

# Dorsal Root Ganglia Neurite Extension Is Inhibited by Mechanical and Chondroitin Sulfate-Rich Interfaces

Xiaojun Yu and Ravi V. Bellamkonda\*

Biomaterials, Cell and Tissue Engineering Laboratory, Department of Biomedical Engineering, Case Western Reserve University, Cleveland, Ohio

Glial scar formation plays a critical role in the regenerative failure in the central nervous system of adult mammals through the formation of mechanical or biochemical barriers as a result of its molecular composition. In this study, we report an in vitro model to study growth-cone behavior at controlled 3D interfaces using layered agarose hydrogels. The behavior of growth cones from embryonic day 9 (E9) chick dorsal root ganglia (DRGs) at interfaces that were mismatched in terms of their elasticity or chondroitin sulfate content was quantitatively determined. A mechanical barrier formed by the elasticity mismatch of layered agarose gels greatly influenced the ability of neurites from E9 DRGs to cross the 3D interface. To form chondroitin sulfate-rich interfaces, chondroitin sulfate B was covalently coupled to agarose hydrogel. Compared with unmodified agarose gels, the presence of CS-B-modified agarose gels at the interface significantly inhibited E9 DRGs neurites. After treatment of CS-B-modified agarose gels with chondroitinase ABC, the inhibitory effects of CS-B at the interface were significantly decreased. The effect of doping CS-B gels with laminin 1 (LN-1)-coupled agarose gels was investigated as a potential strategy to overcome inhibitory interfaces. When CS-B agarose gels were doped with LN-1-coupled agarose gels, DRG neurite's ability to cross 3D interfaces was significantly enhanced compared with that of non-LN-1-containing interfaces presenting equivalent CS-B. Our in vitro model may be used to study the influence of individual components of glial scar on inhibition as well as to design strategies to overcome this inhibition. *J. Neurosci. Res.* 66:303–310, 2001. © 2001 Wiley-Liss, Inc.

**Key words:** glial scar; agarose; controlled 3D interface; glycosaminoglycan; laminin

One of the principal reasons for the regenerative failure of the central nervous system (CNS) is the interface of normal neural tissue and glial scar that forms after injury and prevents axon growth (Windle et al., 1952; Puchala and Windle, 1977). Reactive astrocyte-derived glial scar prevents axon growth either by forming a mechanical barrier or by forming a biochemical barrier as a result of its molecular composition (Guth et al., 1986; Davies et al., 1996, 1999).

Glial scar produced by the astrocytes contains chondroitin sulfate proteoglycans (CSPGs), and CSPGs have been shown to play a crucial role in the inhibition of axon growth (Levine, 1994; Powell et al., 1997). During fetal development, CSPGs repel axons, inhibit neural cell adhesion, and play an important role in boundary formation (Snow et al., 1990, 1992; Powell and Geller, 1999). CSPG expression increases following injury of CNS (McKeon et al., 1991; Davies et al., 1997). Some studies indicate that the inhibitory effects of CSPGs are due to chondroitin sulfate's (CS) glycosaminoglycan (GAG) sugar chain (Snow et al., 1990; Cole and McCable, 1991; Geisert and Bidanset, 1993), whereas other studies show that CSPGs inhibited neurite outgrowth through their core protein (Milev et al., 1994). In addition to their direct inhibitory effects, CSPGs could also interact with cell adhesion molecules or neurotrophic factors to influence neurite outgrowth (Roberts et al., 1988; Ruoslahti and Yamaguchi, 1991; Milev et al., 1994).

Various tissue culture models of glial scar have been developed for studying the inhibitory effects in the nervous system (Reier, 1979; Fawcett et al., 1989; Snow et al., 1990; Fok-seang et al., 1995). These approaches include the design of a mechanical barrier by generating a glial scar in the *Xenopus* visual system (Reier, 1979), the design of a 2D interface by monolayer astrocyte cultures (Snow et al., 1990), the design of a 3D interface by packing astrocytes into millipore tubes (Fawcett et al., 1989), and the design of an interface by culturing clonal astrocyte cell lines (Fok-seang et al., 1995). In these approaches, the inhibitory interfaces of glial scar were generated by glial cells. Therefore, it was hard to distinguish the mechanical effects from chemical effects or to determine the effects of specific molecules in those models, because glial cells produce both inhibitory and growth-

Contract grant sponsor: The Whitaker Foundation; Contract grant number: RG98-0159.

\*Correspondence to: Prof. Ravi V. Bellamkonda, Biomaterials, Cell and Tissue Engineering Laboratory, Department of Biomedical Engineering, Case Western Reserve University, Wickenden Bldg. No. 319, 10900 Euclid Ave., Cleveland, OH 44106-7207. E-mail: rvb@cwru.edu

Received 15 March 2001; Revised 11 June 2001; Accepted 13 June 2001

promoting molecules. The interface model we report allows us to study the mechanical and chemical effects of the 3D interfaces separately and to study the contributions of specific molecules to this inhibition.

The objective of the current study was to design an *in vitro*, 3D interface model that can be used flexibly as a tool to investigate the role of some important elements of the glial scar, particularly CS-B GAG, in influencing growth cone advance. We demonstrate that an *in vitro* model of controlled 3D interfaces can be established by serial layering of appropriate hydrogels. When E9 chick dorsal root ganglia (DRGs) were used as model neurons, mechanical mismatch inhibited neurite crossing 3D interfaces in a manner dependent upon the extent of mismatch. Presence of CS-B-modified agarose gels at the interface contributed significantly to inhibited neurite extension across the interface that could partially be relieved either by digesting the CS-GAG with chondroitinase ABC or by the addition of LN-1.

## MATERIALS AND METHODS

### Assessing the Ability of DRG Neurites To Cross Mechanically Mismatched Interfaces

**Fabrication of Agarose Gels.** Different concentrations of agarose gels, 0.75%, 1.0%, 1.25%, 1.5%, 2.0%, 2.5%, and 3.0% (w/v), were fabricated by methods previously described (Bellamkonda et al., 1995a,b). Briefly, SeaPrep agarose (FMC Corp., Rockland, ME) was dissolved in pH 7.4 Dulbecco's phosphate-buffered saline (PBS; Gibco, Grand Island, NY) by heating and stirring until the solutions became clear. Agarose solutions were sterilized by passing through a 0.45  $\mu\text{m}$  filter (Nalgene Syringe Filters; Nalge Company, Rochester, NY) and were gelled and stored at 4°C. Prior to use, the gel was degelled into clear liquid by heating to 65°C for 10 min and then cooled to 37°C. Once gelled at a temperature below 17°C, agarose remains in gel form until heated to 60°C.

