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IGF-I instructs multipotent adult neural progenitor cells to become oligodendrocytes

Jenny Hsieh,[1] James B. Aimone,[1] Brian K. Kaspar,[1] Tomoko Kuwabara,[1] Kinichi Nakashima,[1,2] and Fred H. Gage[1]

[1]Laboratory of Genetics, The Salk Institute, La Jolla, CA 92037
[2]Department of Cell Fate Modulation, Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto 860-0811, Japan

Adult multipotent neural progenitor cells can differentiate into neurons, astrocytes, and oligodendrocytes in the mammalian central nervous system, but the molecular mechanisms that control their differentiation are not yet well understood. Insulin-like growth factor I (IGF-I) can promote the differentiation of cells already committed to an oligodendroglial lineage during development. However, it is unclear whether IGF-I affects multipotent neural progenitor cells. Here, we show that IGF-I stimulates the differentiation of multipotent adult rat hippocampus-derived neural progenitor cells into oligodendrocytes. Modeling analysis indicates that the actions of IGF-I are instructive. Oligodendrocyte differentiation by IGF-I appears to be mediated through an inhibition of bone morphogenetic protein signaling. Furthermore, overexpression of IGF-I in the hippocampus leads to an increase in oligodendrocyte markers. These data demonstrate the existence of a single molecule, IGF-I, that can influence the fate choice of multipotent adult neural progenitor cells to an oligodendroglial lineage.

Introduction

Multipotent cells with the ability to divide and differentiate have been shown to exist throughout the central nervous system (CNS), yet neurogenesis appears to be restricted to two specific brain regions within the adult CNS: the subventricular zone and the hippocampal subgranular zone (Gage, 2000). Beyond these two regions, most of the dividing cells in other areas give rise to new glial cells and not neurons (Horner et al., 2000; Kornack and Rakic, 2001; Rakic, 2002). However, when these dividing cells are isolated from different regions of the CNS (Reynolds and Weiss, 1992; Lois and Alvarez-Buylla, 1993; Palmer et al., 1997), including nonneurogenic areas (Shihabuddin et al., 1997; Palmer et al., 1999; Kondo and Raff, 2000), they all retain the ability to self-renew and differentiate into neurons, oligodendrocytes, and astrocytes in culture. Remarkably, even proliferating cells isolated from nonneurogenic areas can differentiate into neurons when transplanted back into the hippocampus (Suhonen et al., 1996; Shihabuddin et al., 2000), suggesting that cues from the local environment influence their fate determination and differentiation programs.

Recent work by Song et al. (2002) directly examined the idea that different environments can influence the fate specification of neural stem cells. Astrocytes from neurogenic regions (but not nonneurogenic regions) can preferentially direct multipotent adult neural stem cells to differentiate into neurons. Interestingly, these neural stem cells appear to preferentially differentiate into oligodendrocytes when cultured together with hippocampus-derived neurons. These results suggest that specific signals from different CNS environments exist for lineage-specific differentiation. Examples of extrinsic factors important for neuronal and astroglial differentiation of multipotent adult neural stem cells have already been identified (Takahashi et al., 1999; Lim et al., 2000; Tanigaki et al., 2001). However, little is known regarding the control of oligodendrocyte differentiation of multipotent adult neural stem cells.

Insulin-like growth factors (IGFs) comprise one class of molecules that have effects on oligodendrocyte biology during development (D'Ercole et al., 1996). IGFs (IGF-I, IGF-II) and insulin can all independently promote the survival of purified oligodendrocytes in culture (Barres et al., 1993). Furthermore, IGFs have important roles in the proliferation and differentiation of cells that have already committed to an oligodendroglial lineage during development (McMorris et al., 1986; McMorris and Dubois-Dalcq, 1988). In addition,

Address correspondence to Fred H. Gage, The Salk Institute, 10010 N. Torrey Pines Road, La Jolla, CA 92037. Tel.: (858) 453-4100. Fax: (858) 597-0824. email: gage@salk.edu

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Abbreviations used in this paper: AAV, adeno-associated virus; BMP, bone morphogenetic protein; CNS, central nervous system; IGF, insulin-like growth factor; MBP, myelin basic protein; RA, retinoic acid.

