BMP4 and CNTF are neuroprotective and suppress damage-induced proliferation of Müller glia in the retina

Andy J. Fischer,a,* Mike Schmidt,b Ghezal Omar,a and Thomas A. Rehb

aDepartment of Neuroscience, Ohio State University, Columbus, OH 43210-1239, United States
bDepartment of Biological Structure, University of Washington, Seattle, WA 98195, United States

Received 7 April 2004; revised 12 July 2004; accepted 11 August 2004
Available online 28 September 2004

In response to acute damage, Müller glia in the chicken retina have been shown to be a source of proliferating progenitor-like cells. The secreted factors and signaling pathways that regulate this process remain unknown. The purpose of this study was to test whether secreted factors, which are known to promote glial differentiation during development, regulate the ability of Müller glia to proliferate and become retinal progenitors in response to acute damage in mature retina. We made intraocular injections of BMP4, BMP7, EGF, NGF, BDNF, or CNTF before or after a single, toxic dose of N-methyl-D-aspartate (NMDA) and assayed for proliferating progenitor-like cells within the retina. We found that injections of BMP4, BMP7, or CNTF, but not EGF, NGF, or BDNF, before NMDA treatment reduced the number of Müller glia that proliferated and gave rise to progenitor-like cells. CNTF and BMP4, but not NGF or BDNF, greatly reduced the number of cells destroyed by toxin treatment indicating that these factors protect retinal neurons from a severe excitotoxic insult. Injections of CNTF 5 days before NMDA treatment prevented neurotoxin-induced cell death and Müller glial proliferation, while injections of BMP4 had no protective effect. In addition, CNTF injected after NMDA treatment suppressed glial proliferation, while BMP4 did not. We conclude that BMP4 and CNTF, when applied before a toxic insult, act as neuroprotective agents and likely suppress the proliferative response of Müller glia to retina damage by attenuating the retinal damage; protecting bipolar and amacrine neurons from NMDA-induced cell death. When applied after a toxic insult, CNTF suppressed glial proliferation independent of levels of retinal damage.

© 2004 Elsevier Inc. All rights reserved.

Introduction

Glial responses to neural damage have both positive and negative implications for the recovery of neuronal function, regeneration of neurons, and regrowth of axons. In response to damage, glia are known to produce increased levels of secreted factors that may promote neuronal survival and attenuate the loss of neurons (reviewed by Anderson et al., 2003; Garcia and Vecino, 2003). By contrast, glia are also known to proliferate, form a scar, and produce factors that prevent axonal regeneration in response to neuronal damage (reviewed by Anderson et al., 2003; Chen et al., 2002; Silver and Miller, 2004). Recently, neurogenesis from glial cells has become recognized as a promising possibility for neural regeneration. Radial glia and astrocyte-like glial cells in neurogenic zones of the brain are known to be neural progenitors during embryonic development and adulthood, respectively (reviewed by Kreigstein and Götz, 2003; Fischer and Reh, 2003; Steindler and Laywell, 2003). The mechanisms that regulate the glial proliferation and neurogenic potential are beginning to be understood.

We have recently demonstrated in the avian retina that Müller glia respond to neurotoxic damage by proliferating and dedifferentiating into a neural progenitor-like cell. The cells produced by Müller glia-derived progenitors include some glia and neurons, but the majority of newly generated cells remain as undifferentiated progenitor-like cells. To better understand the molecular basis of the response of Müller glia to neurotoxic damage, we tested whether specific growth factors were sufficient to elicit the response. We found that the combination of insulin and FGF2, in the absence of damage, causes Müller glia to become progenitor-like cells and proliferate in a manner similar to that observed in toxin-damaged retina (Fischer et al., 2002). This finding is consistent with the hypothesis that an up-regulation of mitogenic factors within the retina following damage might be responsible for their reentry into the cell cycle.

In the course of these studies, we found that the response of the Müller glia to damage was not uniform across the retina; the region of retina that contains proliferating Müller glia shifts from central to peripheral regions with increasing age of the animal (Fischer and Reh, 2003). This parallels the manner in which retinal cell types exit the cell cycle and differentiate during the histogenesis of the retina; first in the central retina, with differentiation progressively spreading into the peripheral retina (McCabe et al., 1999; Prada et al., 1991). Taken together, these
data suggest that the Müller glia lose their potential to reenter the cell cycle after neurotoxic damage as the retina matures, leaving only the most peripherally located Müller glia to retain a regenerative potential in older animals.

