Limited Growth of Severed CNS Axons After Treatment of Adult Rat Brain With Hyaluronidase

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Many chondroitin sulfate proteoglycans (CSPGs) have been shown to influence CNS axon growth in vitro and in vivo. These interactions can be mediated through the core protein or through the chondroitin sulfate (CS) glycosaminoglycan (GAG) side chains. We have shown previously that degrading CS GAG side chains using chondroitinase ABC enhances dopaminergic nigrostriatal axon regeneration in vivo. We test the hypothesis that interfering with complete CSPGs also limit axon growth in vivo. Neurocan, versican, aggrecan, and brevican CSPGs may be anchored within extracellular matrix through binding to hyaluronan glycosaminoglycan. We examine whether degradation of hyaluronan using hyaluronidase might release these inhibitory CSPGs from the extracellular matrix and thereby enhance regeneration of cut nigrostriatal axons. Anesthetized adult rats were given knife cut lesions of the right hemisphere nigrostriatal tract and cannulae were secured transcranially thereby allowing repeated perilesional infusion of saline or saline containing hyaluronidase once daily for 10 days post-axotomy. Eleven days post-transection brains from animals under terminal anesthesia were recovered for histological evaluation. Effective delivery of substance was inferred from the observed reduction in perilesional immunoreactivity for neurocan and versican after treatment with hyaluronidase (relative to saline). Immunolabeling using antibodies against tyrosine hydroxylase was used to examine the response of cut dopaminergic nigral neurons. After transection and treatment with saline, dopaminergic nigral neurons sprouted in a region lacking astrocytes, neurocan and versican. Axons did not regenerate into the lesion surround that contained astrocytes and abundant neurocan and versican. After transection and treatment with hyaluronidase, there was a significant increase in the number of cut dopaminergic nigral axons growing up to 800 μm anterior to the site of transection. However, cut dopaminergic nigral axons still did not regenerate into the lesion surround that contained reduced (albeit residual) neurocan and versican immunoreactivity. Thus, partial degradation of hyaluronan and chondroitin sulfate and depletion of hyaluronan-binding CSPGs enhances local sprouting of cut CNS axons, but long-distance regeneration fails in regions containing residual hyaluronan-binding CSPGs. Hyaluronan, chondroitin sulfate and hyaluronan-binding CSPGs therefore likely contribute toward the failure of spontaneous axon regeneration in the injured adult mammalian brain and spinal cord.

Key words: proteoglycan; nigrostriatal; brain; regeneration; extracellular matrix

After injury in the adult mammalian central nervous system (CNS), cut axons fail to regenerate through regions of scar formation containing putative inhibitory extracellular matrix molecules. Chondroitin sulfate proteoglycans (CSPGs) including NG2, neurocan, versican, aggrecan and brevican are present at sites of CNS injury, at least during the first month post-injury (Levine, 1994; McKeon et al., 1999; Jaworski et al., 1999; Asher et al., 2000; Lemons et al., 2001; Moon et al., 2001; Asher et al., 2002). All of these CSPGs have been shown to limit neurite growth in vitro, depending on context (Asher et al., 2001).

The inhibitory effects of many CSPGs depend upon either their core proteins or upon their chondroitin sulfate glycosaminoglycan side chains (CS GAGs), or both (Iijima et al., 1991; Oohira et al., 1991). Regarding the latter, degradation of CS GAGs using chondroitinase ABC frequently reduces growth inhibition in vitro (Snow et al., 1990; Brittis et al., 1992; Dou and Levine, 1994; McKeon et al., 1995; Smith-Thomas et al., 1995; Powell et al., 1997; Fidler et al., 1999; Niederost et al., 1999), disrupts axon guidance in the embryo (Chung et al., 2000), enhances growth of CNS neurons into peripheral nerve implants (Yick et al., 2000) and enhances regeneration of cut CNS axons in vivo (Moon et al., 2001; Bradbury et al., 2002). At present, however, very few experiments have
examined whether interfering with complete CSPGs also promotes CNS axon growth in vivo.

We examine this possibility. Because several CSPGs (including neurocan, aggregcan, versican and brevican) are bound to the extracellular matrix molecule hyaluronan glycosaminoglycan (hyaluronic acid, hyaluronate) (Margolis and Margolis, 1993), we set out to determine whether degradation of hyaluronan using the enzyme hyaluronidase might interfere with CSPG binding and function, thereby enhancing CNS axon regeneration in vivo. Indeed, it has previously been shown that a single intracerebral injection of hyaluronidase potentiates sprouting of crushed optic nerves in adult rats (Tona and Bignami, 1993). Because hyaluronan-binding CSPGs become more abundant over the weeks after injury (Moon et al., 2002), we test whether administering hyaluronidase once daily for ten days post-injury might potentiate this effect.

Anesthetized adult rats were given unilateral knife cut lesions of the nigrostriatal tract using a Scouten wire knife, and cannulae were secured transcranially allowing repeated perilesional infusion of saline or saline containing hyaluronidase once daily for 10 days post-axotomy. Sections of brains recovered at different times post-transection were immunostained to confirm the specificity of treatments and to visualize the response of cut dopaminergic nigral axons.

MATERIALS AND METHODS

Animals

All procedures were carried out using adult female Sprague-Dawley rats. All animals were treated in accordance with the Animals Scientific Procedures Act, 1986. Animals were bred in-house and kept in groups on a 12/12 hr light/dark cycle and were given food and water ad lib as well as playthings to reduce boredom.

