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Soluble Factor Effects on Glial Cell Reactivity at the Surface of Gel-Coated Microwires

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Abstract

A basal lamina gel preparation was incorporated into a modified neuroinflammation cell culture model to test the system as a characterization tool for surface-modified microwires. The extent of gliosis at the surface of gel-coated microwires was quantified in response to titrating the cell culture with a number of soluble factors reported to be involved in reactive gliosis. Positive control conditions (1% FBS, 10ng/ml bFGF, Neural Basal medium with B27 supplement) induced a layer of GFAP-expressing astrocytes to accumulate on all gel-coated microwires. Serum, inflammatory cytokines IL-1 α and IL-1 β and the neural progenitor cell (NPC) proliferating growth factors bFGF and PDGF all increased the number of reactive cells on the gel, in agreement with their reported roles in cell activation, migration and proliferation at the site of injury. Technically, this study shows that a fetal neuron-glia culture system is well suited for characterizing the impact of electrode coatings designed to mitigate implant-associated gliosis, and for screening the effect of multiple soluble factors that promote and/or inhibit implant-associated gliosis. Scientifically, this study points to essential roles of serum and inflammatory factors to induce NPC activation and migration to the site of injury, where growth factors like bFGF and PDGF induce proliferation of cells that will eventually form the glial scar.

Introduction

We recently reported an in vitro, mixed cell culture based system that reproduces many of the characteristic hallmarks of the in vivo glial scar that forms around microwire electrodes (Polikov et al., 2009). This model recapitulated in vivo behavior such as microglial activation and attachment to stainless steel microwires, astrocyte activation beyond the microglial layer in the form of GFAP upregulation, neural precursor cell migration to a site of injury and differentiation into GFAP-expressing astrocytes, and the presence of healthy, electrically active neurons. An important and novel observation in the 2009 study was that a robust glial response occurred in culture every time around mock microwire electrodes *only* with the presence of the conditions that allow for the maintenance and growth of neural precursor cells (NPCs): serum, Neurobasal media, B27 supplement, bFGF.

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Mounting evidence suggests that the glial scar forms as a result of NPC migration to the site of injury, proliferation at the injury site, and differentiation into activated GFAP expressing astrocytes (Alonso, 2005; Hampton et al., 2004; Kernie et al., 2001; Magnus et al., 2007b; Wang et al., 2004). The idea that NPCs may play a critical role in the glial scarring around neural implants runs counter to the predominantly held view that surviving astrocytes in the wound area proliferate and upregulate GFAP to form the glial scar (Polikov et al., 2005). Consequently, strategies targeted solely at inhibiting the activation of resident astrocytes may be inadequate if NPC migration, proliferation, and differentiation are ignored.

An important assessment tool in the design of long-term implantable neuroelectrodes is the development of an in vitro system for testing electrode coatings that mitigate implant-associated gliosis (Polikov et al., 2006; Polikov et al., 2009; Achyuta et al., in press). Since basal lamina is thought to play an important role in gliosis (Landis 1994), we coated stainless steel microelectrodes with a basal lamina mimic to directly study the reactive astroglial layer that forms around a gel comprised of the ECM preparation Matrigel® blended with alginate hydrogel. In this study the conditions that promote NPC proliferation served as a stable positive control, and removal of just one of these conditions, bFGF, results in minimal reactivity and therefore acted as a negative control. The cellular responses to gel-coated wires were analyzed microscopically using previously reported protocols for GFAP, Vimentin and DAPI staining (Polikov et al., 2006; Polikov et al., 2009).