**Rheological Test for Gel Elasticity.** To compare the mechanical stiffness of the gels, the complex shear modulus of each gel concentration was determined by rheometry (RFS-8500 Fluid Spectrometer; Rheometric Scientific, Inc., Piscataway, NJ). Two milliliters of liquid gel samples including different concentrations of agarose gels (0.75%, 1.0%, 1.25%, 1.5%, 2.0%, and 2.5%) were added to the sample chamber at room temperature. The gel solution was cooled to 4°C for 15 min to allow for gelling. The gel was then warmed to 37°C, and the shear modulus was determined.

**Layering of Gels to Generate Controlled 3D Mechanical Boundaries.** To form 3D mechanically mismatched interfaces, agarose gels were layered in a custom-designed rectangular tissue culture dish (20 mm  $\times$  5 mm  $\times$  25 mm; Fig. 1). Two hundred microliters of freshly made 1% liquid agarose solution were added as the bottom layer (layer 1), and the dish was chilled at 4°C for 15 min to allow gel formation. Next, a mixture of 80  $\mu\text{l}$  of 1.0% agarose and four or five DRGs was added to form the middle layer (layer 2). E9 chick DRGs were explanted and embedded in the middle layer by methods previously described (Bellamkonda et al., 1995a,b). The entire dish was placed at 4°C again for 15 min for gelation. Finally, 200  $\mu\text{l}$

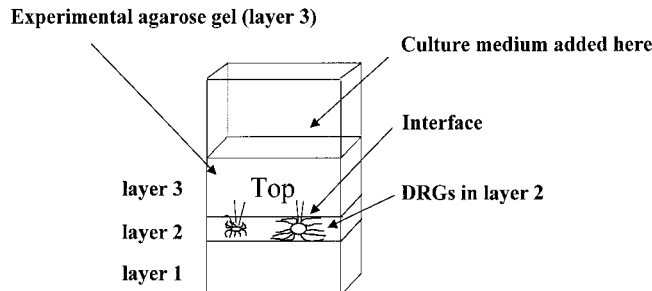


Fig. 1. Diagram illustrating layering of gels in custom-designed rectangular tissue culture dish. Layer 1, 1.0% agarose; layer 2, 1.0% agarose gels with DRGs; layer 3, different concentrations of agarose gels, 2.0% CS-B-modified agarose or 2.0% LN-1-modified agarose.

of agarose gel solution chosen from 1.0%, 1.5%, 2.0%, 2.5%, and 3.0% gel concentrations were added as the top layer (layer 3), and the dish was placed in 4°C for 15 min. As a consequence, different mechanical stiffness mismatches were generated in 3D between layers 2 and 3, and Table I lists the interfaces generated (labeled  $M_{1.0}$  through  $M_{3.0}$ ).

One milliliter of culture medium, consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Gibco), 1% penicillin-streptomycin (Gibco), and 50 ng/ml mouse nerve growth factor (mNGF 2.5S; Alomone Labs, Jerusalem, Israel) was added to the top of the gel. The dishes were kept at 37°C with 95% humidity and 5%  $\text{CO}_2$  in triplicate for each interface type.

**Quantification of DRG Neurite Crossing of 3D Interfaces.** After 5 days of culture, the ability of neurites to cross 3D interfaces was evaluated by quantifying the percentage of DRG neurites that successfully cross the 3D interface for every 100 growth cone encounters of the interface. Briefly, the culture medium was removed, the culture dish was placed on its side (perpendicular to plane of culture), and the gel interfaces were observed under a Nikon Eclipse TE 300 inverted microscope (Nikon Corporation, Tokyo, Japan) at  $\times 100$ . The number of neurites encountering and crossing the 3D interfaces from each explant was counted by serially moving the microscope visual field from one side of the dish to the other along the axis of the interface. The percentage of neurites crossing the 3D interface was calculated. The percentage of neurites crossing the interface from at least four DRGs was measured in each dish. A two-tailed Student's *t*-test was used for statistical analysis. A *P* value less than 0.05 was considered to be statistically significant.

### Assessing the Ability of DRG Neurites To Cross the CS-Rich Interfaces

To provide a CS-rich boundary or interface (instead of the mechanically mismatched interfaces as described above), chondroitin-B-sulfate GAG was covalently coupled to 2% agarose as described below and constituted layer 3 of our serially layered gels.

**Covalent Coupling of CS-B to Agarose Gel.** Protocols for the coupling of bioactive agents including CS-B to agarose gels have been developed in our laboratory (Hearn, 1987; Bellamkonda, 1995a; Dillon et al., 2000). Briefly, 4 ml of

TABLE I. Properties of Interfaces Used\*

Interface notation	Cell layer	Mechanical barrier	CS-rich barrier	LN-1 doped
M <sub>1,0</sub>	1.0% Agarose	1.0% Agarose		
M <sub>1,5</sub>	1.0% Agarose	1.5% Agarose		
M <sub>2,0</sub>	1.0% Agarose	2.0% Agarose		
M <sub>2,5</sub>	1.0% Agarose	2.5% Agarose		
M <sub>3,0</sub>	1.0% Agarose	3.0% Agarose		
C <sub>1</sub>	1.0% Agarose		2.0% CS-B(10)-modified agarose	
C <sub>2</sub>	1.0% Agarose		2.0% CS-B(20)-modified agarose	
C <sub>3</sub>	1.0% Agarose		2.0% CS-B(30)-modified agarose	
C <sub>4</sub>	1.0% Agarose		2.0% CS-B(40)-modified agarose	
C <sub>4C</sub>	1.0% Agarose		2.0% CS-B(40)-modified agarose (chondroitinase ABC-treated)	
L <sub>2,0</sub>	1.0% Agarose			2.0% LN-1-modified agarose
CL <sub>2,0</sub>	1.0% Agarose			Equal volumes of 2.0% LN-1-modified agarose and CS-B(40)-modified agarose

\*Cells were always embedded in 1.0% agarose gels (layer 2). This table lists the gel (layer 3) layered on top of 1.0% agarose to generate either a mechanical or a CS-rich interface.