Immunofluorescence Labeling

Unfixed cryosections of intact adult rat brain and spinal cord were immunolabeled to determine appropriate methods for detecting effective degradation of hyaluronan. Animals were terminally anesthetized (Euthatal, 2 ml/kg intraperitoneal, Roche Meriaux, UK) and the brain or spinal cord was dissected out rapidly and immediately frozen on dry ice in plastic molds containing embedding medium (TissueTek, Raymond Lamb Ltd., UK). Tissue was kept at −80°C before cutting sections on a cryostat maintained at approximately −20°C. Sections were mounted onto presubbed glass slides (1.0% gelatin in PBS or Superfrost, BDH, UK) and air dried for approximately 30 min. Sections were stored in airtight containers at −80°C until required.

In attempts to digest hyaluronan in vitro, series of sections were incubated for 3 hr at 37°C in PBS or in PBS containing hyaluronidase (50 μg/ml, bovine testes, Worthington Biochemical Corp., Freehold, NJ). Next, sections were immunolabeled to determine whether hyaluronan had, in fact, been degraded.

One set of sections was incubated in 3% BSA containing biotinylated hyaluronan-binding protein (8 μg/ml, Seikagaku, Japan) before washing and incubating in 1% BSA containing streptavidin conjugated Cy3 (1:200, Amersham, UK) containing 5μM bisbenzamide nuclear dye (Hoescht 33342, Sigma, UK). Nonspecific binding of the biotinylated hyaluronan-binding protein was controlled for using a biotinylated antibody (8 μg/ml, sheep anti-mouse Ig, Amersham, UK).

In all other cases, a standard immunofluorescent protocol was employed. All incubations were carried out at room temperature for 60 min unless stated otherwise. Between each incubation, sections were washed four times in PBS containing 0.05% Tween–20 (polyoxyethylene–(20)–sorbitan monolaurate, BDH, UK). First, sections were blocked using 3% bovine serum albumen (BSA, Fluka, UK) in PBS containing 10 mM sodium azide (Sigma) and 20 mM L-lysine monohydrochloride (BDH, UK). Second, sections were incubated in 3% BSA containing one of the following primary antibodies: mouse monoclonal antibodies CS56 (1:500, Sigma, UK), 2B6 or 3B3 (1:200, Seikagaku, Japan) or mouse monoclonal antibodies against neurocan (1D1, 1:200, Developmental Studies Hybridoma Bank (DSHB), University of Iowa, USA; 1G2, 1:1, DSHB), phosphacan (3F8, 1:200, DSHB), NG2 (D31-10, 1:1, gift of J. Levine) or versican (12C5, 1:1, gift of R. Asher). Nonspecific binding of the primary antibody was controlled for using an isotype- and species-matched antibody used at the same concentration as the primary (IgG1, Sigma; IgM, Sigma). Third, sections were incubated in 1% BSA containing the biotinylated secondary antibody (sheep antimouse Ig, Amersham). Finally, sections were incubated in 1% BSA containing streptavidin conjugated Cy3 (Amersham) containing 5μM bisbenzamide nuclear dye (Hoescht 33342, Sigma). Sections were washed three times in PBS containing 0.05% Tween–20 and once in PBS before coverslipping using fade-reducing mounting medium (PBS containing 50% glycerol, 2.5% DABCO (1,4 diazobicyclo-(2,2,2) octane, Sigma) and 20 mM azide, pH 8.6).

Immunoblotting

Ice-cold Tris-buffered saline (TBS) was prepared using 50 mM Tris–HCl (pH 7.0), a protease inhibitor cocktail (25× in 0.1 M phosphate buffer, pH 7.0, Complete™, Roche, UK) and pepstatin A (2 μg/ml, Fluka, UK). Adult rats were terminally anesthetized (Euthatal, 2 ml/kg intraperitoneal, Roche Meriaux, UK) and brains were quickly dissected out and homogenized in tris-buffered saline containing 0.15 M NaCl. Homogenates were spun at 13,000 rpm for ten min at 4°C and the supernatant (25 ml) was mixed with rehydrated diethyl amino ethyl (DEAE)–cellulose (1 g in 10 ml distilled water) for anion exchange chromatography. This mixture was spun at 3,000 rpm for 5 min at 4°C and the pellet was resuspended in TBS containing 0.15 M NaCl. The spin/resuspend cycle was then repeated four times using, sequentially, TBS containing 0.15 M NaCl, 0.50 M NaCl, 0.75 M NaCl and 1.0 M NaCl. Aliquots of the fraction eluted using TBS containing 0.75 M NaCl were incubated either in TBS or TBS containing either hyaluronidase (5 μg/ml or 50 μg/ml, bovine testes, Sigma, UK) or hyaluronidase (50 μg/ml, bovine testes, Worthington Biochemical Corp.) or chondroitinase ABC (25 μU per sample, Roche, UK) for 3 hr at 37°C. A half volume of 3× Laemmli sample buffer containing sodium dodecyl sulfate (SDS) was added to the samples before boiling for 5 min. Cooled samples were then separated overnight by sodium dodecyl sul-
and lateral coordinates made relative to bregma and vertical coordinates made relative to dura. After surgery, the wound was closed using absorbable sutures (Vicryl 4/0, Ethicon, Edinburgh, UK) and antiseptic powder was applied (Battle, Hayward & Bower Ltd., Lincoln, UK).