To better understand the developmental restriction in the regenerative response of Müller glia in central retina, we tested whether intraocular injections of growth factors, following or before neurotoxic insult, would modulate the response of the Müller cells. We reasoned that the postmitotic maturation of glial cells may suppress their ability to reenter the cell cycle. Specifically, we focused on factors that have previously been shown to affect glial development and maturation. Secreted factors that promote glial differentiation include epidermal growth factor (EGF)-related molecules, bone morphogenetic proteins (BMP), and leukemia inhibitory factor (LIF) or ciliary neurotrophic factor (CNTF; Burrows et al., 1997; Gross et al., 1996; Johe et al., 1996; Viti et al., 2003). Factors, such as EGF, CNTF, or BMPs, that promote glial differentiation may prevent dedifferentiation and reentry into the cell cycle in response to acute neural damage. In addition to promoting differentiation, these factors may promote the maturation of postmitotic glia and this maturation may suppress the ability of glia to reenter the cell cycle. The purpose of the present study was to test whether damage-induced proliferation of retinal Müller glia is influenced by EGF, CNTF, or BMPs. In addition, we tested whether known neurotrophins, nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF) would protect retinal neurons against excitotoxic cell death, and whether reduced levels of retinal damage prevented glial proliferation. We found that intraocular injections of BMP4, BMP7, or CNTF on the days immediately before a neurotoxic insult greatly reduced the number of Müller glia that reentered the cell cycle, though only CNTF was able to do so when injected several days before the neurotoxin. While consistent with the hypothesis that glial differentiation factors might promote the postmitotic maturation of Müller glia, we also found that BMP4 and CNTF reduced the amount of cell death induced by NMDA. Therefore, our results indicate that glial proliferation in response to neurotoxic damage is suppressed by glial differentiation factors and these factors also render amacrine and bipolar neurons resistant to NMDA-induced excitotoxicity.

Results

NMDA-induced loss of cells in the INL correlates with glial proliferation

To assay whether there is a relationship between the extent of retinal damage and glial proliferation, we performed a dose–response for NMDA-induced excitotoxicity and assayed for proliferation, accumulation of progenitor-like cells, and cell death within the retina. At 2000 nmol, NMDA is known to destroy primarily amacrine and bipolar neurons (Barrington et al., 1989; Tung et al., 1990; Fischer et al., 1998). To assay for proliferating Müller glia in response to doses of NMDA between 80 and 2000 nmol, we made a single injection of BrdU on the second day after NMDA treatment; when Müller glia are known to reenter the cell cycle as a result of NMDA-induced retinal damage (Fischer and Reh, 2001). All BrdU-labeled cells in the INL and ONL in NMDA-treated retinas are derived from Müller glia (Fischer and...
Compared to retinas treated with 2000 nmol NMDA, there was a significant ($P < 0.0001$) decrease in the number of BrdU-labeled cells in the INL and ONL in retinas treated with 1000 nmol NMDA (Figs. 1a, b, c, and f). With a 50% decrease in the dose of NMDA, there was nearly a fourfold decrease in the number of BrdU-labeled cells in the INL and a 10-fold decrease in the number of BrdU-labeled cells in the ONL (Figs. 1e and f). For doses of NMDA at 1000, 400, or 80 nmol, there was not a significant difference in the number of BrdU-labeled cells in the INL or ONL (Figs. 1b–f).

To test whether different doses of NMDA influence the accumulation of Müller glia-derived progenitor-like cells, we assayed for Pax6-labeled nuclei in the outer half of the INL and ONL. We have shown previously that Müller glia express the homeodomain transcription factor Pax6 and migrate into outer layers of the retina in response to NMDA-induced retinal damage (Fischer and Reh, 2001). In retinas treated with saline, there were no Pax6-expressing cells in the outer INL or ONL, with the exception of horizontal cells (Fig. 2a). The Pax6-positive horizontal cell nuclei were relatively small, round in shape, and directly opposed to the OPL at the outer edge of the INL. These cells were excluded from counts of Pax6-expressing cells in NMDA-treated retinas. In acutely damaged retinas, we found that the number of Pax6-labeled nuclei in the outer half of the INL decreased steadily with decreasing doses of NMDA from 2000 to 80 nmol (Figs. 2b–f). There was no statistical difference between the mean number of Pax6-labeled cells in the outer INL of retinas treated with 400 and 80 nmol of NMDA (Fig. 2g). The 80-nmol dose of NMDA resulted in a significant ($P < 0.001$) decrease in the number of Pax6-labeled nuclei in the ONL when compared to numbers in retinas treated with 2000 nmol NMDA (Fig. 2g).

