Published in final edited form as:

Biomed Mater. ; 13(5): 054101. doi:10.1088/1748-605X/aac4de.

Electrospun fiber surface nanotopography influences astrocyte-mediated neurite outgrowth

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Abstract

Aligned, electrospun fiber scaffolds provide topographical guidance for regenerating neurons and glia after central nervous system injury. To date, no study has explored how fiber surface nanotopography affects astrocyte response to fibrous scaffolds. Astrocytes play important roles in the glial scar, the blood brain barrier, and in maintaining homeostasis in the central nervous system. In this study, electrospun poly L-lactic acid fibers were engineered with smooth, pitted, or divoted surface nanotopography. Cortical or spinal cord primary rat astrocytes were cultured on the surfaces for either 1 or 3 d to examine the astrocyte response over time. The results showed that cortical astrocytes were significantly shorter and broader on the pitted and divoted fibers compared to those on smooth fibers. However, spinal cord astrocyte morphology was not significantly altered by the surface features. These findings indicate that astrocytes from unique anatomical locations respond differently to the presence of nanotopography. Western blot results show that the differences in morphology were not associated with significant changes in glial fibrillary acidic protein (GFAP) or vinculin in either astrocyte population, suggesting that surface pits and divots do not induce a reactive phenotype in either cortical or spinal cord astrocytes. Finally, astrocytes were co-cultured with dorsal root ganglia to determine how the surfaces affected astrocyte-mediated neurite outgrowth. Astrocytes cultured on the fibers for shorter periods of time (1d) generally supported longer neurite outgrowth. Pitted and divoted fibers restricted spinal cord astrocyte-mediated neurite outgrowth, while smooth fibers increased 3 d spinal cord astrocyte-mediated neurite outgrowth. In total, fiber surface nanotopography can influence astrocyte elongation and influence the capability of astrocytes to direct neurites. Therefore, fiber surface characteristics should be carefully controlled to optimize astrocyte-mediated axonal regeneration.

Keywords

biomaterials; electrospun fibers; nanotopography; cortical astrocytes; spinal cord astrocytes; GFAP; topographical guidance

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Supplementary material for this article is available online

1. Introduction

Spinal cord injury (SCI) affects 17 000 individuals in the United States every year [1]. The prognosis of SCI is severe, and often results in paralysis below the level of injury. After the initial mechanical injury to the spinal cord, a cascade of secondary injury mechanisms causes additional tissue dieback. Ultimately, a fibrotic glial scar forms, which presents a major barrier to regenerating axons. Individuals with SCI endure life-long paralysis in part due to the glial scar barrier.

Astrocytes are the main cell responsible for the formation of the glial scar. After injury, astrocytes migrate to the edge of the lesion, become hypertrophic, proliferate, overlap, and strengthen their cytoskeleton with intermediate filaments like glial fibrillary acidic protein (GFAP) and vimentin [2, 3]. The resulting dense population of astrocytes at the edge of the lesion reestablish the blood brain barrier to protect undamaged tissue from additional injury. The dense scar remains after the injury has subsided and continues to restrict axonal extension. While this scarring process was once thought to be irreversible, a recent study showed that when scar-like astrocytes are placed into an uninjured spinal cord environment, they revert back to a non-reactive state [4].

The study by Hara and colleagues indicated that astrocyte response is highly dependent on the environment. Therefore, recreating certain aspects of healthy tissue could alter scar formation, or possibly even remove chronic scars. One approach to change the environment after SCI is to bridge the lesion with a biomaterial guidance scaffold that mimics the anisotropy of the uninjured spinal cord. The anisotropy of aligned electrospun fibers directs axonal regeneration and glial migration into the lesion site while limiting glial scar formation in models of SCI [5, 6]. *In vitro*, aligned electrospun fibers promoted elongation of astrocytes [7], which are able to confer the underlying aligned topography to neurons growing along the top of the astrocyte layer [8].

The potential of electrospun fibers for topographical guidance of cells is clear, so additional studies seek to determine how specific fiber physical characteristics alter the cell response in the central nervous system. These physical characteristics mainly include fiber diameter, alignment, and density, which can be adjusted by modifying the electrospinning parameters. Previous studies showed that changing fiber diameter can affect how cells respond to electrospun fiber scaffolds. Electrospun fibers over a certain diameter ($0.4\ \mu\text{m}$) induced differentiation and myelination by oligodendrocyte precursor cells in culture [9], and the diameter dictates the thickness and length of the myelin sheath [10]. With neurons, larger fiber diameters ($>760\ \text{nm}$) promoted increased neurite lengths compared to fibers with diameters near $290\ \text{nm}$ [11]. Fiber alignment also can affect the cell migration rates. On a surface of aligned fibers, crossing fibers restricted neurite extension from a dorsal root ganglion (DRG) [12], and astrocyte migration was increased on aligned electrospun fibers compared to randomly oriented fibers [7]. Fiber density (fibers mm^{-1}) can influence the direction of migration of cells. Neurites respond to low density fibers by following the alignment of the fibers, but when density is increased past a threshold ($\sim 1500\ \text{fibers mm}^{-1}$), neurites begin to migrate perpendicular to fiber alignment [13].

While fiber diameter, alignment, and density are parameters that are known to affect neurite extension and glial migration, electrospun fibers can also contain surface nanotopography. Surface nanotopography is created during electrospinning when the organic solvent evaporates. Evaporative cooling on the fiber surface causes water vapor in the environment to condense on the surface of the fiber. When the water evaporates from the surface, a divot is left behind in its place. This process is known as vapor induced phase separation (VIPS) [14–16] and can be controlled by changing the humidity of the electrospinning chamber. The fibers become increasingly pitted as the amount of non-solvent in the electrospinning solution is increased in the presence of humidity [14]. It is believed that the pits on the fiber surface form when the electrospinning solution is doped with a non-solvent because the VIPS process causes phase separation within the electrospinning jet. This creates pores of polymer-free non-solvent that crystalize in place as the solvent evaporates. However, these pores only form pits in a humidified environment [16–18]. By adjusting the humidity in the electrospinning environment and dimethyl sulfoxide (DMSO) non-solvent concentration, an electrospun poly-L-lactic acid (PLLA) fiber surface nanotopography can take the form of either divots or pits [17].

Surface pits on fibers increase neurite length and alignment compared to smooth surfaces [19]. Surface nanotopography increases human mesenchymal stem cell proliferation [20], reduces RAW 264.7 macrophage elongation [14], and modestly changes cytokine expression from bone marrow derived macrophages [17]. However, it is unknown how nanotopography influences astrocyte behavior.

The goal of this study was to examine how fiber surface nanotopography influenced astrocyte morphology, GFAP expression, and vinculin expression. Three distinct electrospun fiber scaffolds were fabricated and characterized with scanning electron microscopy (SEM). The three distinct scaffolds consisted of fibers without nanotopography (smooth), fibers with shallow grooves (divoted fibers), or fibers with small indentations (pitted fibers). The fiber processing parameters were adjusted so that there were no statistical differences between the diameters, alignments, and densities of the fibers within the scaffolds. This approach allowed us to isolate the variable of fiber nanotopography and study its effect on the astrocyte response. Next, either cortical or spinal cord astrocytes were cultured on the surface of each scaffold type. After 1 or 3 d in culture, astrocyte morphology was assessed using GFAP immunocytochemistry for each fiber type. To determine if surface nanotopography alters the expression of cytoskeletal markers, vinculin and GFAP protein expression were quantified after 3 d in culture using a Western blot. Finally, an astrocyte and dorsal root ganglia co-culture was established to test how fiber surface nanotopography affected astrocyte-mediated neurite guidance.

2. Materials and methods

2.1. Electrospun fiber fabrication and analysis

2.1.1. Electrospinning apparatus—The electrospinning apparatus used is described in detail in previous publications [21]. To create fibers with divots or pits, the electrospinning apparatus was placed in a humidity controlled glove box made from dissipative PVC (Terra universal, Fullerton, CA). A desktop humidifier was used to create a

controlled humidified environment inside the glove box. Once the desired humidity level was reached, all inlets and outlets to the box were closed and relative humidity remained constant within $\pm 2\%$ throughout the duration of electrospinning.

2.1.2. Electrospun fiber fabrication—High molecular weight poly-L-lactic acid (PLLA, part # 6201D) was purchased from Cargill Dow LLC (Minnetonka, MN), and chloroform from Fisher Scientific (Waltham, MA) was used as the organic solvent. Prior to electrospinning, thin PLLA films were cast onto 15×15 mm coverslips (Knittel Glass, Brausenweig, Germany) as previously described [7]. Fibers were electrospun onto the thin-film coated glass coverslips. PLLA films helped secure fibers to the glass coverslips during cell culture experiments. The thin-film-coated coverslips were fixed to a rotating mandrel to collect the aligned fibers. Three different fiber groups were electrospun: smooth fibers, divoted fibers, and pitted fibers. Table 1 shows the electrospinning solution compositions and parameters used to create each fiber type. All fibers were electrospun from a solution of PLLA in chloroform using the following electrospun parameters unless otherwise noted in table 1: 22 $\frac{1}{2}$ G needle (Becton-Dickenson, Franklin Lakes, NJ), 15 kV voltage drop between the needle tip and spinning mandrel, 5 cm needle-to-mandrel distance, 1000 rpm rotating mandrel speed (22 $\frac{1}{2}$ cm diameter mandrel), and a 2 ml h⁻¹ solution flow rate. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) was used as a non-solvent to create pitted fibers.

2.1.3. SEM of electrospun fibers—After fabrication, electrospun fiber scaffolds were imaged via SEM using a FEI Versa 3D DualBEAM SEM (Hillsboro, OR). A low accelerating voltage (2–5 kV) was used while imaging electrospun fibers to avoid fiber melting. Prior to SEM, fiber scaffolds were sputter coated with a 5 Å Au–Pd layer using a Technics HummerVsputter coater (Anatech Ltd, Denver, NC).

2.1.4. Electrospun fiber morphological characterization—The purpose of this study was to examine how fiber surface topography influenced astrocyte morphology and protein expression. Thus, it was important to ensure that fiber diameter, alignment, and density remained constant between the different sample types. Scanning electron micrographs of fibers were analyzed to verify consistency in fiber diameter, alignment, and density.

To analyze fiber diameter, images were analyzed using FIJI software (Bethesda, MD). Each fiber image was loaded into FIJI, and a line was drawn perpendicular to fiber orientation spanning the width of one fiber. The pixel length of this line was then converted to a micrometer length using the image's accompanying scale bar. This measurement was repeated on 100 fibers per fiber scaffold. Scaffolds were fabricated and analyzed in triplicate ($N = 3$), providing a total of 300 diameter measurements for each fiber type.