After surgery, animals often appeared unkempt due to temporary cessation of grooming and weight loss averaging 20% but recovered within 10 days given the following special care. Animals were handled, inspected and weighed daily both pre- and post-operatively. Postoperative diet was supplemented with dog food and wet mash and to counteract dehydration animals were given 10 ml 4% glucose in 0.18% saline subcutaneously during surgery and thereafter as required. Clean cages were given every 2 days. For analgesia, animals were given soluble paracetamol (1 mg/ml, Cox Pharmaceuticals, UK) in their drinking water for 3 days post-operatively.

**Unilateral Transection of the Nigrostriatal Tract**

The right medial forebrain bundle (including the nigrostriatal tract) of adult rats was transected using an extruding wire ‘Scouten’ knife (David Kopf Instruments). The tip of the wire knife was lowered to the starting point (A −3.0; L +3.0; V −8.0) and the wire blade was extruded. Previous calibration ensured that this procedure forms the blade into a smooth curve in the coronal plane that reaches medially to the midline and ventrally to the base of the brain. The assembly was then withdrawn vertically 4 mm and the blade retracted and reextruded. Finally, the assembly was relowered 4 mm, the blade was retracted and the entire assembly was withdrawn from the brain. This procedure twice transects the right nigrostriatal tract on average 650 μm from the anterior border of the substantia nigra (Brecknell et al., 1995).

**Transcranial Cannulation**

An in-dwelling cannula was secured transcranially to allow repeated infusion of saline or saline containing hyaluronidase into the site of axotomy. Stainless steel tubing (Coopers Needle Works, Birmingham, UK) was used to make in-dwelling cannulae (7 mm 23 ga) and occlusion stylets (12 mm 30 ga stainless steel bent to a 45° angle at the 8 mm mark). In-dwelling cannulae were certified free of obstructions before use by checking patency with the occlusion stylets. A small hole was drilled to allow placement of the cannula (A −2.5; L −2.5; V −3.0).

Stainless steel screws (1.6 mm, Semat Technical UK Ltd.) were inserted into each of three drill holes placed around the cannula site to form an equilateral triangle with sides of length 3 mm. A 5 mm length of 2 ml syringe barrel (Plastipak, Becton Dickinson, UK) was placed around the screws and through this the in-dwelling cannulae were stereotactically lowered 3.0 mm below dura such that 3.0 mm protruded above the 1 mm-thick skull. Quick setting acrylic cement (Simplex rapid acrylic powder immediately premixed with methyl methacrylate, Associated Dental Products Ltd., Swindon, UK) was poured into the syringe barrel thereby firmly securing the in-dwelling cannulae to the intracranial screws and creating a transcranial infusion “cap.” A stylet was inserted into each in-dwelling cannulae to occlude the cylinder when not in use.
Transcranial Infusion

On post-transection days 1–10 inclusive, animals were given a 3 μl transcranial injection of either saline or saline containing 120 μg hyaluronidase (40 mg/ml, bovine testes, Worthington Biochemical Corp.). This concentration was selected on the basis of a pilot study which demonstrated effective hyaluronic degradation in vitro using hyaluronidase (30 μg/ml, bovine testes, Worthington Biochemical Corp.); we therefore decided to administer at least 50 μg hyaluronidase per ml adult rat brain (approximately 2 cm³) to allow for effects of dilution through brain. Aliquots of stock solutions were kept frozen at −70°C and thawed immediately before use.

Infusion cannulae were made from 11 mm stainless steel tubing (30 ga, Coopers Needle Works, Birmingham, UK). The tubing was bent by 45° 4 mm from one end and this short arm tubing (30 ga, Coopers Needle Works, Birmingham, UK). The 10 mm internal diameter, 0.61 mm outer diameter, Portex, UK) to a 30 cm of clear polythene tubing (0.28 mm); this tubing was connected via 30 cm of clear polythene tubing (0.28 mm/H11002) to a nipulator (sp220i, World Precision Instruments, New Haven, CT). Before loading solutions, infusion kits were cleaned using 70% ethanol and sterile 0.9% saline and unpaired flow was verified by checking for bubbles after immersion and flushing with air.

The lack of primary nociceptors in the adult mammalian CNS allows infusions to be made into the awake, unanesthetized animal (restrained gently in a towel) with no observable discomfort. Stylets were removed and the infusion cannula was inserted fully up to the bend through the in-dwelling cannula such that the ventral tip of the infusion cannula reached the relevant infusion site. Infusions were made at 1 μl per minute, allowing 2 min diffusion time before slow removal of the infusion cannulae and reocclusion of the in-dwelling cannula with the stylet. Use of clear polythene tubing allowed delivery of equal volumes of substance to be confirmed by eye.

Histology

Eleven days post-transection, animals were terminally anesthetized. The majority of rats (n = 10) were perfused fixed using 4% paraformaldehyde and consecutive series of 1:12 40 μm-thick parasagittal sections were cut on a freezing microtome and processed for free-floating immunoperoxidase histology as described below. The remaining rats (n = 2) were used to provide unfixed parasagittal cryosections for immunofluorescence histology as described above. Sections were immunolabeled either to visualize axons of the dopaminergic nigrostriatal tract or to establish the effects of hyaluronidase upon various extracellular matrix molecules.