1.5% agarose gel was dehydrated by a series of two 5 min washes in each of an increasing concentration of acetone (30%, 50%, 70%, 90%, and 100%) and dry acetone (dehydrated over 4 Å molecular sieves; Sigma Chemical Co., St. Louis, MO). Five milliliters of 30 mg/ml 1,1-CDI (Aldrich Chemical Co., Inc., Milwaukee, WI) in dry acetone were reacted with the dehydrated agarose gels for 1 hr. The CDI-activated gel was washed three times with dry acetone to remove the unreacted CDI and dissolved in 3 ml sodium bicarbonate buffer, pH 8.5. Ten, twenty, thirty, or forty milligrams of CS-B (Sigma) in 1 ml of the same buffer were added, and the reaction was allowed to proceed at room temperature for 40 hr under gentle stirring. The reaction mixture was allowed to gel at 4°C, and 5 ml of sodium bicarbonate buffer was added for an overnight quench of unreacted groups. The gels were then extensively washed 10 times with PBS, lyophilized, and stored until needed. These CS-B “modified” gels were reconstituted into 2.0% (w/v) gel solutions when needed, as described previously. The modified gels corresponding to addition of 10, 20, 30, and 40 mg of CS-B are referred to as CS-B(10)-, CS-B(20)-, CS-B(30)-, and CS-B(40)-modified agarose gels, respectively.

#### Characterization of CS-B-Modified Agarose Gels.

The amount of CS-B coupled to agarose gel backbone was quantified by a method modified from that described by Farn-dale et al. (1986). Briefly, a color reagent was made by dissolving 16 mg of 1,9-dimethyl-methylene blue (DMMB; Aldrich Chemical) in 1 liter of distilled water, with the addition of 2.37 g NaCl and 95 ml 0.1 M HCl. A sample of 100 µl of 1.0% CS-B-modified agarose (w/v) was mixed with 2.5 ml of the color reagent for 15 sec. The absorbance at 525 nm was measured by using a spectrophotometer (Spectronic Genesys; Milton Roy Co., Rochester, NY). The amount of CS-B per milliliter of 1.0% CS-B-modified agarose gel (w/v) was calculated through a calibration curve for measuring CS-B that was established by mixing known concentrations of CS-B into 1.0% liquid agarose (w/v; Dillon et al., 2000). To determine the mechanical effects of our coupling chemistry, the shear modulus of the 2.0% CS-B(40)-modified agarose gels was tested by methods described earlier.

#### Layering of Gels: Forming CS-Rich 3D Interfaces.

CS-rich 3D interfaces were generated by methods similar to those used to generate 3D mechanical interfaces. The only difference was that the top layer (layer 3) was made of 2.0% CS-B-modified agarose gels instead of different concentrations of agarose gels. Interfaces were generated with increasing amounts of coupled CS-B as described earlier and are listed in Table I (C<sub>1</sub> to C<sub>4</sub>). Neurite extension across the interface was quantified as described above. We have previously demonstrated that CDI chemistry for modifying agarose gels does not affect DRG neurite outgrowth (Bellamkonda et al., 1995a; Yu et al., 1999); the interface formed by 2% agarose (M<sub>2,0</sub>) was used as control.

#### Testing the Specificity of CS-B in Influencing Neurite Extension.

To liberate CS-B from CS-B-coupled agarose gels, 5 units of chondroitinase ABC (Sigma) were added to 2 ml of 2.0% (w/v) CS-B(40)-modified agarose gel in PBS solution at 37°C for 3 hr. After boiling for 1 min, the solution was then put at 4°C for 15 min for gelation. The resulting small molecules and excess chondroitinase ABC were removed by repeated washing (10 times) with PBS. The completeness of CS-B removal was tested by DMMB dye assay. The chondroitinase ABC-treated 2.0% CS-B(40)-modified agarose gels were then gelled as the top layer (layer 3) to form 3D interfaces (C<sub>4C</sub>; Table I), with layers 1 and 2 remaining the same as those described earlier, and the effect on DRG neurite extension into chondroitinase ABC treated gel was quantified.

#### Ability of LN-1 To Mask CS-B Effects

The coupling chemistry to bind LN-1 to agarose gels covalently was similar to that used for coupling of CS-B to agarose gels. The only difference was that 2 mg of mouse laminin 1 (Gibco) was added to the reaction instead of CS-B.

The CDI-LN-1-coupling chemistry was analytically verified using BioRad protein microassay kits (BioRad, Hercules, CA), the absorbance at 595 nm being detected by a spectrophotometer (Spectronic Genesys; Milton Roy; Yu et al., 1999). To determine the mechanical effects of our coupling chemistry, the

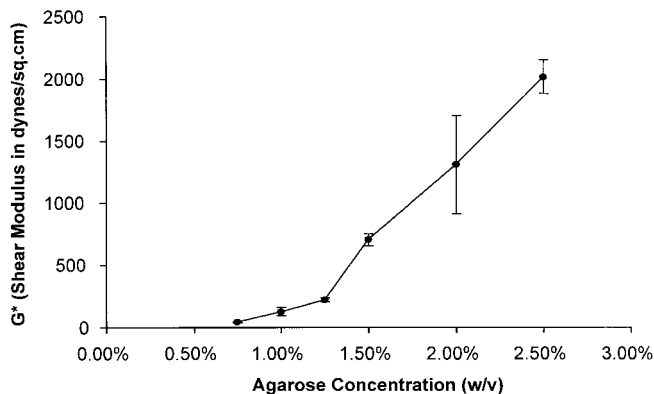


Fig. 2. Complex shear modulus of different concentrations of agarose gels as assessed by rheology.  $N = 3$ . Error bars denote standard deviation from mean.

shear modulus of 2.0% LN-1-modified agarose gels was determined by rheometry.

Similar to the method of forming 3D mechanical interfaces, the 2.0% LN-1-modified agarose gels or mixed gels consisting of equal volumes of 2.0% CS-B(40)-modified agarose and 2.0% LN-1-modified agarose were used as the top layer (layer 3) to form an LN-1 interface ( $L_{2,0}$ ) or LN-1 agarose-doped  $C_4$  interface ( $CL_{2,0}$ ; Table I). The CS-B(40) and LN-1 gel mixture had the same number of sulfated groups as did the CS-B(20) gels when CS-B(20) alone was presented at the interface.

## RESULTS

### Assessing the Ability of DRG Neurites To Cross Mechanically Mismatched Interfaces

**Shear Modulus of Agarose Gels as Determined by Rheometry.** The shear modulus of agarose gel was  $42.7 \pm 4.7$  dyne/cm<sup>2</sup>,  $128.0 \pm 30.8$  dyne/cm<sup>2</sup>,  $220.1 \pm 12.7$  dyne/cm<sup>2</sup>,  $704.7 \pm 46.1$  dyne/cm<sup>2</sup>,  $1,301.8 \pm 390.8$  dyne/cm<sup>2</sup>, and  $2,006.8 \pm 136.2$  dyne/cm<sup>2</sup>, respectively, corresponding to agarose gel concentrations of 0.75%, 1.0%, 1.25%, 1.5%, 2.0%, and 2.5% (Fig. 2). The shear modulus of the agarose gel increased as the agarose gel concentration increased, demonstrating that the stiffness of agarose gel increased with the increase in gel concentration.

