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Immature astrocytes promote CNS axonal regeneration when combined with chondroitinase ABC

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Abstract

Regeneration of injured adult CNS axons is inhibited by formation of a glial scar. Immature astrocytes are able to support robust neurite outgrowth and reduce scarring, therefore, we tested whether these cells would have this effect if transplanted into brain injuries. Utilizing an *in vitro* spot gradient model that recreates the strongly inhibitory proteoglycan environment of the glial scar we found that, alone, immature, but not mature, astrocytes had a limited ability to form bridges across the most inhibitory outer rim. In turn, the astrocyte bridges could promote adult sensory axon re-growth across the gradient. The use of selective enzyme inhibitors revealed that MMP-2 enables immature astrocytes to cross the proteoglycan rim. The bridge-building process and axon regeneration across the immature glial bridges were greatly enhanced by chondroitinase ABC pre-treatment of the spots. We used microlesions in the cingulum of the adult rat brains to test the ability of matrix modification and immature astrocytes to form a bridge for axon regeneration *in vivo*. Injured axons were visualized via p75 immunolabeling and the extent to which these axons regenerated was quantified. Immature astrocytes co-injected with chondroitinase ABC induced axonal regeneration beyond the distal edge of the lesion. However, when used alone, neither treatment was capable of promoting axonal regeneration. Our findings indicate that when faced with a minimal lesion, neurons of the basal forebrain can regenerate in the presence of a proper bridge across the lesion and when levels of chondroitin sulfate proteoglycans (CSPGs) in the glial scar are reduced.

Keywords

immature astrocytes; chondroitinase ABC; chondroitin sulfate; brain injury; regeneration

Introduction

Injury to the adult CNS induces reactive gliosis (Silver and Miller, 2004), which curtails axon regrowth and consists of astrocyte hypertrophy (Bignami and Dahl, 1976; Barrett *et al.* 1981) and the production of inhibitory extracellular matrix (ECM) molecules such as chondroitin sulfate proteoglycans (CSPGs) (Ramón y Cajal, 1928; Pallini *et al.*, 1988; McKeon *et al.*, 1991, 1999; Stichel *et al.*, 1995; Fitch and Silver, 1997; Houle and Jin, 2001; Jones *et al.*, 2003a; Tang *et al.*, 2003). Studies have shown that the digestion of the inhibitory chondroitin sulfate chains with chondroitinase enhances neuronal regeneration (Krekoski *et al.*, 2001; Moon *et al.*, 2001; Bradbury *et al.*, 2002; Fouad *et al.*, 2005;

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Steinmetz *et al.*, 2005; Houle *et al.*, 2006; Pizzorusso *et al.*, 2006; Cafferty *et al.*, 2008; Tester and Howland, 2008).

In vivo, the up-regulation of CSPGs by adult reactive astrocytes occurs as a gradient, with the greatest levels at the lesion epicenter and decreasing towards the penumbra (Davies *et al.*, 1999; Fitch *et al.*, 1999). Although progenitor cells within the lesion core increase the production of growth promoting molecules, such as laminin (Liesi *et al.*, 1984, Busch *et al.*, 2010), inhibitory ECM molecules increase to a much greater extent, limiting the ability of the lesion environment to support regeneration (McKeon *et al.*, 1991; Tom *et al.*, 2004a). Also, the intrinsic ability of astrocytes to promote axon growth decreases as these cells mature (Smith *et al.*, 1990). On the other hand, immature, but not mature, astrocytes transplanted into adult brains have enhanced axon growth promoting properties and they also have the capacity to suppress glial scar formation and integrate into the host tissue (Smith and Silver, 1988; Smith and Miller, 1991).

In the present series of experiments we tested the hypothesis that combining chondroitinase ABC with transplantation of immature astrocytes might help to promote regeneration of an injured CNS axon tract. We used an *in vitro* model to screen all these potential treatments before translating our findings to an *in vivo* model. Next, we have utilized a microlesion model of the cingulum in which one can make a relatively small lesion but still clearly identify only those axons that have been severed and have potentially regenerated. This is possible because a sub-population of axons within the cingulum that arise from the basal forebrain up-regulate p75, a low affinity neurotrophin receptor, after axotomy. This up-regulation extends retrogradely for up to 500 μm from the lesion site and can be visualized using an anti-p75 antibody (Davies *et al.*, 1996). Also, the microlesion provides a reliable model of regeneration failure in which one can use the micropipette that cuts the axons to inject whatever one chooses upon withdrawal of the pipette from the brain. Here, we demonstrate that immature, but not mature, astrocytes are capable of crossing proteoglycans *in vitro*, and that this occurs by a matrix metalloproteinase-2 (MMP-2) dependent mechanism. Our evidence also shows that injection of chondroitinase combined with immature astrocytes can stimulate regeneration across and clearly beyond the lesion. However, axons that have obviously regenerated beyond the lesion, still only regenerate for short distances.

Materials and Methods

Preparation of aggrecan/laminin gradient spot coverslips

Gradient spot glass coverslips (12 mm) were prepared as described previously by Tom *et al.* (2004b). Briefly, coverslips coated with poly-L-lysine (PLL; Sigma-Aldrich, St. Louis, MO) and nitrocellulose were spotted with 2 μl of a solution of aggrecan (0.7 mg/ml; Sigma-Aldrich) and laminin (5 μg/ml; Biomedical Technologies, Stoughton, MA) in calcium and magnesium free Hank's Balanced Salt Solution (CMF; Invitrogen, Gaithersburg, MD). The spots were allowed to dry and were then covered with laminin (5 μg/ml) in CMF and kept at 37°C until just before cell plating (~3 h). Chondroitinase treated aggrecan/laminin spots were prepared in the same manner, except that 0.5 U/ml chondroitinase ABC (Seikagaku, Tokyo, Japan) was added to the laminin/CMF solution for a 3 h incubation. The laminin solution was removed before cell plating.

Cortical astrocyte preparation

P1 rat pup cortical astrocytes were collected by removing the cortices, then finely mincing and treating them with 0.5% trypsin/EDTA (Sigma-Aldrich). Cells were seeded in DMEM/F12 media supplemented with 10% fetal bovine serum (FBS), 2 mM Glutamax, 100 units/ml

penicillin and 100 μg/ml streptomycin (all from Invitrogen) on T75 flasks coated with PLL, and shaken after 4 h to remove non-adherent cells. Immature astrocytes were maintained in culture for 7–14 days and mature astrocytes were maintained in culture for 35 days. Astrocytes were removed from T75 flasks with 0.1 mg/ml trypsin/EDTA, washed in CMF, and then plated at a density of 75,000 cells per coverslip (150,000 cells/ml) in DMEM/F12 media containing 10% FBS, Glutamax and penicillin/streptomycin. Astrocytes were maintained in culture for 1, 3, or 5 days at 37°C and a 5% CO_2 humidified environment, then fixed using 4% paraformaldehyde (PFA) and stained for GFAP (see below for immunocytochemistry).