Immunoperoxidase Labeling

Tris buffered saline (TBS, pH 7.4) was prepared using (per litre distilled water) 12 g tris (tris(hydroxymethyl) aminomethane, Sigma) and 9 g NaCl. TXTBS was prepared using 0.2% Triton X-100 (Sigma) in TBS. TNS was prepared using 6 g tris per litre distilled water. All incubations were carried out at room temperature on a rotary shaker while washes between incubations were carried out three times using TBS. First, endogenous peroxidase activity within tissue was quenched by incubating sections for 15 min in distilled water containing 10% methanol and 3% hydrogen peroxide. Second, sections were then washed and blocked for 60 min in TXTBS containing 3% normal goat serum (NGS, Dako, UK). Third, sections were incubated overnight in TXTBS containing 1% NGS and one of the following primary antibodies: rabbit anti-tyrosine hydroxylase (anti-TH, 1:4,000, Jacques Boy Institut, France), cow anti-glial fibrillary acidic protein (GFAP, 1:10,000, Dako), a mixture of mouse monoclonal antibodies 2B6 and 3B3 (Seikagaku, 1:100), mouse monoclonal antibodies CS56 (Sigma, 1:100) or mouse monoclonal antibodies against neurocan (1D1, 1:200, DSHB) or versican (12C5, 1:1, gift of R. Asher). Control immunostaining was carried out using appropriate concentrations of mouse IgM (Sigma) or mouse IgG1 (Sigma) in place of the primary antibody. Fourth, sections were incubated in the appropriate biotinylated secondary antibody (goat anti-rabbit IgG, 1:200, Dako; horse anti-mouse IgG (H+L), rat adsorbed, 1:200, Vector Laboratories, UK) in TBS with 1% NGS for 3 hr followed by three washes in TBS. The horse anti-mouse IgG (H+L) cross-reacts with IgM (Vector Laboratories, personal communication) and can therefore be used to detect the CS-56 antibody. Fifth, sections were incubated in streptavidin-conjugated peroxidase (Dako, UK) in TBS with 1% NGS for 2 hr followed by three washes in TBS and two washes in TNS. The complexed primary antibody was visualized by immersing sections in TNS containing the chromagen 3,3′-diaminobenzidine (BDH) in TNS with 0.03% hydrogen peroxide. Finally, sections were washed three times in TNS, mounted on presubbed (1% gelatin in PBS) glass slides, air dried, dehydrated in an ascending series of ethanol, cleared in xylene and coverslipped with DPX (BDH, UK).

Measuring Lesion Dimensions

One series of one-in-twelve sections was immunolabeled using antibodies against GFAP. This allowed lesion cores to be visualized and delineated unambiguously from the lesion surround after axotomy and repeated perielsional infusion. High power (×200) images of the region of interest were captured using a digital camera mounted on a light microscope (Leitz DMRB, Leica) coupled to a PC running an appropriate graphics package (Photoshop, Adobe). Lighting conditions and exposures were equal within capture sessions and results are expressed as parenchymal mean pixel densities (white = 0, black = 255). Images were saved as TIFF files without compression and exported to a PC-compatible freeware version of NIH Image (Scion Image, release beta 3b, Scion Corp.). Differences between animals in means of caviation associated with near-zero pixel densities were corrected for mathematically. The maximum rostrocaudal lesion length and the cross-sectional area of the lesion core was measured in all GFAP-labeled sections, allowing calculation of the estimated mean rostrocaudal lesion length and the total volume of the lesion core per animal.

Counting Procedures

Catecholaminergic neurons including dopaminergic neurons of the nigrostriatal tract were visualized in two series of one-in-twelve paraformaldehyde-fixed free-floating sections immunolabeled using antibodies against tyrosine hydroxylase (TH). The growth response of dopaminergic nigrostriatal axons was assessed at high magnification (×400) by counting the number of TH immunoreactive processes crossing one of four...
imaginary lines drawn anterior and parallel to the plane of transection. Processes crossing this line were only counted if they were also within the conjoined site of axotomy and infusion (i.e., within the lesion core). Counts were made in five sections per animal and counts were tallied per animal. The estimated mean total number of axons per brain was estimated by scaling the counts up by a factor of six (two series of one-in-twelve sections were analyzed). Differences between treatment groups were assessed using one-tailed Students \( t \)-tests and a standard level of significance \( (P < 0.05) \).

**RESULTS**

Preliminary Experiments: How to Detect Hyaluronan Digestion In Vitro

Preliminary experiments (using immunoblotting and immunolabeling strategies) were carried out for two reasons: 1) to determine appropriate methods for detecting effective digestion of hyaluronan in CNS tissue after treatment with hyaluronidase in vitro; and 2) to investigate whether the concentrations of hyaluronidase used in this study (selected on the basis of various studies reported in the literature) were sufficient to degrade CS GAG in vitro.

