Delayed IGF-1 Administration Rescues Oligodendrocyte Progenitors from Glutamate-Induced Cell Death and Hypoxic-Ischemic Brain Damage

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Key Words

Insulin-like growth factor • Trophic factors • Periventricular leukomalacia • White matter damage • Excitotoxicity • Apoptosis • Myelin

Abstract

We previously demonstrated that IGF-1 blocks glutamate-mediated death of late oligodendrocyte progenitors (OPs) by preventing Bax translocation, mitochondrial cytochrome c release and cleavage of caspases 9 and 3. Here, we demonstrate that IGF-1 prevents caspase 3 activation in late OPs when administered up to 16 h following exposure to glutamate. Moreover, late addition of IGF-1 to OPs previously exposed to toxic levels of glutamate promotes oligodendrocyte maturation as measured by myelin basic protein expression. We also demonstrate that intraventricularly administered IGF-1 retains OPs in the perinatal white matter after hypoxia-ischemia when given after insult. These results suggest that delayed administration of IGF-1 will rescue OPs in the immature white matter and promote myelination following hypoxia-ischemia.

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Introduction

White matter damage occurs after ischemia in both the adult and the immature brain. However, in contrast to adult stroke where the predominant pathology is neuronal cell death, many of the neurological problems of the infant subsequent to hypoxia-ischemia (HI) are attributed to white matter damage [Volpe, 2001a, b]. White matter damage in perinatal HI leads to periventricular leukomalacia, which is the neuropathology classically associated with brain injuries in the premature infant [Volpe, 2001a]. As in the adult, glutamate is elevated in the immature rat brain including in the white matter after HI and is thought to have a primary role in the subsequent damage [Benveniste et al., 1984; Hagberg et al., 1987; Andine et al., 1991; Silverstein et al., 1991]. Similarly, glutamate is elevated in the cerebral spinal fluid in term infants after perinatal HI [Hagberg et al., 1993]. Recently, Back et al. [2007] demonstrated that HI causes release of glutamate preferentially from axons and oligodendroglia in the perinatal brain. Multiple lines of evidence support the hypothesis that the AMPA/kainate GluR receptors are primarily responsible for glutamate-mediated death of oligodendrocyte progenitors (OPs) and immature oligodendrocytes both in vitro [Yoshioka et al., 1995; Mat-

ute et al., 1997; Sanchez-Gomez and Matute 1999; Alberdi et al., 2002] and in vivo [Follett et al., 2000].

The differential vulnerability of oligodendroglia as they progressively differentiate has led to the view that the immature progenitors are significantly more vulnerable than mature oligodendrocytes. In particular, the late progenitor (late OP) is the most sensitive developmental stage to kainate- and glutamate-mediated death [McDonald et al., 1998b; Fern and Moller, 2000] and to oxidative stress induced by glutathione depletion [Back et al., 1998]. Follett et al. [2000] reported a similar age-dependent vulnerability to white matter lesions after intracerebral injections of AMPA into the pericallosal white matter. These data have led to the proposal that the late OP is intrinsically vulnerable to hypoxic/ischemic insult [Volpe, 1997]. Indeed, the age of highest incidence of white matter damage leading to periventricular leukomalacia in the premature infant directly correlates with the predominance of late OPs in the immature brain [Back et al., 2001; Riddle et al., 2006].

In contrast to the classic NMDA-mediated excitotoxic death of neurons that occurs within a few hours, death of oligodendroglia after HI in the perinatal brain occurs over 24–48 h [Follett et al., 2000; Ness et al., 2001]. This suggests that the initial elevation of glutamate initiates a slow death cascade within the glial cells. Until recently, little was known about the mechanisms by which perinatal HI and glutamate promote death of oligodendroglia or of the ability of trophic factors to prevent this death. Previous in vitro studies suggested that maximal survival of OPs under normal culture conditions could be achieved using a combination of the IGF, neurotrophin and interleukin-6 (IL-6)-like families [Barres et al., 1993]. Several of these factors have been further tested for their ability to protect oligodendroglia against damaging or toxic agents. IGF-1 and CNTF (a member of the IL-6 type cytokines) protect immature oligodendrocytes from death induced by tumor necrosis factor- α [Louis et al., 1993; D'Souza et al., 1996; Ye and D'Ercole, 1999]. Prior to our studies, there was one report suggesting that NT-3 partially inhibited glutamate excitotoxicity of oligodendroglia [Kavanaugh et al., 2000]. Our studies demonstrated that IGF-1 and NT-3, but not CNTF, protect late OP cells from high levels of glutamate through 24 h in vitro [Ness and Wood, 2002]. However, only IGF-1 protects the late OP cells from glutamate toxicity longer than 24 h [Ness and Wood, 2002]. More recently, we determined that IGF-1 blocks glutamate-mediated death in the late OPs by preventing Bax translocation, mitochondrial cytochrome c release and cleavage of caspases 9 and 3 [Ness et al., 2004]. However, IGF-1 has no effect on