Metalloproteinase inhibition

The aggrecan-laminin spot gradient coverslips were prepared as described above. Astrocytes were plated at 25,000 cells per coverslip. At the time of plating, the cells were plated in DMEM/F12 media alone, or in media containing either matrix metalloproteinase-2 (25μM) or matrix metalloproteinase-9 (10μM) inhibitors (MMP-2 Inhibitor I, K_i = 1.7 M, inhibits MMP-2 in a dose-dependent manner; MMP-9 Inhibitor I, IC_{50} = 5 nM, also inhibits MMP-1 and MMP-13 at higher concentrations; Calbiochem). Media with the appropriate inhibitors was replaced every 24h. After 5d, coverslips were fixed with 4% paraformaldehyde and stained for CS56 and GFAP to visualize the spot and the astrocytes. The total number of cells extending processes into the rim were counted by a blinded observer.

Dorsal root ganglion (DRG) dissociation

Following 5 days of astrocyte culture on the spot, DRG neurons were added to the astrocyte cultures according to the following protocol. DRG neurons were harvested as described previously by Davies *et al.* (1999). Briefly, DRG neurons were dissected from adult female Sprague-Dawley rats (Zivic-Miller Laboratories, Harlan). After the roots were trimmed, the ganglia were incubated in a solution of collagenase II (200 U/ml; Worthington Biochemicals, Lakewood, NJ) and dispase II (2.5 U/ml; Roche, Nutley, NJ) in CMF. The ganglia were rinsed several times in fresh CMF and gently triturated. After several low-speed spins (~370 × g), the cells were plated at a density of 2000 cells per coverslip (4000 cells/ml) in Neurobasal A media supplemented with B-27 (both from Invitrogen), Glutamax, 100units/ml penicillin and 100μg/ml streptomycin, following removal of the astrocyte media. DRG neurons were maintained in culture on the astrocyte substrate for 48 hours before fixing with 4% PFA and subsequent staining with GFAP and β-tubulin III antibodies (see below for immunocytochemistry).

GFP lentivirus

The GFP viral expression vector was kindly provided by Mark H. Tuszynski. This vector was a VSV-G-pseudotyped, second-generation HIV-based lentivirus with self-inactivating long terminal repeats (Zufferey *et al.*, 1998) and containing a green fluorescent protein (GFP) transgene run from an internal CMV promoter. The GFP transgene was inserted into the transfer plasmid pHR'CMV.SIN, along with the woodchuck hepatitis virus post-transcriptional regulatory element which was incorporated at the 3' end of the transgene by blunt-end ligation into a unique SmaI restriction site. Lentivirus was produced by co-transfecting 293T cells seeded onto 15-cm plates the day before with plasmids pCMVΔR8.91 (package), pMD.G (envelope), and the GFP containing plasmid. The titer of collected supernatant was determined by diluting on 293T cells and transduction analyzed by flow cytometric determination of GFP expression.

Astrocyte transduction

P1 rat cortical astrocytes were collected as described above. Cells were maintained in PLL coated T75 flasks for 3 days for immature astrocytes and 33 days for mature astrocytes. They were then removed from culture flasks with 0.1 mg/ml trypsin/EDTA in CMF and re-seeded on PLL coated 6 well plates at a density of 80,000 cells per well (40,000 cells/ml). Once 75–90% confluent (the following day), the cells were infected with the lentivirus-GFP (viral titer, 2.5×10^9/ml) in DMEM/F12 containing 10% FBS, Glutamax, penicillin/streptomycin and 4 μg/ml polybrene (Sigma-Aldrich) at a multiplicity of infection of 16. Transduction cultures were maintained at 37°C in a 5% CO_2 humidified environment, and 24h following transduction, the lentivirus containing media was removed and replaced with DMEM/F12 containing 10% FBS, Glutamax and penicillin/streptomycin. The transduced cells were maintained at 37°C in a 5% CO_2 humidified environment for 2 days, then removed from culture plates with 0.1 mg/ml trypsin/EDTA in CMF and re-seeded on PLL coated T25 flasks, and maintained for another 2 days before being transplanted into microlesions or plated on gradient spots.

Immunocytochemistry and microscopy

Cultures were immersion fixed in 4% PFA in PBS for 30 min. After 3 washes in PBS (15 min each wash), the coverslips were incubated in blocking solution (5% normal goat serum, 0.1% BSA in PBS, and 0.1% Triton-X for intracellular proteins) at room temperature for 1 h. Coverslips were then incubated in primary antibodies overnight at 4°C. Primary antibodies used were: anti-GFAP (1:500; Molecular Probes/Invitrogen, Eugene, OR) and anti-β-tubulin III (1:400; Sigma-Aldrich). The coverslips were washed 3 times in PBS and then incubated for 1 h at room temperature in appropriate secondary antibodies (Jackson ImmunoResearch Laboratoris, Inc., West Grove, PA or Molecular Probes/Invitrogen). Following antibody application, the coverslips were washed 3 times in PBS, mounted on glass slides in Citifluor (Ted Pella, Redding, CA) and sealed with nail polish. Negative control immunostaining was performed with omission of each primary antibody and did not yield specific immunostaining (not shown). Coverslips were examined using a Leitz Orthoplan 2 fluorescence microscope. The images were processed in Adobe Photoshop (Adobe Systems, San Jose, CA) to reduce background noise and to improve contrast.

Quantification of astrocyte growth and DRG axons crossing the spot rim

Black and white photomicrographs were collected using a Leitz Orthoplan 2 fluorescence microscope attached to an Optronics digital camera operated by Magnafire software. To measure astrocyte growth, 7 images of GFAP immunostaining were collected; 6 of specific regions of the spot rim and one in the center of the spot. To measure DRG axonal rim crossing, 6 images of β-tubulin III immunostaining on the spot rim were collected. Pixel count was determined using the Metamorph 4.6.4 software. Statistical analysis was completed by using the *Student's t-test* for comparison of astrocyte growth and a One-way ANOVA with a Kruskal-Wallis *post hoc* test for comparison of DRG axonal rim crossing. All axons crossing the rim were counted, both independently and on astrocyte bridges.

Microlesion and transplantation

All animal work was completed according to Case Western Reserve University protocol for animal treatment and wellbeing. Lentivirus-GFP transduced cells were removed from culture plates with 0.1 mg/ml trypsin/EDTA in CMF, washed in CMF, and concentrated to 1×10^5 cells/μl in saline or solution of 5 U/ml chondroitinase ABC. Cingulum microlesions were performed based on the model described by Davies *et al.* (1996), differing in that a 460 μm diameter needle (26G Hamilton) was used instead of a 70 μm glass micropipette. Briefly, adult female Sprague-Dawley rats (body weight 180–220 g) were anesthetized with

a ketamine (70 mg/kg) and xylazine (7mg/kg) solution administered intraperitoneally and microlesions caused by inserting the needle 0.03 cm posterior to bregma, 0.1 cm lateral to bregma, and 0.29 cm ventral to the brain surface through a burr hole drilled with a #5 bit and drill (Fine Science Tools, Foster City, CA). A volume of 1 μl of cells was then injected through the same needle 0.22 cm ventral to the brain surface over a period of 3 min. Control microlesions were made using the same coordinates and with the injection of either 1 μl saline or 1 μl of 5 U/ml chondroitinase ABC. The needle was removed and the skin closed with surgical staples. Animals received Marcaine (1.0 mg/kg) subcutaneously along the incision as well as Buprenorphine (0.1 mg/kg) intramuscularly, and kept warm post-operatively with a heating pad during recovery from anesthesia. Animals were allowed access to food and water *ad libitum*. Five or 7 days later, animals were terminally anesthetized with isoflurane, intracardially perfused with 4% paraformaldehyde, and the brains removed and post-fixed in 4% PFA for immunohistochemistry.