**Immunolabeling.** Unfixed cryosections from intact adult rat brain and spinal cord were immunolabeled to determine appropriate methods for detecting effective digestion of hyaluronan. First, an attempt was made to use a biotinylated hyaluronan-binding protein (BHBP) to detect digestion of hyaluronan directly. Although BHBP reactivity in unfixed or acetone-postfixed cryosections of adult rat spinal cord (Fig. 2A) was abolished by preincubation in hyaluronidase in vitro (Fig. 2B), specific BHBP reactivity could not be detected in unfixed, acetone fixed or perfused fixed (4% paraformaldehyde) cryosections of adult rat brain (Fig. 2C). Consequently, other indirect methods were evaluated for detection and digestion of hyaluronan in adult rat brain. Nevertheless, interestingly, this result indicates that spinal cord and cerebellum may contain more hyaluronan-binding proteins than brain in the adult rat.

A mixture of 2B6 and 3B3 antibodies was used to detect stub epitopes that are created after digestion of chondroitin sulfate glycosaminoglycans using chondroitinase ABC (Hascall et al., 1972; Baker et al., 1991) and at least some preparations of hyaluronidase (Sigma data sheets). Unfixed cryosections of adult rat brain, cerebellum or spinal cord were immunostained using a mixture of 2B6 and 3B3 antibodies either untreated or after digestion in vitro using chondroitinase ABC or hyaluronidase. As expected, whereas no stub reactivity was detectable in undigested unfixed sections of spinal cord (Fig. 3A), brain or cerebellum, stub reactivity was detectable after chondroitinase ABC digestion of unfixed sections of spinal cord (Fig. 3B), brain or cerebellum. Stub reactivity, however, was not detectable after digestion of unfixed sections of spinal cord (Fig. 3C), brain or cerebellum with this preparation of hyaluronidase. This result is therefore consistent with the results obtained by immunoblotting proteins separated from homogenates of adult rat brain; i.e., this preparation of hyaluronidase does not effectively digest chondroitin-4 or chondroitin-6 sulfate GAG.

Versican contains a region that binds hyaluronan (Zimmermann and Ruoslahti, 1989) and effective degradation of hyaluronan with hyaluronidase disrupts versican
(12C5) immunoreactivity in cell culture and liberates versican from homogenates of adult rat brain (Asher et al., 2002). We therefore looked to see whether hyaluronidase might attenuate versican immunoreactivity from unfixed sections of adult CNS. Whereas 12C5 (versican) immunoreactivity was detectable in undigested unfixed sections of adult rat spinal cord (Fig. 4A), brain and cerebellum (Fig. 4B), 12C5 immunoreactivity was abolished in spinal cord (Fig. 4C) and attenuated in brain and cerebellum (Fig. 4D) after digestion in vitro using hyaluronidase.

These in vitro results indicate first that this preparation of hyaluronidase (50 μg/ml, bovine testes, Worthington Biochemical Corp.) is sufficient to degrade hyaluronan in vitro and second that 12C5 immunostaining might be a good way to detect whether treatment of adult rat brain with hyaluronidase was effective in vivo.

**Immunoblotting.** Saline extracts of homogenized adult rat brain were enriched for GAG-bearing proteins by anion exchange chromatography and aliquots were left undigested or digested either with chondroitinase ABC or with hyaluronidase (5 μg/ml or 50 μg/ml, Sigma, UK or 50 μg/ml, Worthington Biochemical Corp.). Proteins were then separated by SDS PAGE, and transferred to nitrocellulose and immunoblotted using the 2B6 and 3B3 monoclonal antibodies. These predominantly recognize “stub” epitopes created after digestion of chondroitin-4 and chondroitin-6 sulfate GAGs respectively with excess chondroitinase ABC (Hascall et al., 1972; Baker et al., 1991) and at least some preparations of hyaluronidase (bovine testes, Sigma, UK). Membranes were also blotted using the monoclonal antibody 1G2 against intact neurocan and neurocan-C.

Immunoblotting demonstrated that, relative to chondroitinase ABC, digestion with hyaluronidase created very few, or no bands immunoreactive for 2B6 (Fig. 5A). Thus hyaluronidase does not effectively digest chondroitin-4-sulfate GAG. Similarly, digestion with hyaluronidase created only a subset of the 3B3-immunoreactive bands observed after digestion with chondroitinase ABC (Fig. 5B). Thus, hyaluronidase is far less effective than chondroitinase ABC in digesting chondroitin-6-sulfate GAG. Digestion with hyaluronidase, however, did cause a shift in the species immunoreactive for 1G2 (Fig. 5C) although this shift was not as great was observed after digestion with chondroitinase ABC. These results indicate that hyaluronidase (at doses that in vitro create “stub” epitopes and that abolish BHABP immunoreactivity) degrades CS GAG less effectively than chondroitinase ABC.

**In Vivo Results**

The following terminology will be used: “axotomy+saline” refers to the group of animals that were given unilateral knife cut lesions of the nigrostriatal tract with repeated perilesional infusions of saline, and “axotomy+hyaluronidase” refers to the group of animals that were given unilateral knife cut lesions and repeated perilesional infusions of saline containing hyaluronidase.

