

# Sema3A Reduces Sprouting of Adult Rod Photoreceptors In Vitro

Frank Kung,<sup>1</sup> Weiwei Wang,<sup>2</sup> Tracy S. Tran,<sup>3</sup> and Ellen Townes-Anderson<sup>2</sup>

<sup>1</sup>Joint Program in Biomedical Engineering, Rutgers University, Graduate School of Biomedical Sciences, New Jersey Institute of Technology, Newark, United States

<sup>2</sup>Department of Pharmacology, Physiology, and Neuroscience, Rutgers University, New Jersey Medical School, Newark, New Jersey, United States

<sup>3</sup>Department of Biological Sciences, Rutgers University, Newark College of Arts and Sciences, Newark, New Jersey, United States

Correspondence: Ellen Townes-Anderson, 185 South Orange Avenue, MSB H592, Newark, NJ 07103, USA; andersel@njms.rutgers.edu.

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**PURPOSE.** Rod photoreceptor terminals respond to retinal injury with retraction and sprouting. Since the guidance cue Semaphorin3A (Sema3A) is observed in the retina after injury, we asked whether Sema3A contributes to structural plasticity in rod photoreceptors.

**METHODS.** We used Western blots and alkaline phosphatase (AP)-tagged neuropilin-1 (NPN-1) to detect the expression of Sema3A in an organotypic model of porcine retinal detachment. We then examined Sema3A binding to cultured salamander rod photoreceptors using AP-tagged Sema3A. For functional analysis, we used a microspritizer to apply a gradient of Sema3A-Fc to isolated salamander rod photoreceptors over 24 hours.

**RESULTS.** Sema3A protein was biochemically detected in porcine retinal explants in the retina 7, 24, and 72 hours after detachment. In sections, NPN-1 receptor was bound to the inner and outer retina. For isolated rod photoreceptors, Sema3A localized to synaptic terminals and to neuritic processes after 1 week in vitro. In microspritized rod photoreceptors, process initiation occurred away from high concentrations of Sema3A. Sema3A significantly decreased the number of processes formed by rod photoreceptors although the average length of processes was not affected. The cellular orientation of rod photoreceptors relative to the microspritizer also significantly changed over time; this effect was reduced with the Sema3A inhibitor, xanthohulvin.

**CONCLUSION.** Sema3A is expressed in the retina after detachment, binds to rod photoreceptors, affects cell orientation, and reduces photoreceptor process initiation in vitro. Our results suggest that Sema3A contributes to axonal retraction in retinal injury, whereas rod neuritic sprouting and regenerative synaptogenesis may require a reduction in semaphorin signaling.

**Keywords:** axon guidance, rod photoreceptor, semaphorin

The first connection in the visual system is the tripartite synapse between photoreceptors and horizontal and bipolar cells in the outer retina. This synaptic connection is essential for retinal function and consequently vision. Much like other synapses in the central nervous system (CNS), the tripartite synapse undergoes morphologic changes during retinal disease and injury.<sup>1</sup> Particularly drastic plasticity occurs at the rod photoreceptor presynaptic terminals in the form of retraction from their postsynaptic partners and formation of long neuritic sprouts. These sprouts can reach all the way into the inner plexiform (synaptic) layer, form varicosities filled with synaptic vesicles, and seem to be guided toward specific cells.<sup>2,3</sup> Previous work in our lab has shown that rod photoreceptor sprouting in vitro is directed toward GABAergic and inner retinal cells that are not normally the synaptic partners of rod photoreceptors.<sup>4,5</sup> Photoreceptor presynaptic retraction and sprouting could be either detrimental, as synapses are broken and subsequent new synaptic connections are inappropriate leading to a loss of visual acuity, or beneficial, as a regenerative response to reform the circuitry of the retina. The factors influencing the guidance of adult rod cell neurites are unknown. Understanding the mechanisms involved could

be key to developing therapies that either take advantage of a regenerative response in rod photoreceptors, or prevent degeneration of rod photoreceptors by ensuring that they remain synaptically connected to their normal bipolar and horizontal cell partners.

We examined the classic guidance cue Semaphorin3A (Sema3A), known to have a role in the development and guidance of axons<sup>6–8</sup> and dendrites.<sup>9,10</sup> In many models of CNS injury, Sema3A is upregulated, producing dramatic effects upon neuronal morphology and hindering regeneration.<sup>11</sup> For example, in spinal cord injury, Sema3A is upregulated in scarred areas, as shown by in situ hybridization, and prevents the regeneration of dorsal column fibers.<sup>12</sup> Similarly, retinal injury upregulates Sema3A expression. In a rat model of retinal detachment, increases in Sema3A expression were detected in the inner retina; peak expression was observed 24 hours and then again 14 days after retinal detachment.<sup>13</sup> In optic nerve axotomy, retinal ganglion cell apoptosis caused by the injury can be rescued via anti-Sema3A antibodies.<sup>14</sup> In ischemia, Sema3A is upregulated in the ganglion cell layer and blocks revascularization of the retina.<sup>15</sup> Although these reports focus on the inner retinal layers, it is likely that upregulated Sema3A



has effects on other parts of the retina as well. Indeed, in a knockout model of one of the Sema3A receptors, Plexin-A4, the rod photoreceptor synaptic triad develops abnormally with a decrease in horizontal cell input.<sup>16</sup>

To our knowledge, our study is the first to look at how a guidance cue, in this case Sema3A, affects sprouting by adult rod photoreceptor terminals. We confirmed that Sema3A increases after retinal injury, showed that Sema3A recombinant proteins bind to rod photoreceptors and, by modifying the turning assay first used by Gunderson and Barret on *Xenopus* spinal neurons,<sup>17</sup> tested for effects on guidance. In our in vitro system, Sema3A influenced rod photoreceptor injury-induced responses and had an inhibitory role in rod cell neuritic sprouting.

## MATERIALS AND METHODS

### Animals

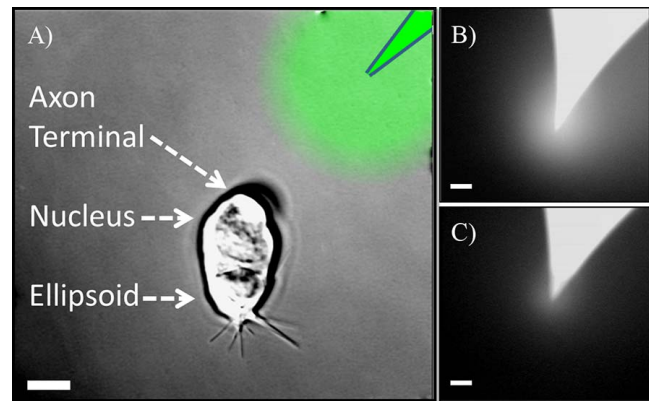
Retinal explants were obtained from American Yorkshire pigs 6 months old weighing between 160 and 200 lbs. The eyes were received from a local abattoir within hours of death and kept on ice before use. Retinal cells were obtained from adult, aquatic-phase tiger salamanders (*Ambystoma tigrinum*, 18–23 cm in length) maintained at 5°C on a 12-hour light/12-hour dark cycle. Animals were adapted to the light cycle for at least 1 week before use. All protocols were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, were approved by the Institutional Animal Care and Use Committee at Rutgers Biomedical and Health Sciences, and followed National Institutes of Health (NIH; Bethesda, MD, USA) Guidelines.

### Retinal Explant Culture and Sectioning

Porcine retinas were obtained and cultured as described previously.<sup>18</sup> Briefly, excess tissue was removed from porcine eyes with surgical scissors. The eye was sterilized in 70% EtOH and washed with sterile PBS. The optic nerve was cut close to the eye and an incision was made into the eye below the cornea. The anterior portion of the eye then was removed using curved scissors and the vitreous removed gently taking care not to detach the retina. Samples were obtained from the eyecup with a 6-mm biopsy trephine. A sterile 6-mm circle of filter paper was placed gently on the ganglion cell side of the retina and the neural retina was detached from the RPE. Neural retinal explants were placed into Dulbecco's modified Eagle's medium (DMEM) +10% fetal bovine serum (FBS) and either immediately scraped gently off the filter paper and flash frozen in optimum cutting temperature (OCT) freezing compound (Catalog No. 25608-930; Sakura Finetek, Torrance, CA, USA) or cultured at 37°C in a 5% CO<sub>2</sub> incubator and then scraped gently off of the filter paper and flash frozen in OCT freezing compound. Samples were stored at –80°C. The retinal samples were cryosectioned at 15 μm and kept at –80°C until labeling ( $n =$  nine retinal explants total, three explants per time point, from three animals).