The screening capability of the culture system was tested against a host of soluble factors that are reported to affect the number of reactive glial cells that accumulate at the specimen surface. The extent of cell accumulation on the gel-coated microwires was significantly increased by titration of the culture with serum, the pleiotropic growth factor bFGF, the inflammatory cytokines IL-1 β and IL-1 α , and the growth factors PDGF and BMP-2. The other fourteen soluble factors tested had little to no effect on the number of cells that attached to the coated microwires, even when administered at concentrations that were often above typical concentrations used in neural cell culture, or sometimes at just below cytotoxic concentrations. Only a specific blocker of the bFGF receptor was able to abrogate the effect of bFGF. This study further supports the essential roles in glial reactivity of serum, which infiltrates brain tissue upon disruption of the blood-brain barrier, and bFGF, which is a necessary growth and survival factor for the neural precursor cells that respond to injury (Maynard et al., 2000; Wu et al., 1998; Stichel et al., 1999).

Materials and Methods

Reagents

Cell culture ingredients were obtained from Invitrogen (Carlsbad, CA, USA). Certified serum (Invitrogen Cat# 16000) contained less than 0.06 units/ml endotoxin and less than 2 mg/dl hemoglobin. Polyclonal antibody against glial fibrillary acidic protein (GFAP) was bought from DAKO Corporation (Carpinteria, CA, USA). The Vectastain ABC kit and biotinylated secondary antibodies were purchased from Vector Laboratories (Burlingame, CA, USA). 50 μ m diameter stainless steel microwire was bought from A-M Systems (Carlsborg, WA, USA). Basic Fibroblast Growth Factor (bFGF) was obtained from R&D Systems (Minneapolis, MN, USA). The various soluble factors and the concentrations used are presented in Table 1.

Animals

Timed-pregnant Fisher F344 rats were obtained from Charles River Laboratories (Raleigh, NC, USA). Housing and breeding of the animals were performed in strict accordance with

the National Institutes of Health guidelines at the National Institutes of Environmental Health Sciences (Research Triangle Park, NC, USA).

Primary mesencephalic neuron-glia cultures

Neuron-glia cultures were prepared from the ventral mesencephalic tissues of embryonic day 14–15 rats, as described in the “Control Protocol” in (Polikov et al., 2009) with minor modifications. 24-well plates were coated with poly-D-lysine for one hour, and washed three times with PBS. Dissociated cells were seeded at 1×10^6 cells/well. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air, in Neurobasal (NB) medium supplemented with B27 serum free supplement containing 1% Fetal Bovine Serum (FBS), 10 ng/ml of bFGF, 2 mM L-glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin. Ten-day-old cultures were used for treatment. Treatment consisted of placement of four microwires coated with Matrigel/Alginate hydrogel in each well after a media change to NB with B27, 2 mM L-glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin. FBS (1%) and 10 ng/ml of bFGF were also added at treatment for “With bFGF” soluble factor studies, while only FBS (1%) was added for soluble factor studies “without bFGF.” Cultures were fixed with 3.7% formaldehyde 7 days after treatment (T=17d). Data shown are of at least 3 different culture preparations.

Basal lamina gel coating

Hydrogel coating was made of basal lamina ECM and sodium alginate. The sodium alginate was used exclusively to shape the ECM around the microwires, not to affect cellular behavior, as ECM-only preparations did not result in a hydrogel that could be inserted into the culture while alginate-only preparations did not influence cell behavior (cells did not attach, data not shown). A 50–50% mixture of Growth Factor Reduced Matrigel Matrix Basement Membrane (Matrigel) (BD Biosciences, San Jose, CA, USA) and 2% Novatech RGD-coupled ultrapure sodium alginate (Novamatrix, Sandvika, Norway) (1% final sodium alginate concentration) was pipetted into a 15 mm length of Teflon tubing (0.81–0.97 mm inner diameter). Microwire was cut into ~10 cm lengths and soaked in 70% ethanol for at least 30 minutes, after which it was washed with water 3 times. A length of microwire was inserted into the tubing so that a small piece was sticking out of one end and the rest of the microwire was sticking out of the other end. The small piece and half of the hydrogel-filled tube was dipped into a 1.5% solution of calcium chloride. The small piece of microwire was gripped by forceps and withdrawn from the tube so that as the microwire was withdrawn into the calcium chloride solution, the Matrigel/Alginate solution wicked onto the microwire and solidified into a hydrogel as it hit the calcium chloride. The coated length of microwire was then cut into smaller 3–5 mm pieces to be placed into the culture during treatment. At treatment time, 4 pieces of coated microwire were placed into each treatment well at random locations using sterile forceps, so that the pieces would sink and rest atop the cultured cell layer.

