI3K in the AAV-L1 nor AAV-GFP groups.

Previous observations had suggested that GFAP levels in glial cells are affected by the cAMP response element binding protein (CREB) through interacting with transcription factors, which interact with the AP-1 DNA binding sequence in the GFAP promoter (Pennypacker et al., 1996). Furthermore, the neural cell adhesion molecule (NCAM) had been shown to stimulate phosphorylation of ERK1/2 and CREB (Schmid et al., 1999). The CREB phosphorylation induced by NCAM was dependent on the MAPK pathway (Schmid et al., 1999). Furthermore, activation of CREB promoted dorsal root ganglion axon regeneration and improved recovery of locomotor functions in a rat spinal cord injury model (Gao et al., 2004). To study whether L1 overexpression and activation of ERK1/2 would stimulate phosphorylation of CREB, spinal cord homogenates were subjected to Western blotting with antibodies against phospho-CREB and total-CREB. The ratio of phosphorylated/total levels of CREB was indeed significantly higher in the AAV-L1 group (4.18 ± 1.17) than in the AAV-GFP group (1.20 ± 0.20, P < 0.05). This correlation is consistent with the idea that exogenous L1 expression may increase CREB activation.

Injecting a cyclic AMP analogue into lesion site improves axonal regeneration and functional recovery in a rat spinal cord injury model (Lu et al., 2004; Pearse et al., 2004; Spencer and Filbin, 2004). We thus quantified cyclic AMP levels by ELISA (Fig. 8) and indeed found differences between the AAV-L1 (1.90 ± 0.05 pmol/ml) and AAV-GFP (1.48 ± 0.09 pmol/ml, P < 0.05) treated groups.
L1 triggered activation of the ERK1/2 pathway has been shown to induce cell motility-associated gene products, among them the small GTPases Rac-1 (Silletti et al., 2004), which also stimulates neurite outgrowth in vivo (Yip et al., 1998; Schmid et al., 2000; Causeret et al., 2004). We observed that Rac1 expression levels were considerably higher in AAV-L1 transduced spinal cords versus AAV-GFP transduced spinal cords (0.46 ± 0.07 vs. 0.38 ± 0.01, P < 0.05).

The small GTPase RhoA is another regulator of the actin cytoskeleton in neurites and its activation results in growth cone collapse, neurite retraction, and neurite growth inhibition (Lehmann et al., 1999; Wahl et al., 2000). Given that chondroitin sulfate proteoglycans inhibit neurite extension via the Rho pathway in the glial scar (Monnier et al., 2003), we investigated whether decreased astrocytic gliosis accompanied by lower NG2 expression levels in the AAV-L1 transduced spinal cords would correlate with a lower activation level of the Rho pathway (Fig. 8). GTP-RhoA was pulled down with the Rhotekin Rho binding domain, and the ratio of GTP-RhoA/total RhoA was calculated. This ratio was significantly lower in the AAV-L1 treated spinal cords (0.15 ± 0.09) compared with the AAV-GFP treated ones (0.42 ± 0.08, P < 0.05), suggesting that RhoA activation is decreased by overexpression of L1.

**L1 inhibits astrocyte proliferation, migration and process extension in vitro**

To further explore the mechanisms underlying overexpression of L1 on reduced astrogliosis in vivo, we investigated proliferation and migration of astrocytes in AAV transduced cultures. AAV-L1 and AAV-GFP were both found to transduce astrocytes in vitro (Fig. 9A). The level of astrocyte proliferation was significantly lower in AAV-L1 than in AAV-GFP transduced astrocytes. Numbers of AAV-L1 transduced astrocytes (97727 ± 5721 per well) were approximately two times lower than in the AAV-GFP transduced astrocytes (181581 ± 11277 per well in the 96-well-plate, one-way ANOVA, P < 0.01). The MAPK inhibitors U0126 and PD98059 did not significantly change the extent of proliferation of AAV-L1 (112109 ± 4390 per well, P > 0.05) and AAV-GFP (176984 ± 10631 per well, P > 0.05) transduced astrocytes, compared with the corresponding untreated cells (Fig. 9B). This result suggests that L1 does not involve the MAP kinase pathway to inhibit the proliferation of astrocytes.