### Cell Isolation and Culture

Retinal cells were isolated and cultured as described previously.<sup>19</sup> Briefly, retinas from adult aquatic phase salamanders were dissociated via enzymatic digestion with papain and trituration.<sup>19,20</sup> Then, 1-cm holes were drilled into culture dishes and either gridded or plain coverslips were glued on the bottom of the dishes to cover the holes using Sylgard 184. Coverslips in the newly created culture wells were coated with goat-anti-mouse IgG and Sal-1 antibodies to provide an adhesive



**FIGURE 1.** (A) Experimental setup. Photoreceptor with a retracted but still identifiable axon terminal. Rod photoreceptors have a distinct ellipsoid, which is an accumulation of mitochondria. For microspritzing, a pipette was set 45° relative to the cell's axis. Pipette not to scale. (B) Fluorescent image of microspritzer immediately after spritzing is initiated. Pipette is filled with dextran tetramethylrhodamine; highest fluorescence is around the tip. (C) Fluorescent image of microspritzer 24 hours after spritzing was initiated. A gradient still is present, with the highest concentration of dye at the tip of the micropipette. Scale bars: 10 μm.

substrate as described previously.<sup>21</sup> Isolated retinal cells were seeded into these culture dishes filled with a serum-free medium containing 108 mM NaCl, 2.5 mM KCl, 2 mM HEPES, 1 mM NaHCO<sub>3</sub>, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM sodium pyruvate, 0.5 mM MgCl<sub>2</sub>, 16 mM glucose, 1.8 mM CaCl<sub>2</sub>, 7% medium 199 (Invitrogen, San Diego, CA, USA), 1% MEM vitamin mix, 0.1 × MEM essential amino acids, 0.1 × MEM nonessential amino acids, 2 mM glutamine, 2 μg/mL bovine insulin, 1 μg/mL transferrin, 5 mM taurine, 0.8 μg/mL thyroxine, 10 μg/mL gentamicin, and 1.0 mg/mL bovine serum albumin. After plating, rod photoreceptors were identified by their morphology, the shape of their ellipsoid (an accumulation of mitochondria in the inner segment), and the presence of an axon terminal (Fig. 1A). Cone photoreceptors were identified similarly by their distinctive ellipsoids. Müller glia were identified by their characteristic morphology. ( $n =$  three animals, four cultures per animal, at least three cells per cell type, experimental group, and time point).

### Western Blot

Detached porcine retinal explants after different periods of culture were homogenized and lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer supplemented with Complete Protease Inhibitor cocktail, 1 mM Na<sub>3</sub>VO<sub>4</sub> and 10 mM NaF. The lysate was clarified with centrifugation, 18,000g for 10 minutes at 4°C. Protein concentrations were determined with the Bradford protein assay. Total lysate was boiled for 5 minutes in 2× Laemmli sample buffer, and loaded onto an 8% Tris-Cl acrylamide gel. Each lane was loaded with lysate that contained 15 μg total protein. Blots were probed with the primary antibody anti-Sema3A (N-15; sc-1148; Santa Cruz Biotechnology, Dallas, TX, USA) and the secondary antibody peroxidase-conjugated donkey anti-goat IgG (705-035-003; Jackson ImmunoResearch Labs, West Grove, PA, USA). HyGLO Quick Spray Chemiluminescent HRP Antibody Detection Reagent (E2400; Denville Scientific, Inc., Holliston, MA, USA) was used for detection. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control and blots were probed with the primary antibody monoclonal GAPDH (1D4) mouse antibody (sc-59540; Santa Cruz Bio-

technology), and the secondary antibody peroxidase-conjugated goat-anti-mouse IgG + IgM (115-035-068; Jackson ImmunoResearch Labs). All blots were exposed with autoradiographic film (41101001; BioExcell, West Lebanon, NH, USA) scanned, and processed with NIH ImageJ (version 1.46r). Bands were normalized to 0 hour control samples for each eye ( $n = 20$  explants total, five explants per time point, from three animals).

### Colorimetric Labeling

Porcine retinal sections and retinal cultures were labeled using chimeric mouse neuropilin-1-alkaline phosphatase (NPN1-AP) and chimeric human Sema3A-alkaline phosphatase (Sema3A-AP) respectively. Plasmid DNA containing the ectodomain of NPN1 or Sema3A sequence in the AP-tag backbone vector (GenHunter Corp., Nashville, TN, USA) was transiently transfected into HEK293T cells. The supernatant media from the transfected cells were collected 72 hours later and concentrated by centrifugation. Enzymatic activity of the NPN1-AP or Sema3A-AP fusion proteins was confirmed using para-nitrophenylphosphate as the chromogenic substrate, as described previously.<sup>22</sup> To image NPN-1 or Sema3A binding using the AP tag, we used a protocol described previously by Tran et al.<sup>22</sup> Briefly, sections or cells were fixed with  $-20^{\circ}\text{C}$  methanol for 10 minutes and then washed with washing buffer ( $1 \times$  tris-buffered saline [TBS] and  $4 \text{ mM MgCl}_2$ ). Fixed sections or cells then were blocked for 3 hours with washing buffer plus 10% FBS and incubated overnight at  $4^{\circ}\text{C}$  with either  $10 \text{ nM}$  Sema3A-AP diluted in blocking solution,  $3 \text{ nM}$  NPN1-AP diluted in blocking solution,  $10 \text{ nM}$  AP diluted in blocking solution, or with only blocking solution. Samples then were washed vigorously with washing buffer, fixed with an acetone fixative (26.46% paraformaldehyde [PFA], 57.7% acetone, 19.25 mM HEPES) and washed again with washing buffer. Finally, samples were incubated at  $65^{\circ}\text{C}$  for 2 hours to inactivate endogenous AP (endogenous AP is inactivated at  $65^{\circ}\text{C}$ ; however, the AP fused with Sema3A or NPN-1 is the embryonic form, which inactivates at a higher temperature), washed several times with washing buffer, treated with a nitro-blue-tetrazolium/5-bromo-4-chloro-3'-indolylphosphate (NBT/BCIP) solution ( $0.48 \text{ mM NBT}$ ,  $0.56 \text{ mM BCIP}$ ,  $10 \text{ mM Tris}$  and  $59.3 \text{ mM MgCl}_2$ , pH approx. 9.21; Catalog No. 34042; Thermo Fisher Scientific, Waltham, MA, USA) and watched carefully until color developed fully. Sections or cells were imaged with a Zeiss Axiovert 135 under bright field illumination.

### Microspritzing

Gradients of the axon guidance cue Sema3A tagged with human Fc (Sema3A-Fc,  $1 \mu\text{g/mL}$ , Catalog No. GF240; Merck Millipore, Billerica, MA, USA) were created using a setup similar to that used in axon turning assays.<sup>23</sup> The spritzer (Neurophore PPS-2 Medical Systems Corp., Greenvale, NY, USA) was set to approximately 4 psi with an eject time of 3 ms and a pause time of 997 ms. Consistent pressure could not be maintained during spritzing but in general did not vary more than  $\pm 0.5$  psi. Other studies on spritzing have demonstrated that pressure has relatively little effect upon concentration gradients.<sup>24</sup> To verify that our setup could maintain a chemical gradient for 24 hours, we microspritzed dextran tetramethylrhodamine (Catalog No. D3308; Invitrogen) dissolved in PBS into a culture dish containing salamander media at  $10^{\circ}\text{C}$  for 24 hours. We then examined the pattern of fluorescence after 24 hours of spritzing the dye to confirm gradient formation (Figs. 1B, 1C).

Rod photoreceptors were isolated from salamander retina as described above and plated on gridded coverslips. The position of rod photoreceptors with axon terminals on the gridded coverslips was noted. Identifying the rod photoreceptors with axon terminals was necessary because, after a few hours in vitro, rod photoreceptor axon terminals retracted and we were unable to distinguish between cells with or without axon terminals.<sup>25</sup> Cells were maintained in a dark, humidified chamber at  $10^{\circ}\text{C}$  for up to 3 days. Each micropipette was filled with either Sema3A-Fc at  $25 \mu\text{g/mL}$  (a similar concentration to that used previously in turning assays<sup>26</sup>) diluted with PBS or a control solution as described below. Rod photoreceptors with axon terminals were identified using their initial location on the coverslip grid (see above). Only those rod photoreceptors, without other cells in a  $100 \mu\text{m}$  radius, were chosen for microspritzing. Before being maneuvered into their correct positions, micropipettes were placed near cell debris at least  $500 \mu\text{m}$  away from the cell. If cell debris was observed to be moving in rhythm with the spritzing it indicated the micropipette was not blocked. The micropipette then was placed such that it was at a  $45^{\circ}$  angle to the axis of the cell (Fig. 1A). Each cell then was spritzed in the dark for 24 hours at  $10^{\circ}\text{C}$  using a custom-made water cooled jacket around the culture dish to maintain the culture temperature at  $10^{\circ}\text{C}$ . Only cells from 0 to 3 days in vitro (DIV) were used for microspritzing. After spritzing, the micropipette was moved close to cell debris and observed to move cell debris in rhythm with the spritzing. If no movement was detected, the cell was not used for subsequent analysis.