Immunohistochemistry and microscopy

Sagital sections (50 μm) of brains were obtained using a Leica VT2000S vibratome and incubated in blocking solution. For immunofluorescence staining, the tissue slices were incubated with primary antibodies overnight at 4°C. The primary antibodies used were: anti-p75 (low affinity neurotrophin receptor; 1:1000; Covance Research Products, Berkeley, CA), anti-CS-56 (1:500; Sigma-Aldrich), anti-digested proteoglycans (2B6; 1:200; Seigagaku, Tokyo, Japan), anti-GFAP (1:500), anti-GFP (1:500; Molecular Probes/ Invitrogen), anti-MMP-2 and anti-MMP-9 (1:500; Abcam) Tissue sections were washed 3 times in PBS (30 min each wash) and then incubated in appropriate secondary antibody (Jackson Jackson ImmunoResearch or Molecular Probes/Invitrogen) overnight at 4°C. Finally, tissue sections were washed 3 times in PBS (30 min each wash), mounted on glass slides in Citifluor and sealed with nail polish. Negative control immunostaining was performed with omission of each primary antibody and did not yield specific immunostaining (not shown). Photomicrographs were collected using Zeiss LSM 410 or Axiovert 510 laser-scanning confocal microscopes. Image thresholds were used to eliminate background noise and to improve contrast (Adobe Photoshop).

Quantification of regeneration

Black and white photomicrographs of p75 immunostained tissue sections at the lesion site were digitally collected. All sections containing the lesion were quantified for each animal and typically 3–5 sections per animal contained the lesion. Five lines were placed on each image; one at the proximal edge of the lesion, one in the middle of the lesion, one at the distal edge of the lesion, one 50 μm past the distal edge of the lesion, and one 100 μm past the distal edge of the lesion. The photomicrographs were then observed at a higher magnification and the number of distinctly p75 labeled axon segments crossing each of the lines was totaled for each animal. Animal groups were defined as: control (animals that received an injection of saline), chondroitinase (animals that received an injection of chondroitinase ABC), immature astrocytes (animals that received an injection of immature astrocytes), immature astrocytes and chondroitinase 5 days (animals that received an injection of immature astrocytes resuspended in a solution of chondroitinase ABC, and were perfused 5 days later), immature astrocytes and chondroitinase 7 days (animals that received an injection of immature astrocytes resuspended in a solution of chondroitinase ABC, and were perfused 7 days later). The number of animals per group was 14, 16, 10, 8 and 7, respectively. Statistical analysis was completed by comparing treatment groups at each of the 5 distances using a One-way ANOVA followed by a Tukey-Kramer *post hoc* test.

Results

Immature astrocytes grow on high levels of CSPGs to a greater extent than mature astrocytes

We sought to better understand the difference in behavior between immature and mature astrocytes using an *in vitro* model that recreates the strongly inhibitory CSPG gradient observed in the glial scar *in vivo*. This model consists of a gradient spot of a mixture of a CSPG (aggrecan) and laminin in which the proteoglycan levels increase towards the perimeter, forming an outer rim that is inhibitory to axonal crossing (Tom *et al.*, 2004b). P1 rat cortical astrocytes were maintained in culture for 7–14 days (for immature astrocytes) or for 35 days (for mature astrocytes), before being plated on the gradient spots of laminin/CSPG. Using this model, we observed that immature astrocytes were capable of attaching to and growing on the gradient spots of laminin/CSPG to a much greater extent than mature astrocytes (N=16 for all groups). At 1, 3 and 5 days of culture, immature astrocytes were observed on a larger portion of the spot compared to mature astrocytes (Fig. 1). At 5 days of culture, immature, but not mature, astrocytes were capable of completely crossing the inhibitory rim of the spot (Fig. 1). This difference was statistically significant. By pre-treating the spot with chondroitinase ABC (ChABC) both immature and mature astrocytes were present within the outer rim of the spot; however, immature astrocytes were capable of almost covering the entire spot rim but mature astrocytes were not (Fig. 1).

Immature astrocytes cross the inhibitory rim using an MMP-2 dependent mechanism

Unlike the mature astrocytes, immature astrocytes were able to enter into and occasionally cross the inhibitory rim of the spot gradient. We hypothesized that immature astrocytes may be using a matrix metalloproteinase to degrade the proteoglycans and enter the rim. We observed that both immature and mature astrocytes express MMP-2 and MMP-9 (Fig. 2A–L). To see if the presence of these proteases is, in part, responsible for permitting immature astrocytes into the inhibitory rim, immature astrocytes were plated on the spot gradient in the presence of an MMP-2 inhibitor, an MMP-9 inhibitor, or control media (Fig. 2M,N). After 5d, the number of cells that entered into the rim was quantified (Fig. 2O). The presence of the MMP-2 inhibitor significantly reduced the ability of immature astrocytes to enter the inhibitory rim. The MMP-9 inhibitor had no significant effect, which is not surprising based on previous work by Muir *et al*., (2002) which demonstrated that MMP-9 was not capable of degrading CSPGs. The action of the protease is evident, when examining the proteoglycan rim beneath the astrocytes. When immature astrocytes were treated with either control media or with the MMP-9 inhibitor, visible degradation of the aggrecan substrate occurred. In contrast, the proteoglycan rim remained much more intact beneath immature astrocytes treated with MMP-2 inhibitor. Although mature astrocytes express MMP-2 and MMP-9 (Fig. 2K,L), their presence in the older cells is somehow insufficient in allowing these cells to migrate into dense proteoglycan deposits (data not shown). Therefore, immature astrocytes use MMP-2, at least in part, to break down the proteoglycan rim and enter into the most inhibitory region of the spot gradient.

Immature astrocytes enhance DRG axonal crossing of the inhibitory rim

Adult dorsal root ganglion (DRG) neurons cultured on the gradient spot form dystrophic growth cones and, for the most part, are incapable of extending axons across the inhibitory rim (Tom *et al.*, 2004b). These dystrophic growth cones represent a stalled growth state achieved in the presence of CSPGs (Ramón y Cajal, 1928). By plating adult DRG neurons on a substrate of immature astrocytes which had been growing on the gradient spot for 5 days, an increase in DRG axonal crossing of the inhibitory rim was observed (compare Figs. 3A, D and G; Fig. 4H; N=8). The immature astrocytes provided a bridge across regions of high levels of CSPGs (Figs. 3A–C). In contrast, mature astrocytes did not significantly

enhance DRG axonal crossing of the inhibitory rim (Figs. 3D–F and Fig. 4H; N=8). Significantly greater DRG axonal crossing of the inhibitory rim was observed for DRG axons cultured on immature astrocytes compared to those cultured on mature astrocytes (Fig. 4H). There was no significant difference in the density of overall neurite outgrowth (Fig. 3A,D, and H) between the two astrocyte populations, indicating that the glial cultures did not alter growth or survival rates.