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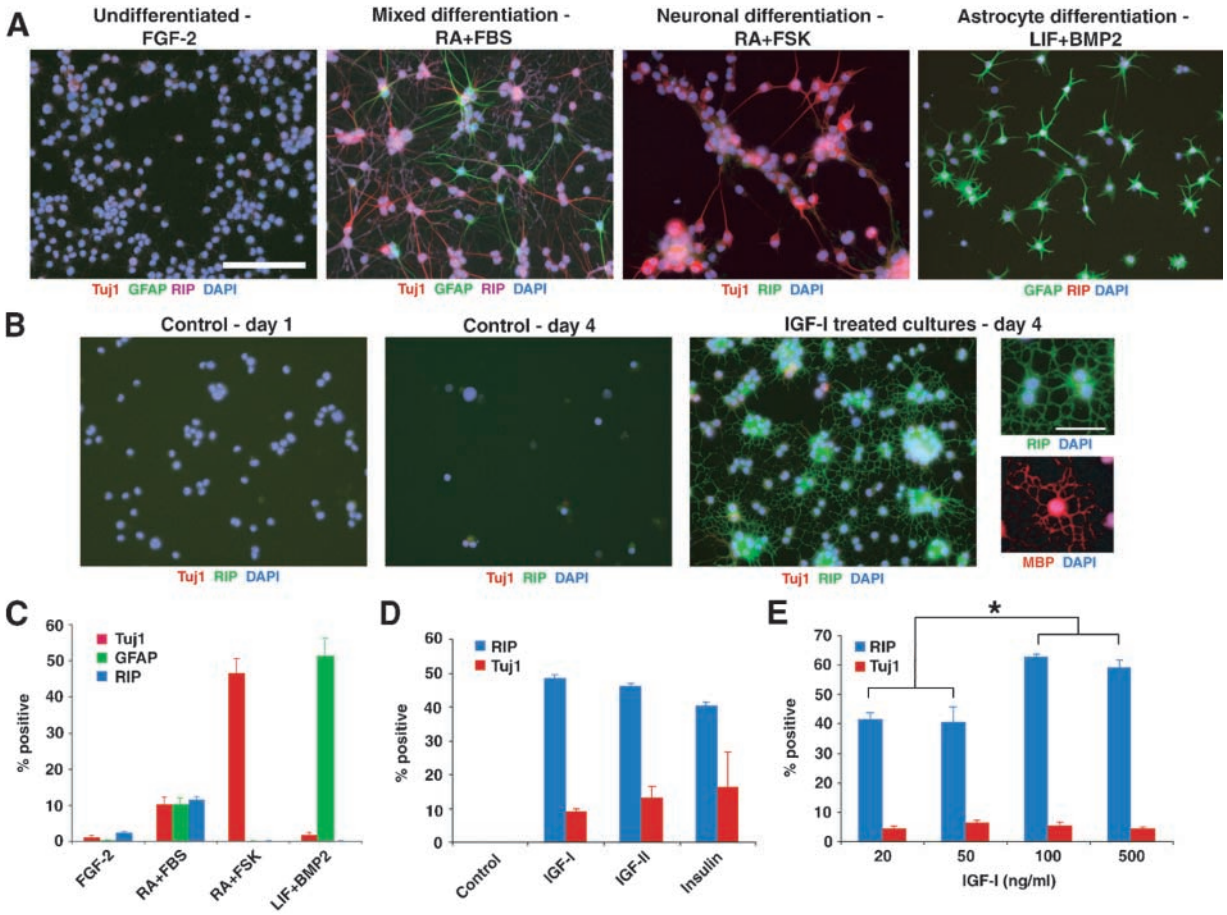