Control conditions included spritzing with PBS alone, with heat treated Sema3A-Fc, or with human Fc fragment alone. For the heat treated control, Sema3A-Fc ( $25 \mu\text{g/mL}$ ) was boiled for 10 minutes and stored at  $-20^{\circ}\text{C}$  until ready for use. The Fc fragment control ( $1 \mu\text{g/mL}$ , Catalog #: AG714; Millipore) was spritzed at  $5 \mu\text{g/mL}$  to match the molarity of Sema3A-Fc. Additional tests included xanthofulvin, which inhibits the action of Sema3A by binding to Sema3A to prevent binding to neuropilin-1 and Plxn-A4.<sup>27</sup> Xanthofulvin was solubilized in DMSO at  $500 \mu\text{M}$  before each experiment. DMSO or xanthofulvin then was mixed with Sema3A-Fc solution at 5% vol/vol. Sema3A and control conditions were masked from the researcher before the start of each experiment except for the Sema3A in dimethyl sulfoxide (DMSO) and the Sema3A, DMSO, and xanthofulvin mixture. The identification of xanthofulvin and DMSO solutions was impossible to mask due to the yellow color of the xanthofulvin solution and the transparent color of the Sema3A, DMSO solution. Each photoreceptor was imaged at the start and end of microspritzing using Nomarski optics ( $n = 63$  cells, from 29 animals, at least nine cells per group).

A few rod cells also were imaged with video time lapse for 24 hours during microspritzing. Cells used for time lapse were exposed to light throughout the period; however, since the cells had no outer segments, their sensitivity to light can be assumed to be very low.

### Analysis of Growth

The angle of growth was examined by drawing a line from the center of the cell to the tip of each neurite and measuring the angle of this line relative to a line drawn for the original axis of the rod photoreceptor, which was determined as explained below. The angle of growth was measured only on the basal side (axon-bearing side) of the photoreceptor where the gradient of the guidance cue was applied directly and, therefore, most assured. The amount of growth was quantified

with a modified Scholl analysis: by counting all neurites that had grown 25  $\mu\text{m}$  from the center of the cell using NIH ImageJ. This analysis was performed on the entire cell circumference since processes can be initiated from any point along the cell surface. Using ImageJ, the length of every neurite was measured from the cell body. Angles were tested for significance using the Watson-Williams *U* test while number and length of neurites were tested using 1-way ANOVA and the Tukey-Kramer test.

The axis of the photoreceptors was determined by drawing a line drawn between the center of the ellipsoid and the nucleus. Changes in orientation after spritzing from the original orientation of the ellipsoid and nucleus relative to the micropipette were measured as changes in the angle of the axis from the original axis. Changes in orientation were tested using 1-way ANOVA and Tukey-Kramer. All data were expressed as the mean plus the standard deviation. Significance was considered to be achieved at  $P \leq 0.05$ .

### Fc Fragment as a Substrate

To determine if the Fc fragment of Sema3A had any effect on adhesion in cultures, we coated plain coverslips used to form wells in the culture dishes with Sal-1, washed the coverslips twice with PBS, and then coated the coverslips with human IgG Fc Fragment at 0, 5, 50, and 500  $\mu\text{g}/\text{mL}$  diluted in PBS for 24 hours at 10°C. The dishes then were washed twice with salamander Ringer and filled with 2 mL of salamander media. Freshly isolated retinal cells were plated onto the coverslips as described previously, cultured for 3 days and then fixed with 4% PFA in PBS. Cells were labeled for rod opsin using methods described previously<sup>28</sup> using a monoclonal anti-opsin antibody 4D2 generously donated by Robert Molday (University of British Columbia, Vancouver, Canada). Cells were imaged with a Zeiss Axiovert 200M microscope under fluorescent and brightfield optics ( $n = 3$  animals, at least 10 cells per experimental or control group).

## RESULTS

### Increased Semaphorin Expression After Retinal Detachment

To confirm the reports of increased semaphorin expression after injury in the retina, porcine retinal explants were detached from the RPE and maintained in vitro.<sup>18</sup> Retinal explants were lysed for Western blots and labeled with an anti-Sema3A antibody. The antibody labeled a prominent 95 kDa band, the reported molecular weight of Sema3A in other species.<sup>29</sup>

In Western blots, detached retina did not initially display Sema3A expression immediately following injury. However, after 7 hours in vitro, expression of the 95 kDa isoform was present (Fig. 2A) and continued to increase for at least 72 hours after detachment. Levels at 24 and 72 hours were significantly greater than those immediately after detachment (Fig. 2B,  $P < 0.05$  as tested by repeated measures 1-way ANOVA and Bonferroni's post hoc analysis); moreover the increases appeared to be linear (post hoc test for linear trend).

To localize Sema3A expression in retina after detachment, we probed the frozen, methanol-fixed sections of detached retina with the ectodomain of the NPN-1, from mouse, tagged with a placental AP. NPN-1 is the obligate binding receptor for Sema3A.<sup>30</sup> Immediately after detachment, low levels of NPN-1-AP binding were detected in detached porcine retinal explants (Fig. 2C, DIV 0). After 1 and 3 days in culture, NPN-1-AP binding

was stronger and present in the outer retina, inner nuclear layer, and ganglion cell layer (GCL; Fig. 2C, DIV 3). No endogenous AP activity or binding of exogenously applied AP could be detected in any porcine retinal explant regardless of time in culture (Fig. 2C, AP only). Thus, semaphorin expression appears to increase after retinal injury.

### Binding of Sema3A to Isolated Retinal Cells

To establish the cell types to which Sema3A binds, a culture system of adult isolated salamander retinal cells was used. Cell isolation begins with a retinal detachment followed by gentle enzyme digestion and finally mechanical agitation. Cells are isolated with varying degrees of injury. A human Sema3A protein tagged with a placental AP (Sema3A-AP) was used to bind semaphorin receptors.

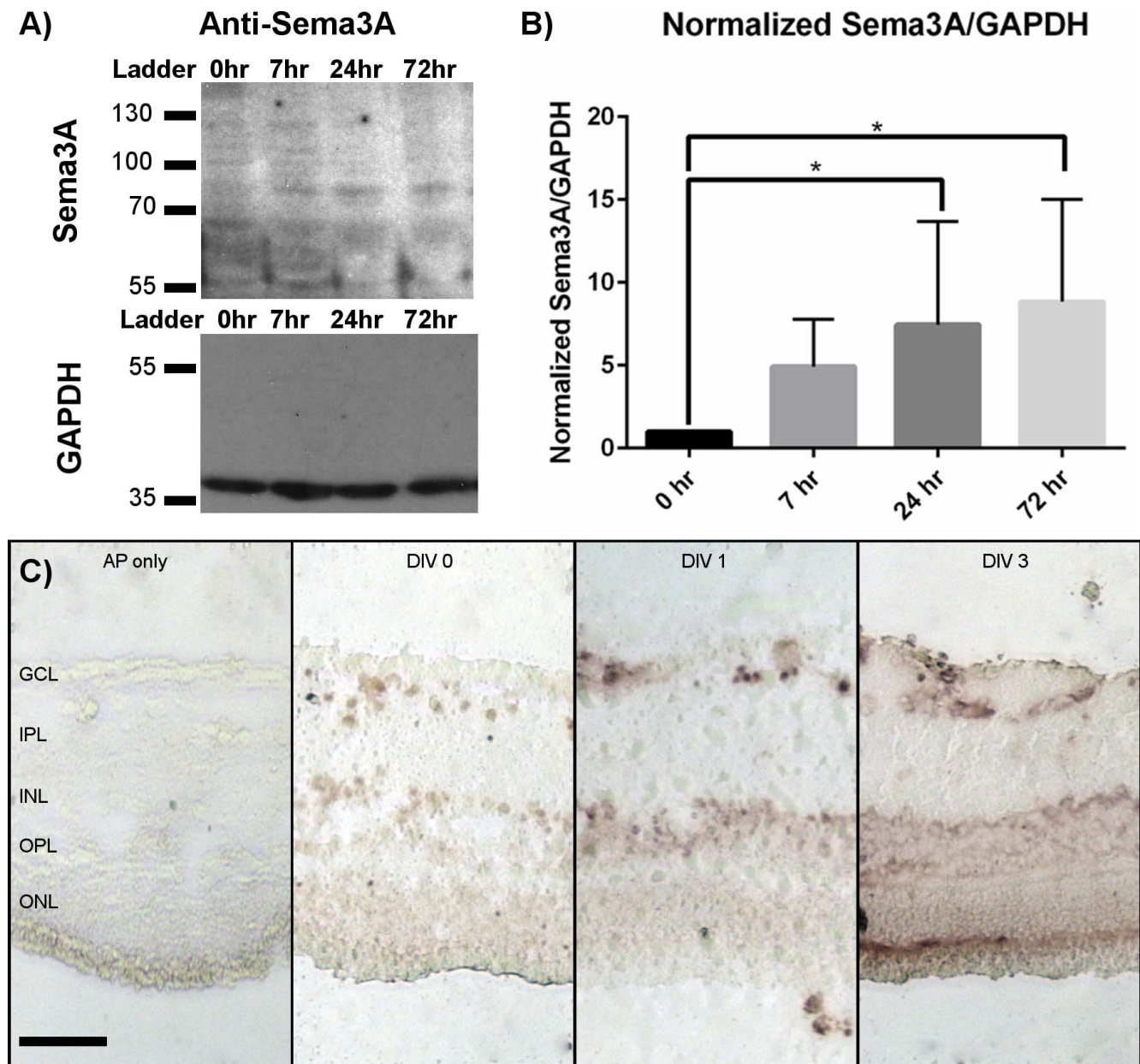
For freshly isolated rod photoreceptors, the chimeric protein was observed at the axon terminal (also called pedicle) as well as in the inner segment particularly around the ellipsoid region, an accumulation of mitochondria; controls had no staining (Figs. 3A, 3B). After 3 days in vitro, rod photoreceptors demonstrated increased binding of Sema3A to all surfaces of the cell including along long processes present in these cells (Fig. 3C). Thicker neuritic processes and those processes with presynaptic varicosities were labeled more strongly than thinner processes.