initial calcium influx into the late OPs after exposure to glutamate nor does it promote recovery of intracellular calcium levels [Ness et al., 2004] as has been reported for IGF-1 trophic actions in neurons [Cheng and Mattson, 1991, 1992, 1994; Cheng et al., 1993; Mattson and Cheng, 1993; Mattson et al., 1993].

There are limited data concerning the ability of IGF-1 to prevent white matter damage and OP death in vivo following HI in the immature brain. Recently, Lin et al. [2005] demonstrated that IGF-1 given prior to the insult partially rescues OPs and myelin basic protein (MBP) expression following bilateral HI in the perinatal rat brain. However, there is no information as to the effectiveness of IGF-1 to rescue white matter OPs if given after the insult. The goal of the studies presented here was to further understand the timing and mechanisms for IGF-1 protection and rescue of the late OPs from insult both in vitro and in vivo.

Materials and Methods

Minimal essential media (MEM), fetal bovine serum (FBS), and trypsin were purchased from Gibco/Invitrogen (Carlsbad, Calif., USA). Cell culture supplements and glutamate were purchased from Sigma Chemical Company (St. Louis, Mo., USA). Recombinant rat IGF-1 was purchased from Upstate Biochemicals (Lake Placid, N.Y., USA). Recombinant human fibroblast growth factor-2 (FGF-2) was purchased from R&D Systems (Minneapolis, Minn., USA). Rabbit polyclonal antibodies to activated caspase 3 and Olig 2 were obtained from Cell Signaling Technologies (No. 9661; Danvers, Mass., USA) and Chemicon International (Temecula, Calif., USA), respectively. Antibodies to MBP and to β -actin were obtained from Chemicon International (rat monoclonal to MBP a.a. 82–87) and from Sigma-Aldrich Corp. (mouse monoclonal; St. Louis, Mo., USA), respectively. Goat anti-rabbit and mouse horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson Laboratories (West Grove, Pa., USA).

Cell Culture and Treatment Conditions

Newborn Sprague Dawley rat forebrain cortices were enzymatically digested with trypsin and DNase I and then mechanically dissociated and plated in MEM containing 10% FBS with antibiotics as previously described [Levison and McCarthy, 1991]. The mixed glial cells were grown in T75 flasks until they were confluent (10–14 days). Microglia were separated from the cultures by shaking the flasks on a rotary shaker for 1.5 h at 260 rpm. OP cells were isolated following an additional 18-hour shake as previously described [McCarthy and de Vellis, 1980]. OP cells were seeded into poly-D-lysine-coated T75 flasks at a density of $1.5 \times 10^4/\text{cm}^2$ in a chemically defined medium, N2S, composed of: (1) 66% N2B2 media [DMEM:F12 (Gibco, Grand Island, N.Y., USA; containing 15 mM HEPES, 2 mM glutamine) supplemented with 0.66 mg/ml BSA, 10 ng/ml d-biotin, 5 $\mu\text{g}/\text{ml}$ insulin, 20 nM progesterone, 100 μM putrescine, 5 ng/ml selenium, 50 $\mu\text{g}/\text{ml}$ apo-transferrin, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin], (2) 34% B104-conditioned medium (N2B2 preconditioned

by B104 neuroblastoma cells), (3) 5 ng/ml FGF-2, and (4) 0.5% FBS. OP cultures were amplified for 4–10 days and passaged once using papain [Young and Levison, 1997] prior to performing experiments. The lineage stages of oligodendrocyte differentiation have been extensively characterized in vitro using stage-specific monoclonal antibodies as markers [Ranscht et al., 1982; Bansal et al., 1989; Bansal and Pfeiffer, 1992, 1997; McMorris and McKinnon, 1996; Orentas and Miller, 1999; Baumann and Pham-Dinh, 2001]. The early OP is bipolar, mitotic and can be identified by expression of ganglioside antigens (detected by immunostaining with either A2B5 or R24 antibodies). Using these isolation procedures, we consistently obtain greater than 95% purity of early OP cells (A2B5+/O4–) as previously reported [Jiang et al., 2001].