Figure 1. **IGF-I–induced differentiation of multipotent neural progenitor cells.** (A) Adult hippocampus-derived neural progenitor cells cultured in insulin-containing N2 media with 20 ng/ml FGF-2 (undifferentiated), 1 μM RA, and 1% FBS for 4 d (mixed), 1 μM RA and 5 μM forskolin for 4 d (neuronal), or 50 ng/ml leukemia inhibitory factor and 50 ng/ml BMP2 for 6 d (astrocytic). Cells were stained for markers for neurons (Tuj1), astrocytes (GFAP), or oligodendrocytes (RIP), and also with DAPI. Bar, 50 μm. (B) Differentiation of neural progenitor cells in insulin-free N2 media without IGF-I (control) or with IGF-I for 4 d. Insets show cells of typical oligodendrocyte morphology stained with RIP or MBP. Bar, 25 μm. (C) Quantification of cells in either proliferating or differentiating conditions. (D) Quantification of cultures grown in insulin-free N2 media (control) or treated with 500 ng/ml IGF-I, IGF-II, or insulin after 4 d. (E) Quantification of cells in response to different doses of IGF-I (20–500 ng/ml) in 4-d cultures. All data shown are from at least three experiments in parallel cultures with error bars representing SDs. Significant differences are indicated with an asterisk ($P < 0.005$).

transgenic and knockout mouse models have revealed in vivo effects of IGFs on oligodendrocyte development and myelination. Overexpression of IGF-I in transgenic mice results in increased brain size and myelin content (Carson et al., 1993; Ye et al., 1995). Conversely, IGF-I knockout mice have smaller brains, reduced oligodendrocyte numbers, and CNS hypomyelination (Baker et al., 1993; Carson et al., 1993; Beck et al., 1995; Ye et al., 2002).

In this paper, we show that IGF-I can preferentially induce the differentiation of multipotent adult neural progenitor cells into oligodendrocytes. Using a modeling approach, we show that the IGF-I–induced increase in oligodendrocyte numbers is attributable to an instructive differentiation of uncommitted cells to an oligodendroglial fate, not to a selective proliferation or survival of committed oligodendrocyte progenitors. The IGF-I–induced oligodendrocyte differentiation appears to be mediated, at least in part, by the inhibition of bone morphogenetic protein (BMP) signaling. Furthermore, overexpression of IGF-I in the hippocampus led to an increase in immunoreactivity for oligodendrocyte markers. These results provide evidence that IGF-I is an important regulator of oligodendrocyte differentiation from multipotent adult neural progenitor cells.

Results

IGF-I–induced oligodendrocyte differentiation

First, we tested the action of exogenous IGF-I on the differentiation of hippocampus-derived adult neural progenitor cells. These neural progenitor cells have stem cell properties in vitro: (1) they self-renew in the presence of basic FGF-2; (2) single genetically marked clones can differentiate into all three main CNS cell types in vitro (neurons, oligodendrocytes, and astrocytes) and when grafted back to adult hippocampus in vivo; and (3) they express progenitor cell markers such as nestin, but lack markers of lineage-specific differentiation (Fig. 1 A; Gage et al., 1995; Palmer et al., 1997). To confirm that the adult neural progenitor cell population in our model system is indeed multipotent, we first used various standard differentiation paradigms and evaluated the ex-

pression of lineage markers. Differentiation with 1 μM retinoic acid (RA) and 1% FBS resulted in mixed numbers of $Tuj1^+$ neurons, $GFAP^+$ astrocytes, and RIP^+ oligodendrocytes (Fig. 1 A). Neuron-enriched differentiation can be achieved with 1 μM RA and 5 μM forskolin (Chu, V., personal communication), and astrocyte-enriched differentiation with 50 ng/ml leukemia inhibitory factor and 50 ng/ml BMP2 (Fig. 1 A; Nakashima et al., 1999). The quantification of lineage-specific differentiation is also shown (Fig. 1 C). These results are in agreement with previous reports (Palmer et al., 1997), and indicate that the majority of the adult neural progenitor cells within the bulk population are multipotent progenitors, and not restricted neuronal or glial progenitors.