Freshly isolated cone photoreceptors demonstrated some labeling at the inner segment but very little at the pedicle that lies opposite the ellipsoid (Fig. 3D). After 3 days in culture, cone photoreceptors showed patterns of binding similar to what was seen in rod cells (i.e., stronger labeling in thicker neuritic processes with varicosities; Figs. 3E, 3F).

Sema3A-AP also bound to freshly isolated inner retinal neurons and Müller cells. Bipolar cells had binding at the axon terminal and the Landolt club (a primary dendrite) whereas amacrine and ganglion cells demonstrated Sema3A-AP binding along processes (usually dendrites) that remained after dissociation as well as at the cell body (Fig. 3H, I). After 3 DIV, most but not all multipolar cells labeled for Sema3A-AP. However, because it was difficult to distinguish among ganglion, amacrine, and bipolar cells after culturing, we cannot conclude with certainty that all inner retinal cells continued to bind Sema3A-AP. Freshly isolated Müller cells had Sema3A-AP binding to all areas of the cell with comparatively high density of staining at their apical microvilli (Fig. 3J). Müller cells do not survive for 3 DIV in our defined medium. Controls, blocking solution with or without AP, showed no labeling on any retinal cells at any DIV. Thus, Sema3A bound to most retinal cell types, and binding remained or increased with time in culture.

### Photoreceptor Sprouting Is Repelled by Gradients of Sema3A

To determine if photoreceptor neuritic growth is sensitive to Sema3A, we used a technique well-established for projection neurons: microspritizing. In vivo, salamander rod photoreceptors have one or more processes that normally extend approximately 15  $\mu\text{m}$ ; after isolation, salamander photoreceptors regrow multiple processes, some as long as 50  $\mu\text{m}$ .<sup>19</sup> Most growth is initiated after the original axon is retracted and absorbed into the cell body, which occurs in the first 24 hours of culture.<sup>31</sup> First numerous filopodial processes form; thicker neuritic processes are usually identified by day 3 in vitro.<sup>19</sup> Notably growth can be initiated from any point on the cell's circumference. Growth continues for more than a week in culture but more slowly.<sup>32</sup> All cells examined had an original

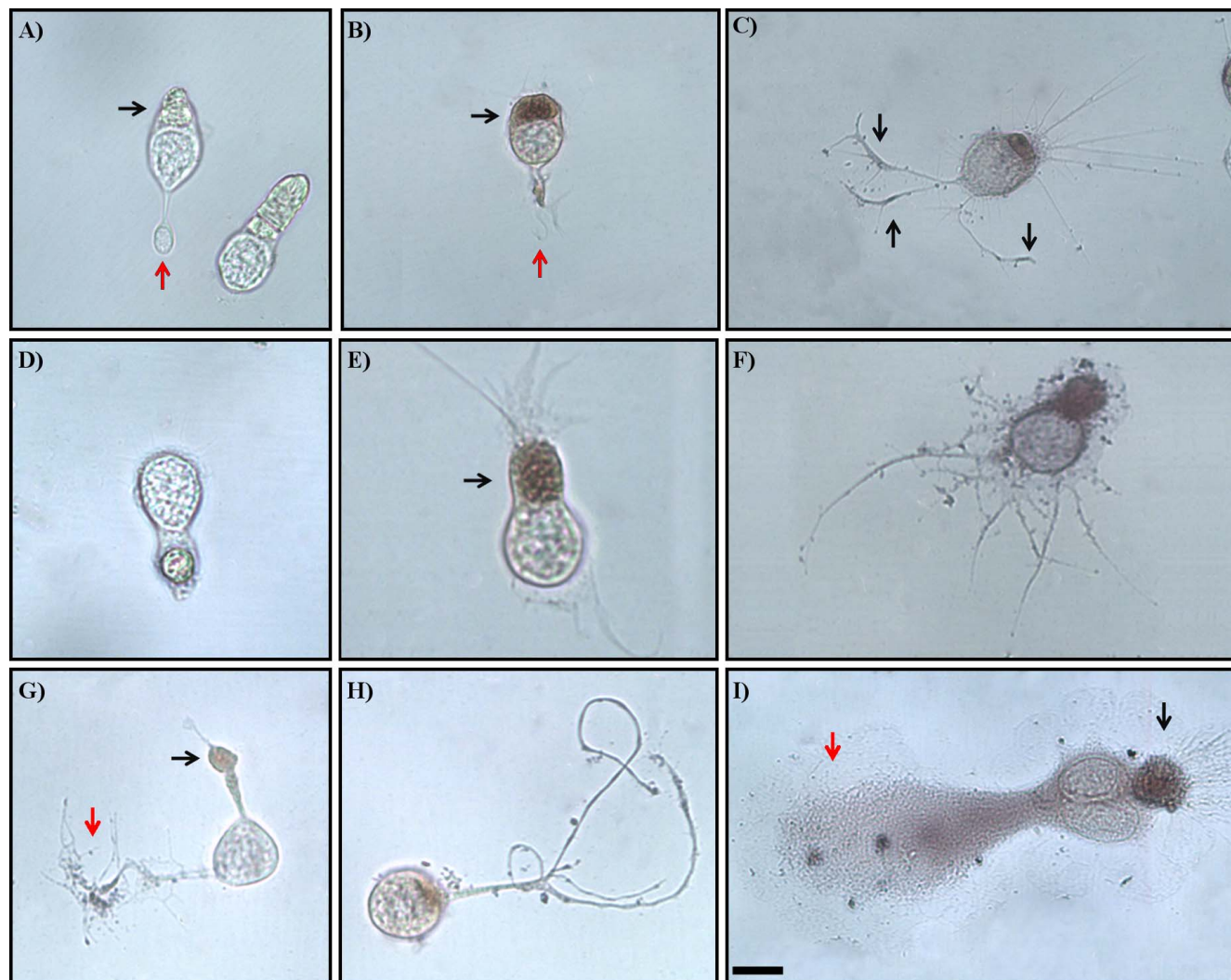


**FIGURE 2.** (A) Western blots for Sema3A. Retinal explants from the same eyes were incubated for 0, 7, 24, and 72 hours and compared. Antibody against Sema3A labeled a 95-kDa band in 7-, 24-, and 72-hour groups, but not in the 0-hour group. GAPDH, from the same SDS-PAGE gel, served as an internal loading control.  $n = 5$  explants per time point, from five eyes from three animals. (B) Quantification of 95 kDa band in Western blots of 0-, 7-, 24-, and 72-hour retinal explant lysates.  $*P < 0.05$ , as determined by repeated measures 1-way ANOVA and Bonferroni post hoc analysis. Additional post hoc analysis showed a significant ( $P < 0.05$ ) linear trend in the increases in expression, from 0, 7, 24, and 72 hours. (C) Representative sections from porcine retinal explants demonstrate binding of NPN1-AP to semaphorin. DIV 0 section treated with 9 nM AP shows no binding (*AP only*). DIV 0 section treated with 9 nM NPN1-AP shows low levels of binding (*DIV 0*). Sections treated with 9 nM NPN1-AP after 3 days in vitro show stronger but variable binding in the inner and outer retina (*DIV 3*). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.  $n = 3$  retinal explants per time point, from three eyes from three animals. Scale bar: 50  $\mu$ m.

axon (see Methods); however, their age in vitro varied from 0 to 3 days, the period during which there is most active process initiation and growth. Cells older than 3 DIV do not form many new processes and these processes tend to remain static. Most rod cell processes grow in a straight line so, once growth is initiated and there is substrate adherence, processes generally do not change direction. Only processes that form on the top surface of the cell will drastically change direction as they approach and flatten onto the substrate (data not shown). However, this is rare. Effects on directional sensitivity,

therefore, were expected to come primarily from the location of process initiation.