L1 and fibroblast growth factor receptor (FGFr) signaling pathways regulate neuronal survival and neuritogenesis by modulating gene transcription through activation of MAPK, PKA and PKC (Loers et al., 2005). One of the FGFrS, FGFr3, is expressed by
astrocytes and their precursors, and GFAP expression was upregulated in the spinal cords of FGFr3-null mice (Pringle et al., 2003), raising the possibility that activation of the FGFr3 signaling pathway inhibits the expression of GFAP. We thus investigated whether overexpression of L1 activates the FGFr3 pathway to reduce astrocyte proliferation and to downregulate GFAP expression. Taking advantage of a FGFr3 neutralization antibody, we inhibited FGFr3 activity in AAV-L1 or AAV-GFP transduced astrocytes. After treatment of cultures with the antibody for 72 hours, we found that after AAV-L1 transduction astrocyte proliferation was significantly elevated (134860 ± 8493 per well, P < 0.05). However, after AAV-GFP transduction astrocyte proliferation was also elevated by anti-FGFR3 treatment (232077 ± 16710 per well, P < 0.05), compared with non-treated astrocyte cultures (Fig. 9B). Thus L1 and the FGFr may act independently of each other.

To investigate migration of astrocytes, we used an in vitro scratch assay in confluent AAV-L1 and AAV-GFP transduced cultures. After placing the scratch, cultures were maintained for 4 or 12 hours. Four hours after the scratch, more AAV-GFP transduced astrocytes migrated into the denuded area than AAV-L1 transduced astrocytes. Moreover, AAV-GFP transduced astrocytes at the edges of the scratch had extended cellular processes into the scratched area, while their cell bodies had remained at the edge (Fig. 9C). AAV-L1 transduced astrocytes barely extended cellular processes into the free space (Fig. 9C). Twelve hours later, the initially cell-free area (about 300µm) was largely closed by the AAV-GFP transduced astrocytes, while the space in the AAV-L1 transduced cultures had remained empty. These observations show that L1 reduces astrocyte migration in vitro.

**L1 changes GFAP but not NG2 expression levels in astrocyte cultures**

L1 mediates neurite outgrowth through homophilic or heterophilic interactions (Appel et al., 1993; Beggs et al., 1997; Kamiguchi and Yoshihara, 2001). Our in vivo experiments showed that L1 overexpression reduced GFAP expression in the injured spinal cord. To explore whether this reduction is due to homophilic and/or heterophilic interaction of L1, we measured GFAP levels in cultured astrocytes after transduction with AAV-L1 or treatment with substrate-coated L1-Fc. As reported previously, cultured astrocytes do not express L1 as confirmed by Western blotting (Fig. 10) and immunocytochemistry (data not shown). When the astrocytes are cultured on the L1-Fc coated substrate, L1 predominantly affects the putative receptors on astrocytes by
heterophilic binding. However, when AAV-L1 transduced astrocytes express L1 at their cell surface, L1 can bind to L1 molecules on adjacent cells via homophilic (L1 binds to other L1 molecule) or heterophilic (L1 binds to a non-L1 molecule) interactions, or both. AAV-L1 transduced astrocytes showed lower levels of GFAP expression (0.78 ± 0.033) than AAV-GFP transduced astrocytes (1.04 ± 0.080, one-way ANOVA, P < 0.05), the L1-Fc treated group (1.09 ± 0.059), or the Fc treated group (1.08 ± 0.086) (Fig. 10). GFAP levels in astrocytes cultured on the L1-Fc substrate were not different in the L1-Fc and Fc treated groups. These results suggest that L1 reduces GFAP levels by homophilic, but not heterophilic interactions.