As the early OP begins to differentiate, it progresses through an intermediate progenitor stage that is still mitotic but multipolar. Late OPs can be identified by the surface antigen POA (detected by immunostaining with the O4 antibody). Under differentiation conditions, the late OP differentiates into a postmitotic immature oligodendrocyte that can be identified by immunostaining for galactocerebroside (GalC, detected by the O1 or Ranscht antibodies). Ultimately, the immature oligodendrocyte will further mature into a fully differentiated oligodendrocyte characterized by production of myelin proteins including MBP and proteolipid protein. To obtain highly enriched cultures of late OPs for experiments, early OP cells were replated at a density of 4.0×10^4 cells/cm² onto poly-D-lysine-coated 60-mm dishes in N2B2 with 0.5% FBS and 10 ng/ml FGF-2 for 48 h. Under these conditions, we obtain cultures that contain 90–95% O4⁺ late OPs, 6–8% R24+/O4– OPs, 1–2% Ranscht⁺ immature OLs, <2% GFAP⁺ astrocytes and <0.01% OX42⁺ microglia [Ness and Wood, 2002]. For glutamate and trophic factor treatments, a hormone-supplemented medium was used that was identical to the N2B2 medium with the absence of insulin. Treatment media did not contain FGF-2. Cells were treated for various time points with 500 μ M glutamate and/or IGF-1 (10 ng/ml), and media were replaced every 18 h.

Western Blotting

Following treatments, OP cells were washed in ice-cold PBS and total cell lysates were isolated in SDS sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM DTT, 1/100 protease inhibitor cocktail (Sigma), 1 mM Na₃VO₄, and 1 mM NaF) and sonicated for 10–15 s on ice. Protein assays were performed to determine protein concentrations (BioRad, Hercules, Calif., USA). 15–25 μ g of protein was heated to 100°C for 5 min, cooled, and separated on 10, 12 or 3–8% mini-SDS polyacrylamide gels (Invitrogen). Proteins were then electrotransferred to nitrocellulose membranes. Membrane blocking and primary and secondary antibody incubations were done in 5% milk (or 5% BSA for some antibodies) in TBS 0.05% (or 0.1%) Tween-20. Protein was visualized using an enhanced chemiluminescence system (New England Nuclear, Boston, Mass., USA) following incubation with appropriate horseradish peroxidase-conjugated secondary antibodies. Images were digitized and quantified using NIH Image 1.62.

HI Model and Olig2 Immunocytochemistry

The left common carotid artery of PND7 outbred Wistar rats was ligated under anesthesia. Following recovery, the pups were exposed to 60 min of hypoxia in a humidified chamber at 36°C with $7.77 \pm 0.01\%$ oxygen in nitrogen. Immediately after HI, pups were treated with human recombinant IGF-1 (50 μ g; Genentech,

Inc.) or vehicle (NaCl 0.9 mg/ml) i.c.v. and then this dose was repeated once daily for 2 days as described previously [Brywe et al., 2005]. The dose of IGF-1 was chosen based on our previous studies in 7-day-old rats where this dose showed neuroprotection [Brywe et al., 2005] and on similar studies in adult rats [Guan, 1993] where this dose showed the most significant neuroprotection without affecting systemic glucose concentrations or cortical temperature. The injection procedure was well tolerated by the pups and there was no mortality. Animals were perfused at PND10 and the brains removed from the skull and immersion-fixed at 4°C for 24 h, dehydrated and embedded in paraffin.