To observe directional effects of Sema3A on photoreceptor neuritic growth patterns we established a gradient of the guidance cue for a 24-hour period. Micropipettes were placed at 45° angle to the apical-basal axis of the cell (Fig. 1A). To verify that our setup could maintain a chemical gradient for 24 hours, we microspritzed dextran tetramethyl-rhodamine. Examining the pattern of fluorescence in the dish before and after 24 hours of microspritzing, we observed that a gradient was



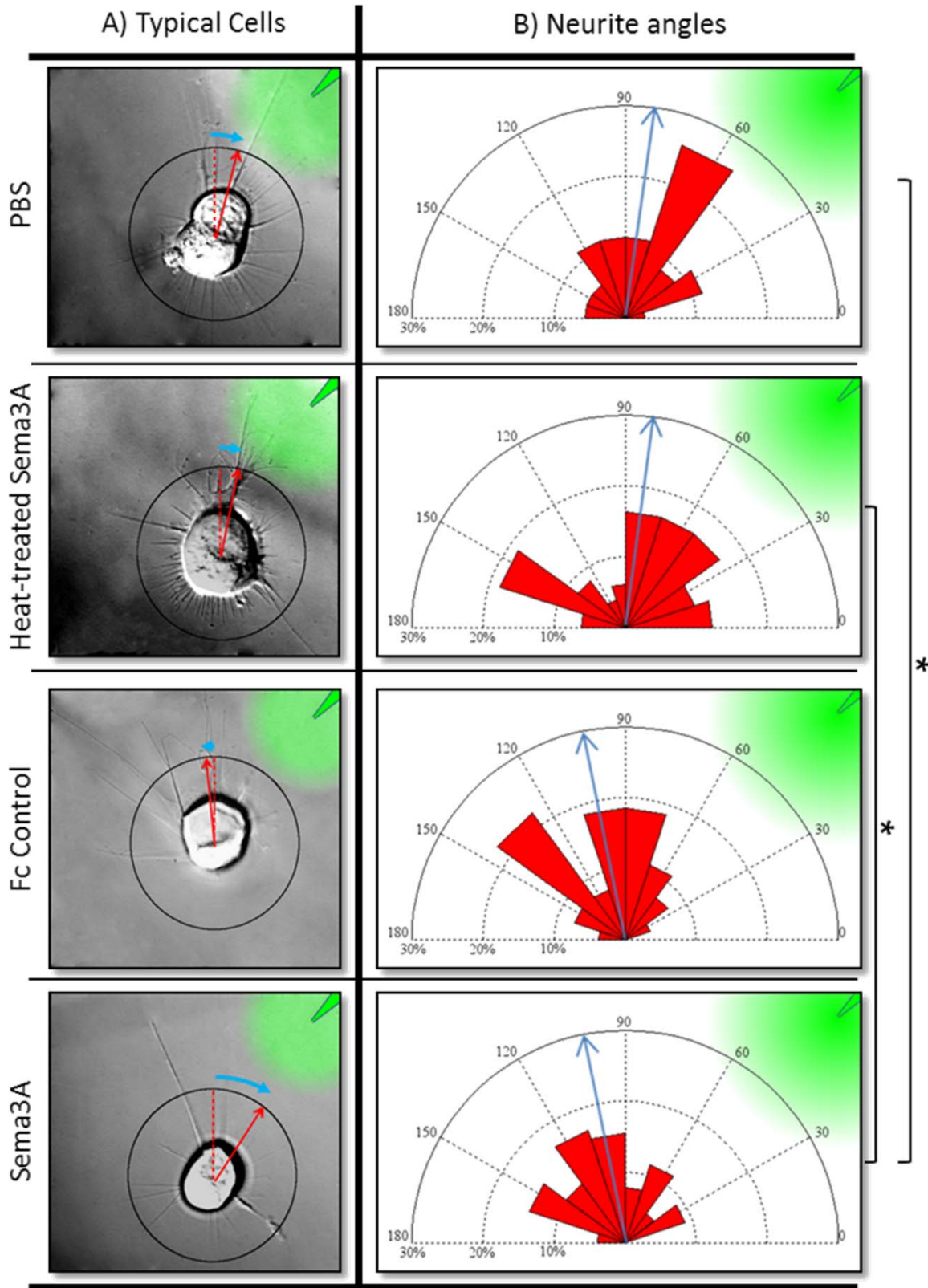
**FIGURE 3.** Isolated salamander retinal cells bind Sema3A-AP chimera. Rod cells: (A) freshly isolated rod cells from a control culture reacted for endogenous phosphatase activity. No staining is present in the cell body and inner segment that contains the ellipsoid (*arrow*) and axon terminal (*red arrow*). (B) Freshly isolated rod photoreceptor shows binding of Sema3A-AP to its surface most prominently over the inner segment (*arrow*) and at the axon terminal (*red arrow*). (C) After 3 days in vitro, Sema3A-AP binds along the cell body, inner segment and neuritic extensions. *Arrows* indicate stained varicosities. Cone cells: (D) freshly isolated cone photoreceptor demonstrates little endogenous phosphatase activity. (E) Freshly isolated cone photoreceptor treated with Sema3A-AP has binding primarily to the area of the ellipsoid (*arrow*). (F) After 3 days in vitro, cone cell shows staining of Sema3A-AP along the cell body, inner segment, and neuritic extensions. Examples of other freshly isolated cells: (G) a bipolar cell binding Sema3A-AP at the primary dendrite, the Landolt club (*arrow*), as well as the axon (*red arrow*). (H) A ganglion cell with binding of Sema3A-AP to cell body and processes. (I) A Müller cell with binding of Sema3A-AP along its entire surface. Apical villi and endfeet are indicated with *black* and *red arrow*, respectively. Controls for nonrod cell types had no staining (data not shown). Representative images from  $n = 3$  animals, four cultures per animal and at least three cells per cell type per time point. *Scale bar*: 10  $\mu\text{m}$ .

established and maintained in the cell culture chamber for 24 hours (Figs. 1B, 1C). Other researchers have examined the concentration gradient more thoroughly during spritzing and found similar results.<sup>24</sup>