Immunostaining

Microglia were detected with the OX-42 antibody, which recognizes the CR3 receptor as described [24], or anti-IBA-1 antibody, which recognized a calcium binding protein specific to microglia. Astrocytes were detected with an antibody against GFAP, and neurons were imaged by staining with MAP-2 as described previously (Polikov et al., 2006). Immature glia were detected with an antibody against vimentin. Briefly, formaldehyde (3.7%) -fixed cultures were sequentially incubated with blocking solution (20 min), primary antibody (overnight, 4°C), biotinylated secondary antibody (1 h), and ABC reagents (1 h). Color was developed with 3,3'-diaminobenzidine. Nuclear staining was with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) from Sigma (St. Louis, MO, USA). Secondary

fluorescent antibodies Alexa-fluor 546 goat anti-mouse and Alexa-fluor 488 goat anti-rabbit were obtained from Invitrogen (Carlsbad, CA, USA).

Normalized Cell Counts

To develop a quantitative measure of the effect of various factors on the cellular response to the hydrogel coated microwires, the relative number of cells present on or within the hydrogel (almost entirely on the outside surface) was quantified (Figure 1). After immunostaining, each microwire in the well was removed from the well using forceps under a dissecting microscope and placed on a blank microscope slide. The stainless steel microwire was separated from the cell-coated hydrogel and placed on one end of the microscope slide under its own coverslip. The cell-coated hydrogel was placed under another coverslip and the coverslip was pressed down firmly to break apart the hydrogel into one 2-d plane for further analysis. Images of the DAPI labeled cells were recorded with a Nikon Eclipse TE2000-U inverted microscope (Nikon; Tokyo, Japan) connected to a Nikon Digital Sight DS-2MV camera (Nikon; Tokyo Japan) operated with the Nikon NIS-Elements software (Nikon; Tokyo, Japan). Each hydrogel image of DAPI stained nuclei was thresholded and the total DAPI-stained area was recorded. Cell nuclei area, rather than nuclei counts, was used because the software did not accurately perform cell counts when several nuclei were clustered together and manual counting was not feasible since a typical hydrogel might contain 5,000–10,000 cells. Several manual counts were performed to validate this method and it was found that measuring cell nucleus area was a good proxy for cell counts. No significant variation in nuclei sizes was visible to the authors, although this was not specifically measured. The total cell nucleus area for each microwire segment was divided by the length of the microwire segment since microwire lengths varied from 3–5 mm on average. The normalized cell counts presented in the figures are calculated as (sum of cell nucleus area within hydrogel in μm^2)/(microwire length in μm). At least three wells, each from a different dissection were used to generate the data, with 1–4 microwires in each well. The minimum number of wire segments used to generate a data point was 4, the maximum was 12, and the average n was 9.6.

Statistical Analysis

Statistical significance was determined by analysis of variance (ANOVA) with a $p < 0.05$ significance level and Dunnett's Multiple Comparisons post-hoc tests. Statistical tests were conducted using the GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA, USA).

Results

In the current paper stainless steel microwires were coated with a basal lamina gel preparation, and then introduced to the cell culture that was titrated with different soluble factors suspected to be involved with reactive gliosis. Following cell culture, the gel-coated microwires were removed from the culture with adherent cells attached. The gel coatings were slipped off the microwires, and the adherent cells were quantified microscopically. This method allowed for removal and direct ex vitro analysis of the responding cells attached to the basal lamina extracellular matrix.

Under the appropriate culture conditions (Polikov et al., 2009), a layer of responding cells accumulated at the gel surface. Although a more thorough quantification the different cell types and expressed proteins is warranted in future studies, Figure 2 captures the general observation that the majority of cells covering the gel-coated microwire were GFAP-expressing astrocytes with enlarged, activated, scar-like phenotypes.