Fiber alignment was analyzed by measuring the angle of orientation of each fiber in the SEM images using FIJI software. The angle of 100 fibers was measured per fiber scaffold for three independently fabricated scaffolds ($N = 3$). The mean fiber alignment was calculated from the population and was then subtracted from each fiber angle measurement to determine the angle each fiber deviated from the mean alignment. The angle of deviation

from the mean was compiled into a histogram with the mean alignment set to 0 and the 'Degree Variation from Mean' grouped into 10° bins. Each histogram represents the entire population of the three independently produced scaffolds (at least 300 measurements).

Fiber density was determined using FIJI software. For aligned electrospun fibers, the density was measured as the number of fibers that intersect with a 1 mm line drawn perpendicular to the fiber alignment. This was repeated for three independently fabricated scaffolds ($N=3$). The length of the line was used to calculate fibers mm^{-1} . The diameter density product (DDP) was determined by multiplying the average fiber density by the average fiber diameter calculated above.

2.2. Cell culture

2.2.1. Primary rat cortical and spinal cord astrocyte isolation

All animal procedures for this study followed NIH guidelines for the care and use of laboratory animals. Procedures used to obtain primary astrocyte cultures were approved by the Institutional Animal Care and Use Committee at Rensselaer Polytechnic Institute.

2.2.2. Cortical astrocyte isolation—The cortical astrocyte isolation protocols followed those previously published by Zuidema and colleagues [7]. Briefly, four P2 Sprague-Dawley rat pups (Taconic Biosciences, Hudson, NY) were euthanized and the cortices were removed and placed in sterile OptiMEM (Invitrogen, Grand Island, NY). The meninges, hippocampi, and basal ganglia were removed using a dissecting microscope. Cells were extracted from the cortices in three, 10 min incubations in a solution of TrypLE (Invitrogen): OptiMEM (1:1 by volume) warmed to 37 °C. The second incubation was supplemented with 1 mg ml^{-1} DNase I (Sigma-Aldrich). Suspensions were pelleted by centrifugation (5 min at 0.5 RCF), then raised in astrocyte media (50 U ml^{-1} penicillin, 50 $\mu\text{g ml}^{-1}$ streptomycin, 10% by volume heat inactivated horse serum in Dulbecco's modified eagle medium (DMEM)) (all from Invitrogen). The cell concentration was determined using a hemocytometer, and astrocytes were plated at a concentration of 200 000 cells/ 75 mm^2 on poly-D-lysine (Sigma-Aldrich) coated flasks in astrocyte media. The cortical astrocytes were cultured for 2 weeks, with full media changes every 3–4 d. The astrocytes were seeded onto fibrous scaffolds when the flasks were approximately 90% confluent.

2.2.3. Spinal cord astrocyte isolation—Ten P2 Sprague-Dawley rat pups were euthanized, and spinal cords were removed. All meninges and spinal nerves were removed, and spinal cords were placed in ice-cold OptiMEM. Spinal cords were then dissociated mechanically using trituration. Spinal cords were drawn into a 30 ml syringe through a 16 ½ gauge needle three times to perform a rough dissociation of the tissues while taking care not to introduce air bubbles to the slurry. The tissue slurry was then drawn through an 18 ½ gauge needle three times to further dissociate the tissue. Needles (16, 18 G) and syringes (5 ml and 30 ml) were purchased from Becton Dickinson (Franklin Lakes, NJ). The slurry was then centrifuged at 0.5 RCF for five minutes, and the supernatant was vacuum aspirated. The cell pellet was reconstituted in 100 ml of DMEM culture media containing 10% HIHS, 50 U ml^{-1} penicillin, and 50 $\mu\text{g ml}^{-1}$ streptomycin. The cell suspension was dispensed into ten plug-seal T75 tissue culture flasks that were pre-coated with poly-D-lysine to promote

astrocyte attachment. Cells were grown to confluency (~2.5 weeks). Prior to seeding onto the fiber scaffolds, the T75 flasks were shaken overnight at 200 rpm in an incubated shaker to remove microglia, oligodendrocyte precursor cells, or neurons attached to the top of the astrocyte layer. After shaking, astrocytes were supplied with fresh media and allowed to equilibrate for at least 48 h prior to culture on fiber scaffolds.

2.3. Astrocyte culture onto electrospun fibers

Each astrocyte experiment was conducted in biological and material triplicate. For each fiber condition (smooth, pitted, or divoted), at least three independently prepared astrocyte cultures from the spinal cord or cortex were generated. The fibers were treated with 70% ethanol in water (by volume) for 10 min then dried before the astrocytes were seeded—to sterilize the fibers and remove residual solvent [22]. After the 70% ethanol solution evaporated for 2 h in a sterile tissue culture hood, the fiber samples were treated in a plasma cleaner (Harrick Plasma, Ithaca, NY) on the medium setting for 90 s to increase the hydrophilicity of the fibers to better support cell attachment. Astrocyte cultures were lifted from the T75 culture flask with warm (37 °C) TrypLE for 10 min, pelleted by centrifugation (0.5RCF for 5 min), and resuspended in astrocyte media. Astrocytes were counted using a hemocytometer and plated at a seeding density of 1 000 cells mm⁻². This low seeding density allowed us to observe individual astrocyte morphoglogy. Only individual cells, as determined by the nuclei, were used for morphological analysis. Astrocyte morphology was assessed at two time points to determine how astrocytes respond initially to fibers (1 d) and whether astrocytes change their morphology over time (3 d).

2.3.1. Cell fixation, immunocytochemistry, and imaging—After 1 or 3 d in culture, astrocytes were fixed for 15 min with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) by volume in 25% PBS, 50% astrocyte culture media, and DI water (37 °C). Astrocytes were permeabilized and blocked for 1 h at room temperature with a solution of 0.4% by volume Triton-X100 (Sigma-Aldrich), 5% wt/vol bovine serum albumin (BSA, Sigma-Aldrich), in phosphate buffered saline (PBS, Fisher Scientific). After blocking, the astrocytes were incubated at 4 °C overnight with a 1:800 dilution of rabbit anti-GFAP primary antibody (DAKO, Carpinteria, CA) in 0.1% by volume Tween-20 (Sigma-Aldrich), 5% wt/vol BSA, PBS incubation solution. Astrocytes were washed using a 0.1% by volume Tween-20, PBS buffer three times for 5 min before incubation with a 1:1000 dilution of Alexa Fluor 488 goat anti-rabbit (Invitrogen) secondary antibody in an incubation solution for 1 h at room temperature. The astrocytes were washed three times for 5 min in PBS, then incubated for 15 min in PBS with a 1:1000 dilution (vol/vol) of DAPI (Sigma-Aldrich) at room temperature. Samples were washed in PBS three times for 5 min each before imaging. Images were taken with a 20× objective using an inverted Olympus IX-81 confocal microscope (Olympus, Tokyo, Japan). Images were stacked by maximum intensity projection, filtered with a rolling ball background subtraction algorithm (radius = 50), and assembled using the MosaicJ plugin on FIJI (Adobe, San Jose, CA).

2.3.2. Metamorph image analysis and elongation ratio—Astrocyte morphology was analyzed using Metamorph (Molecular Devices, Sunnyvale, CA) image analysis software. GFAP and DAPI channel maximum intensity projections of astrocytes were

overlaid to differentiate individual astrocytes from clustered astrocytes. Cell images were thresholded against light objects, which segmented the foreground from the background. The threshold was used to define the perimeter of the astrocytes. From the perimeter, the length and width of the cells were calculated in Metamorph software. The longest cellular aspect was divided by the shortest cellular aspect to determine the aspect ratio. An aspect ratio of 1 shows a rounded morphology, while an aspect ratio greater than 1 indicates the cell is elongated. To eliminate potential bias, individuals performing the analysis were blinded to the astrocyte cell source and fiber type.

2.4. Western blot

2.4.1. Astrocyte culture and lysis—Western blots were performed using a higher cell density of 666 cells mm⁻². In order to achieve a high enough protein concentration for the blot, four coverslips were pooled for each data point. Either spinal cord or cortical astrocytes were cultured on the smooth fiber control, pitted fibers, divoted fibers, or tissue culture plastic (TCPS) as a second control. Western blot analysis was performed after 3 d in culture. The 3 d time point was determined after measuring GFAP intensity in astrocyte immunocytochemistry at the 1 and 3 d time points. There were no significant differences in GFAP intensity between the 1 and 3 d time points (data not shown), so GFAP was measured at the 3 d time point only. After culture, the astrocytes on the coverslips were washed three times with 37 °C PBS to remove residual media. Astrocytes were then lysed for 5 min on ice with RIPA cell lysis buffer 3 (Enzo Life Sciences, Farmingdale, NY) doped with complete ULTRA enzyme inhibitors (Roche, Branford, CT) and PhosSTOP phosphatase inhibitors (Roche). Samples were run through a 28 gauge needle (Becton-Dickenson) to break up any DNA complexes. Sample concentrations were measured using a nanodrop spectrophotometer (Thermo Scientific, Rockford, IL), then 4× concentrated Laemmli sample prep buffer containing β-mercaptoethanol (RPI Research Products, Mt. Prospect, IL) was added to achieve 1× sample prep buffer concentration. Samples were frozen at −80° until the Western blot procedure was performed.

2.4.2. Western blotting technique—4%/10% SDS-PAGE gels were prepared in 1 mm plastic single use cassettes (BioRad, Hercules, CA) according to the Abcam procedure (Abcam, Cambridge, MA). Gels were stored at room temperature until use. Astrocyte lysates were thawed and prepared for gel loading by adding appropriate amounts of water and sample prep buffer to achieve 2 μg protein in each 20 μl loading sample. Prior to loading, samples were heated in a 100 °C heat block for 2 min, then centrifuged for 2 min at 14 000 rpm. Gel cassettes were washed with MQ water, samples were loaded into the wells along with the molecular weight standard (BioRad), and the gel was run for 60 min at 150 V in 1 l of running buffer (0.025 M Tris-Base, 0.192 M Glycine, 0.1% wt/vol SDS).