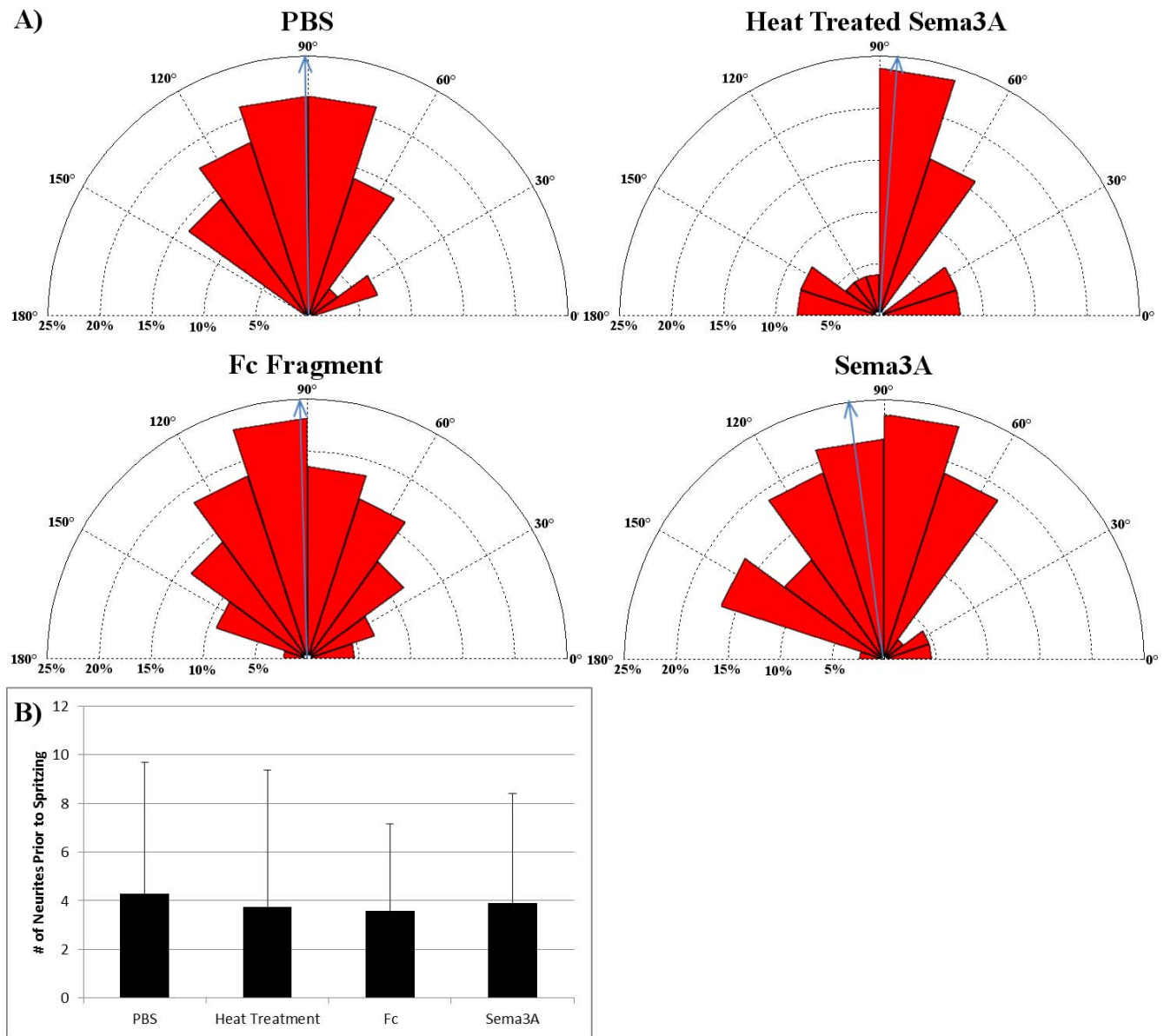
We then used our microspritizer device to examine the effect of Sema3A upon adult rod photoreceptor sprouting. For this experiment we used Sema3A-Fc and not Sema3A-AP to avoid any potential interference due to enzymatic activity from the alkaline phosphatase portion of the Sema3A-AP. At least 3 cells from each DIV were analyzed for each condition. Data from each DIV (0–3 DIV) were combined since this is the active growing period and Sema3A-AP staining was present on neurites throughout this entire time. Examination of processes on the basal side of the photoreceptor (the side from which the axon normally develops and which receives

the gradient of Sema3A directly) showed that Sema3A spritzing caused the angle of processes to become, on average, 20° further away from the pipette than from pipettes spritzing PBS or heat-inactivated Sema3A (Fig. 4,  $P < 0.05$ ).

Rod cells subjected to human IgG Fc fragment formed sprouts at an angle similar to rod photoreceptors spritzed with Sema3A-Fc and significantly different from PBS or heat-treated controls (Fig. 4). To test whether the Fc fragment interfered with the antibody substrate used for the retinal cells, we seeded retinal neurons on our substrate, Sal-1, treated with the Fc fragment at 0.5–50  $\mu\text{g}/\text{mL}$  diluted in PBS for 24 hours. PBS only treatment served as a control. No difference in growth of rod photoreceptors was seen in cells grown on Sal-1 or Sal-1 after Fc fragment treatment as measured by Scholl analysis or



**FIGURE 4.** Change in direction of process growth and in cell orientation. **(A)** Typical rod cells after 24 hours of spritzing. 55 μm diameter circles surrounding cells indicate the line from which number of neurites was calculated. *Red dashed line* indicates original cell axis while the *red arrow* indicates the final axis. *Blue arrow* demonstrates amount of change in orientation. Spritzer pipette included in diagram for reference, not to scale. **(B)** *Rose plots* indicating cumulative percentages of the direction of growth of all neurites on the basal (axonal) side of the photoreceptor. *Blue arrow* indicates the mean angle of growth of all of neurites. Neurites grew away from Sema3A-Fc compared to the heat-treated Sema3A-Fc and PBS controls.  $n = 298$  neurites from 63 cells, from 29 animals, at least nine cells per group.  $*P < 0.05$ .



**FIGURE 5.** Analysis of neurites before spritzing. **(A)** There was no statistical difference between the average angle of processes before spritzing. **(B)** There was no statistical difference in the number of processes present in rod photoreceptors before spritzing. *Scale bars:* Standard deviation.  $n = 63$  cells, 29 animals, nine cells per group.

length of the longest neurite (data not shown). Therefore, how the Fc fragment affected the direction of rod photoreceptor sprouting is unclear. The Fc fragment did not, however, have other effects upon rod photoreceptor growth as reported below.

We concluded that photoreceptors do show changes in the direction of established growth and initiation of process growth following injury: processes are repelled by high concentrations of Sema3A. However, we also concluded that this assay is not highly sensitive as differences between Sema3A and controls were only significant between the treatment and two of the three controls.

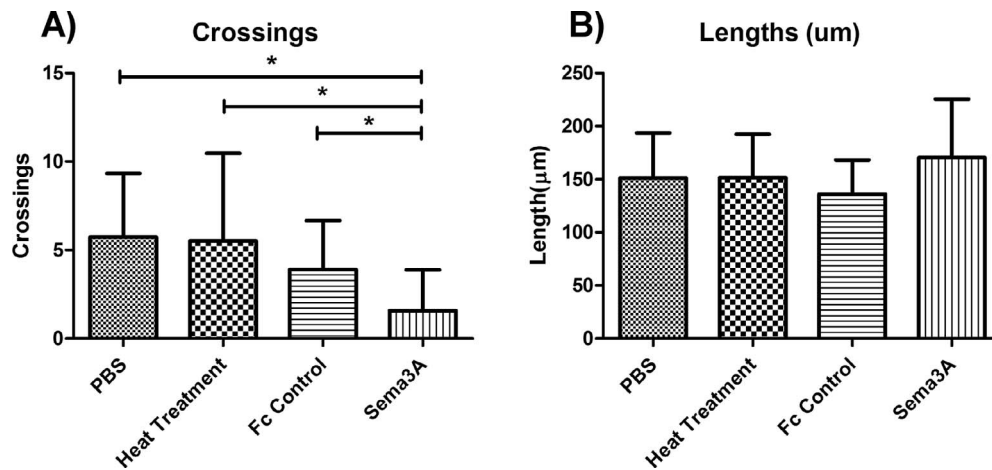
Finally, to insure that prior growth of photoreceptors did not influence sprouting during spritzing, sprouts were analyzed before spritzing. Sprouts that formed before spritzing showed no difference in the direction of growth between experimental or control groups, indicating that prior growth did not affect final process formation (Fig. 5A).

### Sema3A Reduces Number but Not Length of Rod Processes

In addition to looking at the direction of neuritic sprouting, we examined the total number of sprouts from the entire circumference of the cell formed during spritzing via modified Scholl analysis, since process initiation can occur anywhere on the cell body. Before spritzing, no difference was seen between the control and experimental groups (Fig. 5B). After applying a Sema3A gradient, rod cells sprouted on average only approximately 1.6 new processes after 24 hours compared to the controls, PBS, heat-treated Sema3A-Fc, and Fc fragment, which formed on average 5.7, 5.5, and 3.9 new sprouts, respectively (Fig. 6A,  $P < 0.05$ ).

We then measured the length of each individual process from the cell body to the tip of each process of all cells subjected to microspritizing. Average process length for each of the spritzed groups was not significantly different (Fig. 6B).





**FIGURE 6.** Significantly fewer processes were present on cells treated with Sema3A-Fc. **(A)** Number of processes per cell was defined as number of crossings by processes of the 55  $\mu\text{m}$  circle (see Fig. 4). Number of processes was evaluated after spritzing with Sema3A-Fc or controls, PBS, heat-treated-Sema3A-Fc, or Fc alone for 24 hours. **(B)** Rod photoreceptor neuritic length is not affected by Sema3A application. The average length of all the neurites that grew beyond the 55  $\mu\text{m}$  circle was the same in all conditions. Scale bars: standard deviation.  $n = 59$  cells, 29 animals, at least 10 cells per group. \* $P < 0.05$ .

Therefore, Sema3A seems to inhibit sprouting, but does not have an effect on the length of the processes that develop.

### Rod Cell Polarity Is Affected by Sema3A

The cellular axis of rod photoreceptors was determined by drawing a line between the center of the cell nucleus and the center of the cell's ellipsoid and used to examine its position relative to the spritzer. As previously stated, all cells originally were placed with their axis at a  $45^\circ$  angle to the pipette (see Fig. 1). After 24 hours of microspritizing with Sema3A-Fc, the rod photoreceptor orientation changed from its initial position to approximately  $30^\circ$  away from or toward the spritzer (Fig. 7A). This change in orientation was achieved via constant agitation of the ellipsoid and nucleus inside the cell, seen with video time lapse, while processes remained in place. This change in orientation was caused by Sema3A-Fc as little to no change in orientation was seen in rod photoreceptors spritzed with PBS, heat-treated Sema3A-Fc, or the Fc fragment (Fig. 7,  $P < 0.05$ ).

