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Different aspects of gliosis in retinal Müller glia can be induced by CNTF, insulin, and FGF2 in the absence of damage

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Purpose: In response to acute damage, Müller glia in the retina have been shown to dramatically alter their expression of filamentous proteins. Since damaged retinal cells are known to produce growth factors such as insulin-like growth factor (IGF), ciliary neurotrophic factor (CNTF) and fibroblast growth factor (FGF), the altered expression of filaments in Müller glia in response to retinal damage may be induced by some of these factors. The purpose of this study was to assay whether growth factors influence the expression of filamentous proteins in Müller glia in the intact retinas of postnatal chickens.

Methods: We assayed for changes in expression levels of IGF-I, IGF-II, CNTF, FGF1, and FGF2 in N-methyl-D-aspartate(NMDA) damaged retinas by using quantitative PCR. In undamaged retinas, we assayed whether intraocular injections of insulin, CNTF, or FGF2 influenced glial expression of glial fibrillary acidic protein (GFAP), neurofilament, RA4, vimentin and β3 tubulin by using immunocytochemistry on frozen sections.

Results: We demonstrated that levels of mRNA for IGF-II, FGF1, FGF2, and CNTF were increased in the postnatal chicken retina in response to neurotoxic damage. This was coincident with increased glial expression of GFAP and filamentous neuronal proteins. The combination of insulin and FGF2 caused postmitotic Müller glia to transiently increase their expression of vimentin and putative neuron specific filamentous proteins such as neurofilament, β3 tubulin and RA4. By comparison, insulin or FGF2 alone had minor effects on glial expression of cytoskeletal proteins. Although neurofilament expression was not induced by CNTF, this growth factor stimulated Müller glia to express GFAP.

Conclusions: We conclude that the phenotype of postmitotic Müller glia is plastic and can be regulated by retinal damage, and these damage induced changes in phenotype can be induced by exogenous growth factors in the absence of damage.

In response to damage, growth factors, including ciliary neurotrophic factor (CNTF) and fibroblast growth factors (FGF's), are produced by retinal neurons and Müller glia [1-6]. The functions of these damage induced growth factors within the retina remain unknown. It is possible that these factors act to promote neuronal survival and attenuate neuronal death in response to retinal damage. Consitent with this hypothesis, CNTF and FGFs have been shown to support the survival of retinal neurons in a variety of damage paradigms [7]. Alternatively, CNTF or FGFs may stimulate Müller glia to become reactive and proliferate as a result of retinal damage. Damage is known to cause Müller glia to become reactive [3,8-11]. Symptoms of reactive glia include increased expression levels of filamentous proteins such as glial fibrillary acidic protein (GFAP) and vimentin. It is likely that secreted factors mediate glial responses to damage. Consistent with the hypothesis, intraocular injections of growth factors have been shown to cause Müller glia to alter their expression of cytoskeletal proteins. For example, FGF2 stimulates the expression of GFAP and vimentin in Müller glia of rabbit and cat retinas [12] and CNTF stimulates the expression of GFAP in Müller glia of the rodent retina [13]. Taken together, these findings indicate that Müller glia change expression levels of different proteins in response to retinal damage, and that these changes may be mediated by secreted factors.

We have reported that in response to excitotoxic retinal damage, Müller glia in the postnatal chicken retina de-differentiate, proliferate, express transcription factors normally expressed by retinal progenitors, and transiently express neurofilament [14,15]. The response of Müller glia to retinal damage can also be induced by intraocular injections of growth factors [16]. Although neurotoxic damage causes proliferating Müller glia to transiently express neurofilament, it remains unknown whether injections of insulin and FGF2 stimulate the expression of neurofilament in mature, postmitotic Müller glia. Accordingly, the purpose of this study was to examine the effects of intraocular injections of growth factors on the expression of filamentous proteins by Müller glia in the chicken retina.

We report here that Müller glia in toxin damaged retinas are capable of transiently expressing a variety of filamentous proteins in response to exogenous factors. By using quantitative PCR we found that NMDA induced retinal damage leads to increased levels of mRNA for CNTF, IGF-II, FGF1, and FGF2, confirming previous findings in the rodent retina. In the absence of retinal damage, the combination of insulin and

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FGF2 caused Müller glia to transiently express neurofilament, β3 tubulin, and RA4, with no expression of GFAP. In response to injections of CNTF, neurofilament expression appeared unaffected, whereas GFAP was upregulated in Müller glia. Our data suggest that the expression of filamentous proteins by Müller glia in response to damage can be mimicked by different combinations of exogenous growth factors.

METHODS

Animals: The use of animals in these experiments was in accordance with the guidelines established by the National Institutes of Health, the University of Washington, and The Ohio State University. Newly hatched leghorn chickens (Gallus gallus domesticus) were obtained from H and N Highline International (Seattle, WA) or the Department of Animal Sciences at The Ohio State University. Animals were kept on a cycle of 12 h light, 12 h dark (lights on at 7:00 am). Chicks were housed in a stainless steel brooder at about 28 °C and received water and Purina™ chick starter ad libitum.

Injections: Chicks were anesthetized and injected as described elsewhere [17]. Unless specified otherwise, all injection paradigms began at postnatal day 8 (P8). The left eye (control) was injected with 20 µl of vehicle (sterile saline plus 0.1 mg/ml bovine serum albumin) and the right eye (treated) was injected with an excitotoxin or growth factors. We used a single toxic dose of N-methyl-D-aspartate (NMDA) at 2 µmol to induce retinal damage. Growth factors used included: purified bovine insulin (2 µg per injection); purified bovine fibroblast growth factor 2 (FGF2; 100 ng per injection); recombinant human epidermal growth factor (EGF; 100 ng per injection); recombinant rat ciliary neurotrophic factor (CNTF; 100 ng per dose); and purified bovine FGF1 (100 ng per injection). All growth factors were obtained from R & D Systems (Minneapolis, MN) and were dissolved in saline plus 0.1 mg/ml BSA. 100 µg/ml 5-Bromo-2-deoxyuridine (BrdU; Sigma, St Louis, MO) was added to the growth factor diluent for experiments that involved assaying for proliferation (i.e., in all doses for 3 consecutive daily injections of insulin and FGF2). At least 4 animals were used for each injection paradigm.

Fixation and sectioning: Dissection, fixation and sectioning where performed as described elsewhere [17-19]. In short, tissues were fixed for 30 min at room temperature in 4% paraformaldehyde plus 3% sucrose in 0.1 M phosphate buffer pH 7.4. Tissues were washed in 0.05 M phosphate buffered saline (0.9% NaCl; PBS), cryoprotected in 30% sucrose in PBS, embedded and frozen in O.C.T. Compound (TissueTek, Sakura Finetek, Torrance, CA). Cryostat sections were cut at 12 µm in thickness, thaw mounted on to Super-Frost™ Plus slides (Fisher Scientific, Hampton, NH), and stored at -20 °C until use.

Immunocytochemistry: Standard immunocytochemical techniques were applied as described elsewhere [17-19]. Working dilutions and sources of antibodies used in this study included; mouse anti-vimentin at 1:50 (H5; Developmental Studies Hybridoma Bank [DSHB], University of Iowa, Iowa City, IA), rabbit anti-glutamine synthetase (GS) at 1:2000 (Dr. P. Linser, University of Florida, Gainesville, FL), rabbit anti-neurofilament at 1:1000 (recognizes the non-phosphorylated 145 kDa isoform (NF-L) of neurofilament; Chemicon, Temecula, CA), mouse anti-neurofilament at 1:2000 (recognizes the non-phosphorylated 160 kDa isoform (NF-M) of neurofilament; RMO270; Zymed, South San Francisco, CA), mouse anti-neurofilament at 1:80 (recognizes the phosphorylated 200 kDa isoform (NF-H) of neurofilament; RT97; DSHB), mouse anti-β3 tubulin at 1:1000 (TUJ-1; Covance, Princeton, NJ); mouse anti-RA4 at 1:200 (Dr. S. McLoon; University of Minnesota, Minneapolis, MN), rabbit anti-GFAP at 1:1000 (glial fibriliary acidic protein; Dako, Carpinteria, CA), rat anti-BrdU at 1:80 (Accurate Chemicals, Westbury, NY), and mouse anti-BrdU at 1:80 (G3B4; DSHB). Secondary antibodies included goat-anti-rabbit-Alexa568, goat-anti-mouse-Alexa568, goat-anti-mouse-Alexa488 and goat-anti-rat-Alexa488 (Molecular Probes Inc., Eugene, OR) diluted to 1:500 in phosphate buffered saline (PBS; 0.05 M phosphate buffer, 145 mM NaCl, pH 7.4) plus 0.3% Triton X-100.