Pre-treating the gradient spot with ChABC to reduce chondroitin sulfate levels enhanced the ability of adult DRG axons to cross the inhibitory rim for all groups (compare Fig. 3 to 4; Fig. 4H; N=16 for all groups). However, rim crossing of DRG axons cultured on immature astrocytes plated on spots pre-treated with chondroitinase was significantly greater than that observed for any other DRG growth condition examined (Fig. 4). When DRG neurons were cultured on mature astrocytes plated on chondroitinase pre-treated spots, rim crossing of axons was similar to that observed for DRG neurons cultured on immature astrocytes plated on untreated spots (Fig. 4H). When we compared rim crossing of axons of DRG neurons cultured on mature astrocytes plated on chondroitinase pre-treated spots to that of DRG neurons plated directly on pre-treated spots without astrocytes, we could see that the number of axons crossing in the presence of the mature glia decreased, indicating that mature astrocytes actually impaired rim crossing (compare Fig. 4D to G; Fig. 4H). Also, we observed that mature astrocytes located on and off the pre-treated spot did not meet to form contiguous highways (Fig. 4E).

These findings indicated that the most effective method of promoting DRG axonal crossing of inhibitory levels of CSPGs was a combination of immature astrocytes and ChABC treatment.

Cingulum microlesions

To test our hypothesis that one method for promoting axonal regeneration would be a combination of co-injecting immature astrocytes and ChABC, we used the microlesion model described by Davies *et al.* (1996) with a slight modification as described in Materials and Methods. This model consists of a small lesion that penetrates through the deeper layers of the cingulate cortex, severs a narrow band of basal forebrain projection fibers in the cingulum, and penetrates into the corpus callosum. Interestingly, fibers from the basal forebrain within the cingulum express low levels of p75+ and this receptor is dramatically upregulated following injury. Using a 460 μm diameter needle, we were able to lesion a number of these axons in the cingulum while maintaining a nearly continuous glial path proximal and distal to the lesion. Injured axons, identified by intense p75 immunostaining, were unable to cross the lesion despite its small size (Figs. 5A and B) and despite the nearly intact glial pathway (Figs. 5C and D) as had been described by Davies et al. (1996). The injured axons extended towards the lesion and into the gradient of upregulated CSPG before taking on a dystrophic appearance (arrows in Fig. 5B). On rare occasion they turned away from the CSPG territory (arrowhead in Fig. 5B). Few axons were capable of extending beyond the middle of the lesion, and no axons were able to extend beyond the distal edge of the lesion after 7 days, in untreated control animals (Figs. 5B and D; Fig. 9; N=14).

Chondroitinase ABC treatment alone does not promote regeneration

To test whether chondroitinase treatment alone can promote regeneration in this diminutive CNS injury, we injected 1 μl of 5 U/ml ChABC directly into the lesion at the time of needle insertion and withdrawal. This single injection of ChABC was effective in degrading CSPGs present in the lesion as well as beyond the confines of the lesion (Figs. 6A and B). Additionally, the injection of ChABC did not alter the glial cell alignment or the glial response to injury (compare Fig. 5D to 6D), or the severity of the immune response to injury

(data not shown). Despite reducing inhibitory CSPGs, ChABC delivered once at the time of injury was unable to promote regeneration of p75+ axons beyond even the proximal edge of the lesion after 7 days (Figs. 6 and 9; N=16), where axons took on a dystrophic appearance (arrows in Figs. 6B and D) and occasionally even turned (arrowhead in Fig. 6D).

Immature astrocyte transplants alone do not promote regeneration

The failure of ChABC treatment to promote regeneration led to the hypothesis that in addition to removing inhibitory CSPGs, regenerating axons would need a bridge across the lesion site. Hypothetically, astrocytes transplanted into the lesion would integrate into the lesion and serve as a bridge for regenerating axons. In order to test this, we initially transplanted immature and mature astrocytes into cingulum microlesions at the time of injury in the absence of ChABC treatment. Prior to transplantation, the cells were infected with a lentivirus containing the gene encoding GFP so that cells could later be located in brain slices. Immature astrocytes transplanted into the microlesions survived, integrated into the lesions, and even migrated along the cingulum/corpus callosum junction (Figs. 7A and B). Despite the integration and bridging potential of the transplanted immature astrocytes, few p75+ axons were able to extend beyond the middle of the lesion and no p75+ axons were able to extend beyond the distal edge of the lesion after 7 days (Figs. 7A and 9; N=10). Although in some instances the transplanted immature astrocytes interacted with the axons and appeared to promote meager regeneration (asterisks in Fig. 7A), most of the time this was insufficient to prevent the formation of dystrophic endballs and turning of axons (arrows in Fig. 7A).

Mature astrocytes transplanted into the lesion did not survive well over the 5 days of the experiment; only a few viable mature astrocytes were found in the transplanted animals (Figs. 7C and D). The cells that survived were unable to promote regeneration of p75+ axons beyond even the proximal edge of the lesion (dashed line in Fig. 7C). The transplanted mature astrocytes also induced CSPG up-regulation (compare Fig. 7B to D), and in fact, the severity of the CSPG up-regulation, increase in GFAP+ cells and macrophage invasion resulted in cavitation, enlarging the lesion to a size well beyond that of a microlesion alone (data not shown). As a result of the massive host response observed following transplantation of mature astrocytes, we chose to focus our attention on the use of immature astrocytes in transplant experiments.

A combination of immature astrocytes and chondroitinase ABC promotes regeneration

Considering immature astrocytes were able to survive and integrate into the lesion, we sought to further study their potential to provide a bridge across the lesion for regenerating axons. We hypothesized that by co-injecting immature astrocytes and ChABC, we would simultaneously obtain a bridge across the lesion and a reduction in CSPG levels. When immature astrocytes were transplanted in the presence of ChABC, these cells were still capable of integrating into the lesion and provided a bridge across the lesion (Fig. 8A). Furthermore, the injected chondroitinase digested chondroitin sulfate in the lesion and in a wide-reaching area adjacent to the lesion (Figs. 8B and C). At 5 days post-lesion, this combinatorial treatment promoted regeneration of p75+ axons, which were able to extend up to 100 μm beyond the distal edge of the lesion (asterisks in Fig. 8A; Fig. 9; N=8). This was the only treatment capable of promoting regeneration clearly beyond the lesion (Fig. 9). Interestingly, 7 day survival groups were not significantly different from 5 day survival groups in terms of number of axons regenerating beyond the distal edge of the lesion (Fig. 9; N=7). When immature astrocytes and ChABC were co-injected into the portion of the microlesion affecting the callosum, they provided a bridge for regenerating axons, which allowed the p75+ axons to extend from the cingulum through the corpus callosum, a highly myelinated structure, and exit on the other side (Figs. 8D–F).