Since the NG2 levels were also reduced after AAV-L1 transduction of injured spinal cords in vivo, we investigated whether AAV-L1 transduction or L1-Fc treatment would influence NG2 expression in cultured astrocytes as measured by Western blot analysis. In contrast to the findings on GFAP expression levels, neither AAV-L1 nor L1-Fc treatment affected NG2 expression (data not shown).
Discussion

L1 overexpression mediated by adeno-associated virus transduction beneficially affects spinal cord regeneration.

During nervous system in development L1 is abundantly expressed by growth cones and axons and becomes down-regulated when the brain matures and is, for instance, hardly detectable in the spinal cord of healthy or lesioned adult mice. On the basis of experiments showing that L1 is beneficial to axon regrowth and neuronal survival (Roonprapunt et al., 2003; Xu et al., 2004; Chen et al., 2005b), we have overexpressed L1 in different neural cell types in the lesioned spinal cord using transduction by the adeno-associated virus AAV-5. As opposed to control adeno-associated virus treatment, L1 overexpression significantly improved recovery of locomotion, regeneration and/or sparing of serotonergic descending axons and protection of cortical spinal axons from retraction from the lesion site. Interestingly, AAV-5 transduces the three major neural cell types in the injured spinal cord, most predominantly neurons, followed by astrocytes and oligodendrocytes. The infected area extends up to 10 mm away from the lesion site, both rostrally and caudally. The positive outcome of L1 overexpression is thus likely due to its tipping the balance from an inhospitable environment to a more hospitable one, being presented not only by neurons, but also by otherwise L1 negative glial cells, the astrocytes and oligodendrocytes. Interestingly, the fibroblast-like cells containing lesion core consisting of infiltrating meningeal and/or proliferating vascular endothelial cells was not transduced to overexpress L1. Nevertheless, better regeneration is observed.

Possible mechanisms underlying improved functional recovery after AAV-L1 treatment

Using a set of tests to evaluate different motor abilities, we found significant overall improvement in AAV-L1 treated mice compared to control animals. The enhanced overall motor performance of AAV-L1 treated mice at 5 weeks after lesion was mainly due to better abilities to walk as indicated by three different measurements: the BMS, the foot-stepping angle and the rump-height ratio. In adult mammals, the abilities to stand and walk after spinal cord injury barely recover spontaneously despite preservation of circuitries distal to the injury capable of initiating and controlling rhythmic and coordinated movements (Edgerton et al., 2004; Fouad and Pearson, 2004). Initiation of activity in locomotor pattern generating centers in the injured spinal cord can be
efficiently achieved by pharmacological treatments activating aminergic transmitter systems (Fouad and Pearson, 2004). Among the transmitters effectively influencing locomotion in rodents is serotonin. The serotonergic system in the spinal cord affects rhythmic efferent discharges in hind limb nerves (Viala and Buser, 1969), participates in initiation and modulation of locomotor patterns during walking (Barbeau and Rossignol, 1991), and enhances learning-related plasticity in the injured spinal cord (Crown and Grau, 2005). The degree of serotonin innervation of the injured spinal cord correlates with motoneuron excitability as estimated by analyses of H-reflex responses (Lee et al., 2005). Other research has also shown a positive correlation between regrowth of serotonergic axons and degree of functional recovery (Ribotta et al., 2000; Pearse et al., 2004; Fouad et al., 2005). We observed robust serotonergic axonal regrowth in the spinal cords of AAV-L1, but not AAV-GFP treated mice, which could well explain the enhanced improvement of walking associated with exogenous L1 expression. AAV-L1-associated expression of L1 may also positively influence lesion-induced alterations in synaptic connectivity. The importance of such alterations in the injured spinal cord has recently been indicated by studies on enhanced structural plasticity of synaptic inputs to motoneurons in mice deficient in the growth-inhibiting molecule tenasin-R correlating with better recovery of the animals' walking abilities (I. Apostolova, A. Irintchev and M. Schachner, unpublished observations). In contrast to walking, no significant effects of the AAV-L1 treatment were found for motor abilities requiring higher levels of supraspinal control as evaluated by the extension-flexion ratios and performance during ladder climbing. These findings are consistent with our observation that cortico-spinal tract axons, although retracting less in AAV-L1 treated mice than in AAV-GFP treated mice, fail to regenerate beyond the lesion site indicating that the AAV-L1 treatment does not promote regrowth of these types of severed myelinated axons.