Immun-blot PVDF membranes (BioRad) were soaked in methanol for 15 s to activate the membranes, then soaked in transfer buffer (25 mM Trisbase, 192 mM glycine, 20% methanol) (Sigma-Aldrich) for 5 min. Gels were removed from the gel cassettes and washed with MQ water to remove SDS. Gels were then assembled into the transfer cassette with the activated membrane and run at a constant current of 0.2 Amp for 90 min in transfer buffer. Care was taken to keep the cassettes cool by packing ice around the system during transfer.

2.4.3. Western blot immunostaining and imaging—After transfer of protein from the gel to the membrane, the cassettes were disassembled and the PVDF membranes were transferred to a blocking buffer of freshly prepared 1% Tween-20 (Sigma-Aldrich), 20 mM Tris, 150 mM NaCl (TBST, Thermo Scientific) with 5% wt/vol instant nonfat dried milk, pH 7.6. The membranes were blocked for 1 h at room temperature on a rocking platform shaker (VWR). The blocking buffer was removed and replaced with a primary antibody in incubation solution (TBST, 5% wt/vol instant nonfat dried milk). The primary antibody was incubated overnight at room temperature on the rocking platform shaker. After the primary antibody incubation, the incubation buffer was removed and the membranes were washed three times for 5 min with TBST. The final wash was replaced with a horseradish peroxidase conjugated secondary antibody diluted 1:10 000 in TBST with 5% (wt/vol) instant nonfat dried milk. The secondary antibody was incubated with the membrane on the rocking platform shaker for 2 h, then removed and washed three times for 5 min. Meanwhile, the Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, IL) was prepared according to the manufacturer's instructions. After washing, the detection reagent was applied to the surface and incubated for 2 min. The detection reagent was removed, and the membrane was imaged using a ChemiDoc XRS system with the Chemi Hi Resolution protocol in the ImageLab Software (BioRad). The molecular weight standard was imaged immediately after (without moving the membrane) using the colorimetric protocol in the Image-Lab Software.

Immediately after imaging, the samples were returned to TBST for the stripping procedure to remove the primary and secondary antibodies. The membranes were stripped with two 10 min incubations in stripping buffer (200 mM glycine, 3.5 mM SDS, 1% Tween-20, pH 2.2) all from Sigma-Aldrich. After stripping, the membranes were washed three times for 5 min in PBS, then two times for 5 min in TBST. The membranes were then re-probed for GAPDH using the immunostaining procedure above to serve as a loading control.

Vinculin (117 kDa) was detected with a primary monoclonal mouse anti-vinculin antibody (1:500, Sigma) followed by a HRP-conjugated donkey anti-mouse antibody (1:10 000, Jackson Research). GFAP (55 kDa) was detected with a rabbit anti-GFAP (1:2000, DAKO) primary antibody, and a (HRP)-conjugated Donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA). GAPDH(36 kDa) was detected with a goat anti-GAPDH (1:1000, Sigma) primary antibody, and a (HRP)-conjugated donkey anti-goat secondary antibody (1:10 000, Invitrogen). The resulting vinculin, GFAP, and GAPDH bands and molecular weight standard band intensities were analyzed using FIJI software. At least three independently produced fibers and cell cultures were used to determine the mean for each condition. TCPS served as a flat surface control.

2.5. Co-culture with astrocytes and dorsal root ganglia

Astrocytes were co-cultured with DRG to determine if the changes observed in astrocyte morphology after 1 and 3 d in culture on the three fiber types significantly affected neurite outgrowth. DRG were seeded on to astrocytes that had been attached to the surfaces for either 1 d (hereafter referred to as '1 d old') or 3 d (hereafter referred to as '3 d old'). The

purpose was to determine if surface nanotopography influenced the astrocyte's ability to neurite outgrowth.

2.5.1. Astrocyte culture—Astrocytes were seeded in reverse order to allow the 1 and 3 d old astrocyte cultures to be seeded with DRG at the same time (for protocol timeline, see figure 5(S)). Three days before DRG isolation, astrocytes were seeded on smooth, pitted, and divoted electrospun fibers (3 d old), as described in section 3.3. Astrocytes were cultured at a higher density ($444 \text{ cells mm}^{-2}$) to generate an astrocyte monolayer. One day before DRG isolation, a second set of electrospun fiber scaffolds was seeded with astrocytes (1 d old).

2.5.2. DRG isolation—After removing the spinal cords from P2 rats as described in section 2.2.3, the spinal columns were cut down the midline of the ventral and dorsal aspects to obtain two separate halves. DRG were then carefully removed from each half of the spinal column using Dumont #5 jeweler's forceps. Thoracic and Lumbar DRG were isolated and pooled together in ice-cold sterile Ham's F12 nutrient mixture (ThermoFisher) until ready for co-culture with astrocytes.

2.5.3. Astrocyte and DRG co-culture on electrospun fibers—To determine if the observed changes in astrocyte response over time to the different surfaces would affect neurite extension, DRG were placed on the 1 d old and 3 d old astrocyte cultures on the three different electrospun fiber groups. DRG were halved using jeweler's forceps and placed on top of the astrocytes that were growing on the fibers. At this time, the previously described astrocyte media was changed to serum-free, neurobasal media supplemented with 1% B-27 (ThermoFisher) and 50 ng ml^{-1} nerve growth factor (Invitrogen) to promote neurite outgrowth. DRG were co-cultured with astrocytes for five days prior to fixation and immunocytochemistry.

2.5.4. Co-culture fixing and staining—Astrocyte-DRG co-cultures were fixed in 4% paraformaldehyde for 15 min after the DRG were in culture for 5 d. After fixation, cultures were blocked for 1 h at room temperature with a solution of 5% BSA in 0.01% Triton-X100 in PBS (all from Sigma). The percentage of Triton-X100 was reduced in the co-culture staining protocol, compared to the astrocyte-only protocol, to reduce overexposure for the more fragile neurites. After blocking, co-cultures were incubated overnight with primary antibodies against GFAP (1:800 dilution) to label astrocytes and against RT-97 (1:200, DSHB, University of Iowa, Iowa City, IA) to label neurofilament in DRG. Primary antibody solutions were then washed three times with PBS, 0.1% Tween-20 and a secondary antibody solution containing 1:1000 dilutions of Alexa Fluor 488 goat anti-rabbit, an Alexa Fluor 594 donkey anti-mouse (Life Technologies) were added to the cultures for two hours. During the last half hour of this incubation, $1 \mu\text{g ml}^{-1}$ DAPI, and $1 \mu\text{g ml}^{-1}$ Phalloidin were added to the co-cultures. Phalloidin was used in order to visualize the full body of the astrocytes. At the end of the two hour incubation, cells were washed three times with PBS and held at 4°C until being imaged. Co-culture images were taken with $4\times$ and $20\times$ objectives. Images were stacked by maximum intensity projection, filtered with a rolling ball background subtraction algorithm (radius = 50), and assembled using the MosaicJ pairwise stitching plugin on FIJI.

2.5.5. DRG neurite and area analysis—Co-culture images were analyzed to quantify neurite extension and the area of DRG coverage to see if changes in astrocytes among the different fibers impacted either of these metrics. DRG neurite extension was calculated by calculating the average of the ten longest neurites from each DRG using FIJI. DRG images were then manually thresholded to separate the RT-97 signal (neurites) from the background, producing a binary image. The total area of neurofilament immunofluorescence was quantified in FIJI, and the area of the DRG body was removed from each area calculation to give the metric referred to as coverage area. For all experiments, data was collected from at least five independent DRG, three individual astrocyte cultures, and five independently fabricated electrospun fiber scaffolds. Statistical significance ($p < 0.05$) was determined using an ANOVA followed by a Tukey HSD.

2.6. Statistics

Statistical analysis was performed using Sigma Plot 11.0 software (Systat Software Inc., San Jose, CA). Data in the text are reported as mean $\pm$ standard deviation, while the error bars in bar graphs represent the standard error. All groups were tested for normality using a Kolmogorov–Smirnov test. If the data was normally distributed, an ANOVA was run with a Tukey HSD post hoc analysis. If the samples were not normally distributed, a non-parametric ANOVA was run on ranks. Statistical significance was assigned for all groups where $p < 0.05$. All data was analyzed by using at least three independently fabricated fiber replicates containing three independently isolated batches of astrocytes unless otherwise specified. Analysis of fiber alignment differences between fiber groups was performed using the Brown–Forsythe test in JMP software (SAS, Cary, NC).

3. Results

3.1. Preparation of smooth, divoted, and pitted fibers

Smooth, pitted, and divoted fibers were prepared to determine if fiber surface features influenced astrocyte morphology and GFAP production. Smooth fibers result when the electrospinning solution was electrospun in a low humidity chamber ($<21\%$) (figure 1(A)). Pitted fiber surfaces are formed by incorporating the non-solvent (DMSO) and electrospinning in a higher humidity chamber ($28\%–32\%$) (figure 1(B)). Shallow divoted fiber nanotopography was generated by electrospinning at the higher ($28\%–32\%$) humidity, but without including the non-solvent DMSO (figure 1(C)). Fiber diameter, alignment, density, and solvent retention were measured and controlled in order to analyze the ability of surface nanotopography to influence astrocyte morphology and GFAP production.

3.2. Adjusted electrospinning process to increase the smooth fiber diameter

The initial attempts to create smooth fibers yielded fibers with smaller diameters than either the divoted or pitted fibers. Since the pitted and divoted fibers had similar diameters, the smooth fiber diameter was adjusted by increasing the weight percentage of PLLA content in the electrospinning solution (figure S1 is available online at stacks.iop.org/BMM/13/054101/mmedia). As weight percentage of PLLA in chloroform increased, fiber diameter increased. The average diameters for the 8%, 9%, and 10% wt/wt PLLA solutions was $1.64 \pm 0.37 \mu\text{m}$, $1.75 \pm 0.38 \mu\text{m}$, and $2.11 \pm 0.36 \mu\text{m}$, respectively (figure S1). Electrospinning the 10%

PLLA solution required the inclusion of a more volatile solvent. Chloroform solution was doped with the more volatile dichloromethane to help facilitate solvent removal from higher weight percentage PLLA solutions. The resulting smooth fibers had a mean fiber diameter of $2.11 \pm 0.36 \mu\text{m}$ which was not statistically different when compared to the mean fiber diameters of pitted and divoted fibers ($2.02 \pm 0.40 \mu\text{m}$ and $2.06 \pm 0.31 \mu\text{m}$, respectively) using an ANOVA (figure 1(G)).