**Quantification of Neurites Crossing Mechanically Mismatched Interfaces.** A light micrograph of DRG neurites crossing the 3D interface  $M_{2,0}$  is shown in Figure 3A,B. There was no significant difference in the percentage of neurites crossing the interface of  $M_{1,0}$ ,  $M_{1,5}$ , and  $M_{2,0}$  (Fig. 4). The percentage of neurites crossing the 3D interfaces of  $M_{2,5}$  and  $M_{3,0}$  was significantly lower ( $P < 0.01$ ) than that of  $M_{2,0}$ . The shear modulus data indicate that there is no significant difference in the percentage of neurites crossing the 3D interfaces up to a shear modulus mismatch of  $1,173.8$  dyne/cm<sup>2</sup> ( $M_{2,0}$ ). At  $M_{2,5}$ , when the mechanical mismatch of the interfaces is at the difference of shear modulus of  $1,878.8$  dyne/cm<sup>2</sup>, the percentage of neurites crossing 3D interfaces is significantly lower ( $P < 0.01$ ), as is the case at  $M_{3,0}$ .

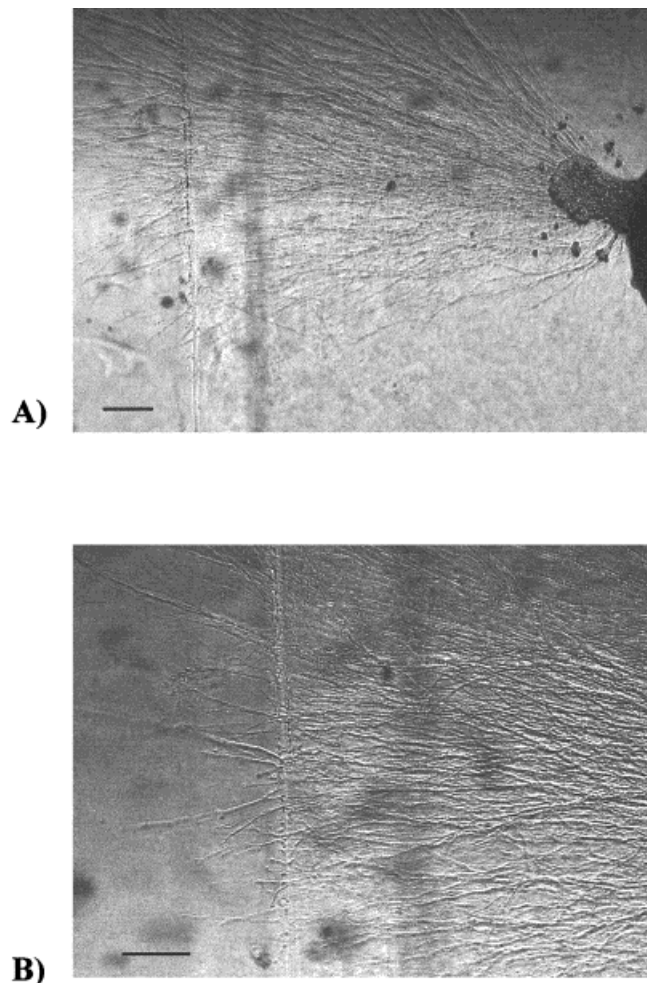


Fig. 3. Phase-contrast photomicrograph showing E9 chick DRG neurites crossing the interface  $M_{2,0}$  after 5 days culture at lower magnification (A) and higher magnification (B). The line showing the interface is due to refraction of light at the interface. Scale bars = 100  $\mu$ m.

### Assessing the Ability of DRG Neurites To Cross the CS-Rich Interfaces

**Characterization of CS-B-modified Agarose Gels.** After extensive washing with PBS to remove unbound CS-B, DMMB dye assay shows that 0.33, 0.80, 1.15, and 1.40 mg of CS-B are covalently coupled per milliliter of CS-B(10)-, CS-B(20)-, CS-B(30)-, and CS-B(40)-modified agarose gel, respectively. The amount of CS-B originally added was 1.67, 3.33, 4.50, and 6.67 mg/1 ml of 1.0% agarose gel, so the efficiency of CS-B coupling to agarose through CDI chemistry is  $22\% \pm 3\%$ . The shear modulus of the 2.0% CS-B(40)-modified agarose was  $167.8 \pm 31.6$  dyne/cm<sup>2</sup> and close to the modulus of 1.0% unmodified agarose gel ( $128.0 \pm 30.8$  dyne/cm<sup>2</sup>).

**Quantification of Neurites Crossing CS-Rich Interfaces.** A light micrograph of DRG neurites crossing the 3D interface  $C_4$  is shown in Figure 5. The percentage of neurites crossing the 3D interfaces of  $C_1$ ,  $C_2$ ,

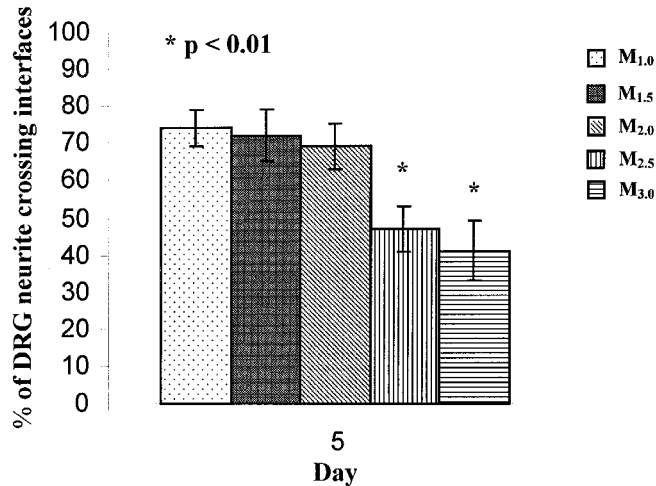


Fig. 4. Graphic plot of E9 chick DRG neurite crossing 3D mechanical interfaces formed by different concentrations of agarose gels. Asterisks denote a statistically significant, lower percentage ( $P < 0.01$ ) compared with DRGs crossing interfaces M<sub>2.0</sub>. Four DRGs from each group were counted, with the experiment repeated at least twice. Error bars denote standard deviation.