In situ hybridization: Tissues were dissected and immediately embedded and frozen in O.C.T. medium (Tissue-Tek). Fourteen µm thick sections were cut in the naso-temporal plane, thaw mounted onto Super-Frost™ Plus slides (Fischer Scientific), and stored dessicated at -80 °C until use. Upon thawing, slides were immediately fixed for 10 min in 4% paraformaldehyde in DEPC treated PBS, followed by two 15 min washes in 0.1% active DEPC in PBS, and a 15 min wash in DEPC treated 5X SSC (standard sodium citrate). Sections were prehybridized for 2 h at 60 °C in 50% formamide, 5X SSC, 5X Denharts, 250 µg/ml yeast RNA, and 500 µg/ml herring sperm DNA. This solution was replaced with fresh hybridization buffer that was added with 1 µg/ml DIG labeled

Table 1. Primer sequences that were used for quantitative PCR

Primer name	Primer sequence (5'-3')	Amplification product (bp)
CNTF	F: CAGCCAGGTGATGCTCTG	102
	R: ATTCCTAAGCCGCCTTTCAG	
IGF1	F: CCCACTGCACTCCCTGTAA	104
	R: GCAGTTTGAAGGACATTGTTG	
IGF2	F: TACGTGCCAAGTCAA	135
	R: CTGCCACACGTTGTACTTGG	
FGF1	F: TACTGTGCCAAGTCAA	118
	R: CATGCACTGGCTGTGAGTTC	
FGF2	F: TGCAGCTTCAAGCAGAAGAA	173
	R: CTTCCGTGACCGGTAAGTGT	
GFAP	F: CCAACGAGAAGGTGGAGATG	151
	R: TGGTACACATCACCCAGACG	
GAPDH (control)	F: CATCCAAGGAGTGAGCCAAG	188
	R: TGGAGGAAGAAATTGGAGGA	

Sequences for the forward (F) and reverse (R) primers were designed to chicken CNTF, IGF1, IGF2, FGF2, FGF2 and GAPDH using Primer3. The PCR product sizes are given in the column on the right side of the table.

riboprobe and sections were incubated over night at 60 °C in a humidified chamber. Sections were rinsed with 2X SSC at 65 °C and washed for 1 h in 0.2X SSC at 72 °C. Sections were processed for DIG immunolabeling as described elsewhere [20]. Riboprobes to neurofilament were made from base pairs 207 to 1760 by using an in vitro transcription kit (New England Biolabs, Inc.; Beverly, MA).

Quantitative PCR: Retinas from eyes treated with CNTF or NMDA and their contralateral saline injected controls were dissected as described above. Control and treated retinas from 3 animals were each pooled and placed in 1 ml Trizol Reagent (Invitrogen, Carlsbad, CA) for storage at -80 °C. Total RNA was isolated according to the Trizol protocol and resuspended in 50 µl RNAse free water. RNA cleanup and on-column DNAse treatment (Qiagen RNeasy kit, Valencia, CA) was performed on 10 µl of each total RNA sample. Cleaned and DNAse treated RNA was eluted from the RNeasy column in 30 µl water. Part of this sample (10 µl) was then used for cDNA synthesis using SuperScript II reverse transcriptase (Invitrogen) and oligo dT primers according to the manufacturer's protocol. Parallel reactions were performed using all components with the exception of the reverse transcriptase as negative controls. Both cDNA syntheses and negative controls were diluted 1:50 for subsequent real-time PCR reactions. PCR primers were designed using the web based program Primer3 from the Whitehead Institute for Biomedical Research, Cambridge, MA. Primer sequences are in Table 1. Real-time PCR reactions were performed using an MJ Opticon thermal cycler (MJ Research Inc., South San Francisco, CA). Each reaction (20 µl volume) contained 1 pM each of forward and reverse primer, 1 µl of diluted cDNA from CNTF treated or control retina, and 10 µl of 2X SYBRgreen Master Mix (BioRad, Hercules, CA). The reactions were run using the following protocol: 10 min at 94 °C, followed by 42 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min. SYBRgreen fluorescence was measured at each cycle during the 72 °C annealing step. Determination of C_T values for amplified products was automated using the MJ Opticon analysis software. C_T values were normalized against GAPDH for each experimental condition. PCR products were run on an agarose gel to verify the predicted product sizes.

Measurements, cell counts, and statistical analyses: Errors were calculated as the standard deviation of each sample that was comprised of at least 5 individuals per group. To compare data from treated and control eyes statistical significance was assessed by using a two tailed Student's t test or ANOVA and post hoc Student's t test. All measurements were made from digital micrographs, whereas all cell counts were made under the microscope on at least 4 different sections per individual.

RESULTS

Müller glia co-express GFAP and neuronal proteins in NMDA damaged retinas: To cause Müller glia to become reactive we induced acute retinal damage by injecting NMDA into the eyes of postnatal chickens. A single toxic dose of NMDA to the postnatal chicken retina destroys primarily amacrine and bipolar cells [18]. To characterize changes in glial expression of filamentous proteins, we immunolabeled retinal sections from eyes treated with saline or NMDA. In retinas from saline injected eyes, levels of GFAP immunoreactivity was minimal and neurofilament immunoreactivity was confined to the somata and axons of ganglion cells, neurites in the inner plexiform layer (IPL), and the axons of efferent target cells at the distal border of the IPL Figure 1A [19,21-23]. The antibody to GFAP cross-reacted with an unidentified antigen present in the axons of efferent target cells (Figure 1A). The GFAP-antibody did not cross-react with neurofilament, RA4, or β3 tubulin because immunolabeling for GFAP was not observed in Müller glia under conditions where the expression of neurofilament, RA4, and β3 tubulin were induced (see below). At 3 days after NMDA treatment, we found that numerous Müller glia across all regions of the retina increased their expression of GFAP (Figure 1D,G). Levels of GFAP immunoreactivity were elevated between 1 and 7 days after toxin treatment and were reduced by 15 days after toxin treatment (results not shown). Although GFAP immunoreactivity was elevated in Müller glia in central regions of toxin damaged retinas, levels of neurofilament immunoreactivity in glial cells were not increased in this region (Figure 1D-F). In peripheral regions of the retina, by comparison, we found that Müller glia were transiently immunoreactive for neurofilament (Figure 1G-I). Neurofilament immunolabeling was apparent at 2 days (data not shown) and 3 days after toxin treatment (Figure 1G-I) and subsided by 5 days after treatment (results not shown), consistent with our previous report [14]. Based on observations made from confocal micrographs, we found that all neurofilament-expressing Müller glia were co-labeled for GFAP immunoreactivity (145 cells counted), whereas some Müller glia were immunoreactive for GFAP alone (Figure 1G-I).

To test whether filamentous proteins in addition to neurofilament were expressed by damage-reactive Müller glia, we immunolabeled retinal sections for β3 tubulin or RA4. β3 tubulin and RA4 are neuron specific filamentous markers known to be expressed shortly after retinal ganglion cells begin to differentiate [24,25]. In saline treated retinas, Müller glia were not immunoreactive for β3 tubulin or RA4 (Figure 1B,C). Three days after NMDA treatment, we found that many Müller glia became immunoreactive for β3 tubulin (Figure 1J-L) or RA4 (Figure 1M-O). Müller glia immunoreactive for GFAP and β3 tubulin/RA4 were concentrated in peripheral regions of the retinas, whereas glia in central regions were immunoreactive for GFAP alone (data not shown). However, not all GFAP positive Müller glia were immunoreactive for β3 tubulin or RA4.

Retinal levels of CNTF, FGF, and IGF are affected in NMDA damaged retinas: Previous reports in the rodent retina have demonstrated that levels of FGFs and CNTF are elevated in response to damage [1-6]. To test whether expression levels of secreted factors are altered in damaged chicken retinas, we used quantitative RT-PCR to assay mRNA levels in NMDA treated retinas. We found that retinal levels CNTF mRNA were increased about three fold at 3 days after NMDA treatment