3.3. Characterization of fiber alignment and density

After the diameter of the smooth fibers was adjusted to have similar diameters to divoted and pitted fiber diameters, the alignment and density of the fibers were measured to guarantee their consistency between fiber groups. The alignment of the fibers within the scaffolds was measured for each group in terms of deviation from the primary axis of orientation (figures 1(D)–(F)). For all groups, the fibers were highly aligned within $\pm 15^\circ$ of the primary axis and none of the groups showed statistical differences in fiber alignment. The densities of the fibers were 181 ± 35 , 192 ± 24 , and $180 \pm 12 \text{ fibers mm}^{-1}$ respectively for the smooth, pitted, and divoted fibers. An ANOVA analysis confirmed that there were no statistical differences between the fiber densities in different groups (figure 1(H)). The fiber coverage of each scaffold was quantified by multiplying the fiber diameter by the fiber density (DDP). The results are displayed in terms of micrometers covered while traveling along a two-dimensional line oriented perpendicular to the long axis of the fibers (figure 1(I)). For example, the smooth fibers covered $392 \mu\text{m}$ of every $1000 \mu\text{m}$, or 39.2% coverage. For smooth, pitted, and divoted fibers, the coverage was $39.2 \pm 5.3\%$, $39.7 \pm 4.7\%$, and $38.4 \pm 2.1\%$, respectively. There were no statistical differences between the coverages for all groups (figure 1(I)).

3.4. Cortical astrocyte changes in length in response to nanotopography

Cortical astrocytes were cultured on the surfaces of the smooth, pitted, and divoted electrospun fiber scaffolds to determine if nanotopography presence influenced astrocyte morphology (figure 2). The purity of both cortical and spinal cord astrocytes were measured to be greater than 98% in culture (figure S3). One important experimental design decision was to not coat the fiber surfaces with specific adhesive peptides or proteins, because adhesive ligands may mask the effects of the surface topography [23, 24]. Astrocyte morphology was selected as a parameter for investigation because astrocytes that quickly elongate on fibers may have an enhanced ability to direct subsequent axonal regeneration [8]. On the smooth fibers, at the 1 d time point, cortical astrocytes had a mean length of $135 \pm 76 \mu\text{m}$ (figure 2(A)), while those on the pitted and divoted fibers had mean lengths of $141 \pm 56 \mu\text{m}$ (figure 2(C)) and $159 \pm 82 \mu\text{m}$ (figure 2(E)), respectively. After 3 d in culture, cortical astrocyte length increased on all scaffold types (figures 2(B), (D), (F), and (G)). The cortical astrocytes on the smooth fibers increased in length the most, and more than tripled in length to $474 \pm 144 \mu\text{m}$ (figure 2(G)) compared to the 1 d time point (figure 2(A)). The cortical astrocytes cultured on fibers with pitted and divoted nanotopography increased in length to $242 \pm 157 \mu\text{m}$ on the pitted fibers, and to $287 \pm 120 \mu\text{m}$ on divoted fibers. These changes in length on the smooth fibers were significantly larger than those seen on the pitted or divoted fibers (figure 2(G)). For cortical astrocytes, nanotopography presence does still allow for astrocyte elongation, but to a lesser extent compared to astrocyte extension on

smooth fibers. It may be that pit presence on the fiber surface is helping to stabilize the astrocytes, and there is reduced need for astrocytes to spread and seek additional attachment points. Conversely, astrocytes on smooth fibers surfaces are seeking additional attachment points, and they may be quickly elongating on the fibers to seek greater stability.

3.5. Cortical astrocyte changes in aspect ratio in response to nanotopography

The aspect ratio evaluates the ratio of length to width of an individual astrocyte, with a value of 1 describing a circular astrocyte, and values greater than 1 describing increasingly long or thin astrocytes. Aspect ratio was assessed to examine the ability of nanotopography to influence an astrocyte to spread along individual fibers or groups of fibers. Additionally, the aspect ratio is a valid metric since a majority of the astrocytes presented a bipolar morphology that extended along the length of the fibers (figures 2(A)–(D)). Aspect ratio differences (figure 2(G)) mirrored differences observed with astrocyte length (figure 2(H)). After 1 d on smooth fibers, the cortical astrocytes had an aspect ratio of 5.7 ± 3.9 (figure 2(H)). The aspect ratios on pitted and divoted fibers were slightly increased with values of 6.7 ± 3.9 and 7.9 ± 5.2 , respectively (figure 2(H)). After 3 d in culture, the aspect ratio of cortical astrocytes cultured on smooth fibers increased significantly to 10.0 ± 4.2 but decreased on the pitted and divoted fibers to 4.7 ± 2.2 and 6.4 ± 2.5 , respectively (figure 2(H)). The decrease in the aspect ratio observed in the cortical astrocytes on the pitted and divoted fibers indicated that the astrocyte was broadening faster than it was lengthening. Both the pitted and divoted nanotopography induced cortical astrocytes to spread perpendicular to fiber alignment.

3.6. Spinal cord astrocyte changes in length in response to nanotopography

Cortical astrocyte response to biomaterials is more commonly studied than that of spinal cord astrocytes. If fibers are meant to direct axonal regeneration in the spinal cord, it is important to understand how spinal cord astrocyte populations respond to fibers with different surface features compared to cortical astrocytes.

After 1 d of culture, spinal cord astrocytes had mean lengths of $215 \pm 87 \mu\text{m}$ on smooth (figure 3(A)), $116 \pm 70 \mu\text{m}$ on pitted (figure 3(C)), and $160 \pm 67 \mu\text{m}$ on divoted fibers (figure 3(E)). There were no significant differences between the mean lengths of any group at the 1 d time point. After 3 d in culture, spinal cord astrocytes cultured on all three fiber types increased modestly in length to $241 \pm 91 \mu\text{m}$, $220 \pm 97 \mu\text{m}$ and $265 \pm 123 \mu\text{m}$ on smooth (figure 3(B)), pitted (figure 3(D)), and divoted fibers (figure 3(F)), respectively. Like the cortical astrocytes, the spinal cord astrocytes increased in length over time, but unlike the cortical astrocytes, there were no differences in the change of length on the different fiber scaffolds (figure 3(G)).

3.7. Spinal cord astrocyte changes in aspect ratio in response to nanotopography

The aspect ratio of spinal cord astrocytes showed opposite trends when compared with cortical astrocytes. After 1 d, the spinal cord astrocytes had an aspect ratio of 5.0 ± 2.3 , 2.8 ± 1.5 , and 5.3 ± 2.8 , respectively, on smooth, pitted, and divoted fibers (figure 3(H)). There were no statistical differences between the aspect ratios of the groups at 1 d. After 3 d in culture, the aspect ratio of spinal cord astrocytes decreased slightly to 4.7 ± 2.2 on smooth

fibers, and increased to 5.2 ± 3.5 and 6.1 ± 3.3 on the pitted and divoted fibers, respectively (figure 3(H)). This trend contrasted that of cortical astrocytes which decreased on smooth fibers and increased on pitted and divoted fibers (figure 2(H)).

3.8. Western blot analysis of cortical and spinal cord GFAP expression

Astrocyte reactivity is thought to exist along a spectrum involving the expression of many different cytoskeletal proteins, soluble factors, surface receptors, and unique extracellular matrix proteins [25]. Since there were differences in elongation, this study focused on two cytoskeletal indicators for reactivity (vinculin and GFAP). Large increases in vinculin and GFAP expression are used as an indicator for astrocytes in a reactive phenotype [2, 25, 26]. Western blotting was performed on 3 d astrocyte cultures to measure changes in astrocyte vinculin and GFAP protein expression in response to fibers with unique surface nanotopography (figure 4). Each value was normalized to the corresponding GAPDH value, then the values in each repeated measure were normalized to the corresponding tissue culture polystyrene (TCPS) control value (smooth surface without curvature). It should be noted that serum proteins in the media likely coated the surface of the fibers soon after the cell suspension was placed on the surface of the fibers. The surface topographies of the fibers were analyzed by SEM after submersion in media and after an 8 h incubation in the humidified environment of the cell culture incubator (37 °C) (figure S4). The images suggested the incubation environment did not alter the morphology of the fibers.

It appears that some of the pits on the pitted fibers may be covered after submersion, while the divoted and smooth fibers remain unchanged. However, this was not quantified because of confounding factors with the drying of salt-containing solutions on the fibers surfaces. The drying process clearly shows crystal growth, and the drying of the salts and proteins may have covered surface features that were available in the aqueous environment.

For each experimental group in the Western blot, the same amount of protein from each sample (2 μ g) was loaded in each lane to reduce variation between groups. The cortical astrocyte GFAP expression on the smooth, pitted, and divoted fibers is 1.63 ± 0.27 , 1.45 ± 0.74 , and 0.95 ± 0.38 , respectively, relative to the TCPS control. The spinal cord astrocyte GFAP expression, when normalized to the TCPS control, was 1.14 ± 0.20 , 1.01 ± 0.24 , and 0.78 ± 0.40 on the smooth, pitted, and divoted fibers, respectively. An ANOVA confirmed that there were no statistically significant differences between any of the groups. Interestingly, both cortical and spinal cord astrocytes had lower GFAP expression values when cultured on divoted fibers, compared to all other fiber surfaces. (figures 4(A), (D)). It is possible that the lack of statistical significance is due to a low sample size.

The cortical astrocyte vinculin expression on the smooth, pitted, or divoted fibers was 2.85 ± 2.26 , 1.79 ± 0.42 , and 0.94 ± 0.61 relative to the TCPS control. The spinal cord astrocyte vinculin expression on the smooth, pitted, and divoted fibers was 0.60 ± 0.33 , 0.95 ± 0.33 , and 0.62 ± 0.12 relative to the TCPS control. There were no statistically significant differences between any of the groups using an ANOVA. Overall, the Western blot results show that the smooth, pitted, and divoted surface topography do not significantly alter the vinculin or GFAP expression compared to a flat surface control.

3.9. Cortical astrocyte co-culture with dorsal root ganglia

We cultured DRG with astrocytes to test how neurite outgrowth was affected by the changes in astrocyte morphology elicited by the fiber surface nanotopography. Two methods were employed to isolate the effects of surface nanotopography-mediated astrocyte-guidance of neurons. First, astrocytes were cultured at a high density ($444 \text{ cells mm}^{-2}$) to create a monolayer and reduce the direct interaction between neurites and the fiber surface. Second, the DRG were co-cultured with the astrocytes for the same period of time (5 d) on all surfaces and treatments (figure 5(S)). By using the same DRG culture time across groups, the changes in DRG neurite length and coverage were the result of astrocyte cues that were produced in response to the surface. In order to improve the clarity of the writing, the '1 d co-culture' will be used to describe the experiment in which the astrocytes were cultured for 1 d on each surface before the DRG were introduced for 5 d. The '3 d co-culture' will be used to describe the experiment in which the astrocytes were cultured for 3 d on each surface before the DRG were introduced for 5 d.