We also investigated the use of xanthofulvin, a Sema3A inhibitor that is thought to work by binding to Sema3A and blocking its ability to bind NPN1 and Plxn-A4.<sup>27</sup> The inhibitor was dissolved in DMSO and mixed with Sema3A-Fc immediately before spritzing. The effect of xanthofulvin was compared to the control solution of Sema3A-Fc in DMSO. Sema3A-Fc in DMSO caused a significant change in orientation of the photoreceptor toward or away from the spritzer whereas xanthofulvin mixed with Sema3A in DMSO significantly curtailed this turning behavior (Fig. 7). However, the inhibitor did not cause significant changes in the growth of processes, either in their direction or number compared to spritzing with Sema3A-Fc in DMSO alone (data not shown).

Combining the data from all groups, we found that rod photoreceptors whose polarity turned in the positive direction "toward" the spritzer grew fewer processes in the direction of the spritzer. Rod photoreceptors that turned "away" from the spritzer tended to grow more processes away from the spritzer (Fig. 8,  $P < 0.05$ ). In addition, those rod photoreceptors that turned away from the spritzer tended to sprout more processes ( $n = 67$  processes, nine cells) than those that turned toward the spritzer ( $n = 37$  processes, nine cells). Therefore, the orientation of the rod photoreceptor correlated with the

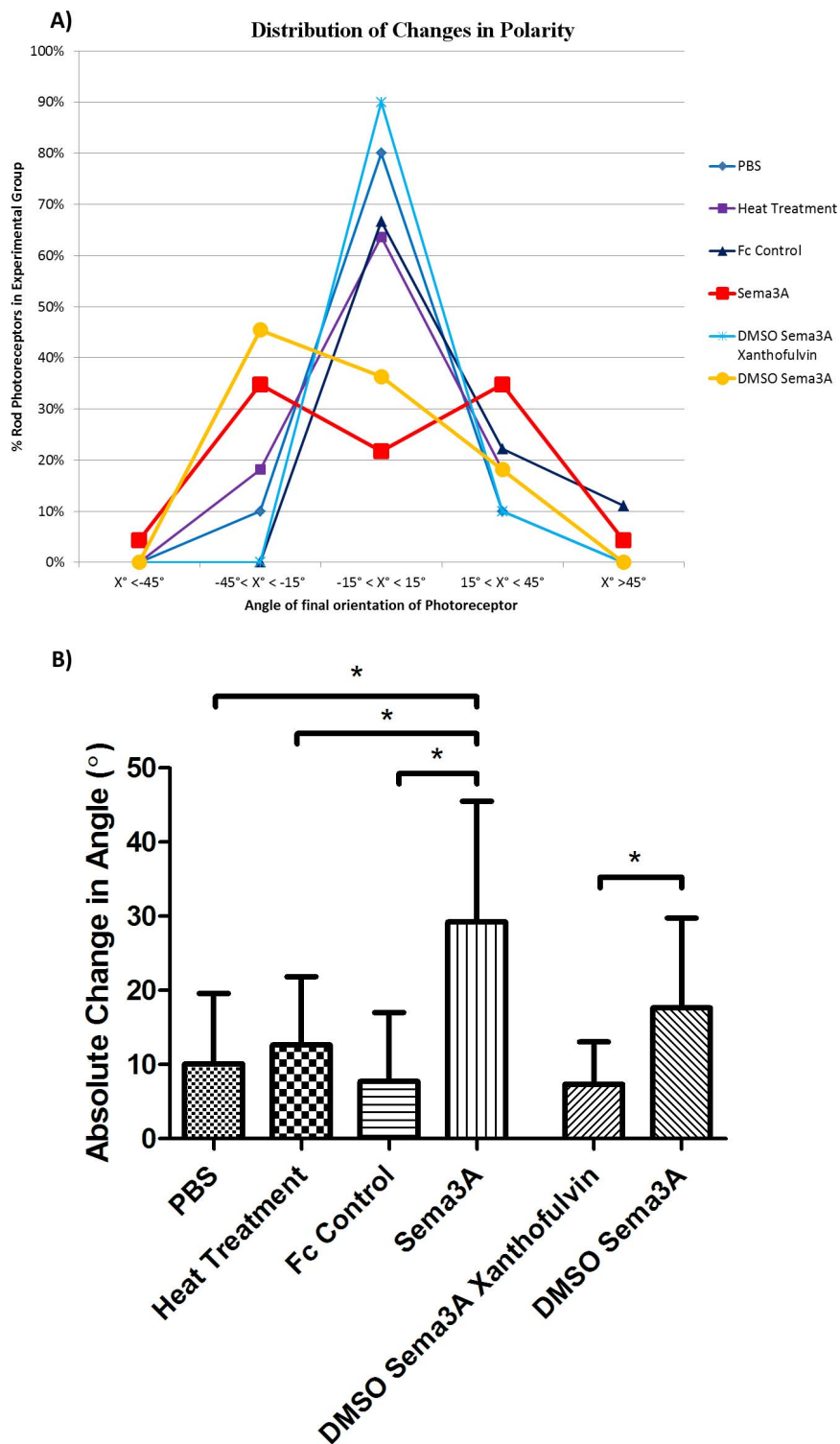
direction of the growth of processes. Moreover, cells with an "away" orientation had more growth, consistent with the data above that Sema3A is inhibitory to growth initiation.

### DISCUSSION

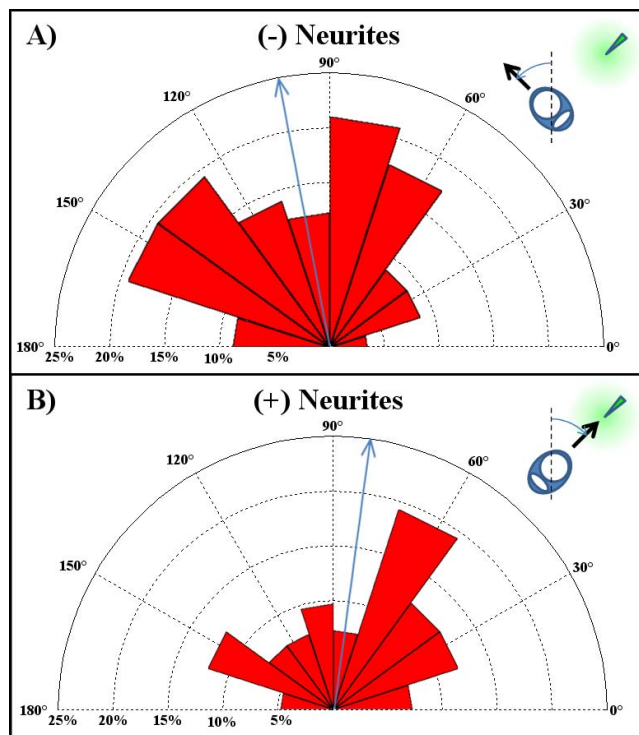
At present, most of the work examining the upregulation of Sema3A in diseased or injured retina has focused on its effects on the retinal vasculature.<sup>13,15,33</sup> To our knowledge, our study is the first to show that adult injured rod photoreceptors are sensitive to a classic guidance cue. Moreover, we demonstrated that Sema3A has the opportunity, based on expression and localization, to affect rod photoreceptor responses to injury.

First, we found that Sema3A levels increase in detached retina as shown by Western blot. However, it is possible that the Sema3A antibody may have nonspecific binding to other semaphorin ligands, such as Sema3B, Sema4D, or Sema3C, since members of the semaphorin family are highly conserved and have significant homology among each other. Our results of Sema3A expression in injured retina were confirmed by increased NPN1-AP binding in an in vitro porcine retinal detachment model. While the labeling protocol does not optimally preserve the structure of the retina, the cellular layers were clear enough to observe that expression of Sema3A localized to outer and inner retinal layers. NPN1-AP staining, initially weak, increased with time after detachment. Although we cannot rule out the possibility that other semaphorins and molecular cues contribute to the binding of NPN1-AP,<sup>30,34</sup> the increased levels of NPN1-AP as well as Sema3A in Western blots indicated a likely increase in Sema3A expression in the retina. Growth factors in FBS, such as FGF2, potentially present in the culture medium, may induce an increase in Sema3A.<sup>35</sup> However, no FBS is present in the salamander photoreceptors where an increase in binding of Sema3A is seen. Moreover, our data of increased Sema3A expression in detached porcine retina agreed with other reports on injury models of retina, including retinal detachment in rat.<sup>13,15</sup>

Second, Sema3A receptors (NPN1-AP) bind to Sema3A in the outer retina in retinal sections and Sema3A-AP, which detects endogenous NPN1, binds to rod photoreceptors at their synaptic terminal endings after isolation. After several days in vitro, Sema3A also binds to rod photoreceptors along the cell body and the newly formed neuritic sprouts



**FIGURE 7.** Cell polarity changed with Sema3A-Fc. Cell axis was determined by drawing a line from the center of the nucleus through the center of the ellipsoid. Change in polarity was defined as the degrees from the initial orientation of the photoreceptor to a new orientation after 24 hours of spritzing. A positive change was defined as moving toward the microspritzer, or clockwise, while a negative change was defined as moving away from the spritzer, or counterclockwise. **(A)** Distribution of changes in polarity after spritzing. Degree change in polarity was binned according to their value.  $X$  = Angle in degrees. **(B)** Average absolute change in polarity after spritzing. The absolute value of each cell's change in polarity for each experimental group was averaged together and compared between groups. Changes in polarity were observed in cells spritzed with Sema3A but not in any of the controls. Scale bars: standard deviation.  $n = 83$  cells, 29 animals, at least 10 cells per group.  $*P < 0.05$ .