Discussion

We have found that adult axons are capable of regenerating at least a short distance across CSPG rich inhibitory regions found in the glial scar *in vivo*, if provided with a growth promoting cell type that is able to both bridge and integrate with the edges of the lesion after high CSPG levels are reduced. Immature astrocytes transplanted along with chondroitinase fulfilled these requirements, allowing a population of axotomized forebrain neurons to cross a narrow lesion. This is important in light of recent findings showing that adult sensory axons also have an intrinsic capacity to regenerate a short distance beyond very small, scar-free lesions (Dray *et al.*, 2009; Ylera *et al.*, 2009). Interestingly, DRG axons were suggested to regenerate upon newly formed blood vessels that bridged the core of this type of diminutive lesion (Dray *et al.*, 2009) suggesting again that a growth-supportive bridge across lesion cavities, even small ones, may be necessary. In other studies, fibroblasts (Kawaja and Gage, 1991; Tuszynski *et al.*, 1997; Jones *et al.*, 2003b), Schwann cells (Xu *et al.*, 1997; Takami *et al.*, 2002; Fouad *et al.*, 2005), skin-derived precursor-derived Schwann cells (Biernaskie *et al.*, 2007), olfactory ensheathing glia (Raisman and Li, 2007; Ibrahim *et al.*, 2009), stem/progenitor cells (Goldman 2005; Liu *et al.*, 2009), bone-marrow derived mononuclear cells (Yoshihara *et al.*, 2007; Coulson-Thomas *et al.*, 2008) a growth-promoting matrix, Matrigel (Lemons *et al.*, 2003), or a peripheral nerve graft (David and Aguayo, 1981) were used to fill or bypass the lesion cavity. Our results suggest that immature astrocytes could also be an ideal bridge-building cell (Davies *et al.*, 2006).

Studies have shown that immature astrocytes have a high capacity to support neurite outgrowth *in vitro* (Bähr *et al*., 1995; Smith *et al*., 1990). We verified not only the potential of immature astrocytes to support axonal outgrowth but also to grow on high levels of CSPGs and modify a lesion by using an *in vitro* model of the glial scar (Tom *et al*., 2004b) as well as the *in vivo* microlesion model (Davies *et al*., 1996). We discovered that the mechanisms that underlie the enhanced growth of immature astrocytes on high levels of CSPGs *in vitro* are MMP-2-dependent. MMPs are produced by astrocytes following injury (Muir *et al*., 2002; Wang *et al*., 2002) and they have been implicated in ECM remodeling and neurite regeneration (Duchossoy *et al*., 2001; Ogier *et al.*, 2006). Although both immature and mature astrocytes express MMP-2 and MMP-9, only immature astrocytes use MMP-2 to cross the inhibitory rim *in vitro*. It is possible that as astrocytes mature they continue to express MMPs, but also express tissue inhibitors of metalloproteinases (TIMPs). The presence of TIMPs would therefore hinder their capacity to use MMPs to cleave CSPGs. This possibility has not yet been tested. Additionally, studies were not conducted to determine if the MMP-2 and MMP-9 were present in their inactivated or activated form. It is, therefore, also possible that MMP-2 is only in its activated form in immature astrocytes. The role of MMP-2 has previously been demonstrated after spinal cord injury (Hsu *et al*., 2006). Hsu *et al*. saw more CSPG immunoreactivty and less functional recovery in MMP-2 null mice versus wild-type mice. Thus, MMP-2 appears to function in regulating CSPGs after injury. Our *in vitro* results, in addition to these observations after spinal cord injury, suggest that immature astrocytes may also use MMP-2 *in vivo* to form a bridge across CSPGs, although this has not been tested. However, even with transplantation of immature astrocytes, axon regeneration is still limited, which may be attributed to the limited growth potential of mature axons. This strategy of bridging the inhibitory environment could be combined with other techniques to enhance the intrinsic ability of the axons to regenerate, such as reducing the effect of the PTEN gene (Park *et al.*, 2008) or the use of a conditioning lesion (Richardson and Issa 1984).

Immature astrocytes transplanted into adult animals either on nitrocellulose (Smith and Silver, 1988) or directly as cell suspensions (Smith and Miller, 1991) have the capacity to integrate with damaged host tissue, reduce glial scar formation and modify the environment

of the mature CNS to begin to resemble the permissive environment of the young CNS. These observations may be explained by the fact that immature astrocytes can use MMP-2 to remodel the ECM, whereas mature astrocytes cannot, even though mature cells produce at least some level of this protease. Why mature astrocytes are so immobile in a proteoglycan environment that they themselves help to create in vivo remains to be answered. These observations also provide a partial explanation for why young animals have a greater capacity to recover after injury than mature animals (Nakamura and Bregman 2001). Despite the effect on scarring, p75+ axons were still unable to regenerate beyond microlesions when only immature astrocytes were transplanted. This is probably due, in part, to CSPGs, which remained at levels sufficiently high to disallow dystrophic axons to re-enter a robust growth state, as well as the presence of activated macrophages which cause axonal dieback (Horn *et al*., 2008; Busch *et al*., 2009).

While the combinatorial treatment of immature astrocytes and chondroitinase could promote the regeneration of p75+ axons clearly beyond the distal edge of the microlesion, the axons still continued to grow for only a short distance. The presence of other inhibitory molecules such as myelin in the CNS could be acting to inhibit extending growth cones beyond the lesion site (Caroni, *et al*., 1988; Savio and Schwab, 1990; Schwab, 2002; Filbin, 2003; McGee and Strittmatter, 2003). However, it is interesting that, while in the presence of immature astrocytes, the regenerating axons were capable of extending from the cingulum through the corpus callosum, which is a highly myelinated structure, suggesting that myelin may not be as potently inhibitory as once believed (Lee 2010).

Other studies have shown that limited regeneration beyond lesions (Richardson and Issa, 1984; Neumann *et al*., 2002; Fournier *et al*., 2003; Lingor *et al*., 2007; Andrews *et al*., 2009) can be enhanced by the delivery of exogenous trophic support (Kawaja and Gage, 1991; Tuszynski *et al*., 1997; Jones *et al.*, 2003b; Kadoya *et al*., 2009). Massey *et al*. (2008) documented that the use of ChABC alone only partially overcame regeneration inhibition of sensory axons into the gracile nucile but when combined with NT-3, this combination strategy dramatically increased axonal regeneration. Therefore, regeneration beyond the microlesion could possibly be further enhanced by injecting growth factors, such as BDNF (Kobayashi *et al.*, 1997; Oudega and Hagg, 1999; Tropea *et al.*, 2003), NT-4/5 (Kobayashi *et al*., 1997), NT-3 (Bradbury *et al.*, 1999; Oudega and Hagg 1999), NGF (Oudega and Hagg, 1996, 1999), or an analog of cAMP (Neuman *et al.*, 2002; Qiu *et al.*, 2002). Growth factor administration could also encourage enhanced regeneration if injected into the region of the cell bodies of lesioned neurons (Kobayashi *et al.*, 1997; Kwon *et al.*, 2002, 2007; Vavrek *et al.*, 2006).