To test for cell death, we assayed for fragmented DNA in situ by using the TUNEL method 1 day after the injection of NMDA. We found no significant difference between the mean numbers of TUNEL-positive nuclei in the amacrine cell layer of the INL in retinas treated with 80–2000 nmol of NMDA (Figs. 3a–e). By contrast, we found a significant decrease in the number of TUNEL-positive nuclei in the bipolar cell layer of the INL in retinas treated with 1000 nmol compared to that observed in retinas treated with 2000 nmol NMDA (Figs. 3a, b, and f). With decreasing doses of NMDA, the mean number of TUNEL-positive nuclei in the bipolar cell layer was not further decreased below that observed with the 1000-nmol NMDA treatment (Figs. 3b, c, d, and f). Thus, there is a correlation with the dose of NMDA that elicits the destruction of bipolar cells and that which elicits the Müller glial response, particularly the proliferative response as assayed by BrdU incorporation.

---

**Fig. 2.** The number of Pax6-labeled cells in the retina is reduced with decreasing doses of NMDA. Intravitreal injections of saline (a) or NMDA at 2000 (b), 1000 (c), 400 (d), or 80 nmol (e) were made at P7. Retinas were harvested 5 days later and labeled for Pax6 immunoreactivity. (f) Histogram illustrating the number of Pax6-positive nuclei in the INL for doses of NMDA between 2000 and 80 nmol. (g) Histogram illustrating the number of Pax6-positive nuclei in the ONL for doses of NMDA between 2000 and 80 nmol. Data sets were obtained from five animals per treatment. Statistical significance was assessed by ANOVA ($P < 0.001$) followed by a post hoc Student’s $t$ test ($^{*}P < 0.05$; $^{**}P < 0.001$). Abbreviations: ONL—outer nuclear layer, INL—inner nuclear layer, IPL—inner plexiform layer, GCL—ganglion cell layer. The scale bar (50 μm) in panel e applies to panels a–e.
Intraocular injections of BMP4 and CNTF prevent Müller glia from proliferating and becoming progenitor-like cells

To test whether secreted factors influence the ability of Müller glia to proliferate and become progenitor-like cells in response to retinal damage, we made two consecutive daily intraocular injections of BMP4, BMP7, CNTF, NGF, BDNF, or EGF before a single injection of NMDA (Fig. 4a). In retinas treated with 10–200 ng of BMP4 before NMDA, there was a significant decrease in the number of BrdU-labeled cells in the outer layers of the retina (Figs. 4b–e). In a dose-dependent manner, the largest decrease was seen for the 200-ng dose of BMP4 (Fig. 4f). Similar to the effects observed for BMP4, 50 ng of BMP7 and 300 ng of CNTF significantly decreased the number of BrdU-labeled cells in the outer layers of the retina, while 30 ng of CNTF had no effect (Fig. 4f). By contrast, EGF, NGF, or BDNF at 200 ng/injection had no significant effect upon the number of BrdU-labeled cells that accumulated in the INL and ONL in NMDA-damaged retinas (Fig. 4f).

When BMP4 and CNTF were applied before NMDA treatment (Fig. 5a), we found that higher doses of BMP4 and CNTF reduced the number of Pax6-expressing cells that accumulated in outer layers of the retina (Figs. 5b–h). While 10 ng of BMP4 had no effect upon the number of Pax6-immunoreactive cells that accumulated in outer layers of the retina, 200 ng of BMP4 significantly decreased the number of Pax6-positive cells in outer layers of the retina, 30 ng of CNTF had no effect (Fig. 4f). By contrast, EGF, NGF, or BDNF at 200 ng/injection had no significant effect upon the number of BrdU-labeled cells that accumulated in the INL and ONL in NMDA-damaged retinas (Fig. 4f).

The result that both CNTF and BMP4 caused a reduction in the Müller glial response to neurotoxic damage was consistent with the hypothesis that these factors promote the maturation of the Müller glia. However, secreted factors such as CNTF have also been shown to promote neuronal survival in different paradigms of retinal degeneration (Cayouette and Gravel, 1997; Chong et al., 1999; LaVail et al., 1998; Liang et al., 2001). Thus, we tested whether injections of CNTF or BMP4 before NMDA treatment affected the number of retinal cells that were damaged.