**Astrocyte response.** The astrocyte response to axotomy and treatment with either saline or hyaluronidase...
was examined eleven days post-transection by immunoperoxidase labeling one series of one-in-twelve paraformaldehyde-fixed free-floating sections using antibodies against glial fibrillary acidic protein (GFAP). After axotomy and treatment with saline, GFAP immunolabeling indicated that the sites of axotomy and infusion were conjoined forming a continuous “lesion core” that essentially lacked GFAP immunoreactive cell bodies and processes (Fig. 6A,B). In “axotomy + saline” animals the lesion core measured a mean of 1,100 μm in the rostrocaudal axis and reached from the midline to 2.5 mm laterally. There were small regions of cavitation but in general there was continuity of tissue through the lesion core. In contrast, the surrounding tissue (“lesion surround”) appeared relatively intact and contained intense GFAP immunoreactivity associated with hypertrophied processes and cell bodies that walled the lesion core off completely from the lesion surround. A similar distribution of GFAP immunoreactivity was observed after axotomy and repeated perilesional infusion of saline containing hyaluronidase. The sites of axotomy and infusion formed a continuous lesion core that essentially lacked GFAP immunoreactive cell bodies and processes (Fig. 6C,D). Again, there was continuity of tissue through the lesion core with only small regions of cavitation and the surrounding tissue appeared relatively intact. This lesion surround contained intense GFAP immunoreactivity associated with hypertrophied processes and cell bodies that walled the lesion core off completely from the lesion surround. In “axotomy + hyaluronidase” animals the lesion core measured a mean of 1,200 μm in the rostrocaudal axis and reached from the midline to 2.5 mm laterally.

There was no difference between groups in mean lesion rostrocaudal length \( (t_{8} = 0.87, P = 0.41) \) or in mean lesion volume \( (t_{8} = 1.60, P = 0.15) \).

Repeated perilesional infusion of hyaluronidase enhances sprouting of dopaminergic nigral axons. Catecholaminergic neurons including dopaminergic nigral neurons were visualized by immunoperoxidase labeling paraformaldehyde-fixed sections using antibodies against tyrosine hydroxylase (TH).

Eleven days post-transection, in “axotomy + saline” animals, local nigral axons sprouted within the

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Fig. 4. Immunofluorescence labeling transverse section using 12C5 antibodies against versican. (A,C) Intact adult rat spinal cord and (B,D) cerebellum, after incubation in (A,B) PBS, or (C,D) hyaluronidase. (A,C) Corticospinal tract occupies V reaching down to middle of image. Scale bar = 45 μm.
lesion core anterior to the plane of axotomy (Fig. 7A–C, 4). Large numbers of TH immunoreactive nigral axons were clearly visible sprouting within the lesion core, particularly within 200 μm of the plane of axotomy (Fig. 3B), but frequently as far as 400 μm. Axons were often fasciculated or oriented parallel to the plane of transection, i.e., perpendicular to the original nigrostriatal tract. TH immunoreactive nigral axons were also observed bordering small cavities (where present) and ventral to brain parenchyme in meninges that had been penetrated during transection. TH immunoreactive debris was scant. Fewer axons grew more than 600 μm (Fig. 7C). An estimated mean total of 118 axons per animal were observed extending anteriorly up to 800 μm. Axons were not observed regenerating, however, beyond the lesion core through distal host tissue.

Thus many (but not all) axons grew along the course of the original nigrostriatal tract within the lesion core.

Relative to “axotomy+saline” animals, in “axotomy+hyaluronidase” animals, nigral axon sprouting assessed 600 μm or 800 μm anterior to the site of transection was enhanced approximately fourfold (Fig. 7D–F, 8). The difference was significant at 600 μm (Fig. 7E, \( t_\alpha = 6.05, P < 0.001 \)) and at 800 μm (Fig. 7F, \( t_\alpha = 2.52, P = 0.036 \)) but neither at 200 μm nor at 400 μm. An estimated mean total of 466 axons per animal were observed having grown up to 800 μm. This represents, however, only a small fraction of the number of axons observed sprouting at 200 μm. Axons were not observed leaving the lesion core to regenerate through the surrounding tissue. Indeed, relative to “axotomy+saline” animals, there was no significant increase in the number of dopaminergic axons

Sigma h’ase and Worthington h’ase created 3B3 immunoreactive species with different AMWs. C: Ch’ase and both preparations of h’ase caused a downshift in the AMW of intact 1G2 immunoreactive neuropean. The effect of Sigma h’ase was dose dependent. Note that 2B6 antibodies recognize degraded chondroitin-4-sulfate GAG whereas 3B3 antibodies recognize degraded chondroitin-6-sulfate GAG.

Fig. 5. Immunoblot analysis of effect of different preparations of hyaluronidase (h’ase) versus chondroitinase ABC (ch’ase) upon GAG-enriched fraction of adult rat brain eluted from a DEAE column. A: Ch’ase but not h’ase created a number of 2B6 immunoreactive species with different apparent molecular weight (AMWs). B: Ch’ase created more 3B3 immunoreactive species than preparations of h’ase.
counted 1,000 μm anterior to the plane of transection. Thus, repeated perilesional infusion of hyaluronidase increased sprouting of cut dopaminergic nigral axons within the lesion core but did not enhance long distance axon regeneration back to the original ipsilateral striatal target.

Repeated perilesional infusion of hyaluronidase reduces the abundance of versican and neurocan in vivo. CSPGs were detected in free floating parasagittal sections of brains ($n = 10$) perfused fixed with 4% paraformaldehyde by immunoperoxidase staining using antibodies against chondroitin sulfate glycosaminoglycans (CS GAGs) and using antibodies against CSPG core proteins. Results were confirmed by immunolabeling unfixed cryosections of brains ($n = 2$) using the same antibodies (data not shown).