To determine the effects of IGF-I on neural progenitor cell differentiation, we compared cells treated with and without IGF-I over a 4-d period. All experiments were performed in insulin-free N2 medium to avoid its actions on insulin and/or IGF-I receptors. When FGF-2 was withdrawn from neural progenitor cells and the cells were cultured in the absence of insulin for 4 d, lineage-specific differentiation was not seen (Fig. 1 B). There was also a dramatic decrease in cell survival, consistent with the important effects of IGFs for preventing cell death (Vincent and Feldman, 2002). We found that neural progenitor cells cultured in the presence of 500 ng/ml IGF-I differentiated into a large percentage of RIP^+ cells (~45–55%; Fig. 1, B and D). RIP is a marker for both immature and mature oligodendrocytes, in that it stains preensheathing oligodendrocytes through myelinating stages and associated myelin (Friedman et al., 1989). In addition to RIP staining, these cells exhibited small and round somata with the characteristic weblike oligodendrocyte morphology within 4 d (Fig. 1 B). Approximately 23% of the total cells were also positive for myelin basic protein (MBP), a late-appearing marker for myelinating oligodendrocytes (Akiyama et al., 2002), at the 4-d time point (Fig. 1 B).

Furthermore, 500 ng/ml IGF-II or insulin could also promote the preferential differentiation of neural progenitor cells into oligodendrocytes (Fig. 1 D). The IGF-mediated oligodendrocyte differentiation is likely to occur through an activation of IGF-I receptors; addition of IGF-I, IGF-II, or insulin at different concentrations (20–1,000 ng/ml) showed a rank order of potency IGF-I > IGF-II > insulin, which is consistent with the pharmacology of the IGF-I receptor (LeRoith et al., 1993). RT-PCR analysis showed IGF-I receptor mRNA expression in neural progenitors (unpublished data). There was also a small percentage of neural progenitor cells that differentiated into $Tuj1^+$ neurons (~5–15%) with IGF-I or -II or insulin treatment (each at 500 ng/ml), with few to no $GFAP^+$ astrocytes (Fig. 1 D). The IGF-I–induced oligodendrocyte differentiation occurred in a dose-dependent manner, whereas the percentage of Tuj1-positive cells did not appear to change at any of the tested concentrations (Fig. 1 E).

The severe and rapid death of neural progenitor cells cultured in the absence of IGF-I left open the possibility that FGF-2 withdrawal alone could be promoting oligodendrocyte differentiation and that the addition of IGF-I merely promoted oligodendrocyte survival. To address this issue, we kept neural progenitor cells alive with the broad caspase inhibitor Q-VD-OPh (Caserta et al., 2003), and assessed neural progenitor differentiation with and without IGF-I in 2-d cultures (Fig. 2, A–C). Neural progenitors that differentiated into definitive oligodendrocytes were scored on the basis of morphological criteria (elaboration of weblike processes), as well as immunoreactivity with various oligodendrocyte markers (O4, NG2, RIP, and MBP). In the absence of Q-VD-OPh in insulin-free N2 medium, there was decreased cell survival, evidenced by the presence of fragmented DAPI-stained nuclei and a general disappearance of cells. Addition of 2 μM Q-VD-OPh to the cells promoted the majority of the cells to survive, as seen by smooth DAPI-stained nuclei and a general persistence of cells in 2-d cultures, without obvious effects on proliferation or differentiation (Fig. 2, A and B). The absolute number of cells does not significantly change in Q-VD-OPh-treated cultures compared with cells just after plating (unpublished data). Although the cells exhibited some degree of O4 and RIP staining in the soma, only a small number of cells (~2–4%)