When injected before NMDA treatment (Fig. 6a), we found that two consecutive daily injections of BMP4, at 10 or 200 ng/dose, and 300 ng of CNTF dramatically decreased the total number of TUNEL-positive cells in the INL (Figs. 6b–f). Since decreasing doses of NMDA selectively spared bipolar cells, but not amacrine cells, we assayed whether BMP4 or CNTF influenced the number of TUNEL-positive cells in the bipolar layer of the INL. We found that numbers of TUNEL-positive cells in the bipolar layer of the INL were reduced by 200 ng of BMP4 and 300 ng CNTF, but not by 10 ng of BMP4 (Figs. 6c–e and g). While the 10-ng dose of BMP4 did not suppress NMDA-induced cell death in the bipolar layer of the INL, there was an obvious reduction in the number of dying cells in the amacrine layer of the INL (Figs. 6c and f). Thus, the effects of BMP4 and CNTF on Müller glial proliferation may be secondary to reduced levels retinal damage because these factors protect amacrine and bipolar neurons from excitotoxic damage. We also tested whether neurotrophins, NGF and BDNF, affected the number of TUNEL-positive cells that resulted from NMDA-induced excitotoxicity. We found that two consecutive daily injections of 200 ng of NGF or BDNF before NMDA treatment did not affect the number of TUNEL-positive cells in the INL (Figs. 6f and g). This finding indicates that the neurotrophins...
NGF and BDNF are ineffective in protecting neurons in the INL of the chick retina from NMDA-induced excitotoxicity. When applied immediately before NMDA, both BMP4 and CNTF decreased the number of TUNEL-positive cells in the retina. It is possible that fewer TUNEL-positive cells were observed at 1 day after NMDA treatment because these factors delayed the onset of cell death, fragmentation of DNA, and commitment to death. Accordingly, we assayed for in situ fragmented DNA at 5 days after NMDA treatment. We found that there was no significant difference in the number of TUNEL-positive nuclei observed in retinas treated with NMDA alone and those treated with CNTF or BMP4 before NMDA (results not shown). These findings suggest that CNTF and BMP4 prevent NMDA-induced neuronal death instead of delaying it.

Fig. 4. Intraocular injections of BMP4, BMP7, or CNTF reduce the number of proliferating cells in NMDA-treated retinas. Panel a illustrates the injection paradigm used to obtain the data presented in this figure. At P1 and P2, eyes were injected with saline (b), 10 ng BMP4 (c), 50 ng BMP4 (d), or 200 ng BMP4 (e). Eyes were injected with 2000 nmol of NMDA at P3 and 2 μg of BrdU at P5. Retinas were harvested at P8 and retinal sections labeled for BrdU immunoreactivity (b–e). (f) Histogram illustrating the number of BrdU-labeled cells in the INL and ONL of retinas treated with NMDA alone or 10–200 ng BMP4, 50 ng BMP7, 30 ng CNTF, 300 ng CNTF, 200 ng EGF, 200 ng NGF, or 200 ng BDNF before NMDA. Data sets were obtained from six animals per treatment. Statistical significance was assessed by ANOVA (P < 0.001) followed by a post hoc Student’s t test (*P < 0.0001). Abbreviations: ONL—outer nuclear layer, INL—inner nuclear layer, IPL—inner plexiform layer. The scale bar (50 μm) in panel e applies to panels b–e.
Injections of CNTF, but not BMP4, after NMDA suppress glial proliferation

If BMP4 and CNTF reduced the number of proliferating Müller glia by rendering retinal neurons resistant to excitotoxicity and suppressing retinal damage, then administration of these factors after the neurotoxic insult, when the retinal neurons are committed to death, should not prevent the glial response. In response to NMDA-induced excitotoxicity, the vast majority of retinal neurons destined to perished are TUNEL positive by 1 day after toxin treatment (see above; Fischer et al., 1998). Fragmented DNA detected by the TUNEL method represents late stages or apoptosis and necrosis when cells can no longer be rescued from death (reviewed by Otsuki et al., 2003). Accordingly, we tested whether injections of BMP4 or CNTF starting 1 day after NMDA treatment (Fig. 7a), when retinal neurons are TUNEL positive and committed to death, reduced the number of Müller glia that accumulate BrdU. We found that injections of CNTF after NMDA treatment significantly reduced numbers of BrdU-labeled cells in central and peripheral regions of the retina, while BMP4 had no effect (Fig. 7). This result indicates
that CNTF can inhibit Müller glial proliferation independent of levels of cell death in the retina.