Versican

In brains of “axotomy + saline” animals, extracellular 12C5 immunoreactivity is present in both grey and
white matter within the lesion surround (Fig. 9A). In contrast, extracellular 12C5 immunoreactivity was absent from the lesion core (i.e., a region extending up to 1,100 μm anterior to the plane of transection). The most intense 12C5 immunoreactivity was present in a 200 μm-thick rim immediately surrounding the lesion core. In “axotomy+hyaluronidase” animals, this rim was not observed (Fig. 9B) and the region lacking 12C5 immunoreactivity extended up to 1,200 μm anterior to the site of transection. Extracellular 12C5 immunoreactivity, however, remained present in the lesion surround. These results indicate that repeated perilesional injection of hyaluronidase attenuated but did not abolish perilesional versican in vivo.

**Neurocan**

Neurocan was detected using the 1D1 monoclonal antibody. In “axotomy+saline” animals, extracellular 1D1 immunoreactivity was present in both grey and white matter within lesion surround (Fig. 9C). In contrast, extracellular 1D1 immunoreactivity was absent from lesion core (that extended up to 1,100 μm anterior to the site of transection). The most intense 1D1 immunoreactivity was present within a 200 μm-thick rim immediately surrounding the lesion core. In “axotomy+hyaluronidase” animals, this rim was not observed (Fig. 9D) and the region lacking 1D1 immunoreactivity extended up to 1,200 μm anterior to the site of transection. Extracellular 1D1 immunoreactivity remained...
present in the lesion surround. These results indicate that repeated perilesional injection of hyaluronidase attenuated but did not abolish perilesional neurocan in vivo.

**2B6/3B3 Immunoreactivity**

A mixture of 2B6 and 3B3 antibodies was used to detect stub epitopes that are created after digestion of chondroitin sulfate glycosaminoglycans using chondroitinase ABC (Hascall et al., 1972; Baker et al., 1991) and at least some preparations of hyaluronidase (Sigma data sheets). In “axotomy + saline” animals, stub immunoreactivity was not observed within the lesion core and only very little stub immunoreactivity was observed in the lesion surround (Fig. 10A). Similarly, in “axotomy + hyaluronidase” animals, stub immunoreactivity was not observed within the lesion core and only very little stub immunoreactivity was observed in the lesion surround (Fig. 10B). This indicates that repeated perilesional injection of hyaluronidase did not completely degrade CS GAGs at the site of adminis-

![Image](image_url)
Fig. 10. Immunoperoxidase labeling for (A,B) degraded CS GAG using 2B6 and 3B3 antibodies, and for (C,D) intact CS GAG using the CS56 antibody after axotomy and treatment with (A,C) saline, or (B,D) hyaluronidase, examined 11 days post-axotomy. Arrow indicates level of transection, arrowhead indicates level of infusion. Nigra is to left of each image, striatum to the right. Scale bar = 1 mm.
tration. These results are consistent with those obtained by immunoblotting and immunolabeling unfixed cryosections of adult rat brain.

CS56

CS GAGs were detected using the CS56 monoclonal antibody that predominantly recognizes intact chondroitin sulfate D glycosaminoglycans (Avnur and Geiger, 1984; Sorrell et al., 1993). In “axotomy+saline” animals, CS56 immunoreactivity was generally absent from the lesion core (Fig. 10C). In contrast, diffuse extracellular CS56 immunoreactivity was present in the lesion surround, being particularly associated with blood vessels and in a 200 µm-thick rim immediately surrounding lesion core. Similarly, in “axotomy+hyaluronidase” animals, diffuse extracellular CS56 immunoreactivity was present in the growth-inhibitory surround and was associated with blood vessels (Fig. 10D). Unexpectedly, strong, dense CS56 immunoreactivity was detected, however, within the lesion core. These results indicate that repeated perilesional injection of hyaluronidase showed additional CS56 epitopes at the site of administration. One explanation is that treatment with hyaluronidase increased the abundance of intact CS GAGs in the lesion core, for example, by stimulation of CS GAG-producing cells. An alternative possibility is that, because the lesion core did not display any 2B6/3B3 immunoreactivity for CS GAG “stub” epitopes after treatment with hyaluronidase, hyaluronidase created additional CS56 epitopes by partial degradation of CS GAG but that CS GAGs were not degraded up to their stub regions (proximal to the core protein). This is consistent with our finding that Worthington bovine testicular hyaluronidase unmasked hidden CS56 epitopes within the lesion core.

DISCUSSION

After unilateral nigrostriatal transection and repeated perilesional infusion of saline (examined 11 days post axotomy), cut dopaminergic nigral axons sprouted within a lesion core that contained few astrocytes and low levels of chondroitin sulfate (CS)-bearing molecules (including neurocan and versican). Very few axons grew more than 800 µm anterior to the plane of transection and essentially none grew into the lesion surround that contained astrocytes and high levels of CS proteoglycans including neurocan and versican.

After transection and repeated perilesional infusion of saline containing hyaluronidase (also examined 11 days post axotomy), there was a significant increase in the number of cut dopaminergic nigral axons that grew up to 800 µm anterior to the plane of transection. Indeed, in one animal, a fascicle of axons grew up to 1,200 µm. Sprouting occurred within lesion cores that contained few astrocytes but, again, essentially no axons grew into the astrocyte-rich lesion surround. Treatment with hyaluronidase reduced the abundance of neurocan and versican in the lesion surround; however, this was partial rather than complete.