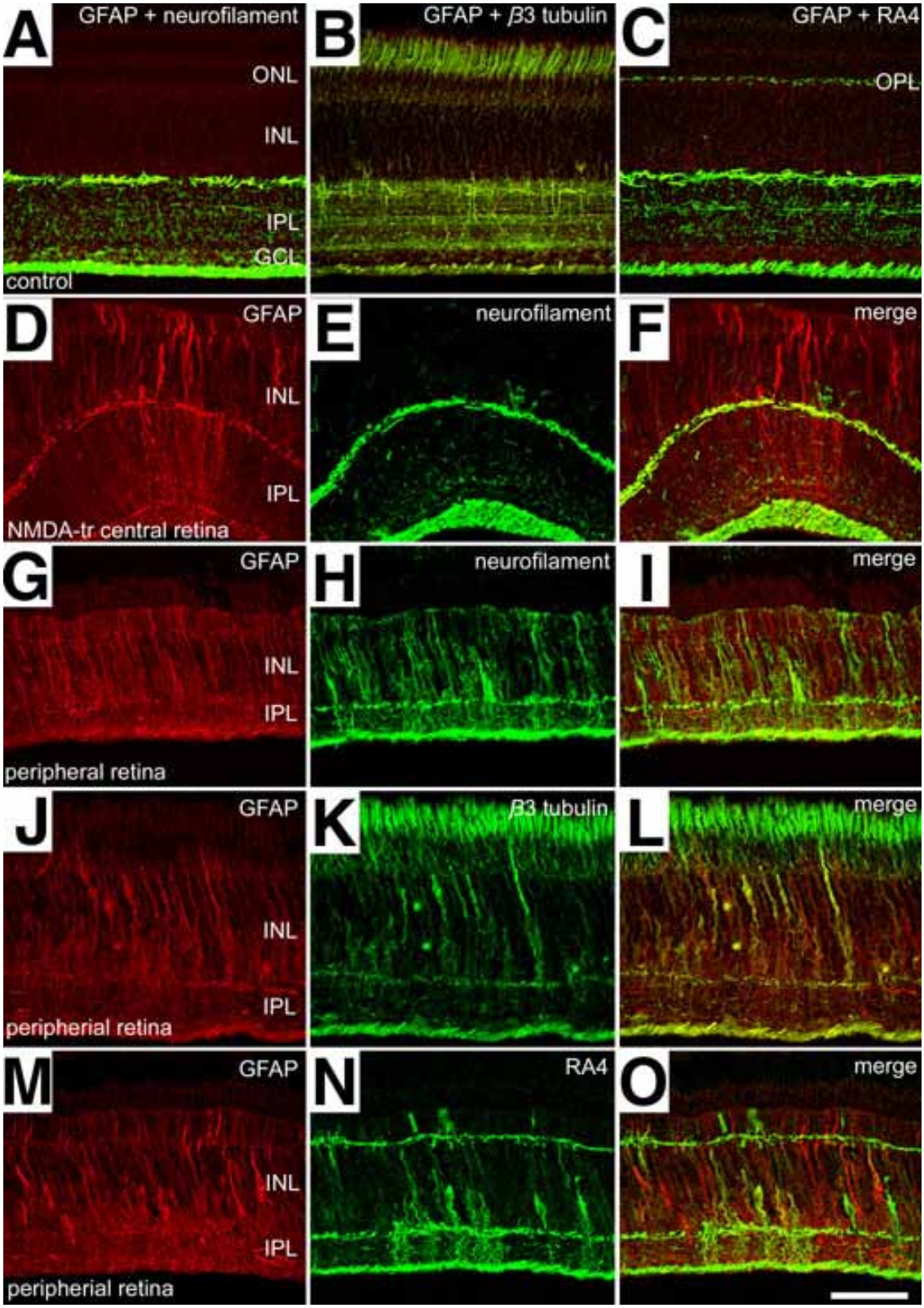


Figure 1. Retinal damage induces glial expression of filamentous proteins. NMDA induced retinal damage causes Müller glia to become immunoreactive for GFAP, neurofilament, β3 tubulin, and RA4. Retinal sections were obtained from eyes 3 days after treatment with saline (**A**-**C**) or NMDA (**D**-**O**). Sections were labeled with antibodies to GFAP and neurofilament (NF-M; **A**,**D**-**I**), GFAP and β3 tubulin (**B**,**J**-**L**), or GFAP and RA4 (**C**,**M**-**O**). Images were taken from central (**A**-**F**) or peripheral (**G**-**O**) regions of the retina. Confocal images were obtained by projecting 7 optical sections that were 1.2 μm in thickness. For all images, identical settings were used on the microscope and for post-acquisition processing to maintain the relative labeling intensity. The calibration bar in **O** represents 50 μm and applies to all panels. The outer nuclear layer (ONL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL) are labeled.

(Figure 2). Similarly, levels of IGF-II, FGF1, and FGF2 were increased in the retina three to four fold at 3 days after NMDA treatment (Figure 2). By contrast, levels of IGF-I were decreased (Figure 2).

Insulin and FGF2 induce the transient expression of neurofilament by Müller glia: To test whether growth factors influence the expression of cytoskeletal proteins in Müller glia we made 1 to 3 consecutive daily injections starting at postnatal day 7 (P7). At different times after the final injection of growth factors we harvested the eyes and processed tissues for immunocytochemical labeling.

In retinas treated with 3 consecutive daily doses of insulin (2 μg per dose), in addition to neurofilament immunolabeled structures observed in saline treated retinas (Figure 3A), we observed a few neurofilament immunoreactive cells that were oriented vertically through the retina (Figure 3B,G). These cells were found only in the far peripheral regions of the retina, within 700 μm of the retinal margin. In retinas treated with 3 consecutive daily applications of FGF2 (100 ng per dose), we also found some neurofilament-immunoreactive cells with vertical orientation in peripheral regions of the retina, within 700 μm of the retinal margin (Figure 3C,G). When insulin and FGF2 were co-injected for 2 consecutive days, we observed a significant ($p<0.005$; n=5) increase in the number of neurofilament-immunoreactive Müller glia-like cells (Figure 3G). These neurofilament-immunoreactive cells were found within 700 μm of the retinal margin. The number of neurofilament-immunoreactive cells with vertical orientation was increased further (significance; $p<0.005$; n=5) in retinas that received 3 injections of both insulin and FGF2 (Figure 3D,F,G). These cells had the morphology of Müller glia (Figure 3F) and were found in the peripheral retina up to 3 mm from the retinal margin.

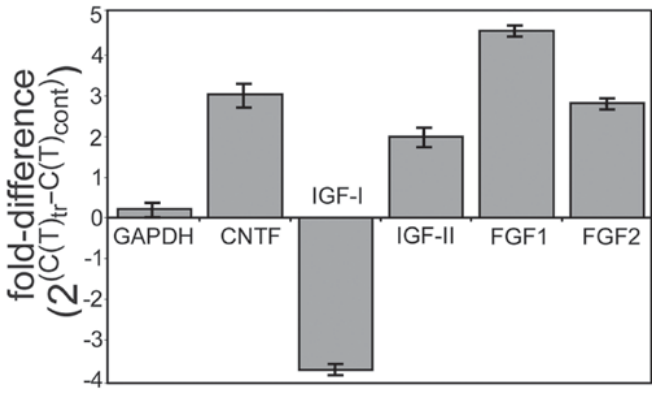


Figure 2. Expression levels of growth factors are affected by retinal damage. At 3 days after NMDA treatment, retinal expression levels of CNTF, IGF-II, FGF1, and FGF2 were increased, whereas the level of IGF-I was decreased. The histogram illustrates the fold difference of the growth factors as measured by quantitative PCR. Fold difference was calculated as 2^x where x equals the $C(T)_{NMDA\ treated}$ minus $C_{(T)control}$. The C_T value is the PCR cycle number at which the reaction enters log phase and is inversely proportional to transcript abundance. C_T values were averages of samples run in triplicate.

The number of neurofilament-expressing Müller glia-like cells was maximal at 2 days after the last of 3 consecutive injections of insulin and FGF2 (Figure 3H). This effect was transient; few neurofilament-expressing Müller glia-like cells were observed 4 days after the final dose of insulin and FGF2 (Figure 3E,H), and by 10 days after the final injection the number of neurofilament-immunoreactive Müller glia-like cells in the peripheral retina was equal to that of untreated eyes (results not shown). Three different antibodies to neurofilament, one raised to the 145 kDa isoform (NF-L), one raised to the 160 kDa isoform (NF-M), and one raised to the 200 kDa isoform (NF-H), gave similar patterns of labeling (results not shown). Insulin and FGF2 stimulated the expression of neurofilament in Müller glia-like cells in younger and older animals; this effect was observed when injections of growth factors began at P1 or P21 (results not shown). Levels of GFAP immunoreactivity were low in Müller glia treated with insulin and FGF2 (data not shown).

To test whether the expression of neurofilament by Müller glia resulted from achieving a concentration threshold after 3 consecutive daily injections of insulin and FGF2, we gave one large dose of these growth factors and made observations 24 h later. We found that a single large dose of insulin (6 μg) and FGF2 (500 ng) did not induce the expression of neurofilament in Müller glia (data not shown).

To test whether the expression of neurofilament in Müller glia was caused by a successive or simultaneous activation of insulin/IGF receptors and FGF receptors, we made injections of insulin and FGF2 in sequence or with a short interval (6 h) between injections. We found that insulin or FGF2 alone, applied 6 h apart, did not induce neurofilament in Müller glia-like cells (Table 2). Similarly, if we applied insulin first and FGF2 second, or in reverse order, neurofilament was not induced in Müller glia (Table 2). However, if we combined insulin and FGF2 and made 2 injections 6 h apart, we found a significant induction of neurofilament in Müller glia-like cells (Table 2).