The effects of CNTF on glial proliferation are long-lasting, while those of BMP4 are short lived

To test whether the effects of BMP4 and CNTF on glial proliferation were long-lasting, we made two consecutive daily injections of 50 ng BMP4, 30 or 300 ng CNTF at P1 and P2, a single toxic injection of NMDA at P7, a single injection of BrDU at P9, and processed retinas for immunocytochemical labeling at P12 (Fig. 8a). While 30 ng of CNTF did not significantly affect numbers of BrDU-labeled cells in outer retinal layers (results not shown), we found that 300 ng of CNTF 5 days before NMDA treatment caused a significant decrease in the number of BrDU-labeled cells in central and peripheral regions of the retina (Figs. 8b–f). When applied 5 days before NMDA-induced retinal damage, BMP4 did not influence the number of BrDU-labeled cells in outer retinal layers (Fig. 8f).

To assay whether the neuroprotective effects of CNTF are long lasting, we made two consecutive daily intraocular injections of 300 ng CNTF 5 days before NMDA and assayed for TUNEL-positive cells 1 day after NMDA treatment (Fig. 8a). With 5 days between the last application of CNTF and the application of NMDA, there was ample time for the CNTF to be cleared from the eye. We found
significantly ($P < 0.0001$) reduced numbers of TUNEL-positive cells in retinas treated CNTF/NMDA compared to numbers observed in retinas treated with NMDA alone (Figs. 8g–i). This effect was comparable to that seen when CNTF was applied immediately before NMDA treatment; each injection paradigm resulted in approximately an 80% decrease in the number of TUNEL-positive cells (compare Figs. 6 and 8). These findings suggest that the neuroprotective effects of CNTF are long lasting. Intraocular injections of 30 ng of CNTF had no effect on numbers of TUNEL-positive cells in this paradigm (data not shown).

**Discussion**

In response to sufficient levels of NMDA-induced damage, we found accumulations of proliferating cells that express Pax6 in the retina. Our data indicate that a threshold level of retinal damage that includes the destruction of amacrine and bipolar cells is required for this proliferative response. We have shown previously that these proliferating Pax6-expressing cells are derived exclusively from Müller glia that have reentered the cell cycle (Fischer and Reh, 2001; Fischer et al., 2002). The region of retina that contains proliferating Müller glia in response to neurotoxic damage shifts from the central retina to the peripheral retina with increasing age of the animal (Fischer and Reh, 2003). We hypothesized that Müller glia undergo a maturation process during postnatal development that prevents cells located in central retina from reentering the cell cycle. We tested four factors that have been implicated in glial differentiation, BMP4, BMP7, CNTF, and EGF, for their ability to promote the maturation of Müller cells and prevent the proliferative response to neurotoxic damage. Only BMP4, BMP7, and CNTF caused a significant reduction in the
proliferation of Müller cells in response to retinal damage; however, the interpretation of these results is complicated by the fact that these factors significantly reduced the amount of neuronal death resulting from neurotoxic damage.

It is possible that reduced glial proliferation resulted from reduced cell death. We found that when CNTF and BMP4 were applied before NMDA, there were fewer dying and proliferating cells within the retina. Numbers of TUNEL-positive cells in the bipolar layer of the INL were reduced by 200 ng of BMP4 and 300 ng CNTF, while 10 ng reduced numbers in the amacrine cells layer of the INL. Our data indicate that BMP4 is preferentially protective for amacrine cells, while CNTF is protective for amacrine and bipolar cells. In the chick retina, receptors for BMPs are widely distributed with higher levels observed in ventral regions of the retina and in the amacrine layer of the INL (Belecky-Adams and Alder, 2001). Our findings are consistent with other reports demonstrating that BMPs influence the survival of neurons in the retina and different regions of the CNS. For example, BMP4 has
been shown to promote the survival of newly generated olfactory receptor neurons (Shou et al., 2000). BMP6 and BMP7 have been shown to promote the survival and suppress apoptosis of cultured cerebellar granule cells (Yabe et al., 2002). In the rodent retina, the absence of BMP receptor 1b results in elevated apoptotic cells in the amacrine cell layer, suggesting that BMP-mediated signaling is normally required to promote the survival of inner retinal neurons (Liu et al., 2003). By contrast, BMP4 has been shown to promote apoptosis in dorsal regions of the early embryonic chick retina (Trousse et al., 2001). Similarly, BMP4 has been shown to promote apoptosis of cultured sympathetic neurons and progenitors (Gomes and Kessler, 2001; Song et al., 1998). Taken together, these findings suggest that early during retinal development BMPs promote cell death and that this role is reversed in the mature retina with BMP acting as survival factor.