L1 affects signal transduction mechanisms in regrowing axons and/or surrounding tissue

We observed elevated levels of cyclic AMP, phosphorylated CREB and MAPK in AAV-L1 treated spinal cords. These elevations are likely to reflect the function of L1 in transduced cells rostral and caudal to the lesion site and possibly lesioned neurons. Cyclic AMP is capable of improving axonal regrowth and functional recovery after spinal cord injury by local administration (Pearse et al., 2004; Spencer and Filbin, 2004). Also activated CREB promotes spinal axon regeneration (Gao et al., 2004). Activated MAPK
mediates regulation of neurite outgrowth and regeneration (Chierzi et al., 2005). Activated MAPK transiently inhibits phosphodiesterase 4, the enzyme that hydrolyses cAMP (Gao et al., 2003). From our present experiments we cannot determine whether L1 overexpression is more beneficial to the neural cells in the local lesion context or whether it acts mainly through the regenerating axonal tracts, whose cell bodies locate in brain. It is thus unknown whether phosphorylation of MAPK or CREB is beneficial in the local motoneurons or interneurons and glial cells. However, it is likely that CREB can regulate astrogliosis by binding to the AP-1 DNA binding sequence in the GFAP gene, thereby regulating GFAP expression and thus reactive astrogliosis (Pennypacker et al., 1996).

RhoA is at a convergent point of many inhibitory molecules’ pathways. Inhibitory molecules, such as Nogo-A, myelin associated glycoprotein, ephrins, semaphorins, and chondroitin sulfate proteoglycans can activate the RhoA pathway, hence block regrowth of axons (Mueller et al., 2005). Studies in EphA4-deficient mice have shown that RhoA activation correlated with low capacity of spinal axon regeneration and high reactive astrogliosis (Goldshmit et al., 2004). In the present study, we found that GTP-RhoA levels were decreased in L1 overexpressing spinal cords, while the total RhoA levels were not changed.

Numb is an important cell fate determinant which was originally identified as an antagonist of Notch signaling (Uemura et al., 1989). Although Numb is barely detectable in adult rodent spinal cords, it is highly elevated after spinal cord injury (Chen et al., 2005a). Numb is associated with L1 and functions in endocytosis of L1 in growth cones to promote axonal growth (Nishimura et al., 2003). We here report that L1 overexpression is able to elevate Numb levels in the injured spinal cord, providing further evidence for a relationship between Numb and L1.

**L1 overexpression reduces changes in reactive astrogliosis in vivo and in vitro**

Astrocytes are inhibitory to growing axons when presented to these axons in the vicinity of the fibronectin immunoreactive lesion core. *In vitro* experiments have shown that L1-modified astrocytes provide to axons a more permissive substrate for growth (Adcock et al., 2004). We here report that L1 overexpression limits reactive astrogliosis and downregulates the expression of the main inhibitory chondroitin sulfate proteoglycan NG2 *in vivo*. Upregulation of the intermediate filament protein GFAP is indicative of morphological changes of activated astrocytes, such as hypertrophy of cellular
processes (Pekny et al., 1999) and thus astrogliosis (Pekny and Pekna, 2004). It is not known whether decreased GFAP expression in astrocytes is directly linked to promoting axon regrowth (Wang et al., 1997; Xu et al., 1999; Menet et al., 2000, 2001). Our observation that L1 overexpressing spinal cords show lower GFAP expression strongly suggests a less severe reactive astrogliosis, and hence an ameliorated microenvironment at the lesion site. Another observation vindicating a more friendly environment is that NG2 is downregulated in L1 overexpressing spinal cord. NG2 is upregulated predominantly in astrocytes, oligodendrocyte precursors and macrophages after spinal cord injury and RhoA/ROCK are the downstream effectors of its inhibitory effect (Monnier et al., 2003). Our observation that RhoA activation is decreased in L1 overexpressing spinal cords is consistent with the downregulation of NG2 and amelioration of reactive astrogliosis.