We tested whether FGF1, EGF, or CNTF alone or in combination with insulin induced glial expression of neurofilament 24 h after the final injection in the absence of retinal damage. Although FGF1 alone had little effect, co-injection of FGF1 with insulin induced the expression of neurofilament in Müller glia-like cells, in numbers similar to those observed for injections of insulin and FGF2 (Table 2). The expression of neurofilament by Müller glia-like cells was minimal in eyes that received 3 consecutive daily injections of EGF alone (Figure 3G, Table 2), CNTF alone, or the combination of CNTF and insulin (Table 2). However, we observed numerous Müller glia that expressed neurofilament in retinas treated with the combination of EGF and insulin, but to a lesser extent than that observed in retinas treated with equal doses of FGF2 and insulin (Figure 3G, Table 2).

To further confirm that neurofilament was being expressed by Müller glia-like cells, we probed for neurofilament mRNA by using in situ hybridization. In the retinas treated with saline or insulin alone, neurofilament mRNA was detected in orthotopic and displaced ganglion cells (Figure 4A), consis-

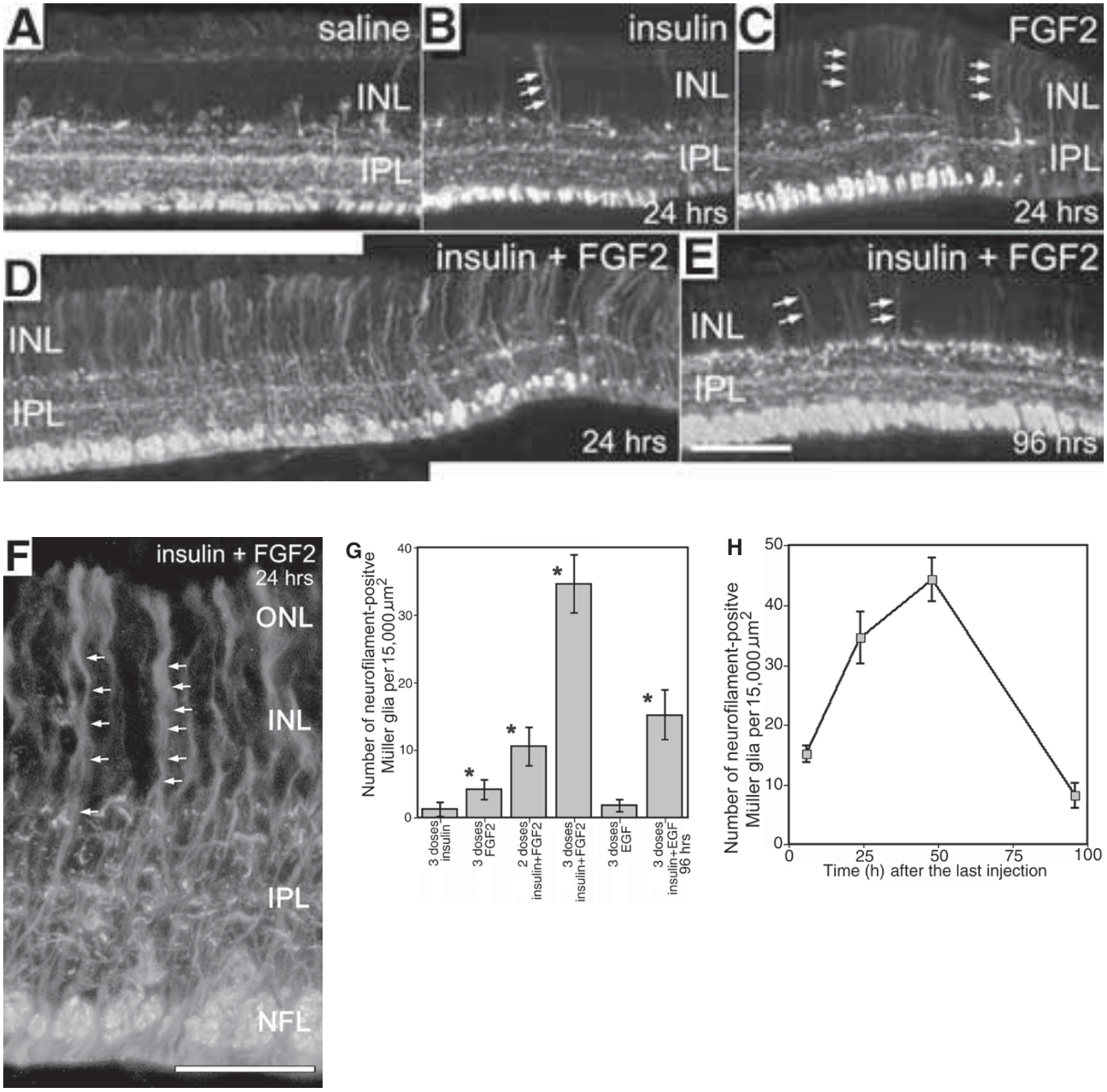


Figure 3. Neurofilament expression can be induced in Müller glia by insulin and FGF2. Exogenous insulin and FGF2 induces the expression of neurofilament in Müller glia in peripheral regions of the retina. Vertical sections of the peripheral retina were labeled with antibodies to neurofilament (NF-M). Retinas were obtained from eyes that were injected with 3 doses of saline (**A**), insulin alone (**B**), FGF2 alone (**C**), insulin and FGF2 (**D**,**E**,**F**). Injections were made at postnatal day 7 (P7), P8, and P9. Retinas were dissected and processed for immunocytochemistry 24 h (**A-D**,**F**) or 4 days (**E**) after the last injection. **F** is a high power field of view of retina that demonstrates neurofilament-expressing Müller glia-like cells. Arrows indicate neurofilament immunoreactive cells with the morphology of Müller glia. The calibration bar in **E** represents 50 μm and applies to **A-E**; the bar in **F** represents 50 μm. ONL indicates the outer nuclear layer; INL indicates the inner nuclear layer; IPL indicates the inner plexiform layer; NFL indicates the nerve fiber layer. **G** is a histogram demonstrating the number of Müller glia, neurofilament-expressing cells in the peripheral retina from eyes treated with combinations of exogenous insulin and FGF2. **H** is a plot demonstrating that the number of neurofilament-immunoreactive cells in the retina increases between 6 and 48 h after the final injection of insulin and FGF2, but decreases thereafter. ANOVA was done to determine significance ($p<0.005$) of difference among the data sets and a post hoc two tailed Student's t-test used to determine significance (asterisk is $p<0.005$) of difference between the mean numbers of neurofilament positive Müller glia in insulin treated retinas and those treated with FGF2 alone, insulin combined with FGF2, and insulin combined with EGF.

tent with the labeling observed with antibodies to neurofilament. In retinas treated with insulin and FGF2, in addition to the cells observed with insulin alone, we detected neurofilament mRNA in vertically oriented cells in the middle of the INL (Figure 4B,C).

To confirm that the cells that express neurofilament after growth factor treatment were Müller glia, we double labeled retinal sections for neurofilament and the Müller glial marker glutamine synthetase (GS). In retinas treated with both insulin and FGF2, many neurofilament immunoreactive processes were also immunoreactive for GS (Figure 5A-C). These double labeled cells were found up to 3 mm central to the retinal margin. Some neurofilament positive Müller glia-like cells were not immunoreactive for GS. To further confirm that neurofilament is expressed by Müller glia, we double labeled retinal sections with antibodies to neurofilament and a second Müller glial marker vimentin. In insulin treated retinas, there were no cells that co-express vimentin and neurofilament (Figure 5D-F). One day after the last of 3 consecutive daily injections of insulin and FGF2, we found that all neurofilament-expressing Müller glia co-expressed vimentin (142 of 142 cells from 4 individuals; Figure 5G-I). The distribution of vimentin immunoreactivity appeared to be increased in the vitread endfeet of the Müller glia in retinas treated with insulin and FGF2 (Figure 5H) compared to the distribution of vimentin in untreated or insulin treated retinas (Figure 5E).