Figure 2A is a 3-dimensional fluorescent image of the end of one such gel coated microwire still in culture, along with the cells that accumulated on top of the gel coating. Figure 2B contains rotated variations of the same image with GFAP (green) Vimentin (red) and DAPI (blue). The levels of GFAP and Vimentin in the astroglial cells coating the gel were much higher than those of the cells in the 2-D layer of neuron-glia culture on the tissue culture plastic below, although the density of nuclei is approximately the same. Of the cells that did not take up labeled markers for astrocytes, microglia, or neuronal cells, a number were successfully labeled with Vimentin, and thus likely to be NPC's that have not yet differentiated into activated astrocytes. As expected, very few microglia were present on the surface of the gel, although a small number could be found on the surface of the microwire between the stainless steel of the microwire and the gel coating. No neurons were found on the coated microwires, although they were present next to the gel in the neuron-glia layer.

The initial application of the technique was to confirm the previously reported observation that titrating the culture with serum and bFGF was found to stimulate glial scar formation in vitro (Polikov et al., 2009). A normalized cell count was measured by summing the nuclear area (DAPI fluorescent area), and then dividing by the length of the gel for each microwire segment. Cultures were incubated with varying levels of serum (0%, 0.1%, 1.0%, 5.0%, 10%, and 20%) and of bFGF (0 ng/ml, 1ng/ml, 10 ng/ml, 50 ng/ml) in the media at treatment time, when the coated microwires were placed on top of the neuron-glia cell layer. The number of cells coating the gel increased with increasing levels of both bFGF and serum with maximum values at 10% FBS and 50ng/ml bFGF, with a slight decrease from the maximum levels with 20% FBS and 50ng/ml bFGF (Figure 3). The effect of both serum and of bFGF were statistically significant to $p < .0001$ in a 2-factor ANOVA although there was no significant interaction between serum and bFGF.

The technique next was used to screen for a broad list of soluble factors that are suspected promoters or inhibitors of glial reactivity (Table 1). In screening for reactivity promoters, cultures were treated with the negative control 1% serum and no bFGF (low reactivity conditions), and each soluble factor was added separately into the media in different trials (Figure 4). Here, five soluble factors exhibited reactivity significantly above the negative control (black bar on left). Inflammatory cytokines IL-1 β and IL-1 α significantly increased the level of gliosis as measured by the number of cells on the gels ($p < .01$), although IFN- γ had no discernable effect. None of the anti-inflammatory, neurotrophin, inhibitors, or IL-6 family cytokines significantly increased the level of gliosis. The glial growth factors PDGF ($p < .01$) and BMP-2 ($p < .05$) increased the level of reactivity, as did the proliferative growth factor bFGF ($p < .01$).

In screening for reactivity inhibition, cultures were treated with the positive control of 1% serum, plus 10 ng/ml of bFGF in the media (high reactivity conditions) as the soluble factors were again added separately in different trials (Figure 5). In this study, only one factor significantly inhibited cell reactivity compared to the positive control (black bar on left), while two factors significantly augmented the positive control. The bFGF inhibitor SU5402 significantly blocked the effect of the bFGF in the culture ($p < .05$) and reactivity dropped to levels observed with low reactivity conditions without bFGF (control in Figure 4). The anti-inflammatory steroid dexamethasone, anti-inflammatory cytokine IL-10, and the IL-6 transduction pathway blocker Tyrphostin all showed decreased but not significantly different changes in reactivity. In contrast, the inflammatory cytokines IL-1b ($p < .05$) and IL-1a ($p < .01$) both significantly increased the level of reactivity above the high reactivity control.