C<sub>3</sub>, and C<sub>4</sub> was  $38\% \pm 9\%$ ,  $28\% \pm 8\%$ ,  $30\% \pm 7\%$ , and  $26\% \pm 5\%$ , respectively, all of which are significantly lower ( $P < 0.01$ ) than the percentage of neurites crossing the 3D interface of M<sub>2.0</sub> (Fig. 6). Although C<sub>1</sub>, C<sub>2</sub>, C<sub>3</sub>, or C<sub>4</sub> interfaces significantly inhibited DRG neurites, no CS-B dose-dependent effect was observed.

**Specificity of CS-B in Influencing Neurite Extension.** After treatment of CS-B(40)-modified agarose gels with chondroitinase ABC, the CS-B could not be detected by DMMB dye assay. The percentage of neurites crossing the C<sub>4C</sub> interface was  $66\% \pm 2\%$ , which is significantly higher ( $P < 0.01$ ) than untreated control,  $26\% \pm 5\%$  (Fig. 7). There is no significant difference for the percentage of neurites crossing the C<sub>4C</sub> interface and M<sub>2</sub> interface (2% agarose, without any CS presence).

#### LN-1 Masks CS-B Effects

The BioRad protein assay showed that the efficiency of laminin 1 coupling to agarose through CDI chemistry was  $20\% \pm 3\%$ . The shear modulus of 2.0% LN-1-modified agarose gel was  $357.7 \pm 59.3$  dyne/cm<sup>2</sup> as determined by rheometry and was between the shear modulus of 1.25% and 1.5% unmodified agarose gels.

The percentage of neurites crossing the L<sub>2.0</sub> interface ( $90\% \pm 3\%$ ) is significantly higher ( $P < 0.01$ ) compared with the percentage of neurites crossing the M<sub>2.0</sub> interface (Fig. 8), indicating that laminin 1 can facilitate increased crossing of the interface in the absence of significant mechanical or CS content mismatch. When CS-B gels were doped with LN-1 (CL<sub>2.0</sub>), the percentage of neurites crossing the 3D interfaces of CL<sub>2.0</sub> ( $67\% \pm 7\%$ ) was significantly greater ( $P < 0.01$ ) than the percentage of neurites crossing the 3D interfaces of C<sub>2</sub> (Fig. 8).

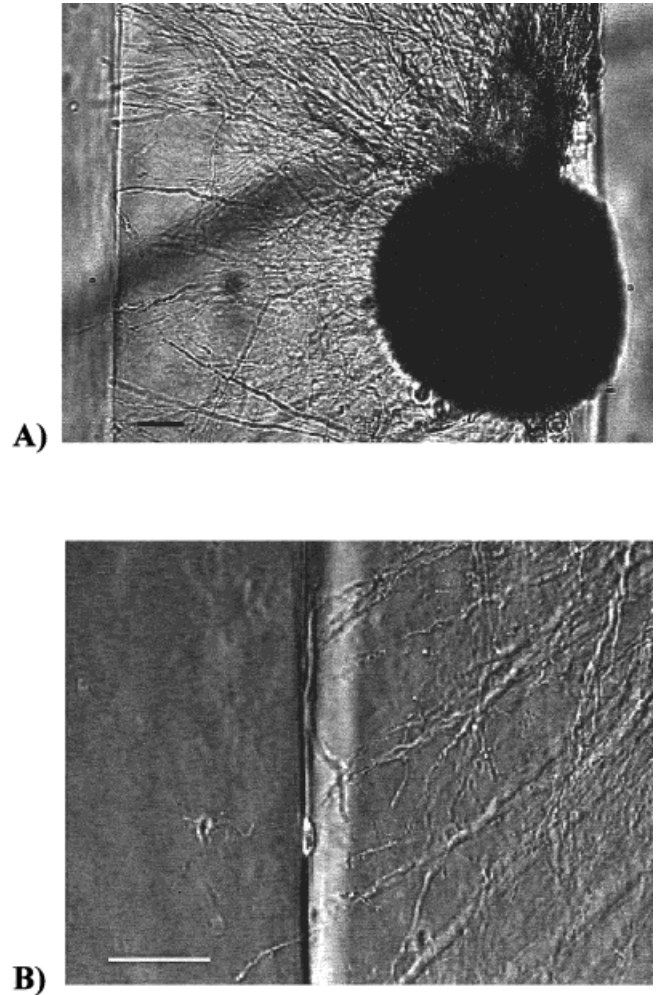


Fig. 5. Phase-contrast photomicrograph showing E9 chick DRG neurites crossing the interface C<sub>4</sub> after 5 days of culture at lower magnification (A) and higher magnification (B). The line showing the interface is due to refraction of light at the interface. Scale bars = 50  $\mu$ m.

#### DISCUSSION

We have previously demonstrated that low concentration of agarose gels supported DRG neurite extension in 3D, and the neurite outgrowth was inhibited by increasing the gel concentration (Dillon et al., 1998). We are interested in probing the behavior of neurites at the 3D interfaces with increased mechanical mismatch in the current study. Studies analyzing the mechanical properties of brain tissues from several species have been published (Fallenstein et al., 1969; Galford et al., 1970; Donnelly and Medige, 1997; Bilston et al., 1997; Arbogast et al., 1997). In these studies, the complex shear modulus of brain tissues ranged from 6,940 dyne/cm<sup>2</sup> to 238,260 dyne/cm<sup>2</sup>, whereas the complex shear modulus of our agarose gels used in this study ranged from 128 dyne/cm<sup>2</sup> to 2,007 dyne/cm<sup>2</sup>. Although the complex shear moduli of agarose gels were lower than those of the brain tissue, they were of

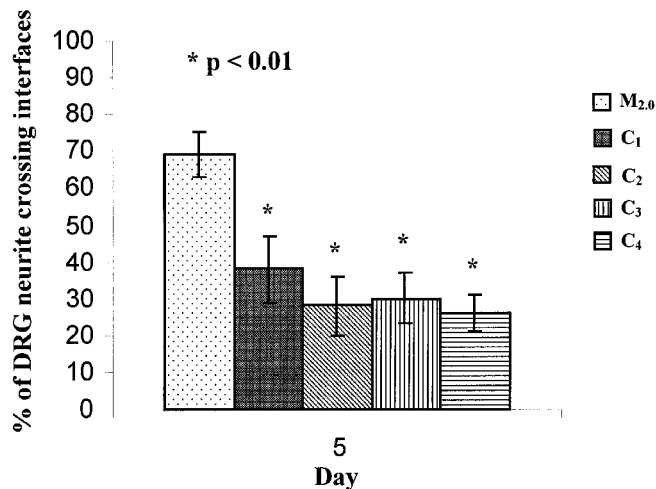


Fig. 6. Graphic plot of E9 chick DRG neurites crossing CS-rich interfaces formed by CS-B-modified agarose gel (C<sub>1</sub>–C<sub>4</sub>). Asterisks denote a statistically significant, lower percentage ( $P < 0.01$ ) compared with DRG neurite crossing interfaces M<sub>2.0</sub>. Four DRGs from each group were counted, with the experiment repeated at least twice. Error bars denote standard deviation.