Cortical astrocytes aligned along the orientation of the fibers after both 1 and 3 d of culture on the surface. DRG neurites extending along the surface followed the orientation of the underlying fibers through the astrocyte monolayer (figure 5). The $20\times$ images show the DRG neurites preferred the astrocyte surface over void spaces without astrocytes (figures 5(A), (D), (G), (J), (M), (P)).

Based on the astrocyte morphology data collected earlier, we hypothesized that the 3 d cortical co-cultures would result in the longest neurite outgrowth. The ten longest neurites from each DRG were measured and averaged to determine the longest neurite value (figure 5(T)). In the 3 d co-cultures, the neurite length was longest on the smooth fiber/astrocyte surfaces, however the differences were not statistically significant. Therefore, the hypothesis that neurite outgrowth would be longest on 3 d old cortical astrocytes on smooth fibers was not upheld. Surprisingly, the DRG neurite outgrowth was longer on the 1 d cortical co-cultures compared to the 3 d cortical co-cultures. Over time (1 versus 3 d cortical co-cultures), the neurite outgrowth length was significantly decreased on the pitted and divoted surfaces. However, the decrease in neurite length was not significantly different on the smooth fibers when comparing the 1 and 3 d co-culture time points. The DRG neurite area coverage was also measured, but did not yield any statistically significant differences (figure 5(U)).

3.10. Spinal cord astrocyte co-culture with dorsal root ganglia

Spinal cord astrocytes also aligned along the orientation of the fibers, and the DRG neurites extending on the astrocyte monolayer aligned with the underlying topography. The $20\times$ images (figures 6(A), (D), (G), (J), (M), (P)) are included to show that the neurites grow along the astrocyte layer. In a similar manner to the cortical astrocytes, there was a significant decrease in length of the longest neurites on the pitted and divoted co-culture surfaces after 3 d, compared to the 1 d spinal cord astrocyte surfaces (figure 6(T)). However, the smooth fiber surfaces resulted in the opposite trend. The 3 d spinal cord co-culture on smooth fibers resulted in significantly increased neurite length compared to the 1 d spinal cord co-culture. As a result, the neurite length on the 3 d spinal cord co-culture was

significantly increased on the pitted and divoted surfaces relative to the smooth fiber control. These data indicate that the smooth fiber surfaces modified the spinal cord astrocytes to increase the neurite outgrowth length.

In both the cortical astrocyte and spinal cord astrocyte co-cultures, the pitted and divoted surfaces yielded a significant decrease in neurite growth length after 3 d, but not on the smooth fibers. The DRG neurite outgrowth was shorter overall on the spinal cord astrocytes (1700–3800 μm , figure 6(T)), compared to the cortical astrocytes (2100–4900 μm , figure 5(T)).

There was a high degree of variation in the DRG area coverage on the spinal cord astrocytes (figure 6(U)), however the trends were similar to the longest neurite data. The area covered by the neurites increased in the 3 d spinal cord co-cultures on the smooth fiber surfaces, but showed a decrease over time on the pitted and divoted surfaces.

4. Discussion

Electrospun fibers have emerged as a tool to modulate astrocyte behavior by adopting phenotypes that are less reactive and more supportive of CNS tissue regeneration. Astrocytes cultured on electrospun fibers maintain an *in vivo*-like phenotype [27], increase glutamate transporters and glutamate uptake [7], induce embryonic astrocytes to mature into a protoplasmic phenotype [28], and upregulate genes associated with brain derived neurotrophic factor and the antioxidant glutathione [29]. In order to further probe the effects of topography, our current study examined the ability of fiber surface nanotopography to influence astrocyte morphology, GFAP production, and neurite guidance. The method for controllably engineering nanotopography on the surface of PLLA fibers was previously developed in our laboratory [14], but the effects of fiber nanotopography have not been studied in astrocyte populations or in astrocyte-neuron co-cultures.

In vivo, astrocytes are most commonly classified into two primary phenotypes, protoplasmic and fibrous astrocytes, which are characterized by their location and morphology. Protoplasmic astrocytes are located in the gray matter and appear in the classic, highly ramified morphology. Fibrous astrocytes reside in the myelinated white matter and have fewer but longer processes [2, 30]. The spinal cord has a higher proportion of white matter to gray matter, while the cortex has a higher proportion of gray matter to white matter. Astrocytes perform similar functions in both the white and gray matter by maintaining the blood brain barrier, adjusting glutamate levels in the synaptic cleft, buffering ions after action potentials, and providing energy to neurons [2, 30]. On a flat surface *In vitro*, astrocytes lose the ramified morphology and exhibit a polygonal morphology (figure S3). The phenotypic shifts in astrocytes on electrospun fiber scaffolds involve changes toward an elongated and ramified morphology and reduced GFAP expression [7, 27, 28], so these measures were used to study the effects of the surface nanotopography. To our knowledge, it is not known how astrocyte vinculin is affected by electrospun fiber nanotopography.

The results of this study can be summarized in five points: 1. Three fiber scaffolds were created with unique surface topographies, but statistically similar diameters, alignments, and

densities; 2. Cortical astrocytes extended to a greater extent along smooth fibers compared to divoted or pitted fibers; 3. Spinal cord astrocytes were not significantly affected by the surface nanotopography, but also did not extend as far as cortical astrocytes on the smooth fibers; 4. There were no significant differences in GFAP or vinculin expression between cortical and spinal cord astrocyte on any surface. However, there tended to be a decrease in both vinculin and GFAP expression in astrocytes on the divoted fibers compared to those on smooth fibers; and 5. Astrocytes residing on the fibers for shorter periods of time supported longer neurite outgrowth. Three day old spinal cord astrocytes on smooth fibers increased neurite extension, while those on pitted or divoted fibers stunted neurite extension.

Fiber physical properties such as diameter, alignment, and density, modify neuron and glial cell behavior in culture [7, 11, 12, 31–34]. These variables were measured and controlled to remove them as confounding variables that may affect astrocyte behavior observed here. The fiber diameter required the most adjustment. When the smooth fibers were prepared with the same electrospinning solution as the pitted fibers, the smooth fibers had a smaller diameter than both the pitted and divoted fibers. To match the diameters of the pitted and divoted fibers, the PLLA concentration in the smooth fiber electrospinning solution was increased to 10% of the solution weight. However, the higher polymer content caused the fibers to flatten during collection, so dichloromethane was added to allow the fibers to solidify. The resulting fibers had a smooth surface with diameters that were statistically similar to the pitted and divoted samples (figure S1). Since dichloromethane was a new solvent introduced during the diameter adjustment process, TGA analysis was used to verify that ethanol sterilization removed all solvents (figure S2). Ethanol effectively sterilizes the fibers for culture and removes organic solvents from fibers after electrospinning [22]. TGA confirmed that the ethanol wash removed the different organic solvents as a potential confounding variable in this study.

The fiber alignment and density were controlled by the collection wheel rotation speed and the collection time. Zuidema and colleagues observed that aligned fibers promoted directional astrocyte migration, while unaligned fibers restricted migration [7]. As a result, fiber alignment was controlled by maintaining a consistent collection wheel rotation speed for all groups during electrospinning. The resulting fibers were aligned within 15° of the mean fiber alignment, and there were no statistical differences between groups (figures 1(D)–(F)).

Fiber density has previously been shown to affect the direction of extending neurites [34]. Xie and colleagues prepared submicron diameter fibers that ranged in density from 100 to 3000 fibers mm⁻¹. Xie *et al* found that neurites from dorsal root ganglia extended parallel to the topography of low density fibers (<1500 fibers mm⁻¹), but grew perpendicular to the alignment of high density fibers (>1500 fibers mm⁻¹). The fibers in this study were kept at a low density to encourage astrocytes to interact with more isolated single fibers and each group was electrospun for the same amount of time to keep the fiber density consistent. There were no statistical differences between fiber groups for both the fiber density (figure 1(H)) and the DDP—a measure of fiber coverage (figure 1(I)). With statistically similar fiber diameters, alignments, densities, and coverage between the three experimental groups, the

changes observed in astrocytes between fiber groups is likely due to the presence of nanotopography.

Previously, electrospun fibers with pitted surfaces were shown to reduce macrophage elongation compared to smooth fibers [14]. Thus, we hypothesized that pits and divots on the fiber surfaces would reduce the elongation of astrocytes relative to the smooth fiber control surfaces. Cortical astrocytes are known to respond to aligned smooth electrospun fibers with an elongated bipolar morphology [7]. Here, the cortical astrocytes on the smooth fibers were used as the standard to which the experimental fiber surfaces and spinal cord astrocytes were compared. The hypothesis that surface nanotopography reduces astrocyte elongation was upheld in cortical astrocyte populations cultured for 1 and 3 d (figure 2). The increase in length of cortical astrocytes seen on smooth fibers over the three days was significantly reduced on both pitted and divoted fibers (figure 2(G)). The aspect ratio gives a more descriptive measure of cell shape by accounting for the width of the cells. The aspect ratio of cortical astrocytes significantly increased on smooth fibers, indicating long thin astrocytes, but the aspect ratio decreased on pitted and divoted fiber surfaces, indicating that the astrocytes were spreading perpendicular to the fiber alignment to a greater degree than they were lengthening parallel to the fiber alignment (figure 2(H)). Both of these measures show that pitted and divoted fiber surfaces limit cortical astrocyte length and aspect ratio when compared to cortical astrocytes on smooth fiber surfaces.

In the co-culture, all experiments maintained equal DRG culture times, so the differences in neurite outgrowth were due to the longer astrocyte exposure times to each surface type (figure 5(S)). In 2003, Biran and colleagues found a direct relationship between astrocyte extension on grooved surfaces and neurite migration over the astrocytes [8]. Since we determined that astrocytes extended further when cultured on fiber surfaces for longer periods of time (3 d), we hypothesized that the extended morphology would allow longer neurite extension on the 3 d old astrocyte cultures. Surprisingly, neurite extension on astrocytes cultured on pitted and divoted fibers was significantly shorter in the 3 d old cortical co-cultures, compared to the 1 d old cortical co-cultures (figure 5(T)). These findings suggest that extended astrocyte morphology may not entirely predict whether an elongated astrocyte induces longer neurite outgrowth. Furthermore, there were more neurites on average (greater coverage) on astrocyte-coated pitted and divoted fibers on the 1 d old cultures compared to the 3 d old cultures (figure 5(U)). However, there were no statistical differences in neurite coverage between any of the groups in figure 5(U).