Some of the neurofilament expressing Müller glia are proliferating: We have reported elsewhere that insulin and FGF2 stimulate Müller glia to re-enter the cell cycle [16]. To test

Table 2. Müller glial expression of neurofilament is influenced by growth factors

Growth factor	Number of injections	Interval between injections (h)	Number of NF+ Müller glia per 15,000 square μm (±SD)
saline	3	24	1.4±0.9
insulin	3	24	1.2±1.0
FGF1	3	24	3.6±1.8
FGF2	3	24	4.1±1.5
insulin + FGF2	1		1.3±1.4
insulin + FGF2	2	24	10.5±2.8
insulin + FGF2	3	24	34.6±4.3
insulin + FGF1	3	24	29.7±6.2
EGF	3	24	1.8±0.9
insulin + EGF	3	24	15.2±3.7
CNTF	3	24	0.1±0.1
insulin + CNTF	3	24	2.1±1.2
insulin	2	6	2.1±1.4
FGF2	2	6	2.3±1.2
FGF2 1st-insulin 2nd	2	6	2.3±1.4
insulin 1st-FGF2 2nd	2	6	2.0±1.0
insulin+FGF2	2	6	12.8±1.2

The expression of neurofilament in Müller glia is preferentially induced by repeated doses of the combination of insulin and FGF1/FGF2. Intraocular injections of EGF, CNTF, insulin, or FGF2 alone have little effect on glial expression of neurofilament.

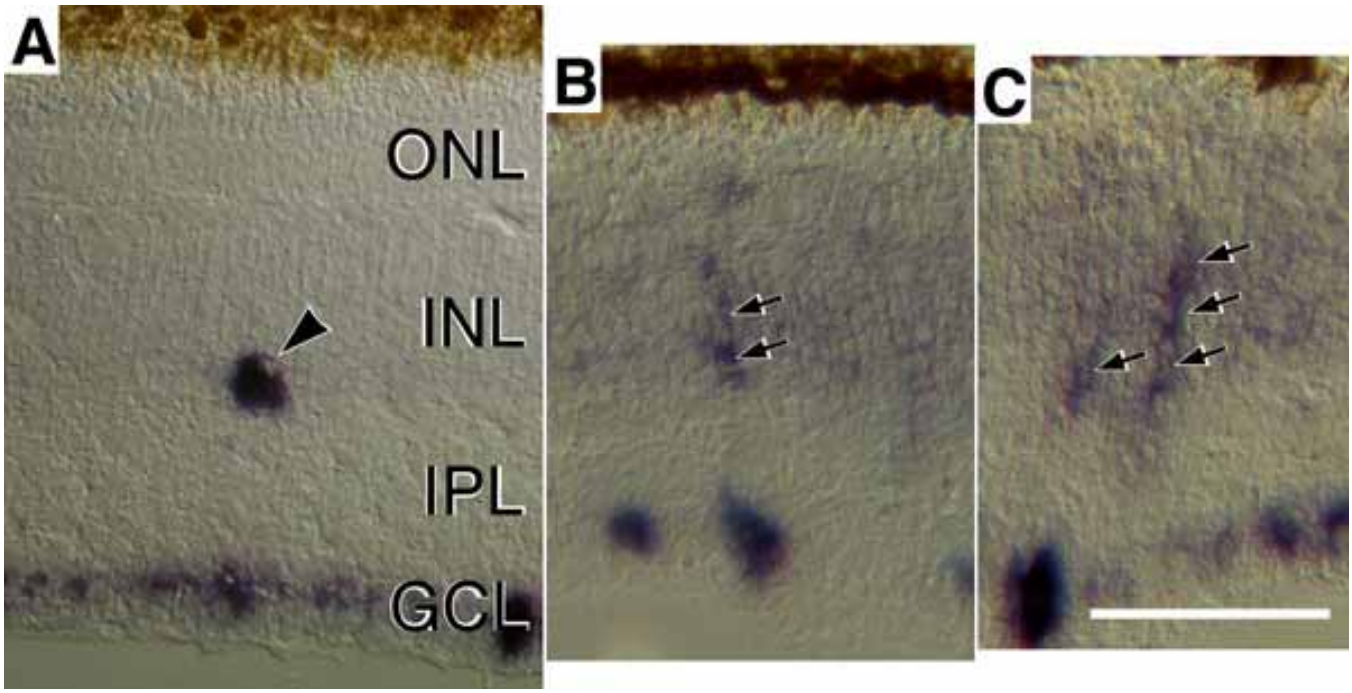


Figure 4. In situ hybridization for neurofilament in retinas treated with insulin and FGF2. Intraocular injections of insulin and FGF2 induce the expression of neurofilament mRNA in vertically oriented cells within the retina. Retinas were obtained from eyes that received 3 consecutive daily injections of insulin (**A**) or insulin and FGF2 (**B**,**C**). In situ hybridization was used to detect neurofilament transcripts. Arrows indicate vertically oriented cells that express neurofilament mRNA and the arrowhead in **A** indicates a displaced ganglion cell. The calibration bar in **C** represents 50 μm and applies to all panels. The outer nuclear layer (ONL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL) are labeled.

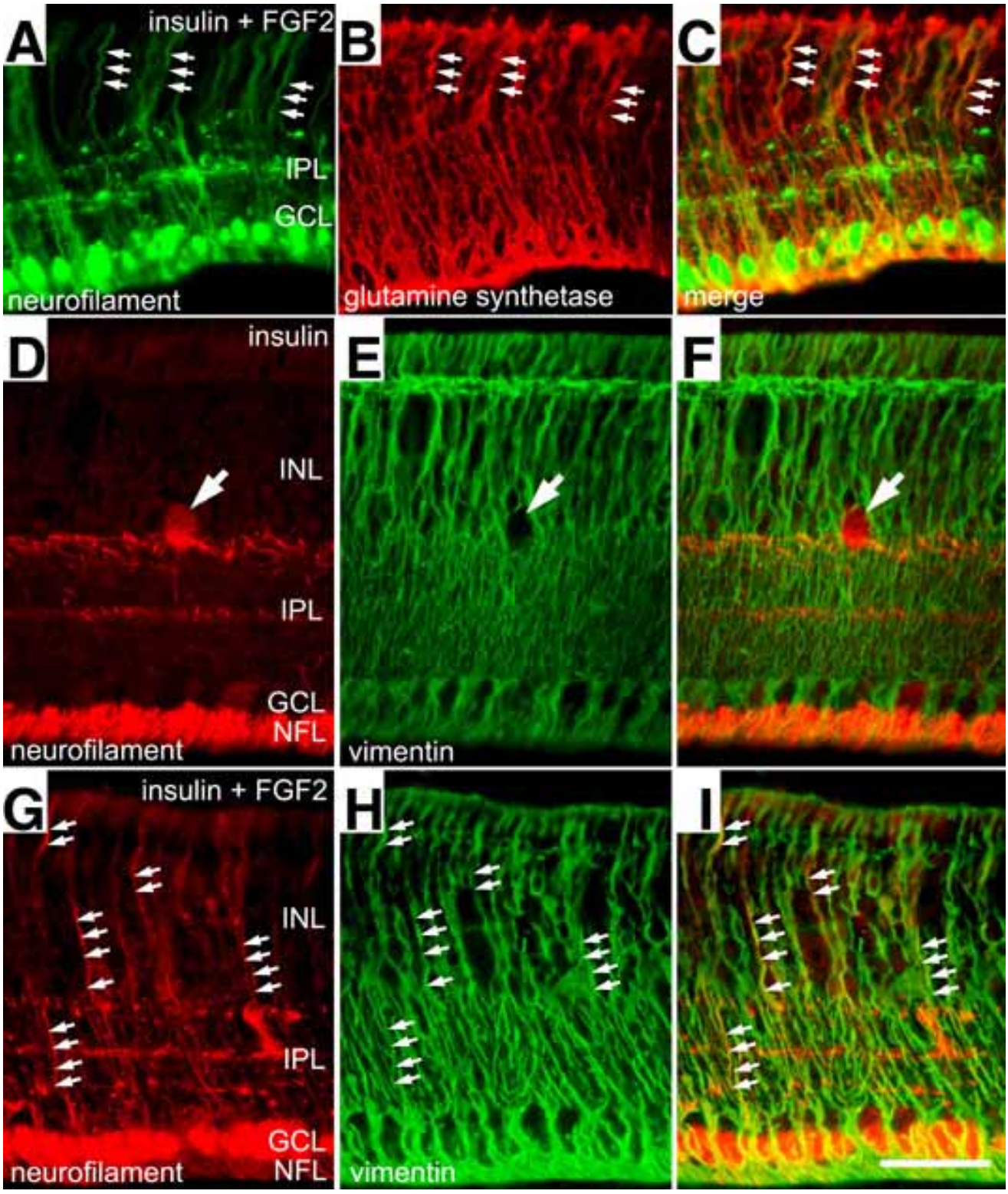


Figure 5. Neurofilament expression co-localizes with glutamine synthetase and vimentin in growth factor treated retinas. Intraocular injections of insulin and FGF2 induce the expression of neurofilament in Müller glia that express glutamine synthetase and vimentin. Retinas were treated with 3 consecutive daily doses of insulin and FGF2 (**A**-**C** and **G**-**I**) or insulin alone (**D**-**F**). Retinas were fixed and processed for immunocytochemistry 24 h after the final dose of growth factors. **A**-**C**: Vertical section of the peripheral retina that was labeled with antibodies to neurofilament (NF-M; in green) and glutamine synthetase (in red). **D**-**I**: Vertical sections of the retina that were labeled for neurofilament (NF-L; in red) and vimentin (in green). Arrows in **A**-**C** and **G**-**I** indicate double labeled structures and the arrow in **D**-**F** indicates a displaced ganglion cell that is immunoreactive for neurofilament. The inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL), and nerve fiber layer (NFL) are labeled. The calibration bar in **I** represents 50 μm and applies to all panels.