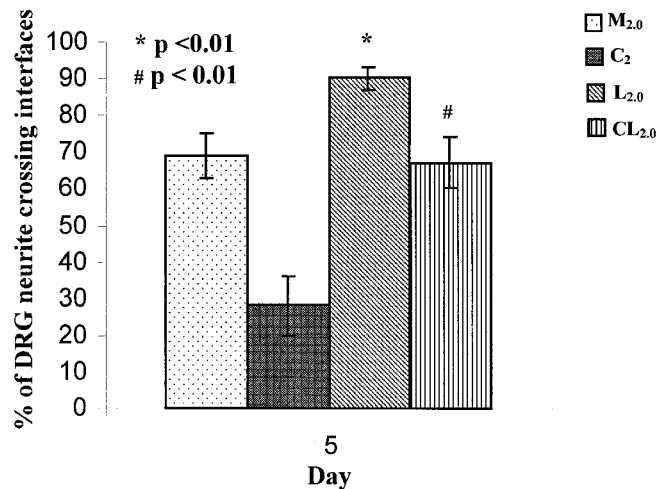


Fig. 8. Graphic plot of E9 chick DRG neurite crossing interfaces of C<sub>2</sub>, L<sub>2.0</sub>, and CL<sub>2.0</sub>. Asterisk denotes a statistically significant, higher percentage ( $P < 0.01$ ) compared with DRG neurite crossing interfaces M<sub>2.0</sub>. Number sign denotes a statistically significant, higher percentage ( $P < 0.01$ ) compared with DRG neurite crossing C<sub>2</sub>. Four DRGs from each group were counted, with the experiment repeated at least twice. Error bars denote standard deviation. Note: CL<sub>2.0</sub> is not significantly different from M<sub>2.0</sub>.

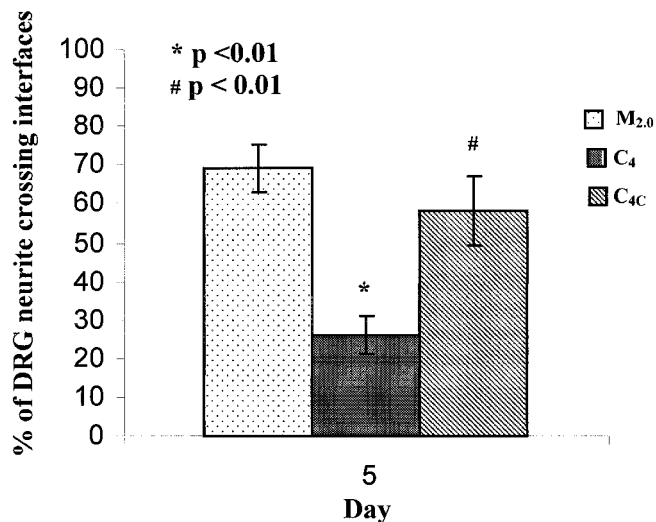


Fig. 7. Graphic plot of E9 chick DRG neurite crossing interfaces of C<sub>4</sub> and C<sub>4C</sub>. Asterisk denotes a statistically significant, lower percentage ( $P < 0.01$ ) compared with DRG neurite crossing interfaces M<sub>2.0</sub>. Number sign denotes a statistically significant, higher percentage ( $P < 0.01$ ) compared with DRG neurite crossing C<sub>4</sub>. Four DRGs from each group were counted, with the experiment repeated at least twice. Error bars denote standard deviation.

similar magnitude (in thousands of dyne/cm<sup>2</sup>), as reported in some studies of brain mechanical properties.

After CNS injury, various cellular and molecular events occur. As cell proliferation and molecules secreted alter the environments, the glial scar tissue is different from the normal neural tissue (Stichel and Muller, 1998; Qiu et al., 2000). The interface between uninjured and scar tissue

forms a physical barrier to inhibit the crossing of neurites in vivo and leads to the failure of CNS nerve regeneration (Reier and Houle, 1988; Maxwell et al., 1990; Davies et al., 1997). Results from mechanically mismatched interfaces M<sub>1</sub> through M<sub>3</sub> demonstrate that the percentage of neurites crossing 3D interfaces was relatively high and did not decline up to a stiffness mismatch of agarose up to 1.0–2.0%, which corresponded to a mismatch of shear modulus of 1,173.8 dyne/cm<sup>2</sup> (Fig. 2). When the mismatch in agarose gel stiffness increased from 1.0% to 2.5%, with the correspondent mismatch of shear modulus of 1874.8 dyne/cm<sup>2</sup>, the capability of neurites to cross the resulting mechanical interface was greatly inhibited. These data indicate that the “other side” of the 3D interface should be at least as stiff as 2.5% agarose gels before neurite extension across 3D interface is inhibited. With our gel system, by extrapolating data from Figures 2 and 4, we estimate that a mismatch of 6,500 dyne/cm<sup>2</sup> will completely inhibit neurites from crossing mechanically mismatched gels. The in vivo, glial scar formation is a very complex process, and scant information is currently available regarding the stiffness of glial scar tissue relative to neural tissue. Therefore, additional information is needed before the relevance of these data to the actual in vivo situation of reactive glial inhibition in the CNS is established.