The hypothesis that pits and divots limit astrocyte elongation was not upheld in spinal cord astrocyte populations. There were no significant differences in the spinal cord astrocyte changes in length or aspect ratio between the smooth, pitted, or divoted fibers. While all spinal cord astrocytes lengthened over time, the changes were not significantly different between groups. After 3 d in culture, the spinal cord astrocytes on all fiber surfaces had similar lengths to the cortical astrocytes cultured on the pitted and divoted fibers. These astrocyte lengths ranged from 240 to 290 μm , while the cortical astrocytes on smooth fibers were the single exception, extending to 474 μm (figures 2, 3). The trends in aspect ratio in spinal cord astrocytes were opposite those of cortical astrocytes. The aspect ratio in spinal cord astrocytes decreased between 1 and 3 d on smooth fibers, but increased in spinal cord

astrocytes cultured on the pitted and divoted fibers. Overall, the spinal cord astrocytes were less responsive, in terms of morphology, to the changes in surface nanotopography than were the cortical astrocytes.

The length and of neurite outgrowth coverage results from DRG co-cultured with spinal cord astrocytes were similar to the results from cortical astrocyte/DRG coculture experiments. Neurites extending on spinal cord astrocyte-coated pitted and divoted fibers were again significantly shorter on 3 d old astrocytes compared to 1 d old astrocytes (figure 6(T)). There were also fewer neurites (less coverage) on the 3 d old pitted and divoted co-cultures compared to the 1 d old co-cultures (figure 6(U)). Unique to spinal cord astrocytes, the neurites on the astrocyte-coated smooth fiber scaffolds increased in length between the 1 and 3 d time points. Furthermore, in the 3 d old spinal cord co-cultures, the neurites grew significantly longer on the smooth fibers compared to those on the pitted or divoted fibers.

The neurite length results (figures 5(T), 6(T)) do not completely correlate with the astrocyte morphology results (figures 2(H), 3(H)). These findings complicate the theory proposed by Biran and colleagues of a direct relationship between astrocyte elongation and neurite growth length. However, it is important to note that the findings by Biran *et al* were observed on grooved features that ranged from 45 to 492 nm [8], while the fiber here were approximately 2000 nm in diameter. The differences in scale indicate that the relationship between astrocyte extension and neurite elongation may not hold above a certain threshold. Astrocytes support neurite extension by producing glycoproteins like fibronectin and laminin and releasing growth factors [8]. Future work will explore the capability of unique surface topographies to influence astrocyte production of these molecules.

Similarly, further analysis should be performed to understand how the changes in astrocyte morphology relate to changes in astrocyte phenotype, gene, or protein expression. Astrogliosis is the most commonly studied shift in phenotype in astrocytes (the shift from a naive to a reactive to a scar-like phenotype) [4], because of its association with the pathology of traumatic brain injury, traumatic SCI, and diseases like multiple sclerosis. Increases in GFAP, vimentin, cell hypertrophy, and overlapping cell processes are the accepted indicators of astrogliosis [2, 4].

We showed that the fiber surface nanotopography affected cortical astrocyte and spinal cord astrocyte morphology differently when the astrocytes are cultured at a low enough density to distinguish individual cells. Western blot was next performed to correlate the single cell morphology observed at a low cell density to cytoskeletal markers of astrocyte populations at a higher cell density. The higher cell density is more representative of regenerating glia migrating into an electrospun fiber scaffold [6]. We assessed the effects of fiber surface nanotopography on two cytoskeletal markers: vinculin and GFAP. Vinculin serves as a marker for both cell-extracellular matrix adhesion and astrogliosis, and GFAP is the most common marker for astrogliosis [6]. There are two theories for how engineered nanotopography may influence cell adhesion and elongation. Since cell attachment to the surfaces is mediated by focal adhesion, both theories involve vinculin. Vinculin is an important protein in focal adhesions that aids in transducing mechanical signals from the

extracellular matrix to the actin cytoskeleton [35, 36]. Therefore, a Western blot of vinculin may provide evidence of how the astrocyte populations respond to the topography.

The first theory is that the increased surface area of the pitted topography increases surface protein adsorption and provides more adhesion sites for the astrocytes. Leong and colleagues found that electrospun poly-D,L-lactic acid fibers with pitted surfaces adsorbed 80% more protein from a 10% fetal bovine serum solution compared to fibers with a smooth surface [37]. The increase in protein adsorption was correlated with an increase in attachment of porcine epithelial cell to the pitted fibers compared to the smooth fibers. Our smooth, pitted, and divoted electrospun fibers were likely coated with serum proteins shortly after submersion into the astrocyte media with 10% heat inactivated horse serum. Based on the findings by Leong *et al* we expected to observe an increase in the amounts of astrocyte vinculin on the pitted and divoted fibers, compared to the smooth fibers. The Western blot results did not support this theory (figure 4).

The second theory is that the pits and divots on the surface interrupt the astrocyte focal adhesions, diminishing the astrocyte's ability to apply a force to elongate. We hypothesized that if the pits or divots interrupted the cell adhesion enough to stop formation of focal adhesions, there would be less vinculin in those cultures. The Western blot for vinculin did not reveal significant differences between the groups for the spinal cord or cortical astrocyte populations. However, there appeared to be a trend toward fewer vinculin-associated focal adhesions in the cortical astrocyte populations as the substrate changed from smooth to pitted to divoted fibers (figure 4(B)). There was no observable trend in the spinal cord astrocytes on each of the surfaces (figure 4(E)).

The vinculin results are useful because vinculin can also be used as a measure for astrocyte reactivity. The most well-known characteristic of reactive astrocyte is a strengthening of the cytoskeleton with intermediate filaments like GFAP, vimentin, and microfilaments like actin [3, 26, 30]. But activated astrocytes also anchor themselves to the extracellular matrix by increasing the number and size of focal adhesions [26, 38]. The focal adhesion complex is linked to the actin cytoskeleton by vinculin [26, 38, 39]. As a result, an upregulation of vinculin has been observed around lesion sites *in vivo* [38], and vinculin increased 3–4 fold *In vitro* using a Western blot and a proteomic array when astrocyte cultures were stimulated with the activation agent endothelin-1 [25]. The Western blot results showed an increase in normalized vinculin expression in cortical astrocytes on smooth and pitted fibers (2.85 and 1.79, respectively) but a slight decrease on divoted surfaces (0.94) on the fiber surfaces compared to the TCPS control (1.00) (figure 4(B)). Spinal cord astrocyte vinculin expression ranged from 0.60 to 0.95, a decrease relative to the TCPS control (1.00) (figure 4(E)). Overall, none of the experimental groups were significantly different from the control.

The vinculin protein has an actin binding region that transmits mechanical sensations from the ECM to the cytoskeleton of the cell [40]. Since actin and vinculin are intricately linked, it is not surprising that actin—in particular F-actin—is also upregulated in reactive astrocytes [39, 41]. As a result, GAPDH was used instead of actin as a housekeeping protein for the Western blot, to remove the risk of using a protein that may vary with astrocyte reactivity. It is important to note that Abd-El-Basset *et al* found that F-actin is most

significantly affected by astrocyte reactivity [39], and the pan-actin control should not be affected. Western blotting was also performed using pan-actin as the control and data were reported in the supplemental figures to examine the relationship between GFAP and actin (figure S5).

The final cytoskeletal protein that was measured in this study is the most well-known marker for astrocytes. In astrocytes, GFAP is linked to both positive and negative responses for treatment of CNS injury [42, 43]. GFAP is important for glutamate transporter trafficking. For example, GFAP-null mice show a 25%–30% decrease in neuroprotective glutamate uptake [44]. But, large increases in GFAP are also linked to astrocyte reactivity and glial scar formation [3]. There is not a specific GFAP value that defines a reactive astrocyte because GFAP is expressed at different levels in both local and regional astrocyte populations within the healthy CNS. However, GFAP is necessary for reactive gliosis and glial scar formation, and pronounced increases in GFAP are associated with astrocytes entering a reactive state [45]. Therefore, significant increases in GFAP expression from astrocytes on any of the experimental surfaces would indicate that the surface induces reactivity in astrocytes.

GFAP has also been linked to increased astrocyte process extension and migration [45, 46]. Since cortical astrocytes grew significantly longer on smooth fibers than those on pitted or divoted fibers, we hypothesized that the cortical astrocytes would have the highest GFAP expression on the smooth fiber surface nanotopographies. The results favored this hypothesis, but the values were not statistically significant. The GFAP expression was greatest on the smooth fiber surfaces in both cortical and spinal cord astrocytes. In cortical astrocytes, the increased GFAP expression correlated with the significantly increased astrocyte extension. In spinal cord astrocytes, there were no significant differences and no correlation. Compared to the smooth fiber controls, GFAP expression was decreased on the pitted and divoted fiber surfaces in both spinal cord and cortical astrocytes (figures 4(A), (D)). These values are still below GFAP levels associated with astrocyte reactivity. Lipopolysaccharide (LPS) treatment, known to induce traumatic brain injury, have induced a reactive astrocyte phenotype that more than doubled the amount of GFAP present [47]. Transgenic mice engineered to study GFAP overexpression exhibited 3–5 fold increases in GFAP after 14 d [48]. The Western blot results show that there were no significant differences in spinal cord or cortical astrocytes on any of the surfaces, but it is possible that the sample size was too low to detect significant differences.

Interestingly, the ratio of GFAP to actin is increased in astrocytes cultured on electrospun fibers compared to those on the TCPS control. The ratio of GFAP to actin was approximately 50% higher on all fiber types compared to the TCPS control, and was similar across all fiber nanotopography groups (figure S5). The increase in the ratio of GFAP to actin in cortical and spinal cord astrocytes may mean that astrocytes on fibers require stronger intermediate filaments instead of a more dynamic cytoskeleton provided by actin. Intermediate filaments provide a strengthened cytoskeleton to resist stresses [49], and GFAP intermediate filaments improve vesicle trafficking [50] and astrocyte motility [45] among other astrocyte functions, reviewed by Middeldorp and Hol [51].