whether neurofilament expressing Müller glia were proliferating, we double labeled sections for neurofilament and BrdU. In peripheral regions of the retina (within 2 mm of the retinal margin), nearly half of the BrdU labeled cells were immunoreactive for neurofilament (43.2±12.1%; mean±standard deviation; 124 BrdU/neurofilament positive cells per 286 total BrdU positive cells in the INL of ONL counted from 4 individuals). By comparison, about one fifth of the neurofilament positive Müller glia were labeled for BrdU (18.2±2.5%; mean±standard deviation; 61 BrdU/neurofilament positive cells per 341 total neurofilament positive Müller glia counted from 4 individuals). In more central regions of the retina (between 2 and 3 mm away from the retinal margin), none of the neurofilament positive glial cells were labeled for BrdU. BrdU/neurofilament immunoreactive cells were usually found with their somata in the INL (Figure 6A-C), and occasionally we found BrdU/neurofilament labeled cells in the ONL (Figure 6D-F).

Müller glia become immunoreactive for β3 tubulin and RA4 following treatment with insulin and FGF2: The expression of neurofilament in Müller glia led us to test whether Müller glia express neuronal markers other than neurofilament after injections of insulin and FGF2. We assayed for the expression of β3 tubulin and RA4. The TUJ-1 antibody recognizes β3 tubulin, which is expressed by ganglion cells in the chick retina [25]. Similarly, the monoclonal antibody RA4 labels an unknown protein in ganglion cells in the chick retina [26]. In saline and insulin treated retinas, β3 tubulin immunoreactivity was observed in displaced ganglion cells in the INL, processes within the IPL, orthotopic ganglion cells, and the nerve fiber layer (NFL; Figure 7A). In retinas treated with insulin and FGF2, immunoreactivity for β3 tubulin appeared in numerous vertically oriented cells (Figure 7B). In retinas treated with saline or insulin alone, RA4 immunoreactivity was detected in processes in the outer plexiform layer (OPL), displaced ganglion cells in the INL, processes within the IPL, orthotopic ganglion cells, and the NFL (Figure 7C). Injections of insulin and FGF2 induced immunoreactivity for RA4 in vertically oriented cells that were abundant in peripheral (Figure 7D) and far peripheral regions of the retina (Figure 7E). Many of these RA4 immunoreactive cells co-localized GS immunoreactivity (Figure 7F-H), indicating that these cells were Müller glia.

CNTF induces the expression of GFAP in Müller glia: We tested whether CNTF caused Müller glia to express increased levels of filamentous proteins in the absence of reti-

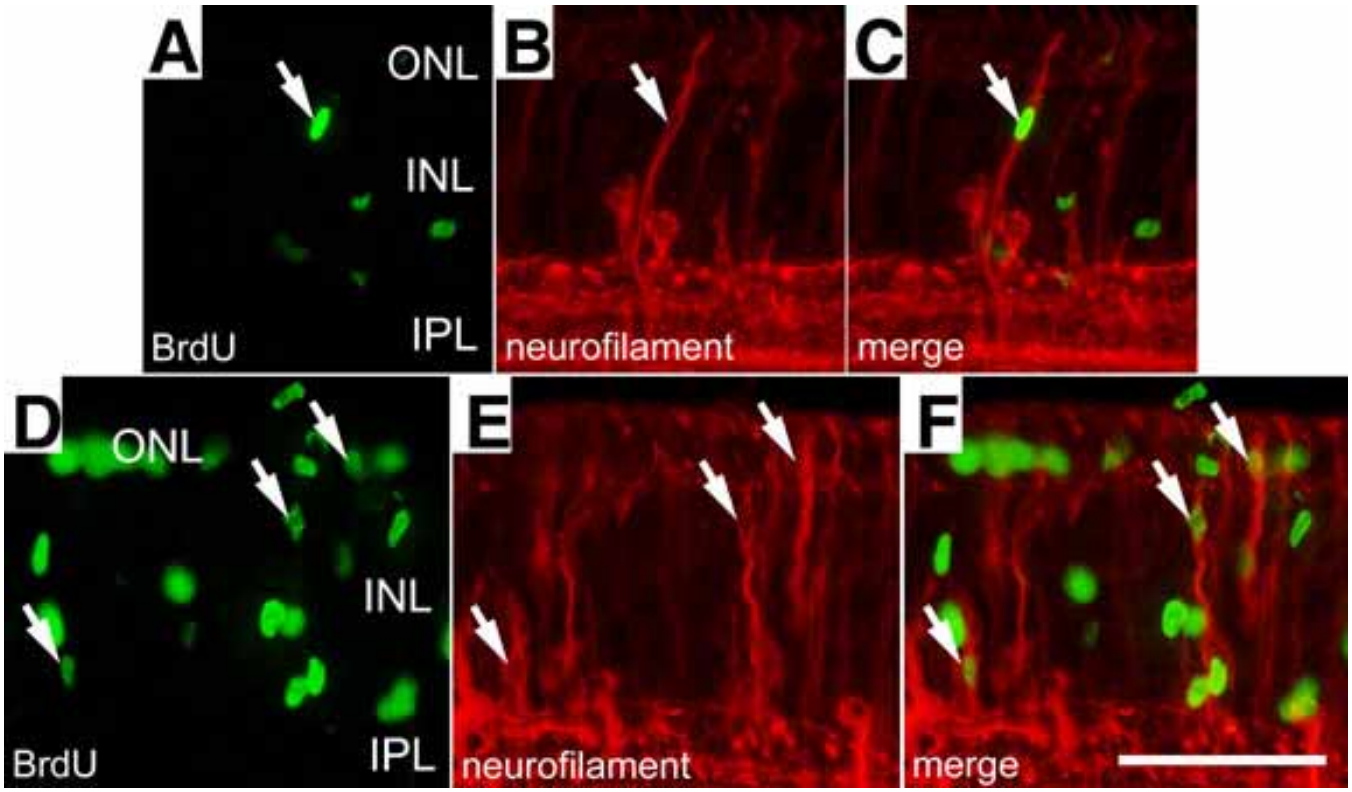


Figure 6. Some of the neurofilament-expressing Müller glia are proliferating. Insulin and FGF2 induce the proliferation of some of the neurofilament expressing Müller glia in peripheral regions of the retina, within 2 mm of the retinal margin. Sections of the retina were labeled with antibodies to BrdU (in green) and neurofilament (NF-M; in red). Eyes received 3 consecutive daily injections of insulin and FGF2 and retinas were obtained at 6 (**A-C**) or 24 h (**D-F**) after the final injection. Arrows indicate cells labeled for BrdU and neurofilament. The outer nuclear layer (ONL), inner nuclear layer (INL), and inner plexiform layer (IPL) are labeled. The calibration bar in **F** represents 50 μm and applies to all panels.