In conclusion, immature astrocytes combined with ChABC is a promising therapy for axonal regeneration in CNS lesions, but for long distance regeneration, this and other bridge building strategies will need to be combined with treatments that markedly increase the intrinsic growth ability of axons.

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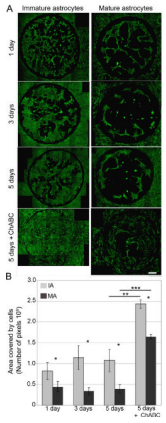

Figure 1.

Immature astrocytes are capable of growing on high levels of CSPGs. Immature and mature astrocytes were cultured on gradient spots of a mixture of CSPG and laminin for 1 day, 3 days and 5 days (A), or on spots pre-treated with chondroitinase ABC for 5 days (A; 5 days + ChABC). Astrocytes were identified by GFAP immunoreactivity (green). Astrocyte growth on the gradient spot was quantified in terms of area of the spot occupied by cells, in Pixel count using the Metamorph software (B), demonstrating the significantly enhanced growth of immature astrocytes compared to mature astrocytes at all time-points (*p<0.0001), as well as the significantly enhanced growth of both immature (**p<1×10^{-9}) and mature astrocytes (***p<1×10^{-15}) following pre-treatment of the spots with chondroitinase ABC (N=16 for all groups; IA: immature astrocytes; MA: mature astrocytes). Scale bar: 300μm.

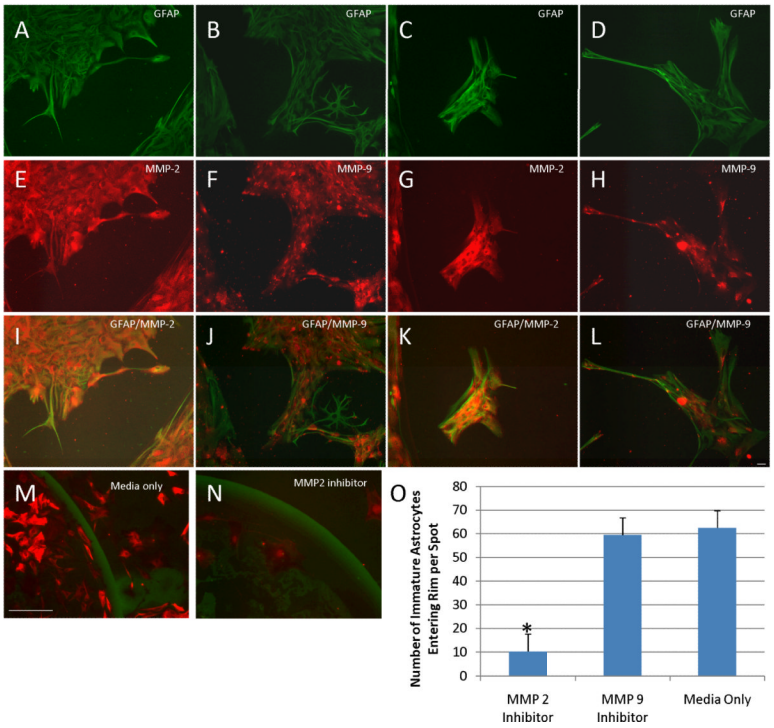

Figure 2.

Immature astrocytes use MMP-2 to cleave CSPGs. Immature (A,B) or mature (C,D) astrocytes were stained for GFAP. Both immature (E,F,I, J) and mature astrocytes (G,H,K,L) express MMP-2 and MMP-9. Immature astrocytes were plated on gradient spot coverslips in the presence of MMP-2-inhibitor (N), MMP-9 inhibitor, or DMEMF12 media alone (M) (N=12, 10, and 6 respectively). The MMP-2-inhibitor significantly decreased the amount of immature astrocytes that extended processes into or across the proteoglycan rim compared to media only (O). MMP-9-inhibitor did not affect astrocyte crossing. Statistical analysis was performed using a one-way Anova (*p< 0.0005). Scale bar: 200 μm.

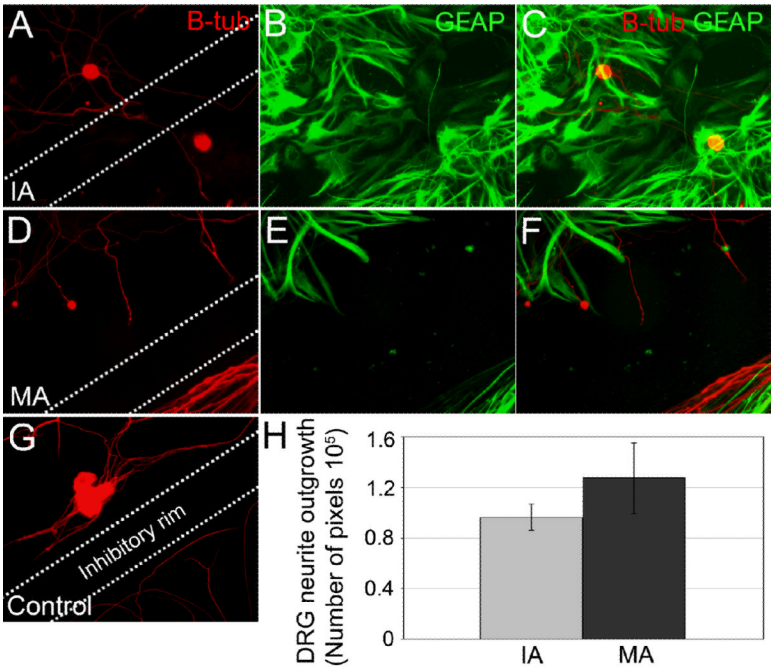

Figure 3.

Immature astrocytes form bridges which adult DRG axons can access to cross inhibitory levels of CSPGs. Adult DRG neurons were cultured for 2 days on 5 day-cultures of immature (A–C) or mature astrocytes (D–F) plated on CSPG gradient spots, or on gradient spots in the absence of astrocytes (Control; G). Axons were identified by β-tubulin III immunolabeling (red) and astrocytes by GFAP immunolabeling (green). DRG neurite outgrowth on immature and mature astrocytes was quantified by Pixel count using the Metamorph 4.6.4 software (H) and indicated no significant difference (N=8 for each; IA: immature astrocytes; MA: mature astrocytes). C and F are merged images of A, B and D, E, respectively. Scale bar: A–G, 50μm.