CNTF is a cytokine that is known to activate the Jak/STAT signaling pathway (reviewed by Inoue et al., 1996). In the retinas of rodents and chicks, CNTF-receptor-α is predominantly expressed by neuronal cells (Fuhrmann et al., 1998; Kirsch et al., 1997). However, Peterson et al. (2000) have demonstrated that exogenous CNTF and stress stimuli induce the accumulation of activated STAT3 in Müller glia, astrocytes, and some ganglion cells. It is possible that Müller glia express an isoform of the CNTF receptor other than the α isoform. Taken together, these findings suggest that exogenous CNTF could act directly or indirectly to render neurons resistant to NMDA-induced excitotoxicity and suppress glial proliferation.

Postmitotic maturation of Müller glia may suppress the ability to reenter the cell cycle

Findings presented in the current study indicate that CNTF prevents glial proliferation when applied before or after a neurotoxic insult, while BMP4 prevent glial proliferation only when applied on the days immediately before the excitotoxic insult. The majority of retinal neurons destinoined to perish as a result of NMDA treatment are TUNEL positive at 1 day after treatment (Fischer et al., 1998). Fragmented DNA detected by the TUNEL method indicates cells that are irreversibly committed to cell death (Osuki et al., 2003). Therefore, the application of CNTF in the days following NMDA treatment would have been too late to rescue the cells from apoptotic or necrotic cell death. This finding indicates that CNTF is capable of suppressing the proliferation of Müller glia independent of suppressing retinal cell death.

The region of retina in which Müller glia proliferate in response to damage shifted from central to peripheral retinal regions during postnatal development (Fischer and Reh, 2003). It is possible that Müller glia in central regions of the retina mature during postnatal development and lose their ability to proliferate. We propose that CNTF-mediated signaling may play a role in this process. CNTF has been shown to promote the differentiation of glial cells in different regions of the developing CNS (Burrows et al., 1997; Joh et al., 1996). In addition, CNTF has been shown to promote photoreceptor differentiation in the chick retina (Fuhrmann et al., 1995) and promote the differentiation of bipolar, amacrine, and Müller cells at the expense of rod photoreceptors in the rodent retina (Ezzidine et al., 1997). It remains uncertain whether endogenous CNTF influences the differentiation of Müller glia in the postnatal chicken retina. Findings presented in the current study indicate that exogenous CNTF prevents glial proliferation when applied 5 days before an excitotoxic insult. This finding suggests that CNTF has long-lasting effects upon suppressing glial proliferation and may permanently render glia incapable of reentering the cell cycle. Further investigations are required to identify the mechanisms by which CNTF promotes glial maturation and suppresses proliferation.

The destruction of amacrine and bipolar cells is needed for glial proliferation

The proliferation of Müller glia may be triggered by the destruction of amacrine and bipolar neurons within the retina. We found large numbers of proliferating Müller glia in retinas treated with 2000 nmol NMDA, but not in retinas treated with 1000 nmol. This correlates with the finding that TUNEL-positive cells were present in the amacrine and bipolar layers of retinas treated with 2000 nmol NMDA but were only present in the amacrine layer in retinas treated with 1000 nmol NMDA. By comparison, pretreatment of the retina with 10 ng BMP4 before injection of NMDA suppressed the proliferation of Müller glia and reduced numbers of TUNEL-positive cells in the amacrine layer, but not the bipolar layer. This finding indicates that the lower dose of BMP4 may suppress glial proliferation by reducing the number of amacrine cells that are destroyed by NMDA, and that the proliferation of Müller glia requires threshold levels of apoptotic cell death of bipolar and amacrine cells.

Müller glia may express Pax6 without reentering the cell cycle

Although decreasing the dose of NMDA from 2000 to 1000 nmol resulted in a steep decline in the number of BrdU-labeled cells in the outer retina and TUNEL-positive cells in the outer half of the INL, the number of Pax6-expressing Müller glia-derived cells in the outer INL and ONL was modestly decreased. In other words, the dose response of NMDA for the accumulation of BrdU- and TUNEL-positive cells within the retina did not parallel the accumulation of Pax6-positive cells in outer retinal layers. Assuming that all BrdU- and Pax6-positive cells in outer retinal layers arise from Müller glia, these findings suggest that proliferation of Müller glia and expression of Pax6 are independent. This is consistent with previous reports indicating that not all Pax6-labeled Müller glia-derived cells that accumulate in the outer retina in response to insulin and FGF2 are colabeled with BrdU (Fischer et al., 2002). In addition, in the current study, we observed large decreases in the number of BrdU-labeled cells in the INL and ONL of retinas treated with 10 ng BMP4 before NMDA, while there was no decrease in the number of Pax6-positive cells in outer retinal layers. Taken together, our data suggest that glial expression of Pax6 and translocation of the nucleus to outer retinal layers can occur without reentry into the cell cycle.