In 2D cultures, neurite outgrowth is inhibited by high concentrations of GAGs (Snow et al., 1990, 1992). GAGs can also inhibit neurite outgrowth from chick DRGs in 3D cultures (Carbonetto et al., 1983; Dillon et al., 2000). The reason we selected CS-B in the current study was that CS-B is the most prevalent GAG in CNS

during development (Margolis et al., 1975). Compared with 2.0% agarose gels, the 2.0% CS-B-modified agarose gels greatly inhibited the percentage of neurites crossing the 3D interfaces. When high dosages of CS-B were used in the modification, no significant difference in the percentage of neurites crossing the 3D interfaces formed by CS-B(10)-, CS-B(20)-, CS-B(30)-, and CS-B(40)-modified agarose gels was observed, perhaps because of saturated inhibitory effects of the coupled CS-B. The stiffness of 2.0% CS-B(40)-modified agarose was between the stiffness of 1.0% agarose and 1.25% agarose. As shown earlier, the percentage of neurites crossing 3D interfaces of agarose gels was not affected by the stiffness up to the agarose gel mismatch of 1.0% agarose to 2.0% agarose. Thus, the inhibitory effects of CS-B-modified agarose gels were not due to mechanical mismatch. Our specificity study with chondroitinase ABC indicated that the inhibitory effects of CS-B-modified agarose gels were lost after treatment of CS-B-modified agarose gels with chondroitinase ABC. These results verify that CS-B is involved in the inhibitory effects of CS-B-modified agarose gels in the CS-rich interfaces. The inhibitory effects may be either due to the high density of negative charges on GAGs (Wight et al., 1992) or due to the spatial pattern of the charged groups on GAGs (Erskine et al., 1997). We are currently investigating, in real time, the ability of CS-B GAG to inhibit neurites compared with CS-2 and CS-6 GAGs.

We have previously reported that coupling of extracellular matrix (ECM) proteins such as LN-1 within agarose gels significantly enhances neurite extension in 3D (Yu et al., 1999). Compared with 2.0% agarose gels, the 2.0% LN-1-modified agarose gels stimulated higher neurite crossing of 3D interfaces. As determined by rheometry, the stiffness of 2.0% LN-1-modified agarose gels was between the stiffness of 1.25% agarose and 1.5% agarose. As stated above, there is no significant difference in the percentage of neurites crossing the 3D interfaces formed by agarose gels up to the mismatch of 1.0% agarose to 2.0% agarose. Therefore, LN-1-modified agarose gels enhance neurite extension across 3D interfaces through mechanisms other than mechanical mismatch.

LN-1 has been demonstrated to prevent the inhibitory effects of brain CSPGs in a concentration-dependent manner (Katoh-Semba et al., 1995). When the ratio of LN-1 to CSPGs was 1:50 (w/w), the inhibitory effects of CSPGs were reduced by 50% (Katoh-Semba et al., 1995). In the present study, LN-1 was able to overcome the inhibitory effects of CS-B in a "mixed" gel of Ag-LN-1 and Ag-CS-B(40). The amount of CS-B per unit volume of mixed gel equaled that of CS-B(20). The data in Figure 6 demonstrate that CS-B(20) also significantly inhibits neurite extension across 3D interfaces. However, the percentage of neurite extension across the LN-1-doped mixed gel was significantly higher than that of CS-B(20) gel, indicating LN-1's ability to overcome CS-B's inhibitory effects. The mechanism by which LN-1 overcomes inhibition resulting from CS of GAGs is not clear. One

possible explanation is that LN-1 competitively binds to membranes of the neurons and thus limits the binding of GAGs. Others have demonstrated LN-1's ability to overcome CSPG-mediated inhibition is due not to LN-1 masking CSPG but to the up-regulation of integrin receptors (Condic et al., 1999). Further studies are underway in our laboratory to clarify the specific mechanisms involved.

Embryonic chick DRGs were used as a cellular model in this study. DRGs have the general properties of nerve cells, and embryonic DRGs are widely used as a cellular model for studying the inhibitory molecules involved in glial scar (Carbonetto et al., 1983; Snow et al., 1990, 1992). The DRGs are also the target cells involved in the peripheral nervous system (PNS)-CNS transition zone, where glial scar inhibits CNS nerve regeneration. Our data with embryonic tissue are also relevant in that CSPGs play important roles in boundary formation during nerve development. Preliminary studies in our laboratory show that E4 chick retinal ganglia are also inhibited by CS-B-modified gels (unpublished data).

By presenting different molecules at the interfaces, the *in vitro*, 3D model we have designed can be used to investigate other specific molecules involved in the inhibitory effects of glial scar as we have done for CS-B GAG in this study. Experiments are underway to study the effects of different GAGs such as CS-2 and CS-6 on neurites' ability to cross the 3D interfaces. By mixing CSPG-producing reactive astrocytes into the top layer, the model can be further used to probe whether the inhibitory effects of glial scar were due to the biochemical barriers or mechanical barriers. Recent studies indicate that chondroitinase ABC delivered to the rat spinal cord injury site *in vivo* can up-regulate GAP-43 expression in DRG neurons and promote functional recovery following dorsal column lesions (Bradbury et al., 2000). Therefore, we suggest that our 3D *in vitro* interface model may find application in design strategies to overcome the inhibitory effects of glial scar *in vivo* as well.

#### ACKNOWLEDGMENT

This work was supported by grant RG98-0159 from The Whitaker Foundation to R.V.B.

#### REFERENCES

- Arbogast KB, Thibault KL, Pinheiro BS, Winey KI, Margulies SS. 1997. A high-frequency shear device for testing soft biological tissues. *J Biomech* 30:757-759.
- Bellamkonda RV, Ranieri JP, Aebischer P. 1995a. Laminin oligopeptide derivatized agarose gels allow three-dimensional neurite extension *in vitro*. *J Neurosci Res* 41:501-509.
- Bellamkonda RV, Ranieri JP, Bouche N, Aebischer P. 1995b. Hydrogel-based three-dimensional matrix for neural cells. *J Biomed Mat Res* 29:663-671.
- Bilston LE, Liu Z, Phan-Thien N. 1997. Linear viscoelastic properties of bovine brain tissue in shear. *Biorheology* 34:377-385.
- Bradbury EJ, Bennett GS, Moon LDF, Patel PN, Fawcett JW, McMahon SB. 2000. Chondroitinase ABC delivered to the site of a spinal cord injury upregulates GAP-43 expression in dorsal root ganglion neurons. *Soc Neurosci Abstr* 26:860.