To study the mechanisms that underlie L1 mediated reduction in astrogliosis, we investigated the effect of L1 on astrocyte proliferation and migration in vitro. Firstly, we showed that proliferation of L1 overexpressing astrocytes is lower than of the control transduced astrocytes. This difference is not changed by adding either MAPK inhibitors or neutralization antibody against the FGF receptor3, which was reported to play an important role in astrocyte development and in inhibiting GFAP expression (Pringle et al., 2003). These results suggest that the inhibition of astrocyte proliferation by L1 does not depend on the MAPK signaling pathway or FGF receptor3 activity. Secondly, we used the scratch assay to investigate migration and process extension of astrocytes in vitro. Indeed, L1 expression reduced migration and process formation of astrocytes, phenomena that may be related to reduced GFAP expression. Although astrocytes cultured from early postnatal brain are different from adult spinal cord astrocytes in vivo, these results may explain why L1 may be able to limit reactive astrogliosis by reducing proliferation, migration and process extension.

Interestingly, overexpressing L1 in astrocytes and culturing of non-virus transduced astrocytes on substrate coated L1-Fc had different effects on GFAP expression levels. Whereas L1 overexpression decreases GFAP expression, L1-Fc does not, suggesting that L1 regulates GFAP expression by homophilic binding. In contrast to GFAP, NG2 expression levels in L1 overexpressing astrocytes and L1-Fc treated astrocytes were not different. It is thus possible that the reduced expression of NG2 in spinal cords in vivo is due to the reaction of other NG2-expressing cell types, such as oligodendrocyte
precursors or macrophages (Jones et al., 2002), with the precaution that astrocytes in vitro and in vivo may also differ in this aspect.

**L1 overexpression enhances expression of myelin basic protein**

Although the oligodendrocyte is not the major cell type transduced by the AAV constructs, we indeed observed that the expression of the larger 21.5 kD isoform of myelin basic protein (MBP) is increased in AAV-L1 versus AAV-GFP treated spinal cords. This isoform was reported to accumulate in nuclei of Hela cells (Staugaitis et al., 1990) and oligodendrocytes (Pedraza et al., 1997) and because of its developmentally early expression it may play an important role in transcriptional regulation and initiation of myelination. Its expression also correlates with remyelination in multiple sclerosis (Capello et al., 1997). Interestingly, L1 was also found to play a role in the initiation of central nervous system myelination by regulating axon-oligodendrocyte interactions (Barbin et al., 2004). It remains to be seen whether L1 overexpression influences expression of 21.5 kD MBP directly or indirectly. Thus, L1 overexpression appears to be beneficial for an important ingredient in recovery after spinal cord injury, namely remyelination.

**Outlook**

In the present study we report that adeno-associated virus is a favorable vector to introduce a beneficial adhesion molecule into the injured spinal cord. By transducing the spinal cord with the cell adhesion molecule L1, we observed improved motor functional recovery, enhanced serotonergic axonal regeneration, and reduced degenerative “dying back” of corticospinal tract axons. We attribute these achievements to the ability of L1 to activate multiple signaling pathways in regrowing neurites and/or surrounding tissue, to limit reactive astrogliosis, and to influence intraspinal circuitry in the rostral and, in particular, caudal parts of the lesioned spinal cord. Results from in vitro experiments agree with the idea that L1 decreases astrocytic proliferation, migration, process extension, and GFAP expression, which are indicators of an astrocytic reaction to injury. Thus, L1 transduction using an adeno-associated viral vector is likely to provide incentives to a therapeutic venue to spinal cord injury in adult mammals, including humans.
Figure legends

Figure 1. Schematic representation of the cellular composition of the lesion site 5 weeks after compression injury of the spinal cord of an adult C57BL/6 mouse. Fibronectin immuno-reactive fibroblasts containing tissue forms a dense cellular core in the center of the lesion. This core center is derived from meningeal and vascular endothelial cells, macrophages and possibly oligodendrocyte precursor cells. Reactive astrocytes form the glial scar surrounding the central core on both sides. Reactive astrocytes are most abundant close to the central core and express high levels of glial fibrillary acidic protein and the chondroitin sulfate proteoglycan NG2. Processes of reactive astrocytes form a boundary with the central core. The area of the lesion site extends over 1 mm on both sides of the lesion center.