Conclusions

We conclude that BMP4/7 and CNTF protect retinal neurons from NMDA-mediated excitotoxicity, with CNTF having long-lasting effects and BMP4 having short-lived effects. Our findings suggest that BMP4/7 and CNTF could protect neurons from a severe excitotoxic insult that may occur as a result of ischemia and stroke. Furthermore, we found that BMP4/7 and CNTF suppress the proliferation and transdifferentiation of Müller into progenitor-like cells in response to acute retinal damage. When these factors are applied before NMDA, the attenuation of glial proliferation
likely was secondary to reduced retinal damage. However, our results suggest that CNTF suppresses glial proliferation independent of suppressing cell death when applied after retinal neurons are committed to die. Thus, factors, such as CNTF, that influence glial differentiation during normal development may regulate the ability of Müller to proliferate in response to retinal damage. This observation suggests that CNTF could be applied to suppress the glial proliferation that may be detrimental to regeneration in an acutely injured central nervous system.

**Experimental 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&N Highline International (Seattle, WA) or the Department of Animal Sciences at Ohio State University and kept on a 12:12 h light–dark cycle (lights on at 7:00 a.m.). Chicks were housed in a clear Nalgene™ cages or a stainless steel brooder at 33°C and received water and Purina™ chick starter ad libitum.

**Injections**

Chicks were anesthetized and injected as described elsewhere (Fischer et al., 1998, 1999). To destroy retinal cells, we injected 2 μmol (42.6 μg) of N-methyl-D-aspartate (NMDA) in 20 μl of sterile saline. Growth factors were injected in 20 μl sterile saline added with 0.1 mg/ml bovine serum albumin as carrier. Growth factors included recombinant human BMP4 (between 10 and 200 ng/injection), recombinant BMP7 (50 ng/injection), CNTF (between 30 and 300 ng/injection), recombinant mouse NGF (200 ng/injection), recombinant human BDNF (200 ng/injection), and recombinant human EGF (200 ng/injection). At 2 days after injection of NMDA, a single injection of 2 μg BrdU in 20 μl of sterile saline was injected into the eye to label proliferating cells. All drugs were obtained from Sigma, and all growth factors were obtained from R&D Systems.

We used three different injections paradigms. Paradigm 1: injections of growth factors were made at postnatal day 1 (P1) and P2, NMDA injected at P3, BrdU injected at P5, and retinas harvested at P4 (to assay for TUNEL-positive cells) or P8. Paradigm 2: NMDA was injected at P7, growth factors at P8 and P9, Brdu at P9, and retinas harvested at P12. Paradigm 3: growth factors injected at P1 and P2, NMDA at P7, BrdU at P9, and retinas harvested at P8 (to assay for TUNEL-positive cells) or P12.

**Fixation, sectioning, whole mounts, and immunocytochemistry**

Retinas were dissected, fixed, sectioned, and immunolabeled as described elsewhere (Fischer et al., 1998, 1999). Working dilutions and sources of antibodies used in this study were as follows: mouse anti-BrdU at 1:50 (Developmental Studies Hybridoma Bank); rat anti-BrdU at 1:80 (Accurate Chemicals); and mouse anti-Pax6 at 1:80 (Dr. A. Kawakami, Developmental Studies Hybridoma Bank). Secondary antibodies included goat-anti-rabbit-Alexa568, goat-anti-mouse-Alexa568, and goat-anti-rat-Alexa488 (Molecular Probes Inc., Eugene, OR) diluted to 1:1000 in 0.05 M phosphate-buffered saline plus 0.3% Triton X-100.

**Labeling for fragmented DNA**

The TUNEL method was used to label fragmented DNA in situ as described previously (Fischer et al., 1998).

**Photography, measurements, cell counts, and statistical analyses**

Photomicrographs were taken by using a Zeiss Axioplan II or Leica DM5000B microscope equipped with epifluorescence, FITC and rhodamine filter combinations, and a Spot™ SliderRT digital camera (Diagnostic Inc.) or Leica DC500 digital camera. Images were optimized for color, brightness, and contrast by using Adobe Photoshop™ 6.0. Cell counts were made on at least three retinal sections per animal and means and standard errors calculated on data sets from at least five individuals. Retinal sections were cut at 14 μm in thickness and cell counts were made from fields that were 140 or 150 μm wide, which is 2000 or 2100 μm² in retinal area. To avoid the possibility of region-specific differences within the retina, cell counts were consistently made from the central or peripheral retina for each data set. Data from treated and control eyes were compared statistically with ANOVA and post hoc Student’s t test.