- Carbonetto S, Gruver MM, Turner DC. 1983. Nerve fiber growth in culture on fibronectin, collagen, and glycosaminoglycan substrates. *J Neurosci* 3:2324–2335.
- Cole GL, McCabe CF. 1991. Identification of a developmentally regulated keratan sulfate proteoglycan that inhibits cell adhesion and neurite outgrowth. *Neuron* 7:1007–1018.
- Condic ML, Snow DM, Letourneau PC. 1999. Embryonic neurons adapt to the inhibitory proteoglycan aggrecan by increasing integrin expression. *J Neurosci* 19:10036–10043.
- Davies SJ, Field PM, Raisman G. 1996. Regeneration of cut adult axons fails even in the presence of continuous aligned glial pathways. *Exp Neurol* 142:203–216.
- Davies SJ, Fitch MT, Memberg SP, Hall AK, Raisman G, Silver J. 1997. Regeneration of adult axons in white matter tracts of the central nervous system. *Nature* 390:680–683.
- Davies SJ, Goucher DR, Doller C, Silver J. 1999. Robust regeneration of adult sensory axons in degenerating white matter of the adult rat spinal cord. *J Neurosci* 19:5810–5822.
- Dillon GP, Yu X, Sridharan A, Ranieri JP, Bellamkonda RV. 1998. The influence of physical structure and charge on neurite extension in a 3D hydrogel scaffold. *J Biomat Sci Polymer Edn* 9:1049–1069.
- Dillon GP, Yu X, Bellamkonda RV. 2000. The polarity and magnitude of ambient charge influences three-dimensional neurite extension from DRGs. *J Biomed Mat Res* 51:510–519.
- Donnelly BR, Medige J. 1997. Shear properties of human brain tissue. *J Biomech Eng* 119:423–432.
- Erskine L, McCaig CD. 1997. Integrated interactions between chondroitin sulphate proteoglycans and weak dc electric fields regulate nerve growth cone guidance in vitro. *J Cell Sci* 110:1957–1965.
- Fallenstein GT, Hulse VD, Melvin JW. 1969. Dynamic material properties of human brain tissue. *J Biomech* 2:217–226.
- Farndale RW, Buttle DJ, Barrett AJ. 1986. Improved quantitation and discrimination of sulfated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta* 883:173–177.
- Fawcett JW, Housden E, Smith-Thomas L, Meyer RL. 1989. The growth of axons in three dimensional astrocyte culture. *Dev Biol* 135:449–458.
- Fok-seang J, Smith-Thomas L, Muir E, Du JS, Housden E, Johnson AR, Faissner A, Geller HM, Keynes RJ, Rogers JH, Fawcett JW. 1995. An analysis of astrocytic cell lines with different abilities to promote axon growth. *Brain Res* 689:207–223.
- Galford JE, McElhaney JH. 1970. A viscoelastic study of scalp, brain, and dura. *J Biomech* 3:211–221.
- Geisert EE, Bidanset DJ. 1993. A central nervous system keratan sulfate proteoglycan: location to boundaries in the neonatal brain. *Dev Brain Res* 75:163–173.
- Guth L, Barrett CP, Donati EJ. 1986. Histological factors influencing the growth of axons into lesions of the mammalian spinal cord. *Exp Brain Res* 13(Suppl):271–282.
- Hearn MT. 1987. 1,1'-Carbonyldiimidazole-mediated immobilization of enzymes and affinity ligands. *Methods Enzymol* 135:102–117.
- Katoh-Semba R, Matsuda M, Kato K, Oohira A. 1995. Chondroitin sulphate proteoglycans in the rat brain: candidates for axon barriers of sensory neurons and the possible modification by laminin of their actions. *Eur J Neurosci* 7:613–621.
- Levine JM. 1994. Increased expression of the NG2 chondroitin-sulfate proteoglycan after brain injury. *J Neurosci* 14:4716–4730.
- Margolis RU, Margolis RK, Chang LB, Preti C. 1975. Glycosaminoglycans of brain during development. *Biochemistry* 14:85–88.
- Maxwell WL, Follows DE, Ashhurst DE, Berry M. 1990. The response of the cerebral hemisphere of the rat to injury. I. The mature rat. *Phil Trans R Soc London [Biol]* 328:479–500.
- McKeon RJ, Schreiber RC, Rudge JS, Silver J. 1991. Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. *J Neurosci* 11:3398–3411.
- Milev P, Friedlander DR, Sakurai T, Karthikeyan L, Flad M, Margolis RK, Grumet M, Margolis RU. 1994. Interactions of the chondroitin sulfate proteoglycan phosphacan, the extracellular domain of a receptor-type protein tyrosine phosphatase, with neurons, glia, and neural cell adhesion molecules. *J Cell Biol* 127:1703–1715.
- Powell EM, Geller HM. 1999. Dissection of astrocyte-mediated cues in neuronal guidance and process extension. *Glia* 26:73–83.
- Powell EM, Fawcett JW, Geller HM. 1997. Proteoglycans provide neurite guidance at an astrocyte boundary. *Mol Cell Neurosci* 10:27–42.
- Puchala E, Windle WF. 1977. The possibility of structural and functional restitution after spinal cord injury. *Exp Neurol* 55:1–42.
- Qiu J, Cai DM, Filbin MT. 2000. Glial inhibition of nerve regeneration in the mature mammalian CNS. *Glia* 29:166–174.
- Reier PJ. 1979. Penetration of grafted astrocytic scars by regenerating optic nerve axons in *Xenopus* tadpoles. *Brain Res* 164:61–68.
- Reier PJ, Houle JD. 1988. Advances in neurology. In: Waxman SG, editor. *The glial scar: its bearing on axonal elongation and transplantation approaches to CNS repair*. New York: Raven Press. p 87–138.
- Roberts R, Gallagher J, Spooner E, Allen TD, Bloomfield F, Dexter TM. 1988. Heparan sulfate bound growth factors: a mechanism for stromal cell mediated haemopoieses. *Nature* 332:376–378.
- Ruoslahti E, Yamaguchi Y. 1991. Proteoglycans as modulators of growth factor activities. *Cell* 64:867–869.
- Snow DM, Letourneau PC. 1992. Neurite outgrowth on a step gradient of chondroitin sulfate proteoglycan (CS-PG). *J Neurobiol* 23:322–336.
- Snow DM, Lemmon V, Carrino DA, Caplan AI, Silver J. 1990. Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth in vitro. *Exp Neurol* 109:111–130.
- Stichel CC, Muller HW. 1998. The CNS lesion scar: new vistas on an old regeneration barrier. *Cell Tissue Res* 294:1–9.
- Wight TN, Kinsella MG, Qvarnstrom EE. 1992. The role of proteoglycans in cell adhesion, migration and proliferation. *Curr Opin Cell Biol* 4:793–801.
- Windle WF, Clemente CD, Chambers WW. 1952. Inhibition of formation of a glial barrier as a means of permitting a peripheral nerve to grow into the brain. *J Comp Neurol* 96:359–369.
- Yu X, Dillon GP, Bellamkonda RV. 1999. A Laminin and nerve growth factor laden three-dimensional scaffold for enhanced neurite extension. *Tissue Eng* 5:291–304.