Discussion

Previously we reported that the three essential culture conditions necessary for obtaining a consistent gliosis response to microwires were the use of Neurobasal (NB) medium, the presence of the growth factor bFGF, and the presence of serum (Polikov et al., 2009). The current study further supports the essential role of these three factors in increasing the level of cell reactivity at the surface of a basal lamina mimic. The basal lamina preparation was also used to measure the extent of cell accumulation on a gel-coated microwire in response to a battery of soluble factors. This preparation mimics the chronic astroglial response after the initial microglial response subsides.

There were also two technical benefits to this experimental method. First, the microwires provided sufficient density to prevent the gel coating from floating at the media surface. Second, the gel coating allowed for easy ex vitro characterization to the cells at the end of each experiment; i.e. the gel-coated microwires were removed from the culture, the gel was slipped off the microwire, and the cells attached to the gel were quantified and characterized outside of the culture.

Under conditions that supported glial reactivity in our model (serum, bFGF, conditions favorable to neural precursor cell maintenance and growth), the gels were covered in GFAP and Vimentin expressing cells. The number of cells responding to the inserted gels rose with rising concentrations of serum and bFGF. Inflammatory cytokines IL-1 β and IL-1 α increased the level of reactivity beyond that seen with just serum and bFGF, but most soluble factors tested (except for PDGF and SU5402, a bFGF inhibitor) had little to no effect, suggesting bFGF and serum are the key drivers of cell migration and proliferation of cells responding to injury by forming a glial scar, with inflammation serving to accelerate the process. Future studies will explore the differentiation state of the cells within the scar in vitro as a result of local and global release of different factors.

The requirement that serum be present also supports the role of NPCs. Without serum, NPCs do not migrate to an injury site in vitro when a portion of the culture is scraped free of cells (Polikov et al., 2006). In vivo models have also shown a strong NPC (Rhodes et al., 2006) and inflammatory cytokine (Streit et al., 1998) response to injuries that involve BBB breakdown but a minimal response to those that do not. Furthermore, serum is often used as a differentiating factor within culture models studying neural and glial precursor cells, with serum driving the differentiation into an astrocytic cell type at the expense of other neural cell fates like oligodendrocytes or even neurons (Hermann et al., 2006; Kondo and Raff, 2000; Magnus et al., 2007a).

Furthermore, if serum is released with every round of device micromotion, it may generate a thicker and thicker scar, pushing away healthy neurons even as nearby neurons are damaged by the electrode motion (Biran et al., 2007). The heparin sulfate proteoglycans in the basal lamina concentrate and present neural precursor cell growth factors such as bFGF and PDGF, so each round of damage through micromotion may create a more potent concentration of growth factors and thus a thicker scar (Cool and Nurcombe, 2006).

Once cells reach the wound site through the early activation/migration induced by serum and inflammatory cytokines, growth factors within the wound site drive cell proliferation. As expected, bFGF and PDGF increased the number of cells accumulating on the basal lamina gel, which contains the heparin sulfate proteoglycans necessary for the presentation of these growth factors to the nearby cells. Only inflammatory cytokines IL-1 α and IL-1 β could increase the level of reactivity (as measured by cell accumulation) beyond the high levels attained through bFGF (Figure 5).

However, this study is not without limitations. First, only the number of cells accumulating on the gel was measured in this study, not the differentiation state of the cells, the cell type or the level of GFAP expression. While our goal was to demonstrate the screening feasibility of the technique, clearly differential staining of cell types rather than non-specific nuclear stain will allow differentiation of cell type in the cells that accumulate at the specimen surface.

Second, this study employs a fetal cell model rather than an adult cell model. The reason for this, which was elaborated upon in previous publications (Polikov et al., 2006; Polikov et al., 2009), is that fetal cells are necessary to form a viable mixed cell culture that is capable of generating a gliosis reaction when titrated with bare or gel-coated microwires. This of course creates a more proliferative and regenerative cell environment than that found in the adult brain.