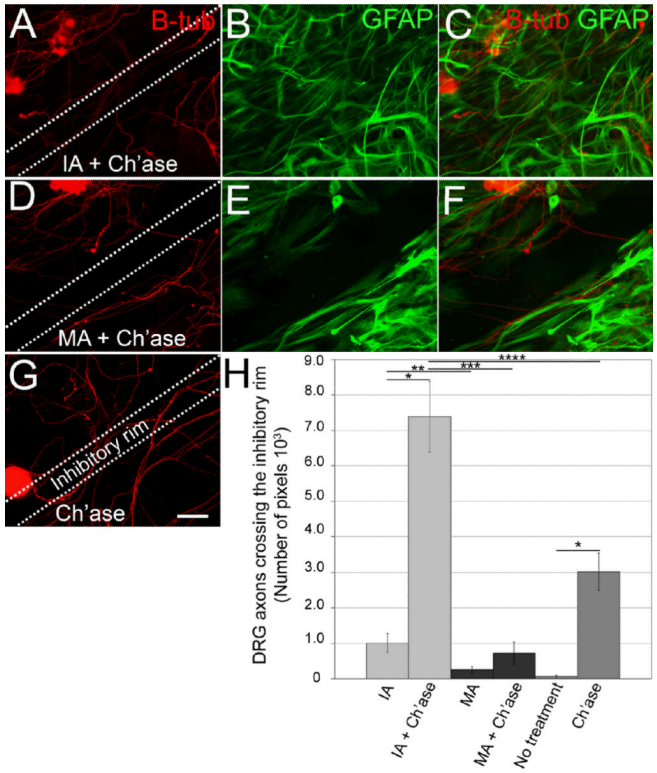

Figure 4.

Pre-treatment of gradient spots with chondroitinase ABC enhances axon rim crossing of DRG neurons cultured on immature astrocytes. Adult DRG neurons were cultured for 2 days on immature (A–C) or mature astrocytes (D–F) which had been plated on gradient spots pre-treated with chondroitinase ABC and in culture for 5 days, or on pre-treated spots in the absence of astrocytes (G). Axons were identified by β-tubulin III immunolabelling (red) and astrocytes by GFAP immunolabelling (green). The Pixel count of β-tubulin III immunostaining in the outer rim of the spot was determined using the Metamorph 4.6.4 software for each group (H) and represents the amount of DRG axons crossing the inhibitory rim (N=16 for all groups). Statistically significant differences were observed for rim crossing of DRG axons cultured on immature astrocytes (IA) compared to rim crossing of axons cultured on immature astrocytes plated on pre-treated spots (IA + ChABC; *$p<0.00001$), and for rim crossing of DRG axons plated on control spots (no treatment) compared to rim crossing of axons plated on pre-treated spots in the absence of astrocytes (ChABC; *$p<0.00001$). A statistically significant difference was observed for rim crossing of DRG axons cultured on immature astrocytes (IA) compared to those cultured on mature astrocytes (MA; **$p=0.01$). No statistically significant difference was observed for rim crossing of DRG axons cultured on mature astrocytes (MA) compared to axons cultured on mature astrocytes plated on pre-treated spots (MA + ChABC). DRG neurons cultured on immature astrocytes plated on pre-treated spots presented enhanced rim crossing compared to all other groups (***$p<0.000001$ and ****$p<0.001$). C and F are merged images of A, B and D, E, respectively. Scale bar: A–G, 50μm.

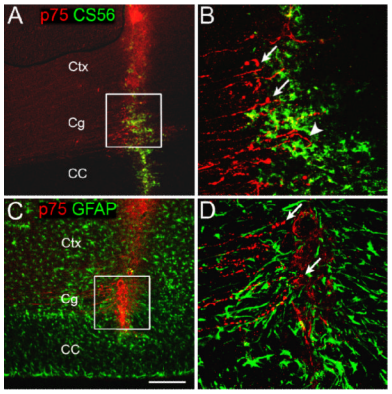

Figure 5.

Injured p75+ axons are unable to regenerate beyond the microlesion. Microlesions were performed in the cingulum and injured axons were analyzed 5 days later by p75 immunostaining (red). The p75+ axons were unable to regenerate beyond the upregulated CSPGs (green) (A and B) and GFAP expressing astroglia (green) (C and D) present in the microlesion, and took on a dystrophic appearance (arrows) or even turned (arrowhead) (Ctx: cortex; Cg: cingulum; CC: corpus callosum). B and D are higher magnifications of the insets in A and C, respectively. Scale bar: A and C, 100μm.

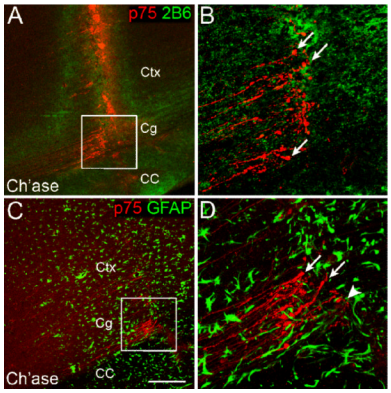

Figure 6.

The p75+ axons in chondroitinase ABC treated microlesions are not capable of regenerating. Microlesions were performed in the cingulum and treated with a single injection of 5U/ml chondroitinase ABC. Chondroitinase treatment resulted in 2B6 immunolabelling of CS stubs left from digested CSPGs (green) (A and B). Injured axons were identified by p75+ immunolabelling (red) and were unable to regenerate beyond the lesion even after CS digestion (A and B) or beyond GFAP expressing astroglia (green) (C and D), taking on a dystrophic appearance (arrows) and even turning (arrowhead) (ChABC: chondroitinase ABC; Ctx: cortex; Cg: cingulum; CC: corpus callosum). B and D are higher magnifications of the insets in A and C, respectively. Scale bar: A and C, 100μm.

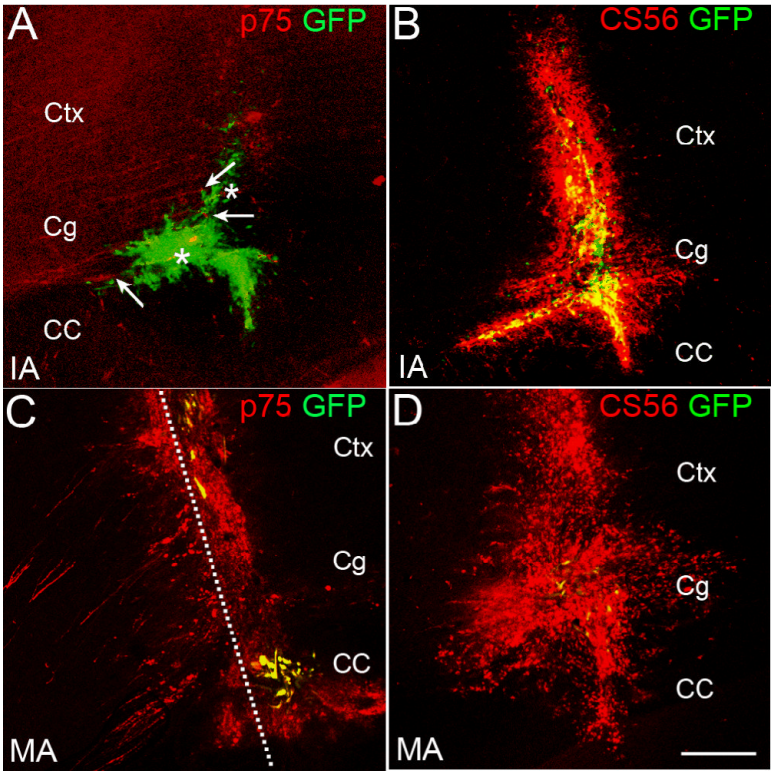

Figure 7.