**FIGURE 8.** Rod cells grew more processes in the direction consistent with the change in polarity. Process analysis was done on cells grouped based on the orientation of the rod photoreceptor after 24 hours of spritzing. (Cells with less than 20° of turning were excluded from analysis.) (A) (−) Neurites: processes from cells where the orientation of the rod photoreceptor changed by turning at least 20° away from the spritzer. This included cells spritzed with Sema3A-Fc or Sema3A/DMSO solutions. Average angle of processes was 100.8°. (B) (+) Neurites: processes from cells where the orientation of the rod photoreceptor changed by turning at least 20° toward the spritzer. This included cells spritzed with Sema3A-Fc or Sema3A/DMSO solutions. Average angle of processes was 82.4°. Angle of processes from positively turning cells was significantly less than angle of processes from negatively turning cells.  $n = 18$  cells, 14 animals.  $P < 0.05$ .

especially those containing presynaptic varicosities. The spread in localization of Sema3A receptors is similar to what is seen for opsin localization after injury. Opsin, the protein responsible for phototransduction, is no longer solely expressed in outer segments and becomes expressed on all areas of the plasma membrane with retinal detachment and disease.<sup>28,36</sup> In addition, Sema3A binds to several other cell types, including Müller cells, after retinal dissociation. Rosenzweig et al.<sup>37</sup> have demonstrated that Sema3A is not expressed by Müller cells in uninjured retina, but how they react after injury remains under investigation. In other types of CNS injury, such as spinal cord or traumatic brain injury, Sema3A is upregulated in the surrounding tissue by glial cells leading to a decrease in neuritic sprouting and regeneration by the injured tissue.<sup>11,38–40</sup>

Third, in the presence of Sema3A-Fc the total number of rod cell sprouts decreased, while the length of each process was not affected. Thus, Sema3A inhibits initiation of process growth and is less involved with process elongation. Compared to most controls, the initiation of sprouts formed by rod photoreceptors was away from high concentrations of Sema3A-Fc. However, processes also grew away from high concentrations of the Fc fragment. The Fc fragment has several potential receptors, including FcγR, FcαR, and FcεR.<sup>41</sup> At this time, only the neonatal Fcγ has been detected in retina

and these receptors were expressed specifically in blood vessels.<sup>42</sup> Whether the Fc fragment itself is responsible for the preferential growth away from the microspritizer is unknown. Xanthofulvin did not reduce Sema3A's inhibition of rod cell process growth or affect guidance. The pharmacokinetics of the inhibitor currently are unknown and the inhibitor has not been shown to block all possible Sema3A receptors.<sup>27</sup> Additionally, DMSO can cause an increase in neuritic growth by photoreceptors in vitro.<sup>31</sup> Therefore, DMSO could mask Sema3A's inhibitory effect on growth and limit any difference between Sema3A and xanthofulvin solutions.

Finally, Sema3A changed the orientation of rod photoreceptors, an activity that was blocked by xanthofulvin, and also not seen in any control group.

### Sema3A and Photoreceptor Polarity

Previous work in our lab has shown that the movement of the nucleus in the photoreceptor cell soma is correlated with photoreceptor neuritic and varicosity formation toward (attractive interactions) or away from (repulsion) specific cell types.<sup>4</sup> While our previous work looked at a time course of 7 days, here we examined a 24-hour period. Thus, the lack of correlation of nuclear movement with repulsion to Sema3A may reflect an initial stochastic stage of nuclear movement before final reorientation.

In embryonic hippocampal and cortical cells, Sema3A has been shown to polarize neurons, creating distinct areas where axon formation is inhibited and dendrite formation encouraged via manipulation of cAMP and cGMP in axons and dendrites.<sup>9,43</sup> Similarly, photoreceptor axon-like sprouting increases with high levels of cAMP and decreases with high levels of cGMP.<sup>28,44</sup> Thus, an effect on cell orientation and inhibition of axonal sprouting by rod cells would be consistent with the effects of Sema3A on polarity in developing neurons.

### Sema3A in Injured and Diseased Retina

Retraction of the rod cell axon occurs in detached retina in animal models and humans.<sup>1,3,45–48</sup> Since Sema3A is upregulated with detachment, it is reasonable to ask whether Sema3A contributes to the retraction.

In active growth cones, repulsion by Sema3A depends on activation of the RhoA signaling pathway leading to phosphorylation of cofilin by LIM kinase.<sup>49,50</sup> In isolated rod photoreceptors, RhoA activation leads to retraction of the synaptic terminal and reduced neuritic growth,<sup>51</sup> whereas inhibition of RhoA, Rho-associated kinase (ROCK), or LIM kinase prevents axonal retraction in isolated salamander rod cells and porcine retinal explants.<sup>25,51,52</sup> Therefore, it seems likely that the RhoA signaling pathway for classic Sema3A repulsion contributes to photoreceptor axon retraction and inhibition of growth in injured retina.

Neuritic sprouting is another feature of degenerate retina. It occurs in virtually all forms of human retinitis pigmentosa (RP).<sup>1–3,53</sup> It also is present after reattachment of a detached retina, in human and animal models.<sup>3,48,54,55</sup> At this time, it is unknown whether Sema3A levels remain elevated after reattachment or if reattachment leads to a reduction of expression of Sema3A. A reduction may remove the “brakes” provided by Sema3A to allow rod photoreceptors to sprout. However, changes in cyclic nucleotide levels intracellularly may contribute to whether rod cells sprout new neurites. In developing dorsal root ganglion neurons, cAMP can compete with inhibition of axon growth by Sema3A and Sema3A-mediated growth cone collapse.<sup>56,57</sup> We have shown that rod cells sprout neurites when cAMP is increased and that this

increase is due to activation of mislocalized opsin.<sup>28</sup> In reattached retina, cAMP levels in rod photoreceptors may increase due to activation of mislocalized opsin on rod cell plasma membranes by 9-cis-retinal, which is supplied by the RPE to the newly attached photoreceptor layer. Thus, after reattachment, conditions of increased intracellular cAMP with decreased extracellular Sema3A and/or receptors could promote rod cell sprouting.

Although neuritic rod sprouting is nearly ubiquitous in human retinal degeneration, sprouting does not occur in mouse models of retinal degeneration. It has been suggested that this is due to a difference in timing of the disease: in rodent models, most photoreceptors die within several weeks or months after disease onset while photoreceptor death in human RP can span a period of years to decades.<sup>1,2</sup> For humans, changes in Sema3A expression or other factors over the decades may come into play to promote sprouting. For future photoreceptor transplantation into degenerate retina, it may be useful to understand the time course of semaphorin expression to insure an environment permissive to neuritic regeneration.

Finally, rod cell orientation can be a problem in retinal disease where photoreceptor rosettes appear<sup>58,59</sup> and in transplantation where integrated rod cells sometimes are misoriented.<sup>60</sup> It is possible that upregulation of Sema3A has a role in the disorientation of photoreceptors.

### Other Guidance Cues

Sema3A is not the only guidance cue active in diseased or degenerate retinal tissue. Other semaphorins may also have a role in photoreceptor sprouting and guidance. For example, in a model of optic nerve crush, Sema3B and Sema3F transiently increased in the retina after injury.<sup>60</sup> Attractive guidance cues also are known to be upregulated and may even overcome Sema3A signaling. These cues include SDF-1,<sup>61</sup> which is upregulated in retinitis pigmentosa and retinal ischemia,<sup>62</sup> and netrin-1, which is upregulated during retinal lesion and optic nerve axotomy.<sup>63,64</sup> More work is required to understand the effects of attractive and repulsive guidance cues and the timing of these effects on synaptic plasticity of photoreceptors within the degenerating retina, on photoreceptor transplants, and on neuronal sprouting in the injured CNS in general.

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