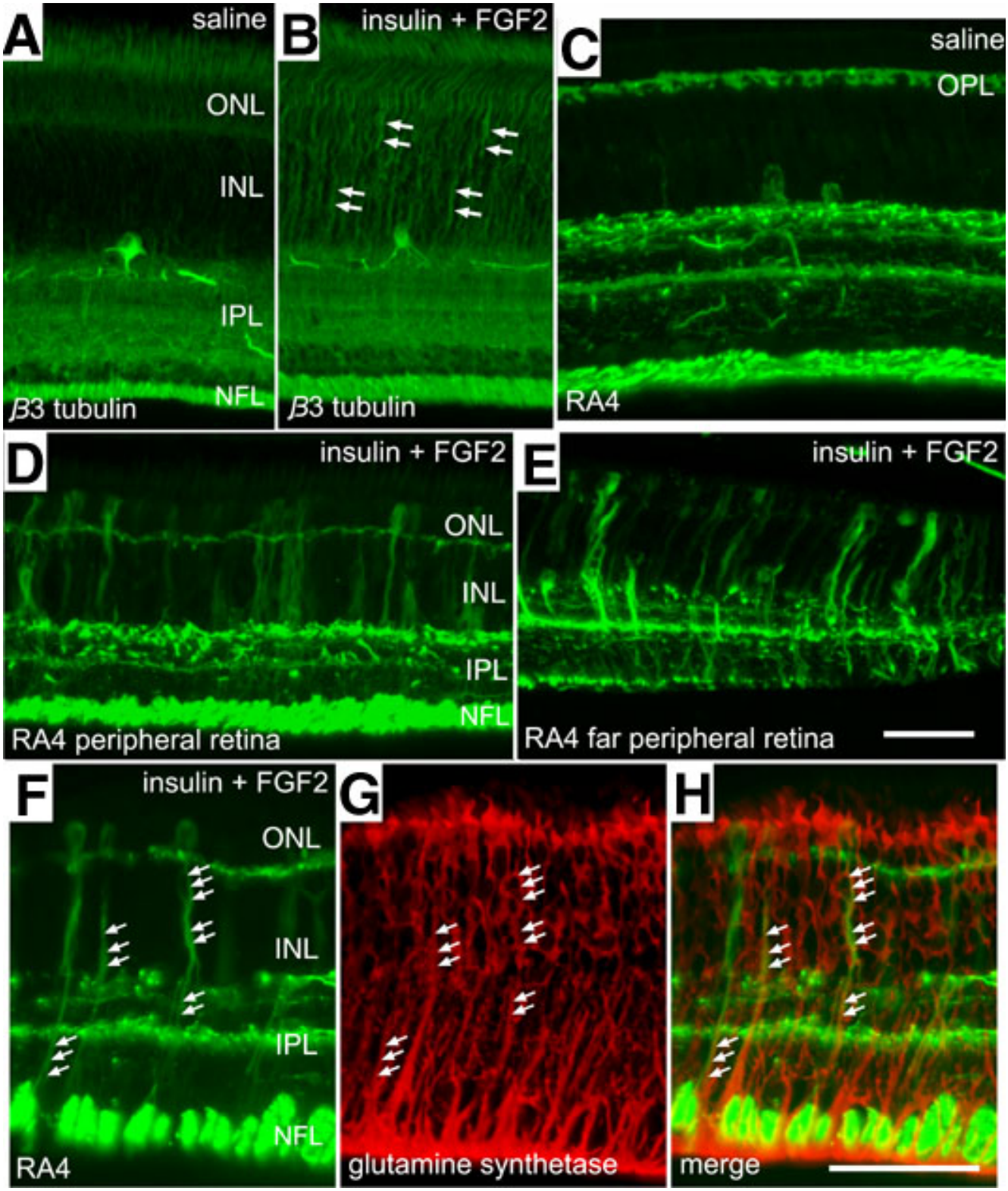
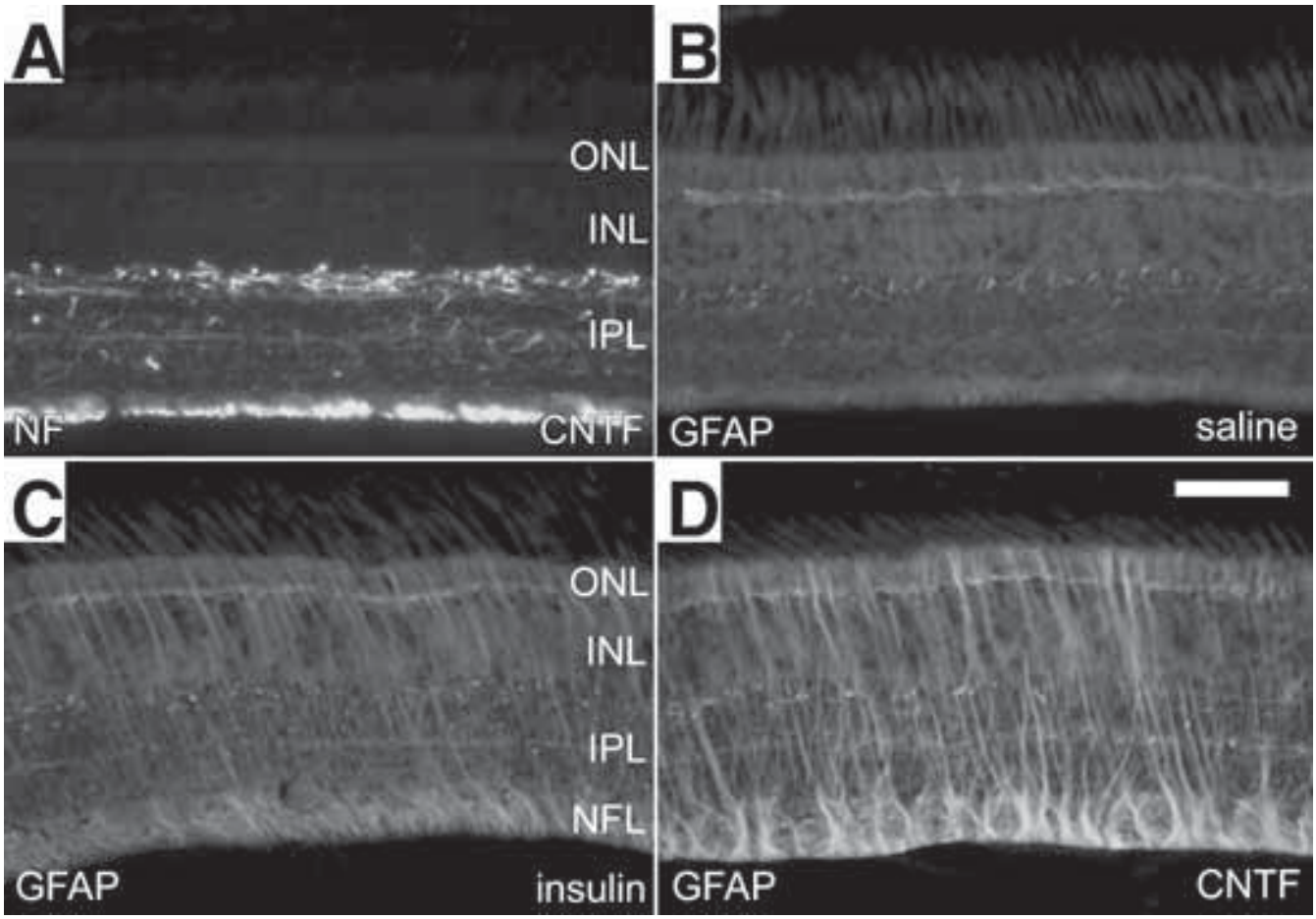


Figure 7. β3 tubulin- and RA4-immunoreactivity can be induced in Müller glia by insulin and FGF2. Insulin and FGF2 induce β3 tubulin and RA4 immunoreactivity in presumptive Müller glia in peripheral regions of the retina. Vertical sections of retina were obtained from eyes that received with 3 consecutive daily injections of insulin alone (**A**,**C**) or insulin and FGF2 (**B**,**D**-**H**). Retinas were processed for immunocytochemistry 24 h after the final injection and labeled with antibodies to β3 tubulin (**A**,**B**), RA4 (**C**-**F**), or glutamine synthetase (**G**,**H**). Arrows in **B** indicate Müller glia that are immunoreactive for β3 tubulin and arrows in **F**-**H** indicate structures double labeled for RA4 and glutamine synthetase immunoreactivity. The outer nuclear layer (ONL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), and nerve fiber layer (NFL) are labeled. The calibration bar in **E** represents 50 μm and applies to **A**, **B**, **D**, and **E**. The bar in **H** represents 50 μm and applies to **C** and **F**-**H**.

nal damage. CNTF did not cause Müller glia to express neurofilament (Figure 8A), β3 tubulin or RA4 (data not shown). Since CNTF is known to promote the expression of GFAP in developing astrocytes [27,28], we probed for GFAP in retinal sections obtained from eyes that received injections of saline, insulin or CNTF. In saline treated retinas, Müller glia had low levels of GFAP immunoreactivity (Figure 8B). In retinas that were treated with insulin alone, GFAP expression was increased in Müller glia (Figure 8C). The level of GFAP expression increased in Müller glia that were in peripheral regions of the retina, within 2000 μm of the retinal margin. In retinas that were treated with CNTF, GFAP expression was increased in Müller glia across all regions of the retina (Figure 8D).