Immature and mature astrocytes transplanted into the microlesion are not capable of promoting regeneration of p75+ axons. Astrocytes were infected with a GFP lentivirus prior to transplantation into the cingulum microlesion and were therefore identified by GFP immunolabelling (green). Immature astrocytes interacted with p75+ axons (red) (asterisks) but were mostly incapable of preventing the formation of dystrophic endballs and turning of axons (arrows) (A). Few mature astrocytes transplanted into the microlesion survived and did not promote regeneration of p75+ axons (red) past the proximal margin of the lesion (dashed line) (C). CSPGs (red) are upregulated at and beyond the lesion site (B and D) (IA: immature astrocytes; MA: mature astrocytes; Ctx: cortex; Cg: cingulum; CC: corpus callosum). Scale bar: A–D, 100μm.

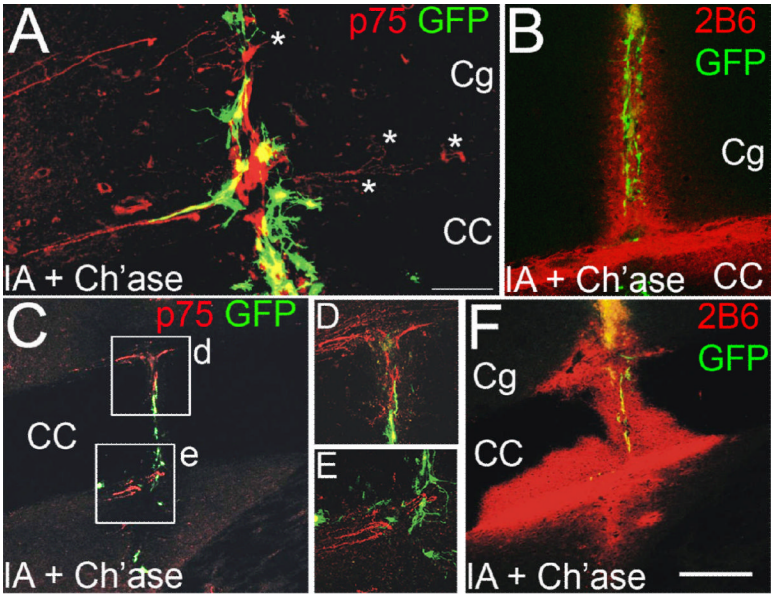

Figure 8.

Immature astrocytes injected with chondroitinase ABC promote regeneration of p75+ axons. Immature astrocytes were infected with a GFP lentivirus prior to transplantation into the cingulum microlesion and were therefore identified by GFP immunolabeling (green). The immature astrocytes were injected along with 5U/ml chondroitinase ABC which was effective in degrading CSPGs (red, 2B6 immunostaining) in a wide area ranging from the margins of the needle tract from the surface of the cortex to the cingulum and the dorsal and ventral surfaces of the corpus callosum (B and F). The immature astrocytes injected along with chondroitinase ABC were capable of promoting regeneration of p75+ axons (red) beyond the lesion after 5 days (asterisks) (A) and through the corpus callosum (C). Figures D and E are higher magnifications of the insets in C (d and e) showing p75+ axons (red) entering into the dorsal surface of the corpus callosum and exiting the ventral surface of the corpus callosum, respectively, in the same location as the transplanted immature astrocytes (green) (IA + Chase: immature astrocytes with chondroitinase ABC; Cg: cingulum; CC: corpus callosum). Scale bars: A, 50μm; B, C and F, 100μm.

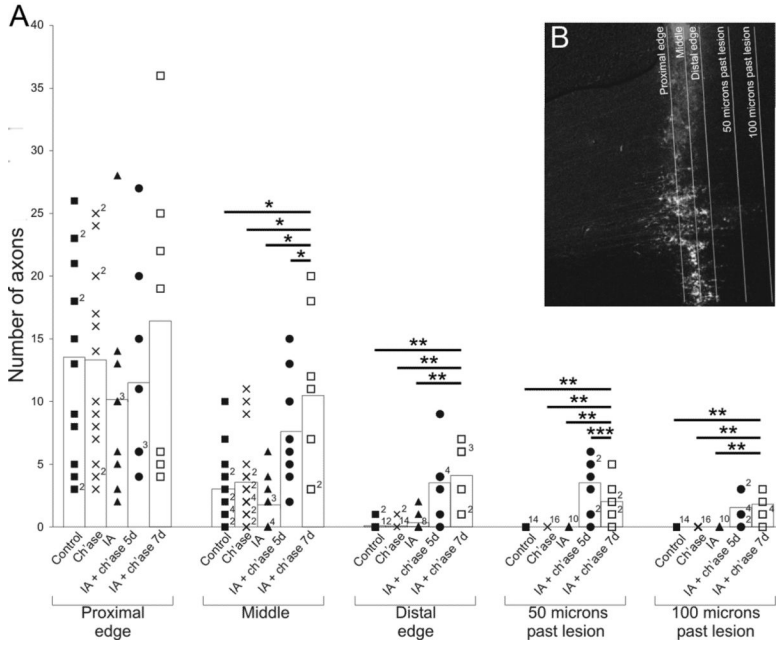

Figure 9.

Quantification of axonal regeneration demonstrates that immature astrocytes combined with chondroitinase ABC would be the best treatment for brain injuries. Axonal regeneration was quantified by counting the number of axons crossing the proximal edge of the lesion, the middle of the lesion, the distal edge of the lesion, 50 μm past the distal edge of the lesion, or 100 μm past the distal edge of the lesion (A). These 5 distance markers are outlined in an example of the lesion shown in B. Within each distance group, the 5 treatment groups outlined as control (control), chondroitinase ABC (ChABC), immature astrocytes (IA), immature astrocytes with chondroitinase ABC at 5 days (IA + ChABC 5d), and immature astrocytes with chondroitinase ABC at 7 days (IA + ChABC 7d), were compared (N=14, 16, 10, 8 and 7, respectively). Animals from the first 3 groups and the fifth group were perfused and the brains removed after 7 days, and those from the fourth group after 5 days. Each data point represents one animal, unless noted with a superscript indicating the number of animals that data point represents, and the bar graphs represent the average number of axons within each treatment group. The y-axis indicates the number of axons observed at the various distances indicated by the x-axis. For example, in the control group, in 2 of the animals, 3 axons had extended past the proximal edge of the lesion, resulting in a total of 24 animals, a total of 189 axons, and an average of 13.5 axons crossing the proximal edge of the lesion per animal. Statistical analysis was completed by comparing treatment groups at each of the 5 distances using a one-way ANOVA followed by a Tukey-Kramer *post hoc* test. There was no statistically significant difference between any of the treatment groups when evaluating the proximal edge of the lesion. The group immature astrocytes with chondroitinase ABC showed a greater number of axons at the middle of the lesion after 7 days, compared to the other 4 groups (*$p<0.005$). At all other distances, the two groups immature astrocytes with chondroitinase ABC 5 days and 7 days showed no statistically significant difference from each other, but were significantly different to the other groups (**$p<0.0001$). The one exception is that at a distance of 50 μm past the lesion, the group immature astrocytes with chondroitinase ABC 5 days was significantly different from the group immature astrocytes with chondroitinase ABC 7 days (***$p<0.0012$).