**Acknowledgments**

We thank Chris McGuire and Melissa Philips for providing expert technical assistance. The Pax6 and BrdU antibodies developed by Drs A. Kawakami and S.J. Kaufman, respectively, were obtained from the Developmental Studies Hybridoma Bank developed under auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. This work was supported by NIH RO1 NS2808 to T.A.R. and start-up funds from The Ohio State University to A.J.F.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2004.08.007.

**References**


Johe, K.K., Hazel, T.G., Muller, T., Dugich-Djordjevic, M.M., McKay,
Gross, R.E., Mehler, M.F., Mabie, P.C., Zang, Z., Santschi, L., Kessler,
Gomes, W.A., Kessler, J.A., 2001. Msx-1 and p21 mediate the pro-
Garcia, M., Vecino, E., 2003. Role of Muller glia in neuroprotection and
Garcia, M., Vecino, E., 2003. Role of Muller glia in neuroprotection and
Gross, R.E., Mehler, M.F., Mabie, P.C., Zang, Z., Santschi, L., Kessler,
Johe, K.K., Hazel, T.G., Muller, T., Dugich-Djordjevic, M.M., McKay,
Evidence for multiple, local functions of ciliary neurotrophic factor
Spanish text:
542
Oligodendrocyte precursor cells: reactive cells that inhibit axon growth
Luthert, P.J., 1999. Repeated injections of a ciliary neurotrophic factor
analogue leading to long-term photoreceptor survival in hereditary
cells fated to become rod photoreceptors can be respecified by CNTF
neurogenic retinal progenitor cells. Glia 43, 70–76.
chemical characterization of NMDA and QA-induced excitotoxicity in
FGF2 activate a neurogenic program in Müller glia. J. Neurosci. 22,
9387–9398.
factor promotes chick photoreceptor development in vitro. Develop-
ment 121, 2695–2706.
Differential regulation of ciliary neurotrophic factor receptor-alpha
expression in all major neuronal classes during development of the
chick retina. J. Comp. Neurol. 400, 244–254.
Steindler, D.A., Laywell, E.D., 2003. Astrocytes as stem cells: nomencla-
Neurosci. 5, 146–156.
induce apoptosis and growth factor dependence of cultured sympathoa-
Tung, N.N., Morgan, I.G., Ehrlich, D., 1990. A quantitative analysis of the
outgrowth of cerebellar granule cell neurons. J. Neurosci. 5, 146–156.
factor receptors control competence to interpret leukemia inhibitory
factor as an astrocyte induced in developing cortex. J. Neurosci. 23,
3385–3393.
BMP-6 and BMP-7 have differential effects on survival and neurite
outgrowth of cerebellar granule cell neurons. J. Neurosci. Res. 68,
161–168.

542
Oligodendrocyte precursor cells: reactive cells that inhibit axon growth
Luthert, P.J., 1999. Repeated injections of a ciliary neurotrophic factor
analogue leading to long-term photoreceptor survival in hereditary
cells fated to become rod photoreceptors can be respecified by CNTF
neurogenic retinal progenitor cells. Glia 43, 70–76.
chemical characterization of NMDA and QA-induced excitotoxicity in
FGF2 activate a neurogenic program in Müller glia. J. Neurosci. 22,
9387–9398.
factor promotes chick photoreceptor development in vitro. Develop-
ment 121, 2695–2706.
Differential regulation of ciliary neurotrophic factor receptor-alpha
expression in all major neuronal classes during development of the
chick retina. J. Comp. Neurol. 400, 244–254.
Steindler, D.A., Laywell, E.D., 2003. Astrocytes as stem cells: nomenclature,
Neurosci. 5, 146–156.
induce apoptosis and growth factor dependence of cultured sympathoadrenal
Tung, N.N., Morgan, I.G., Ehrlich, D., 1990. A quantitative analysis of the
outgrowth of cerebellar granule cell neurons. J. Neurosci. 5, 146–156.
factor receptors control competence to interpret leukemia inhibitory
factor as an astrocyte induced in developing cortex. J. Neurosci. 23,
3385–3393.
BMP-6 and BMP-7 have differential effects on survival and neurite
outgrowth of cerebellar granule cell neurons. J. Neurosci. Res. 68,
161–168.