We conclude that treatment with hyaluronidase potentiates sprouting of cut dopaminergic nigral axons. It should be noted, however, that although statistically significant, the increase in sprouting was very small indeed, and unlikely to be of clinical importance for long distance regeneration of axons after CNS injury.

Our main results extend those reported elsewhere. After optic nerve crush and a single injection of a large dose of Streptomyces hyaluronidase, retinal ganglion cell axons sprouted a maximum of 500 µm (Tona and Bigianni, 1993).

Disappointingly, however, whereas removal of CS glycosaminoglycan (GAG) using chondroitinase ABC enhances regeneration of cut dopaminergic nigral axons through regions of scar formation back to their original targets (Moon et al., 2001), treatment with hyaluronidase did not (here, sprouting was limited to the lesion core). This is despite the fact that rats in the chondroitinase ABC study were each given a total of 2.4 µg enzymatic protein whereas rats in the present study were each given a total of 1.2 mg of enzymatic protein (i.e., a 500-fold difference in total protein delivered). A number of different explanations may be given.

Firstly, although multiple (10) injections of hyaluronidase reduced the abundance of putative inhibitory hyaluronan-binding proteins such as neurocan and versican, these molecules were not removed entirely from the lesion surround. This may be partially due to the method of delivery that itself may have opposed complete removal of neurocan and versican; repeated perilesional infusion of enzyme will induce production of injury-associated molecules after each delivery session. Additionally, versican (and possibly neurocan) can be bound to the ECM by means other than binding hyaluronan (Asher et al., 2002). This proportion of CSPG would presumably not be released by treatment with hyaluronidase. For example, neurocan and versican may also be bound to ECM via their CS GAG side chains or via other ECM molecules such as tenascin–R (Aspberg et al., 1995; Hagihara et al., 1999). Thus, more effective and widespread degradation of hyaluronan and disruption of hyaluronan-binding CSPGs might result in more substantial axon regeneration.

Secondly, other (non-hyaluronan-bound) inhibitory molecules such as phosphacan and NG2 CSPGs likely contribute toward the failure of dopaminergic nigral axons to sprout beyond the lesion core borders in either saline- or hyaluronidase-treated animals. Indeed, we were able to show by immunoblotting and immunolabeling that digestion of adult rat CNS using Worthington hyaluronidase degraded only very little chondroitin–4 and chondroitin–6 sulfate GAG. Treatment with hyaluronidase therefore does not remove all potential growth-inhibitory molecules.

Finally, it is possible that hyaluronidase did not diffuse as well as chondroitinase ABC. We do not believe this to be the case, however. Serendipitous, nonspecific bind-
ing of polyclonal antibodies against tyrosine hydroxylase indicates that ECM was altered up to 4 mm from the site of infusion after treatment with hyaluronidase (but not saline), indicating good diffusion of hyaluronidase. Diffusion may therefore have been similar to that observed for chondroitinase ABC.

How did hyaluronidase treatment potentiate local sprouting in the region lacking (inter alia) astrocytes? Bovine testicular hyaluronidase degrades hyaluronic and, to a lesser degree, chondroitin sulfate but is not known to degrade molecules commonly thought to be permissive for neurite growth such as laminin and heparan sulfate (www.brenda.uni-koeln.de). We favor the explanation that hyaluronidase induced partial degradation of hyaluronic or CS in the lesion core. It is clear that the lesion core that hyaluronidase enhanced sprouting by partially degrading molecules commonly thought to be permissive for neurite growth such as laminin and heparan sulfate. We favor the interpretation that treatment with hyaluronidase was insufficient to degrade CS GAG chains entirely up to the "stub" residue close to the core protein). Treatment with hyaluronidase did not upregulate the levels of intact uncleaved CS GAG, but, rather, partially degraded CS GAG, revealing hidden CS56 epitopes. This possibility carries with it the implication that CS56 immunoreactivity may not unambiguously signal the presence of intact CS GAG.

We favor the interpretation that treatment with hyaluronidase enhanced sprouting by partially degrading hyaluronic or chondroitin sulfate within the lesion core. We also made an incidental observation. Labeling using a biotinylated hyaluronan-binding protein indicated that intact adult rat brain contains less detectable hyaluronic than in cerebellum and spinal cord. This implies that the intact adult rat cerebellum and spinal cord may contain more growth-limiting hyaluronic-binding proteins (e.g., neurocan, versican, brevican and aggrecan) than the brain.

Because the present work indicates that even low levels of residual hyaluronic or hyaluronan-binding proteins may limit regeneration beyond the immediate site of injury, axon regeneration within spinal cord may be especially subject to inhibition by hyaluronic and hyaluronan-binding proteins.

In conclusion, repeated perilesional infusion of hyaluronidase induced a limited increase in sprouting of cut dopaminergic nigral axons within the lesion core but axons did not grow beyond the lesion borders or back to their original targets. The present experiments indicate that hyaluronic acid and hyaluronan-binding CSPGs likely limit spontaneous regeneration in the adult mammalian CNS. In the future, better removal of these molecules might allow clinically relevant amounts of CNS repair; this might be achieved using longer periods of enzymatic administration (e.g., up to 28 days), higher doses of hyaluronidase, different preparations of hyaluronidase (that are known to have differing activities) and through the use of less invasive routes of administration.

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