Third, the health of the cultures after soluble factor addition was not monitored routinely since neurons were not stained. However, through routine GFAP measurement, it was clear that some factors changed the environment of the culture as a whole and may have created conditions that were not healthy for the growth of neurons or microglia, although a confluent layer of astrocytes was present with each factor at the concentration tested. Specifically, IL-1 α seemed to create an unhealthy looking culture with activated astrocytes throughout the culture and cavitation commonly seen in inflammatory environments in vitro (Fitch et al., 1999).

Future studies will explore the differentiation state of the cells within the scar in vitro as a result of different factors and will also explore the effect of multiple factors acting together. This new technique also allows for PCR-based gene profiles and identification of soluble factors released by responding cells. Finally, various extracellular molecules and soluble factors can be incorporated into the gel in future studies.

Conclusions

Technically, the fetal neuron-glia culture system is well suited for characterizing the impact of electrode coatings designed to mitigate implant-associated gliosis, and for screening the effect of multiple soluble factors that promote and/or inhibit implant-associated gliosis. Scientifically, this study points to essential roles of serum and inflammatory factors to induce NPC activation and migration to the site of injury, where growth factors like bFGF and PDGF induce proliferation of cells that will eventually form the glial scar.

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Figure 1.
Procedure for imaging cells accumulating on the hydrogel

Figure 2.

(A) 3-D image of the end of a microwire coated with hydrogel and covered with cells. The layer of cells in the neuron-glia cell culture is on the the plane at the back side of the cube with the hydrogel sticking up above it. The metal of the microwire does not fluoresce so a dark area where the microwire is present can also be seen. The full length of the coated microwire extends up and to the right beyond the picture. (B) Rotated renderings of the image in A with different stains shown. The DAPI images show the entire surface covered by cells, many of which express GFAP and Vimentin. The levels of GFAP and Vimentin are much higher on the “scar” covering the hydrogel than in the neuron-glia culture below. The empty non-fluorescing space formed by the metal microwire can be seen in many of the images. As reference, the unstained microwire is 50 μ m in diameter. (A rotating video of Figure 2A can be found at <http://reichertlab.pratt.duke.edu>.)

Figure 3.

Cultures were exposed to varying levels of serum and of bFGF in the media at treatment time. The number of cells coating the hydrogel increased with increasing levels of both bFGF and serum. The effect of both serum and of bFGF were statistically significant to $p < .0001$ in a 2-factor ANOVA although there was no significant interaction between serum and bFGF.

Figure 4.

Hydrogel coated microwires were placed in the culture (1%FBS, no bFGF) along with various soluble factors. The normalized cell count was determined for each added factor to see the effect on scarring. (ANOVA $p < .0001$, ** signifies $p < .01$ and * signifies $p < .05$ relative to control via Dunnett's Multiple Comparisons post-hoc test).

Figure 5.

Hydrogel coated microwires were placed in the culture (1%FBS, 10ng/ml bFGF) along with various soluble factors. The normalized cell count was determined for each added factor to see the effect on scarring. (ANOVA $p < .0001$, ** signifies $p < .01$ and * signifies $p < .05$ relative to control via Dunnett's Multiple Comparisons post-hoc test).