Coronal brain sections (5 μ m) were cut from paraffin blocks, deparaffinized and dehydrated through a graded series of xylens and ethanol. For analysis of Olig2 expression, sections were analyzed at Bregma –0.3 to –0.9 from all brains. After incubation in proteinase K (10 μ g/ml; Sigma) in 10 mM Tris, pH 8.0 for 15 min, antigen retrieval was performed in 10 mM sodium citrate buffer using the pressure cooker method. Sections were incubated with a rabbit polyclonal antibody to Olig2 (1:100) overnight at 4°C followed by incubation with a goat anti-rabbit IgG alkaline phosphatase (1:250; Southern Biotech, Birmingham, Ala., USA). Immunoreactivity was detected with VectorRed Alkaline Phosphatase Substrate Kit I (Vector Laboratories, Burlingame, Calif., USA) solution for 30 min at room temperature in the dark. Sections were incubated with 4',6'-diamidino-2-phenylindole (1:2,500; Sigma) for 4 min at room temperature in the dark to detect nuclei. Sections were then dehydrated, and mounted in CytosealXYL (Richard-Allan Scientific, Kalamazoo, Mich., USA).

Quantification and Statistical Analyses

Photomicrographic images were captured by an individual blinded to the experimental conditions using a Sensys camera mounted on an Olympus Provis AX70 microscope at 40 $\times$ magnification. DAPI and rhodamine images were captured on each of three, adjacent fields along the white matter tracts (lateral to the subventricular zone) on both left and right hemispheres of sections from IGF-1- (n = 6) or saline-treated (n = 6) animals. Cell counts were performed using a grid overlay. Differences between treatment groups were determined by Student's t test.

Results

Transient Exposure to Glutamate Results in Delayed Death of Late OPs

Our previous studies demonstrated that exposing late OPs to glutamate in the absence of either IGF-1 or insulin induced cell death beginning at 22–24 h [Ness and Wood, 2002; Ness et al., 2004]. Glutamate-mediated death of the late OPs is initiated by calcium influx through AMPA/kainate receptors; however, Bax translocation to the mitochondria was not evident until at least 20 h following glutamate treatment [Ness et al., 2004]. It was not clear from these studies whether continuous exposure to glutamate is required to kill the late OPs. Since we do not use a glutamate receptor desensitizing blocker, we predicted

Fig. 1. Transient (≤ 1 -hour) exposure to glutamate is sufficient to activate caspase 3. Primary cultures of late OPs were exposed to IGF-1 (10 ng/ml), or glutamate (500 μ M) for 24 h (Glu) or to glutamate for 15 min or 1 h only and then switched either to control media (1 h Glu) or to media containing IGF-1 (1 h Glu + IGF) for an additional 23 h. Levels of cleaved caspase 3 were analyzed by Western immunoblotting at 24 and 48 h. **a** Representative Western immunoblot showing cleaved caspase 3 and β -actin on the same blot. **b** Band intensities were quantified using NIH image and normalized to levels of β -actin. ** $p < 0.001$ vs. IGF-1 or 1 h Glu + IGF-1; * $p < 0.04$ vs. IGF-1 or 1 h Glu + IGF-1.

that continuous glutamate exposure is not required for death of the cells. In order to determine whether a transient exposure to glutamate is sufficient to kill the late OPs, we tested whether ≤ 1 h exposure to glutamate is sufficient to induce apoptosis at 24 h. In these experiments, we used cultures enriched for the O4⁺/GalC⁻ stage of the late OP. The late OPs were exposed to 500 μ M glutamate for 1 or 24 h and then the media were replaced with glutamate-free media. Levels of active caspase 3 were determined by Western immunoblotting at 24 and 48 h (fig. 1). Confirming our hypothesis, 1-hour exposure to glutamate was sufficient to activate caspase 3 in the late OPs, and this short exposure was as effective as continuous exposure to glutamate for 24 h. Adding IGF-1 to the

medium immediately after stimulating the cells with glutamate almost completely attenuated caspase 3 activation at both 24 h and 48 h of recovery (fig. 1b). Exposure to glutamate for 15 min similarly activated caspase 3 at 24 h (fig. 1a). These data suggest that a transient exposure to glutamate causes the delayed death of OPs in the absence of a receptor desensitizing agent and of IGF-1R signaling (due to either IGF-1 or micromolar levels of insulin). These results suggest that the initial calcium influx from glutamate exposure initiates a cascade of intracellular events leading to cell death.