Few studies have analyzed GFAP levels of astrocytes cultured on electrospun fibers. However, Lau *et al* showed mouse astrocytes decreased GFAP expression while cultured on 400 nm diameter poly- ϵ -caprolactone fiber surfaces for 12 d (80% decrease in GFAP expression compared to a TCPS control) [29]. It is difficult to relate this study with our current study because the materials, time points, surface treatments, animal models, and fiber diameters are different. However, other studies may help to explain the differences. Puschmann and colleagues observed a similar 75% decrease in mouse astrocyte GFAP when comparing bioactive electrospun fibers coated with laminin and poly-L-ornithine to an untreated glass surface control [27]. These results indicate that the adhesive ligands on the surface also influence on astrocyte GFAP expression. In our study, the TCPS control surfaces were coated with poly-D-lysine to represent the standard treatment scheme for flat surfaces [52, 53]. For the fiber surfaces, only surface plasma treatment was applied before the surfaces were submerged in the cell suspension because the treatment increases the hydrophilicity of the surface without altering the surface nanotopography [54]. But Schaub and colleagues reported that that plasma treatment reduced neurite extension relative to other surface modification schemes [23].

The major goal of this study was to isolate the surface nanotopography as the primary variable affecting astrocyte response. From the results generated here, the presence of nanotopography influenced the morphology of astrocytes differently. Cortical astrocytes extended the longest on smooth fibers while divots or pits interfered with the ability of an astrocyte to extend (figure 2). Conversely, nanotopography presence did not readily influence the ability of spinal cord astrocytes to spread along the fibers (figure 3). Fiber surface nanotopography did not elicit a significant change in GFAP expression in either cortical or spinal cord astrocytes (figure 4) and did not significantly affect vinculin expression. These findings support the theory that nanotopography can influence cell morphology without causing a reactive response from astrocytes or altering major proteins in the cytoskeleton. However, there were some differences in the astrocytes' ability to support neurite extension, especially with the spinal cord astrocytes ability to induce long neurite extension on smooth surfaces but shorter neurite extension in the presence of pitted or divoted fibers.

5. Conclusions

Overall, the pits or divots on electrospun fiber surfaces reduce cortical astrocyte elongation, do not significantly affect spinal cord astrocyte elongation, and do not significantly affect vinculin or GFAP expression in either population. The smooth fiber surfaces improve astrocyte elongation, and the pits and divots slowed cortical astrocyte elongation in favor of a broadened astrocyte shape. The spinal cord astrocyte aspect ratio did not appreciably change over 3 d on smooth fiber surfaces, but increased slightly on pitted and divoted surfaces. None of these changes in morphology were associated with changes in vinculin or GFAP expression. Furthermore, the astrocyte morphology was not a consistent predictor of astrocyte-mediated neurite outgrowth. With spinal cord astrocyte cultures, the pitted and divoted fibers significantly reduced astrocyte-mediated neurite outgrowth, while the smooth fibers did not. The effects of different surface nanotopographies were most significant in the 3 d old spinal cord astrocyte co-cultures, where the astrocytes promoted longer neurite

extension on the smooth fibers, compared to neurite extension in the pitted or divoted fiber co-cultures. Astrocytes cultured on electrospun fibers for shorter time periods supported longer neurite outgrowth. Therefore, these findings suggest that it is important to control both the fiber surface nanotopography and the astrocyte source when designing electrospun fiber scaffolds to maximize neurite extension in central nervous system applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This work was funded by NSF CAREER Award grant (1105125) and NIH R01 grant (NS092754) to RJG. We also wish to acknowledge the funding support provided by The New York State Spinal Cord Injury Research Board (NYSSCIRB) Predoctoral Fellowship Award (Contract# C30606GG) to CDLJ and RJG, and the NYSSCIRB Predoctoral Fellowship (Contract# C32631GG) awarded to ARD and RJG.

References

- [1]. National Spinal Cord Injury Statistical Center 2017 Spinal Cord Injury Facts and Figures at a Glance (Birmingham, AL: National Spinal Cord Injury Statistical Center) (from an online PDF: <https://www.nscisc.uab.edu/Public/Facts%20and%20Figures%20-%202018.pdf>)
- [2]. Sofroniew MV and Vinters HV 2010 Astrocytes: biology and pathology *Acta Neuropathol.* 1197–35
- [3]. Sofroniew MV 2005 Reactive astrocytes in neural repair and protection *Neuroscientist* 11400–7
- [4]. Hara M et al. 2017 Interaction of reactive astrocytes with type I collagen induces astrocytic scar formation through the integrin–N-cadherin pathway after spinal cord injury *Nat. Med.* 23 818–28 [PubMed: 28628111]
- [5]. Zhang Z. et al. 2017; Effect of hierarchically aligned fibrin hydrogel in regeneration of spinal cord injury demonstrated by tractography: a pilot study. *Sci. Rep.* 7:40017. [PubMed: 28067245]
- [6]. Hurtado A et al. 2011 Robust CNS regeneration after complete spinal cord transection using aligned poly-L-lactic acid microfibers *Biomaterials* 32 6068–79 [PubMed: 21636129]
- [7]. Zuidema JM et al. 2014 Enhanced GLT-1 mediated glutamate uptake and migration of primary astrocytes directed by fibronectin-coated electrospun poly-L-lactic acid fibers *Biomaterials* 35 1439–49 [PubMed: 24246642]
- [8]. Biran R, Noble MD and Tresco PA 2003 Directed nerve outgrowth is enhanced by engineered glial substrates *Exp. Neurol.* 184 141–52 [PubMed: 14637087]
- [9]. Lee S et al. 2012 A culture system to study oligodendrocyte myelination processes using engineered nanofibers *Nat. Methods* 9 917–22 [PubMed: 22796663]
- [10]. Bechler ME, Byrne L and French-Constant C 2015 CNS myelin sheath lengths are an intrinsic property of oligodendrocytes *Curr. Biol* 25 2411–6 [PubMed: 26320951]
- [11]. Wang HB, Mullins ME, Cregg JM, McCarthy CW and Gilbert RJ 2010 Varying the diameter of aligned electrospun fibers alters neurite outgrowth and Schwann cell migration *Acta Biomater.* 6 2970–8 [PubMed: 20167292]
- [12]. Wang HB. et al. 2009; Creation of highly aligned electrospun poly-L-lactic acid fibers for nerve regeneration applications. *J. Neural Eng.* 6:016001. [PubMed: 19104139]
- [13]. Xie J, Liu W, MacEwan MR, Bridgman PC and Xia Y 2014 Neurite outgrowth on electrospun nanofibers with uniaxial alignment: the effects of fiber density, surface coating, and supporting substrate *ACS Nano* 8 1878–85 [PubMed: 24444076]

- [14]. Schaub NJ, Britton T, Rajachar R and Gilbert RJ 2013 Engineered nanotopography on electrospun PLLA microfibers modifies RAW 264.7 cell response ACS Appl. Mater. Interfaces 5 10173–84 [PubMed: 24063250]
- [15]. Srinivasarao M, Collings D, Philips A and Patel S 2001 Three-dimensionally ordered array of air bubbles in a polymer film Science 292 79–83 [PubMed: 11292866]
- [16]. Megelski S, Stephens JS, Chase DB and Rabolt JF 2002 Micro and nanostructured surface morphology on electrospun polymer fibers Macromolecules 35 8456–66
- [17]. Schaub NJ et al. 2017 The effect of engineered nanotopography of electrospun microfibers on fiber rigidity and macrophage cytokine production J. Biomater. Sci. Polym. Ed 28 1303–23 [PubMed: 28420296]
- [18]. Dayal P, Liu J, Kumar S and Kyu T 2007 Experimental and theoretical investigations of porous structure formation in electrospun fibers Macromolecules 40 7689–94
- [19]. Yang A, Huang Z, Yin G and Pu X 2015 Fabrication of aligned, porous and conductive fibers and their effects on cell adhesion and guidance Colloids Surf. B 134 469–74
- [20]. Moroni L, Licht R, de Boer J, de Wijn JR and van Blitterswijk CA 2006 Fiber diameter and texture of electrospun PEOT/PBT scaffolds influence human mesenchymal stem cell proliferation and morphology, and the release of incorporated compounds Biomaterials 27 4911–22 [PubMed: 16762409]
- [21]. Johnson CDL, D'Amato AR and Gilbert RJ 2016 Electrospun fibers for drug delivery after spinal cord injury and the effects of drug incorporation on fiber properties Cells Tissues Organs 202 116–35 [PubMed: 27701153]
- [22]. D'Amato AR et al. 2017 Evaluation of procedures to quantify solvent retention in electrospun fibers and facilitate solvent removal Fibers Polym. 18 483–92
- [23]. Schaub NJ. et al. 2015; The effect of surface modification of aligned poly-L-lactic acid electrospun fibers on fiber degradation and neurite extension. PLoS One. 10:e0136780. [PubMed: 26340351]
- [24]. Dolatshahi-Pirouz A et al. 2010 Fibronectin adsorption, cell adhesion, and proliferation on nanostructured tantalum surfaces ACS Nano 4 2874–82 [PubMed: 20443575]
- [25]. Egnaczyk GF et al. 2003 Proteomic analysis of the reactive phenotype of astrocytes following endothelin-1 exposure Proteomics 3 689–98 [PubMed: 12748948]
- [26]. Ostrow LW and Sachs F 2005 Mechanosensation and endothelin in astrocytes—hypothetical roles in CNS pathophysiology Brain Res. Rev 48 488–508 [PubMed: 15914254]
- [27]. Puschmann TB et al. 2013 Bioactive 3D cell culture system minimizes cellular stress and maintains the in vivo-like morphological complexity of astroglial cells Glia 61 432–40 [PubMed: 23292921]
- [28]. Ugbode CI, Hirst WD and Rattray M 2016 Astrocytes grown in alvetex® three dimensional scaffolds retain a non-reactive phenotype Neurochem. Res 41 1857–67 [PubMed: 27099962]
- [29]. Lau CL et al. 2014 3D Electrospun scaffolds promote a cytotropic phenotype of cultured primary astrocytes J. Neurochem 130 215–26 [PubMed: 24588462]
- [30]. White RE and Jakeman LB 2008 Don't fence me in: harnessing the beneficial roles of astrocytes for spinal cord repair Restor. Neurol. Neurosci 26 197–214 [PubMed: 18820411]
- [31]. Nguyen AT, Sathe S and Yim E 2016 From nano to micro: topographical scale and its impact on cell adhesion, morphology and contact guidance J. Phys.: Condens. Matter 28 1–16
- [32]. Baiguera S, Gaudio CD, Fioravanzo L, Bianco A, Grigioni M and Folin M 2010 In vitro astrocyte and cerebral endothelial cell response to electrospun poly(e-caprolactone) mats of different architecture J. Mater. Sci., Mater. Med 21 1353–62 [PubMed: 19957022]
- [33]. Chow WN, Simpson DG, Bigbee JW and Colello RJ 2007 Evaluating neuronal and glial growth on electrospun polarized matrices: bridging the gap in percussive spinal cord injuries Neuron Glia Biol. 3 119–26 [PubMed: 18458759]
- [34]. Xie J, MacEwan MR, Li X, Sakiyama-Elbert SE and Xia Y 2009 Neurite outgrowth on nanofiber scaffolds with different orders, structures, and surface properties ACS Nano 3 1151–9 [PubMed: 19397333]
- [35]. Goldmann WH 2016 Role of vinculin in cellular mechanotransduction Cell Biol. Int 40 241–56 [PubMed: 26909547]