Figure 2. Transduction of cells in the adult mouse spinal cord with adeno-associated virus encoding for green fluorescent protein (AAV-GFP) and adeno-associated virus encoding for full-length mouse L1 (AAV-L1) as monitored 5 weeks after compression injury and concomitant injection of viral constructs into the lesion site. A, AAV-GFP shows expression of the fluorescent indicator in the lesion site with highest expression of neurons and astrocytes and non-detectable expression in the fibroblast-like cell containing central core. GFP label is detectable in cells located up to 10mm rostral and caudal to the injection site (arrow). D, C, Examples of GFP labeled cells taken from the left insert in A for B and right insert in A for C. D Immunohistochemical labeling for L1 after transduction with AAV-L1 as described for AAV-GFP in A. L1 is detectable on average up to 10mm away from the virus injection site and is easily distinguished from endogenous L1 expression, since in the adult non-injured and injured mouse spinal cord L1 is only extremely weakly detectable. E-G An L1 immunoreactive large cell body in the position of motoneurons in the ventral horn of grey matter is shown. H-P Double immunohistochemistry of parasagittal sections with markers for the three major neural cell types. H-J NeuN immunoreactive cells represent approximately 50% of all virus transduced cells. K-M GFAP immunoreactive cells represent approximately 30% of all transduced cells. N-P MBP immunoreactive cells taken from the white matter make up approximately 10% of all transduced cells. N and O are taken from a field that is not representative for average values in that more GFP labeled cells are also immunoreactive for MBP. H-P arrows point to double labeled cells. Scale bars: A, D 200μm; B, C, H-P 50μm; E-G 20μm.
Figure 3. Time course and degree of functional recovery after spinal cord compression in mice treated with AAV-L1 or AAV-GFP. Shown are mean values ± S.E.M of recovery indices for open field locomotion scores (Basso mouse score, BMS) A, foot-stepping angles B, rump-height indices C, extension-flexion ratios D, numbers of correct steps E, and overall recovery indices F at 1 to 6 weeks after injury. Individual values of overall recovery indices at 5 weeks are shown in panel G. Number of mice studied per group are given in panel C. Asterisks indicate significant differences between group mean values at a given time-period (p < 0.05, one-way ANOVA for repeated measurements with Tukey post hoc test).

Figure 4. Immunohistochemical staining of serotonin (5-HT) in AAV-L1 and AAV-GFP transduced adult mouse spinal cord 5 weeks after injury. A, B Serotonergic axons show robust staining in AAV-L1 transduced spinal cord in A, whereas these fibers do not cross the lesion site in AAV-GFP transduced spinal cords in B as seen in parasagittal sections (n = 3). In C, GFAP immunoreactive structures are seen abutting onto the central core of the lesion which is GFAP-immunonegative. In D, AAV-GFP transduced structures around the central core of the lesion site which appears to be larger in this mouse than in AAV-L1 transduced spinal cord C are shown in blue. In E and F the overlay of A, C and B, D, respectively are shown. G, H represent higher magnifications of the insets in E, F, respectively. I and J show serotonergic axons in coronal sections of spinal cords 600μm caudal to the lesion site (n = 3 in AAV-L1 group and n = 2 in AAV-GFP group). More serotonergic axons are observed in the AAV-L1 transduced spinal cords in I, K, than in the AAV-GFP transduced spinal cord in J, M. Scale bars in all images: 200μm.