Late Addition of IGF-1 Can Prevent Glutamate-Mediated OP Death

The delayed death of the OPs following exposure to glutamate suggests the possibility of interfering with the death cascade during the 24-hour time period prior to caspase activation. To determine whether delayed addition of IGF-1 could prevent glutamate-mediated apoptosis of the late OPs, we performed experiments where the late OPs were exposed to glutamate for 12 or 16 h prior to addition of IGF-1 (fig. 2). After this initial period of glutamate exposure, cells were maintained in medium containing either IGF-1 alone or glutamate plus IGF-1. Strikingly, adding IGF-1 almost completely attenuated the cleavage of caspase 3 at both 12 and 16 h after glutamate exposure (fig. 2).

Late Addition of IGF-1 after Glutamate Exposure Promotes Oligodendrocyte Differentiation

While inhibiting caspase 3 activation correlates well with cell survival in the short term, there are noncaspase-mediated cell death cascades that might still be initiated by glutamate that would compromise the survival and differentiation of OP cells. To determine whether IGF-1 can completely enhance OP cell viability, we tested whether exposure to glutamate followed by recovery in medium that would stimulate the IGF-1R would allow the late OPs to survive and then to differentiate. Late OP cells were exposed to 18 h of glutamate in the absence of IGF-1 or insulin and then transferred to medium containing glutamate and/or IGF-1. After 12 h, cells were transferred to differentiation media for 3 days (that contained supra-physiological levels of insulin and thyroid hormone). Differentiation into immature oligodendrocytes was subsequently determined using morphological criteria and the expression of MBP. Cells exposed to glutamate and deprived of IGF-1R stimulation had weak expression of MBP compared to control cells, whereas cells exposed to IGF-1 18 h after exposure to glutamate expressed levels of

Fig. 2. IGF-1 can rescue late OPs after 12- or 16-hour exposure to glutamate. Primary late OPs were treated with IGF-1, glutamate or glutamate + IGF-1 for the times indicated above the immunoblots (**a**) or along the x-axis (**b**). Isolated protein was analyzed for cleaved caspase 3 by Western immunoblotting and normalized to β -actin as for the previous figure. Some cells were treated for 12 or 16 h with glutamate and then switched to media containing either IGF-1 (I) or Glu + IGF-1 (G/I) for 8, 12 or 16 h as indicated. **a** Representative Western immunoblots showing cleaved caspase 3 and β -actin from the same blots from the 28- or 32-hour experiments. **b** Quantification of active caspase 3 after normalization to β -actin. Total treatment times are indicated on the x-axis and coded as 24 h (light gray bars), 28 h (dark gray bars), and 32 h (black bars).

MBP that were higher than the glutamate only-stimulated group, and which were almost equivalent to cells in complete medium that were not exposed to glutamate (fig. 3). Analysis of MBP levels from duplicates of the 30-hour experiment shown in figure 3 showed that MBP levels in the glutamate only treatment were 50–60% of those in the group treated for 18 h with glutamate and then switched to IGF-1-containing media for 12 h prior to differentiation. An additional experiment performed through 36 h with 24 h glutamate plus 12 h of IGF-1 showed similar results: MBP protein levels in the glutamate alone condition were 30–40% of MBP levels in the conditions switched to IGF-containing media. Interestingly, while there was significant death of cells in the cultures exposed to glutamate alone for 30 h until the differentiation treatment, the remaining cells did go on to express MBP; however, at levels lower than levels in the IGF-1 rescue conditions. Our results suggest that the ex-

posure to glutamate does not irreversibly alter OP viability or their capacity to differentiate if they are subsequently stimulated with IGF-1 or high insulin-containing media. Moreover, these cells can go on to produce myelin proteins. These results support the hypothesis that the cascade of events initiated by glutamate-induced calcium influx is reversible within a few hours prior to Bax translocation and cytochrome c release.