Table 1**Soluble Factors Added into the Media**

Soluble Factor	Company/ Catalog #	Concentration	Description
Inflammatory agents			
IL-1b	R&D Systems: 501-RL-010	20 ng/ml	Cytokine released by microglia at the site of injury (Giulian et al., 1994).
IL-1a	R&D Systems: 500-RL-005	10 ng/ml	Cytokine released by microglia at the site of injury (Hamilton et al., 1996; Herx et al., 2000).
IFN- γ	R&D Systems: 585-IF-100	50 ng/ml	Cytokine that acts as a strong activator of immune cells in the body (Rostworowski et al., 1997).
Anti-inflammatory agents			
Dexamethasone	Sigma: D4902	50 μ M	Steroid shown to reduce glial scarring in vivo (Zhong and Bellamkonda, 2007).
IL-10	R&D Systems: 522-RL-005	100 ng/ml	Cytokine shown to reduce glial scarring in vivo (Herx et al., 2000; Woiciechowsky et al., 2004).
Growth factors:			
PDGF	R&D Systems: 520-BB-050	50 ng/ml	Shown to affect NPC's, astrocytes, and fibroblasts (Anderson, 2001; St. Pierre et al., 1996).
BMP-2	R&D Systems: 355-BM-010	50 ng/ml	Known to be produced within the glial scar and to differentiate NPC's into astrocytes (Chang et al., 2003; Deleyrolle et al., 2006; Fukuda et al., 2007; Kondo and Raff, 2004; Morrow et al., 2001; Rajan et al., 2003; Sriram et al., 2004; Turnley and Bartlett, 2000).
BMP-4	R&D Systems: 314-BP-010	50 ng/ml	Known to be produced within the glial scar and to differentiate NPC's into astrocytes (Chang et al., 2003; Deleyrolle et al., 2006; Fukuda et al., 2007; Kondo and Raff, 2004; Morrow et al., 2001; Rajan et al., 2003; Sriram et al., 2004; Turnley and Bartlett, 2000).
bFGF	R&D Systems: 3339-FB-025	10 ng/ml	Known to be produced in the glial scar and essential for the maintenance, migration, and proliferation of NPC's (Ben Hur et al., 2003; Boilly et al., 2000; Clarke et al., 2001; Dailey et al., 2005; FaberElman et al., 1996; Ferretti et al., 2003; Fok-Seang et al., 1998; Joy et al., 1997; Kornblum, 2007; Smith et al., 2001).
GDNF	Millipore: GF030	50 ng/ml	Neurotrophin that helps keep neurons alive (Schmidt and Leach, 2003).
Growth factor inhibitors:			
Noggin	R&D Systems: 1967-NG-025	250 ng/ml	Regulator of BMP's at wound sites (Hampton et al., 2007; Setoguchi et al., 2004).
Su5402	EMD Chemicals: 572631	10 μ m	Blocks the bFGF receptor (Nagao et al., 2007).
DAPT	Sigma: D5942	100 μ m	γ -secretase inhibitor used to block the Notch pathway, which has been implicated in the differentiation of NPC's into astrocytes (Nagao et al., 2007).
GP130/IL-6 family of cytokines upregulated in glial scar			
LIF	Millipore: ESG2206	20 ng/ml	Turns on the genes that convert astrocytes and NPC's into reactive, GFAP expressing astrocytes (Bugga et al., 1998; Kahn et al., 1997b; Mi et al., 2001; Nakanishi et al., 2007; Nakashima and Taga, 2002; Sriram et al., 2004; Turnley and Bartlett, 2000).
IL-6	R&D Systems: 506-RL-010	133 ng/ml	Turns on the genes that convert astrocytes and NPC's into reactive, GFAP expressing astrocytes (Bugga et al., 1998; Kahn et al., 1997b; Mi et al., 2001; Nakanishi et al., 2007; Nakashima and Taga, 2002; Sriram et al., 2004; Turnley and Bartlett, 2000).
CNTF	R&D Systems: 557-NT-010	50 ng/ml	Turns on the genes that convert astrocytes and NPC's into reactive, GFAP expressing astrocytes (Bugga et al., 1998; Kahn et al., 1997b; Mi et al., 2001;

Soluble Factor	Company/ Catalog #	Concentration	Description
			Nakanishi et al., 2007; Nakashima and Taga, 2002; Sriram et al., 2004; Turnley and Bartlett, 2000).
GP130/IL-6 family Inhibitor			
Tyrphostin AG 490	Sigma: T3434	10 μ m	Blocks certain key elements of the IL-6 cytokine family signal transduction pathway (Levitzi, 1999).
Astrocyte Activator:			
N6,2'-O-Dibutyryladenosi ne 3'5'-cyclic monophosphate sodium (dB-cAMP)	Sigma: D0627	1 μ M	Constitutively active analog of the signaling molecule cAMP. Shown to induce GFAP (Eng and DeArmond, 1982) and IL-6 expression in astrocytes (Norris et al., 1994).