Figure 5. Tracing of the corticospinal tract (CST) after transduction with AAV-L1 and AAV-GFP at 5 weeks after spinal cord injury. Corticospinal tract axons were anterogradely labeled by injection of Fluoro-ruby into the sensorimotor cortex. Parasagittal sections show the presence of labeled axons that remain intact close to the lesion site in A, while they appear retracted from the lesion site in B. Arrows in A and B point to the tips of the anterogradely labeled axons in A and B. Double labeling of the same sections for GFAP shows the border of the lesion site as indicated by the dotted lines in C, D. The overlays of the images A, C and B, D are shown in E and F,
respectively. The distance between the tips of the corticospinal tract and the rostral border of the GFAP immunoreactive lesion site is significantly shorter in AAV-L1 transduced spinal cord than in AAV-GFP transduced spinal cords shown in G. Scale bars for A-F, 400μm, n = 3.

**Figure 6.** Western blot analysis of L1, Numb, GFAP, chondroitin sulfate proteoglycan NG2 and myelin-basic protein (MBP), in AAV-L1 and AAV-GFP transduced spinal cords 5 weeks after injury. Representative Western blots are shown in the left panels. Expression levels of proteins were normalized to GAPDH and relative expression levels are shown in the right panel (n = 4). Error bars represent mean ± S.E.M. P < 0.05.

**Figure 7.** Analysis of components of intracellular signaling pathways in AAV-L1 and AAV-GFP transduced spinal cord at 5 weeks after injury. Representative Western blots of PI3K, Rac1, phosphorylated ERK42/44, ERK42/44, phosphorylated CREB, total CREB, phosphorylated PKA and total PKA are shown in the left panel. Expression levels of proteins were normalized to GAPDH and relative expression levels are shown in the right panel (n = 4). Error bars represent mean ± S.E.M. P < 0.05.

**Figure 8.** Analysis of cyclic AMP levels by using ELISA and RhoA pull-down assay in AAV-L1 or AAV-L1 treated spinal cords 5 weeks after injury. Representative results of GTP-RhoA and total RhoA Western blot analyses are shown in A. Rho activation was evaluated by calculating the ratio between GTP-RhoA versus total RhoA (B, n = 4). Cyclic AMP concentrations in AAV-L1 or AAV-GFP transduced spinal cords are shown in C (n = 4). Error bars represent mean ± S.E.M. P < 0.05.

**Figure 9.** Analysis of proliferation and migration of AAV-L1 and AAV-GFP transduced astrocytes *in vitro*. A AAV-L1 and AAV-GFP transduction shows expression of L1 and GFP in L1 immunonegative astrocytes. Co-labeling by immunocytochemistry with GFAP shows the purity of cultures. Since L1 transduced by AAV is expressed at the surface membrane and in the cytoplasm and GFP is mainly localized as a soluble molecule in the cytoplasm, they do not completely co-localize with cytoskeletal GFAP immunoreactivity. B, AAV-L1 transduction inhibits proliferation of astrocytes as measured by detecting total cellular nucleic acids per well. Treatment of cultures with the
MAP kinase inhibitors U0126 + PD98059 do not interfere with proliferation neither in the AAV-L1 nor the AAV-GFP transduced astrocytes. The FGF receptor3 neutralization antibody enhances proliferation both in AAV-L1 and AAV transduced astrocytes. In C migration of astrocytes or extension of astrocytic processes into the denuded space in the scratch assay is shown. Four hours after placing the scratch, AAV-GFP transduced astrocytes extend cellular processes into the free space (arrowheads), while their cell bodies mostly remain on the edge of the scratch (arrows). Twelve hours after placing the scratch, more AAV-GFP infected astrocytes have migrated into the denuded space than AAV-L1 transduced astrocytes. Error bars represent mean ± S.E.M. P < 0.05. Scale bars: 100μm.

**Figure 10.** Analysis of GFAP levels in AAV-L1 and AAV-GFP transduced astrocytes and astrocytes maintained on substrate coated L1-Fc or Fc. L1 expression is only detectable in AAV-L1 infected astrocytes. Representative Western blots of GFAP expression levels are shown. Error bars represent mean ± S.E.M. P < 0.05.
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