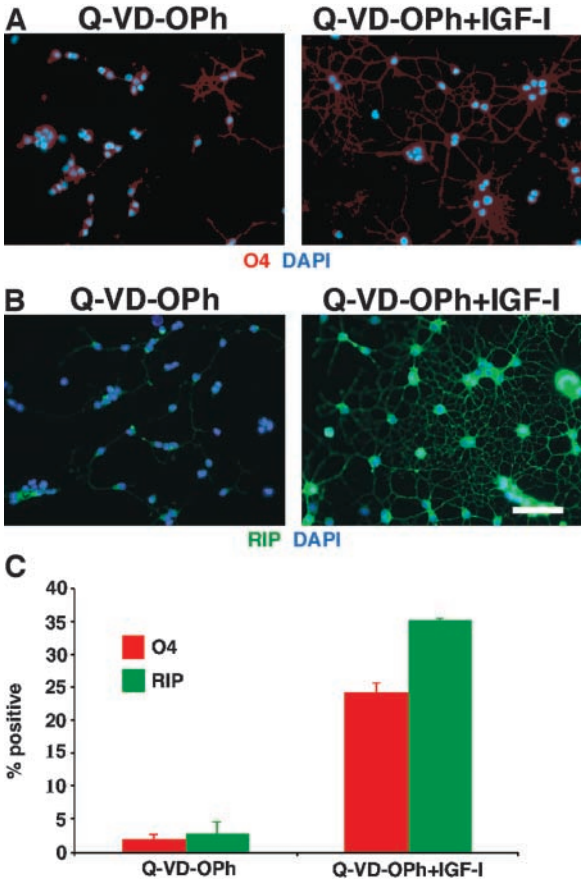

Figure 2. **IGF-I–mediated increase in oligodendrocyte differentiation is independent of effects on neural progenitor cell survival in 2-d cultures.** (A) Treatment of cells with 2 μM Q-VD-OPh resulted in a general absence of apoptosis in short-term cultures. The maintenance of cell survival is shown by a general persistence of cells and a lack of fragmented DAPI-stained nuclei, without obvious effects on cell proliferation or differentiation. Addition of 500 ng/ml IGF-I to the Q-VD-OPh–treated cultures mediated an increase in oligodendrocyte differentiation, as evidenced by O4 staining and morphological criteria. (B) Similar results were seen with RIP. (C) Quantification of O4 and RIP^+ oligodendrocytes. Bar, 25 μm.

exhibited both staining and morphological criteria indicative of a differentiated oligodendrocyte in Q-VD-OPh–treated cultures. This finding is presumably due to spontaneous differentiation upon FGF-2 withdrawal. In addition, we did not detect MBP staining in Q-VD-OPH–treated cultures, further confirming that FGF-2 withdrawal alone could not promote the robust differentiation and maturation of a multipotent neural progenitor cell into a definitive oligodendrocyte. Only when neural progenitors were cultured with 500 ng/ml IGF-1 did we observe a large increase in O4 and RIP^+ cells with the characteristic weblike morphology in 2-d cultures (Fig. 2, A and B). The quantification of oligodendrocyte differentiation is also shown (Fig. 2 C). The presence of $NG2^+$ cells was not observed in Q-VD-OPh–treated cultures, with or without IGF-I, suggesting that adult multipotent neural progenitor cells do not transition through an $NG2^+$ cell upon IGF-I stimulation. These data suggest that IGF-1 can directly induce multipotent neural progenitor cells to differentiate into oligodendrocytes, instead of merely promoting the survival of differentiated oligodendrocytes.

The oligodendrocyte-promoting effects of IGF-I were confirmed with an independently derived rat hippocampus progenitor cell line, as well as a clonally derived line (unpublished data). Furthermore, early-passaged cultures (<5) of multipotent neural progenitor cells derived from the whole brain of P10 ICR mice also exhibited a preferential differentiation into oligodendrocytes upon IGF-I treatment (unpublished data). These data suggest that IGFs (IGF-I and -II) and insulin, through an activation of IGF-I receptors, are important in stimulating the differentiation and maturation of multipotent adult neural progenitor cells into oligodendrocytes.