Since the antibody to GFAP appeared to cross-react with filamentous proteins within retinal neurons, we wanted to confirm that the expression of GFAP was increased by CNTF treatment with an antibody independent method. Accordingly, we designed PCR primers specific to chicken GFAP that produced a single PCR product. We used these primers with real-time PCR to measure GFAP mRNA in samples obtained from saline or CNTF treated retinas. We found that CNTF treated retinas had 32 fold more GFAP mRNA than that of saline treated retinas (Figure 8E). This finding confirms that injec-



E

	GAPDH	GFAP
saline	17.2±0.3	30.0±0.6
CNTF	16.9±0.6	25.0±0.2*

C(T) Value

Figure 8. Ciliary neurotrophic factor and insulin induce the expression of glial fibrillary acidic protein in Müller glia.. Ciliary neurotrophic factor (CNTF) and insulin induce the expression of glial fibrillary acidic protein (GFAP) in Müller glia. Retinas were obtained from eyes that received 3 consecutive daily injections of CNTF (**A**,**D**), saline (**B**), or insulin (**C**). Vertical sections of the peripheral retina were labeled with antibodies to neurofilament (NF-M; **A**), or GFAP (**B**-**D**). Sections were obtained from the peripheral retina >500 μm. The outer nuclear layer (ONL), inner nuclear layer (INL), inner plexiform layer (IPL), and nerve fiber layer (NFL) are labeled. The calibration bar in **D** represents 50 μm and applies to panels **A**-**D**. **E**: GFAP expression levels are increased in retinas from CNTF injected eyes compared to contralateral saline injected eyes. Fold difference was calculated as y^x where y equals to 2 and x equals to $C(T)_{NMDA\ treated}$ minus $C_{(T)control}$. The C_T value is the PCR cycle number at which the reaction enters log phase and is inversely proportional to transcript abundance. C_T values were averages of samples run in triplicate. GAPDH served as a control and showed no significant variation between samples.

tions of CNTF into the vitreous chamber of the eye stimulate Müller glia to dramatically increase their expression of GFAP in the absence of retinal damage.

To test whether GFAP expression was influenced by FGF2 or EGF, we made injections of these growth factors into eyes and assayed for GFAP immunoreactivity in retinal sections. We did not observe an obvious increase in GFAP levels in Müller glia in retinas that were treated with FGF2 alone, EGF alone, or insulin and FGF2 (data not shown).

A summary of the effects of growth factors and acute retinal damage on glial expression of filamentous proteins and glutamine synthetase are provided in Table 3.

DISCUSSION

Here we report that the expression levels of filamentous proteins in Müller glia can be modulated by exogenous growth factors. The filamentous proteins that Müller glia express in response to growth factors include GFAP, neurofilament, β3 tubulin, and RA4. These findings indicate that the phenotype of Müller glia is plastic and that under certain conditions Müller glia are capable of transiently expressing neuronal proteins. Glial expression of neuronal filaments was not observed in central regions of the retina, even in newly hatched birds, but was observed in peripheral regions of the retina. This finding suggests that Müller glia in peripheral regions of the retina have a plastic phenotype, and this plasticity is maintained for at least the first 3 weeks of postnatal development. By contrast, Müller glia in central regions of the retina have a stable phenotype. We propose that the ability of Müller glia to express filamentous proteins in response to exogenous growth factors represents a state of immaturity.

Since we observed the expression of neurofilament by Müller glia following acute damage [14] and following injections of growth factors (see above), it is possible that the injections of growth factors may have induced acute damage and thereby affected Müller glia. However, there were no indications of retinal damage and we have reported elsewhere that at no time after treatment with insulin and FGF2 are apoptotic nuclei detected within the retina [16].

Table 3. The combination of insulin and FGF2 stimulates Müller glial expression of filamentous proteins

	Treatment				
Marker	Acute damage	Insulin	FGF2	Insulin +FGF	CNTF
Neurofilament	+++	+	+	+++	-
β3 tubulin	+++	-	-	+++	-
RA4	+++	-	-	+++	-
GFAP	+++	+	-	-	+++
Vimentin	+	-	-	++	-

Similar to acute retinal damage induced by NMDA, the combination of insulin and FGF2 stimulates glial expression of neurofilament, β3 tubulin, and RA4. In contrast, glial expression of neurofilament, β3 tubulin, and RA4 is not induced by insulin or FGF2 alone. Although CNTF induced glial expression of GFAP, this factor has no effect on the expression levels of other filamentous proteins. In the table, treatments are rated as strongly (+++), moderately (++), weakly (+), or not (-) inducing a marker.

The effects of insulin and FGF2 on Müller glia are likely to be direct, while the effects of CNTF may be indirect. Receptors for FGF2 and insulin/IGF are likely to be co-expressed by Müller glia. Receptors for FGF2 and insulin/IGF are expressed throughout the chick retina by most retinal cell types including Müller glia [29-31]. In the retinas of rodents and chicks, CNTF receptor β is predominantly expressed by neuronal cells [32,33], suggesting that CNTF induced glial expression of GFAP may be mediated by signals provided secondarily through CNTF responsive neurons. However, Müller glia may express an isoform of the CNTF receptor other than the β-isoform. Furthermore, exogenous CNTF and stress stimuli induce the accumulation of activated STAT3, known to be downstream of CNTF signaling, in Müller glia, astrocytes and some ganglion cells [34]. Further, GFAP induced by CNTF is mediated by activation of STAT3 in the rodent retina [35]. Taken together, these findings indicate that CNTF may elicit effects upon Müller glia directly and indirectly.

We found that a single large dose of insulin and FGF2 had no effect upon glial expression of filamentous proteins, suggesting that repeated doses or sustained levels of growth factors may be required for the induction of neurofilament expression. Glial expression of neurofilament was significantly induced only with consecutive injections of the combination of insulin and FGF2, and not with either factor alone. These findings suggest that consecutive and simultaneous activation of insulin/IGF receptors and FGF receptors is necessary to stimulate Müller glia to express neurofilament.

The expression of neurofilament, RA4 and β3 tubulin by Müller glia suggests that these filaments are not always neuron specific. As neurons and glia differentiate they express cell distinguishing proteins such as the intermediate filaments neurofilament and GFAP, respectively [36,37]. Although the expression of cytoskeletal proteins has been used to identify neurons and glia [36,38,39], our findings show that neurofilament, β3 tubulin and RA4 are expressed by retinal glia exposed to insulin and FGF2. The finding that neuron specific proteins are expressed by non-neuronal cells is not without precedence. Neurofilament and tubulin are transiently expressed by Schwann cells during early stages of differentiation or following acute damage [40-42]. In addition, FGF2 has been shown to induce immunoreactivity for RA4 in cultured RPE cells from E6 chick embryos [43]. Taken together, these findings suggest that non-neuronal cells of neuroepithelial origin are capable of expressing neuronal proteins in response to acute damage, culture conditions or treatment with FGFs.

Exogenous growth factors have been shown to influence the expression of cytoskeletal proteins in glial cells in the mammalian retina. For example, FGF2 has been shown to increase the expression of GFAP and vimentin by Müller glia in the retinas of rabbits and cats [12]. Similarly, we observed increased expression levels of vimentin in Müller glia treated with insulin and FGF2. By contrast, insulin and FGF2 did not induce GFAP expression in Müller glia. This may represent a difference between birds and mammals. By comparison, we found that exogenous CNTF stimulated glial expression of

GFAP. This is consistent with the findings of others that have demonstrated that CNTF and JAK/STAT signaling stimulate glial differentiation and expression of GFAP [27,28]. In the adult rat retina, injections of a CNTF analog results in the activation of STAT3 in Müller glia, astrocytes and ganglion cells [34]. Similarly, injections of CNTF or FGF2 into the eyes of mice results in a rapid increase in the phosphorylation of ERK and expression of c-fos, which is followed by increased glial expression of GFAP in CNTF treated eyes [13]. These findings confirm our observations that CNTF mediated signaling stimulates glial expression of GFAP.

The expression of neuronal markers by Müller glia does not represent neural differentiation or de-differentiation into proliferating neural precursors. In the current study, we found that more than 80% of the neurofilament expressing Müller glia were not proliferating, suggesting that re-entry into the cell cycle and expression of neurofilament may be independent. In addition, we found that 2 injections of insulin, and FGF2 stimulate the expression of neurofilament by Müller glia (current study), but fails to stimulate proliferation [16]. We propose that changes in the expression of neurofilament by Müller glia may represent a transition toward a de-differentiated state, but is not equivalent to becoming a proliferating progenitor-like cell. Instead, glial expression of neuronal filaments may indicate heterogeneity among Müller glia. For example, in retinas treated with insulin and FGF2 we found glia that expressed neurofilament and vimentin/GS interspersed among glia that expressed vimentin/GS alone (see Figure 5). Similarly, we found Müller glia that expressed RA4 and GS may be distinct from those that express GS alone.

We conclude that post-mitotic Müller glia can exhibit a great deal of phenotypic plasticity in peripheral regions of the retina for at least 3 weeks after being generated. The expression of cytoskeletal proteins in the Müller glia can be dramatically modulated by CNTF, insulin and FGF2. The expression of filamentous neuronal proteins can be induced in Müller glia by the combination of insulin and FGF2, indicating that under certain conditions neurofilament, β3 tubulin and RA4 are not cell distinguishing markers for neurons. In damaged retinas, we propose that growth factors, including insulin, FGFs, and CNTF, mediate changes in the phenotype of Müller glia.

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