- [36]. John GR, Chen L, Riviaccio MA, Melendez-Vasquez CV, Hartley A and Brosnan CF 2004 Interleukin-1 β induces a reactive astroglial phenotype via deactivation of the Rho GTPase-rock axis J. Neurosci 24 2837–45 [PubMed: 15028778]
- [37]. Leong MF, Chian KS, Mhaisalkar PS, Ong WF and Ratner BD 2009 Effect of electrospun poly(D,L-lactide) fibrous scaffold with nanoporous surface on attachment of porcine esophageal epithelial cells and protein adsorption J. Biomed. Mater. Res. A 89A 1040–8
- [38]. Kálmán M and Szabó A 2001 Immunohistochemical investigation of actin-anchoring proteins vinculin, talin and paxillin in rat brain following lesion: a moderate reaction, confined to the astroglia of brain tracts Exp. Brain Res 139 426–34 [PubMed: 11534866]
- [39]. Abd-El-Basset EM and Fedoroff S 1997 Upregulation of F-actin and α -actinin in reactive astrocytes J. Neurosci. Res 49 608–16 [PubMed: 9302082]
- [40]. Dumbauld DW et al. 2013 How vinculin regulates force transmission Proc. Natl Acad. Sci 110 9788–93 [PubMed: 23716647]
- [41]. Fedoroff S, Ahmed I, Opas M and Kalnins VI 1987 Organization of microfilaments in astrocytes that form in the presence of dibutyryl cyclic AMP in cultures, and which are similar to reactive astrocytes in vivo Neuroscience 22 255–66 [PubMed: 2819774]
- [42]. Pekny M and Nilsson M 2005 Astrocyte activation and reactive gliosis Glia 50 427–34 [PubMed: 15846805]
- [43]. Anderson MA et al. 2016 Astrocyte scar formation aids central nervous system axon regeneration Nature 532 195–200 [PubMed: 27027288]
- [44]. Hughes EG, Maguire JL, McMinn MT, Scholz RE and Sutherland ML 2004 Loss of glial fibrillary acidic protein results in decreased glutamate transport and inhibition of PKA-induced EAAT2 cell surface trafficking Mol. Brain Res 124 114–23 [PubMed: 15135219]
- [45]. Lepekhn EA, Camilla E, Claes-Henric B, Vladimir B, Elisabeth B and Milos P 2008 Intermediate filaments regulate astrocyte motility J. Neurochem 79 617–25
- [46]. Liedtke W et al. 1996 GFAP is necessary for the integrity of CNS white matter architecture and long-term maintenance of myelination Neuron 17 607–15 [PubMed: 8893019]
- [47]. Gong P. et al. 2013; Phosphorylation of mitogen- and stress-activated protein kinase-1 in astrocytic inflammation: a possible role in inhibiting production of inflammatory cytokines. PLoS One. 8:e81747. [PubMed: 24349124]
- [48]. Messing A, Head MW, Galles K, Galbreath EJ, Goldman JE and Brenner M 1998 Fatal encephalopathy with astrocyte inclusions in GFAP transgenic mice Am. J. Pathol 152 391–8 [PubMed: 9466565]
- [49]. Fuchs E and Cleveland DW 1998 A structural scaffolding of intermediate filaments in health and disease Science 279 514–9 [PubMed: 9438837]
- [50]. Potokar M et al. 2007 Cytoskeleton and vesicle mobility in astrocytes Traffic 8 12–20 [PubMed: 17229312]
- [51]. Middeldorp J and Hol EM 2011 GFAP in health and disease Prog. Neurobiol. 93 421–43 [PubMed: 21219963]
- [52]. Silva GA et al. 2004 Selective differentiation of neural progenitor cells by high-epitope density nanofibers Science 303 1352–5 [PubMed: 14739465]
- [53]. Wagner J et al. 1999 Induction of a midbrain dopaminergic phenotype in Nurr1-overexpressing neural stem cells by type 1 astrocytes Nat. Biotechnol 17 653–9 [PubMed: 10404157]
- [54]. Schaub NJ et al. 2015 The effect of surface modification of aligned poly-L-lactic acid electrospun fibers on fiber degradation and neurite extension PLoS One 10 1–19

Figure 1.

Fiber morphology analysis of smooth, pitted, or divoted fibers to verify consistency of alignment, diameter and density between groups. SEM micrographs of (A) smooth, (B) pitted, and (C) divoted fibers show the differences in surface structure. SEM micrographs were used to determine corresponding fiber alignment histograms for (D) smooth, (E) pitted, and (F) divoted fiber groups. There were no significant differences in alignment between groups ($n = 3$). (G) There were no significant differences in fiber diameter between groups. (H) There were no significant differences in fiber collection density between groups. (I) Bar graph of diameter density product to estimate fiber coverage on the surfaces. There were no significant differences between groups. $n = 3$ for all groups. Scale bar = 5 μm .

Figure 2. Cortical astrocyte morphology on smooth (A), (B), pitted (C), (D), or divoted fibers (E), (F). Confocal micrographs of cortical astrocytes at 1 d (A), (C), (E) and 3 d (B), (D), (F) time points after seeding. Astrocytes were immunostained for GFAP (green) and DAPI (blue). Cortical astrocyte morphology measures of length (G) and aspect ratio (H) as a function of surface type are presented as the difference between measurements taken at 1 and 3 d. The * indicates statistically significant differences between astrocytes cultured on smooth compared to pitted or divoted ($p < 0.05$). The data in graphs (G) and (H) show the mean with standard error from three independent astrocyte cultures on three independently fabricated fiber surfaces. Scale bar = 20 μm .

Figure 3. Spinal cord astrocyte morphology on smooth (A), (B), pitted (C), (D), or divoted fibers (E), (F). Confocal micrographs of spinal cord astrocytes at 1 d (A), (C), (E) and 3 d (B), (D), (F) time points. Astrocytes were immunostained for GFAP (green) and DAPI (blue). Spinal cord astrocyte morphology measures of length (G) and aspect ratio (H) as a function of surface type were presented as the difference between measurements taken at 1 and 3 d. There were no statistical differences between groups ($p > 0.05$). The data in graphs (G) and (H) show the mean and standard error from 3 independent astrocyte cultures on three independently fabricated fiber surfaces.

Figure 4.

Western blot of vinculin and GFAP in cortical astrocytes (A)–(C) and spinal cord astrocytes (D)–(F) after 3 d in culture on smooth, pitted, or divoted fibers. The tissue culture plastic (TCPS) was used as a flat topography control. 2 μ g of protein were loaded in each lane. All samples were normalized to their corresponding GAPDH values, then each was normalized to the TCPS control. The graphs represent the mean and standard error from at least three independently fabricated scaffolds, and three separate astrocyte cultures. The differences between groups were not statistically significant.

Figure 5.

Dorsal root ganglia co-culture with cortical astrocytes. Astrocytes were cultured on the surface of either the smooth, pitted, or divoted electrospun fiber surfaces for either 1 or 3 d (S). Then DRG were placed on the surface and cultured for 5 d. DRG co-cultured with 1 d old cortical astrocyte cultures are shown in the first set (A)–(I), and DRG co-cultured with 3 d old cortical astrocyte cultures are shown in the second set (J)–(R). The DRG culture time was the same for all samples, as shown in the timeline for the co-culture protocol (S). The first column (A), (D), (G), (J), (M), (P) shows 20× images of phalloidin (actin, green), RT-97 (neurofilament, red), and DAPI (nuclei, blue). The scale bar for the 20× images represents 50 μm. 4× images are displayed in the next two columns to show the entire DRG outgrowth in the co-culture (B), (E), (H), (K), (N), (Q), and in the RT-97 channel alone (C), (F), (I), (L), (O), (R). The scale bar for the 4× images represents 500 μm. The average of the ten longest neurites from each DRG was determined as a function of surface type and astrocyte culture time (T). The * represents statistically significant differences between groups ($p < 0.05$).

0.05). The area covered by neurite outgrowth from each DRG was also determined as a function of surface type and astrocyte culture time (U).

Figure 6.

Dorsal root ganglia co-culture with spinal cord astrocytes. Spinal cord astrocytes were cultured on the surface of either the smooth, pitted, or divoted electrospun fiber surfaces for either 1 or 3 d. Then DRG were placed on the surface and cultured for 5 d. DRG co-cultured with 1 d old spinal cord astrocyte cultures are shown in the first set (A)–(I), and DRG co-cultured with 3 d old spinal cord astrocyte cultures are shown in the second set (J)–(R). The DRG culture time was the same for all samples, as shown in the timeline for the co-culture protocol (S). The first column (A), (D), (G), (J), (M), (P) shows 20× images of phalloidin (actin, green), RT-97 (neurofilament, red), and DAPI (nuclei, blue). The scale bar for the 20× images represents 50 μm. 4× images are displayed in the next two columns to show the entire DRG outgrowth in the co-culture (B), (E), (H), (K), (N), (Q), and in the RT-97 channel alone (C), (F), (I), (L), (O), (R). The scale bar for the 4× images represents 500 μm. The average of the ten longest neurites from each DRG was determined as a function of

surface type and astrocyte culture time (T). The * represents statistically significant differences between groups ($p < 0.05$). The area covered by neurite outgrowth from each DRG was also determined as a function of surface type and astrocyte culture time (U).

Table 1.

Electrospinning parameters for smooth, pitted, and divoted fibers.

Fiber type	Polymer wt% in solvent	Non-solvent wt% in solvent	Humidity	Fiber collection time
Smooth	10 ^a	N/A	≤21%	10 min
Pitted	8	1.8%	28%–32%	10 min
Divoted	8	N/A	28%–32%	10 min

^aSolvent mixture of 70% CHCl₃ and 30% CH₂Cl₂.