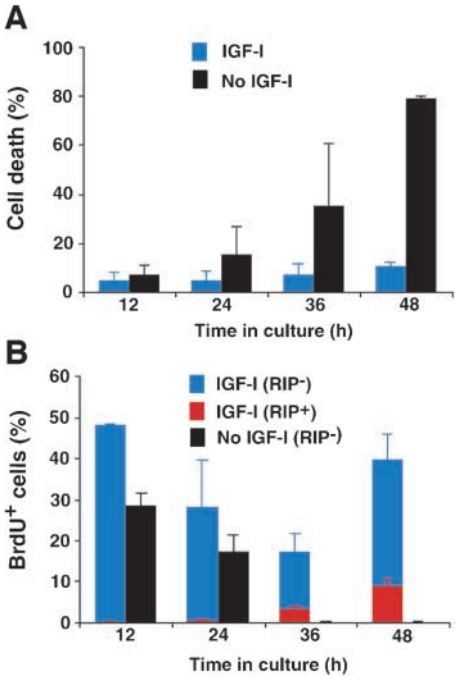

Figure 3. **Effect of IGF-I on the survival, proliferation, and instructive oligodendrocyte differentiation of adult neural progenitor cells.** (A) Quantification of cell death in insulin-free (no IGF-I) and IGF-I–treated (500 ng/ml) cultures, as determined every 12 h by live staining with 1 μg/ml propidium iodide and 1 μg/ml Hoechst 33342. (B) Proliferation in insulin-free (no IGF-I) and IGF-I–treated (500 ng/ml) cultures as determined by BrdU incorporation. Parallel cultures were incubated with 2.5 μM BrdU at the beginning of each 12-h time point, immediately followed by fixation and BrdU staining. $BrdU^+$ cells were counted as either being RIP^- (blue and black columns) or RIP^+ (red columns). All data are from at least three independent experiments in parallel cultures with error bars representing SDs.

IGF-I–induced increase in oligodendrocytes is attributable to an instructive differentiation and a subsequent proliferation of committed oligodendrocytes

Although these results establish IGF-I as an inducer of oligodendrocyte differentiation for a population of multipotent neural progenitor cells, it is more complicated to quantitatively assess the instructive versus selective effects of IGF-I. Therefore, we determined whether the increase of oligodendrocytes after IGF-I treatment could be due to a combination of selective oligodendrocyte survival, increased proliferation of progenitors, and/or instructive differentiation of progenitor cells to an oligodendroglial lineage. First, we directly measured the effect of IGF-I on cell survival. We asked whether a selective decrease in the death of oligodendrocytes (and/or oligodendrocyte progenitors) could explain the net increase in number of oligodendrocytes (defined by the expression of RIP, a marker of both immature and mature oligodendrocytes). If IGF-I had a selective survival effect on oligodendrocytes and/or their progenitors, we would expect to see a change in cell death due to the increased death of other cell types. To quantify cell death of the progeny of neural progenitor cells, living cultures were stained with 1 μg/ml propidium iodide, which stains dead cells, and 1 μg/ml Hoechst 33342, which stains live and dead cells. Staining of live (rather than fixed) cultures was used to avoid underestimating cell death due to possible detachment of dying and/or dead cells from culture substrates. This assay revealed a relatively small percentage of dead/dying cells, and one-way ANOVA analysis revealed no significant change ($P = 0.3930$) in the percentage of cells that died at any of the time points (Fig. 3 A). The amount of cell death in IGF-I–treated cultures was similar to the amount of cell death under standard proliferating conditions with FGF-2 (unpublished data). Cultures grown in the absence of IGF-I exhibited a progressive increase in overall cell death, reinforcing the role of IGF-I as an important factor for cell survival. Because there was minimal death and no significant difference in the percentage of dead/dying cells at each of the time points in the IGF-I–treated cultures, a selective survival of oligodendrocyte progenitors or oligodendrocytes does not appear to have a significant role in the increased net oligodendrocyte differentiation with IGF-I treatment.

Next, we determined whether proliferation of progenitors might contribute to the observed increase in oligodendrocyte differentiation. 2.5 μM BrdU was added to parallel cultures for 12 h at 0, 12, 24, and 36 h after plating, followed by fixation and quantification of total cell numbers and $BrdU^+$ cells. We counted the progenitor cells (evidenced by a lack of RIP staining) that were labeled with BrdU at each time point. For cells grown in the absence of IGF-I, we observed a progressive decrease in the percentage of cells that were proliferating ($BrdU^+$) during the 48-h experiments (Fig. 3 B). There was a marked decrease in proliferation dur-