IGF-1 Administration after HI in the Perinatal Brain Retains Normal OP Numbers

The in vitro studies described above demonstrate that IGF-1 can rescue OPs subsequent to glutamate exposure. While numerous studies have investigated neuronal death and protection following HI in the perinatal brain, few studies have evaluated the vulnerability and rescue of immature oligodendroglia after HI. To test whether addition of IGF-1 after HI can rescue oligodendroglia in

Fig. 3. Late addition of IGF-1 to OPs after glutamate exposure rescues myelin protein expression following differentiation. Primary late OPs were treated for 30 h with full media containing high insulin (N2B2), control media without insulin (Ctl). Additional cultures were treated with glutamate for 18 h followed by an additional 12 h in glutamate alone (Glu/Glu), glutamate plus IGF-1 (Glu/Glu + IGF) or IGF-1 alone (Glu/IGF). After the 30 h of treatment, all cultures were switched to differentiation media for 72 h. Isolated protein was analyzed for expression of MBP or β -actin by Western immunoblotting. Blot is representative of 3 independent experiments with each condition per experiment analyzed in duplicate.

vivo, we analyzed PND10 rat brains 72 h following HI. Differentiation of oligodendrocytes begins between PNDs 10–14 in rats; thus, the oligodendroglia at PND 7–10 are predominantly late OPs. Olig2 is a transcription factor whose expression identifies cells of the oligodendroglia lineage. Therefore, we quantified the number of Olig2⁺ cells in the ipsilateral white matter following administration of either IGF-1 (50 μ g) or saline i.c.v. after HI. A prior analysis of damage and neuronal protection by IGF-1 in these brains revealed that IGF-1 reduced neuronal damage by 40% overall with significant neuroprotection observed in the cortex, hippocampus, and striatum [Brywe et al., 2005]. We analyzed sections from these same brains to evaluate the extent to which IGF-1 could protect the white matter and resident immature oligodendroglia (fig. 4). Cell counts performed by an investigator blinded to the conditions revealed that the total number of cells in the white matter was reduced to 78% in the HI vs. contralateral hemisphere (fig. 4a, b, e; $p = 0.02$). The number of Olig2⁺ cells in white matter of the ipsilateral hemisphere was reduced to 65% of the contralateral hemisphere (fig. 4a, b, e; $p = 0.04$). Administration of IGF-1 completely rescued the loss of Olig2⁺ cells in white matter (fig. 4c–e; $p = 0.04$). Interestingly, while there was a trend towards rescue of total white matter

cells with IGF-1 administration, this did not reach statistical significance suggesting that IGF-1 preferentially rescued the Olig2⁺ cell population (fig. 4e).

Discussion

In previous studies, we demonstrated that death of late OPs from glutamate in vitro involves Bax translocation and a mitochondrial apoptotic pathway that occurs 20–24 h following exposure to glutamate. Here, we demonstrate that 1-hour exposure to glutamate is sufficient to initiate death of the late OPs in the same time frame. Moreover, we found that IGF-1 interferes with the glutamate-induced death pathway in the late OPs when administration is delayed as much as 16–18 h following glutamate exposure. Moreover, these cells can proceed to differentiate and to produce myelin proteins, suggesting that the cells are not irreversibly committed to die until near the time when Bax translocation occurs. We also demonstrate that, when administered following the insult, IGF-1 protects white matter OPs from HI in vivo in the perinatal brain.

Multiple investigators have used cell culture paradigms to characterize the molecular mechanisms of glutamate toxicity on oligodendrocyte lineage cells. There are significant differences in the approaches taken in these in vitro investigations and, thus, in the results obtained and conclusions drawn. The major differences that are critical for interpreting the data and generalizing to the in vivo conditions are the stage of cells utilized, the method for glutamate treatment and the culture conditions. Of note is that the majority of the in vitro systems used to study glutamate toxicity in the oligodendroglial lineage have used cells that are positive for galactocerebroside (GalC⁺), a marker for postmitotic immature or mature oligodendrocytes [Yoshioka et al., 1995; Matute et al., 1997; McDonald et al., 1998a; Sanchez-Gomez and Matute 1999; Alberdi et al., 2002; Sanchez-Gomez et al., 2003]. As discussed previously, increasing evidence suggests that the most vulnerable stage of the lineage in rodent, sheep and human white matter is the O4⁺/GalC[−] late OP. Thus, we developed a culture system to enrich for the late OP cell [Ness and Wood, 2002]. Under conditions that promote progression to the late OP stage but prevent further differentiation, we generate cultures that are greater than 90–95% O4⁺ late OPs with only 1–2% GalC⁺ cells (detected by O1 or Ranscht antibodies), 6–8% early progenitors (A2B5⁺/O4[−]), <2% astrocytes, and <0.01% microglia [Ness and Wood, 2002].

A second distinguishing difference between our in vitro system vs. other paradigms is the absence of the recep-

Fig. 4. IGF-1 administration after HI in the perinatal brain prevents loss of Olig2⁺ cells in white matter. **a–d** Olig2 immunostaining in corpus callosum from PND10 rat brains 72 h after exposure to HI (**a, c**) or hypoxia alone (**b, d**) and treatment with either IGF-1 (**c, d**) or saline (**a, b**). DAPI (blue) was used to detect all nuclei. Double-positive cells appear as pink since the Olig2⁺ transcription factor is present in the nucleus. **e** Graph showing average number of total DAPI+ cells or Olig2⁺ cells per field (average of 3 fields per hemisphere, n = 6 for each condition). * p = 0.02 vs. Ctl for DAPI counts and p = 0.04 vs. Ctl for Olig2 counts; ** p = 0.04 vs. HI.

tor-desensitizing blocker, cyclothiazide, which inhibits desensitization at AMPA-preferring receptors [Yoshioka et al., 1995; Matute et al., 1997; Matute, 1998; McDonald et al., 1998a; Sanchez-Gomez and Matute, 1999; Li and Stys, 2000; Alberdi et al., 2002; Sanchez-Gomez et al., 2003]. In addition, other laboratories use culture media containing micromolar levels of insulin, which activate the IGF-1R. In contrast, the insulin levels in our system are absent or physiological (nanomolar range), which do not activate the IGF-1R. This modification to the culture medium formulation is critical since our previous studies demonstrated that stimulating the IGF-1R antagonizes

glutamate-stimulated OP death through sustained activation of Akt and downstream survival pathways [Ness and Wood, 2002]. The required addition of AMPA receptor-desensitizing agents used in other studies to obtain significant glial cell death is likely a direct consequence of including high insulin in the media as well as to using the postprogenitor stage of immature oligodendrocytes. Without the AMPA-R desensitizing blocker, we observe death of the late OPs after 20–24 h, a time frame consistent with the peak of active caspase 3 in the perinatal HI brain and with the incidence of ISEL⁺ cells in the white matter in the p7 rat brain after HI [Han and Holtzman,

2000; Ness et al., 2001]. HI in the perinatal rat brain also reduces expression of IGF-1 within the first 24 h [Lee et al., 1996]. Taken together, the absence of a desensitizing blocker and reduced IGF-1R signaling as employed in our in vitro studies are likely to better model the in vivo state of the white matter after perinatal HI.

A critical finding of these studies is that administering IGF-1 following HI completely rescues loss of OPs in the white matter of the neonatal rat brain. Moreover, our data suggest that IGF-1 preferentially rescues oligodendroglia vs. other white matter glia after HI in the immature brain. A recent study demonstrated that IGF-1 partially rescued O4⁺ late OPs and MBP expression following bilateral HI in the perinatal rat brain [Lin et al., 2005]. However, in this study, IGF-1 was administered as a single dose prior to the HI insult, which may account for the partial rescue of OPs in this study. Consistent with our results, postinsult administration of IGF-1 following bilateral artery occlusion in the near-term sheep prevented loss of proteolipid protein-positive cells in the white matter [Cao et al., 2003]. While IGF-1 is a poor mitogen for OPs in vitro, our previous studies demonstrate that it significantly enhances OP proliferation induced by mitogens such as FGF-2 and PDGF [Jiang et al., 2001; Frederick and Wood 2004]. HI in the immature brain results in proliferation of stem/pro-

genitor populations [Back et al., 2002; Felling et al., 2006; Yang and Levison, 2006]. Thus, it is possible that rescue of the OP cells in our study was due in part to an increase in proliferation of the OPs that was augmented by infusion of IGF-1. However, our previous studies demonstrated that excitotoxic death of late OPs in vitro occurs over a protracted time course [Ness and Wood, 2002] similar to the time course observed for death of OPs following HI in vivo in the neonatal rat brain [Ness et al., 2001]. Moreover, the in vitro results presented here suggest that IGF-1 treatment is effective in blocking excitotoxic death when administered just prior to the time when we observe initiation of mitochondrial-mediated apoptosis in the late OPs [Ness et al., 2004]. Thus, the results of these studies contribute to the viewpoint that administering IGF-1 will prevent white matter damage and promote myelination in the immature brain even if given several hours after insult.

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