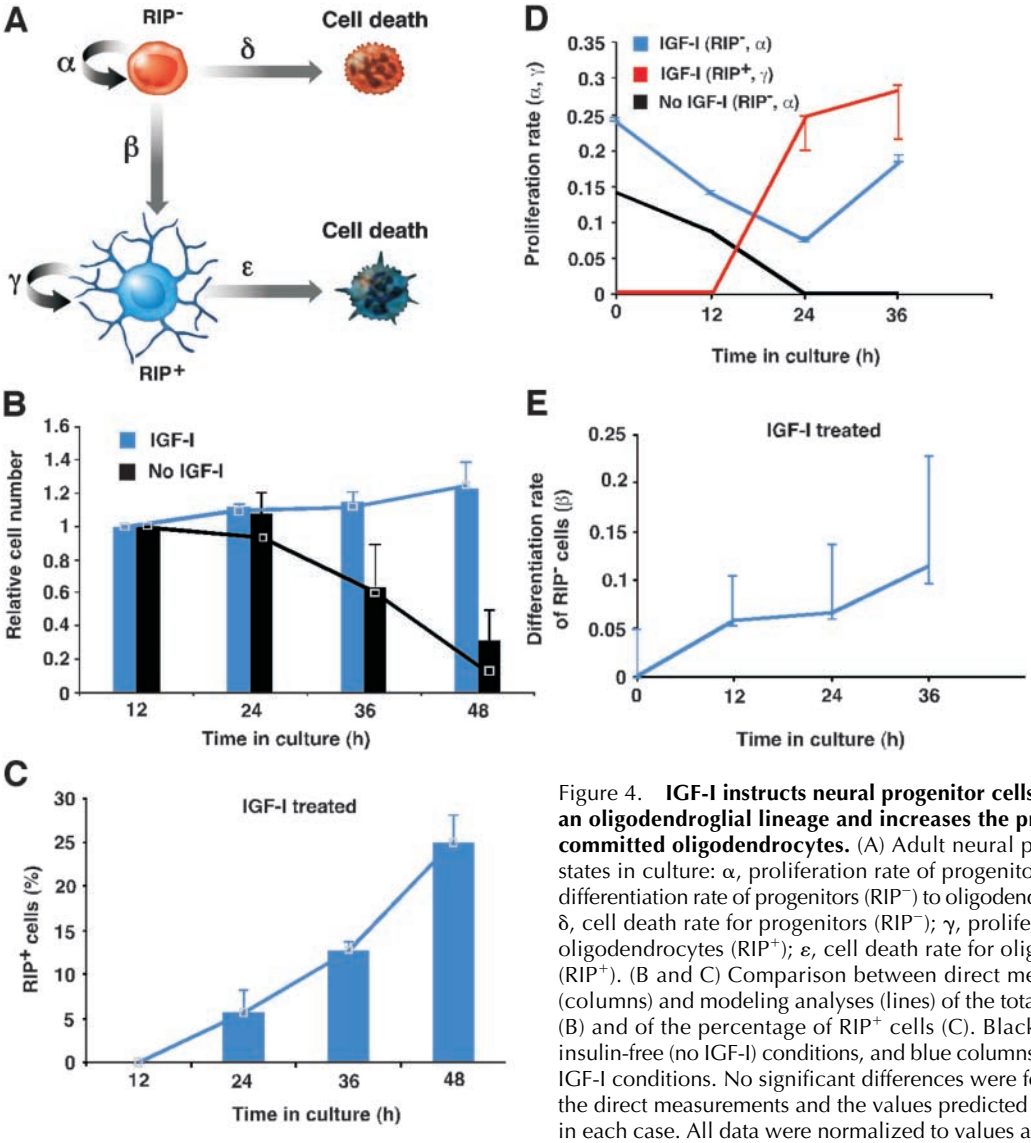

Figure 4. **IGF-I instructs neural progenitor cells to commit to an oligodendroglial lineage and increases the proliferation of committed oligodendrocytes.** (A) Adult neural progenitor cell states in culture: α, proliferation rate of progenitors (RIP^-); β, differentiation rate of progenitors (RIP^-) to oligodendrocytes (RIP^+); δ, cell death rate for progenitors (RIP^-); γ, proliferation rate for oligodendrocytes (RIP^+); ε, cell death rate for oligodendrocytes (RIP^+). (B and C) Comparison between direct measurements (columns) and modeling analyses (lines) of the total cell numbers (B) and of the percentage of RIP^+ cells (C). Black columns are insulin-free (no IGF-I) conditions, and blue columns are 500 ng/ml IGF-I conditions. No significant differences were found between the direct measurements and the values predicted by the model in each case. All data were normalized to values at the 12-h time point within each condition due to variability in cell behavior at time 0 (4 h after plating). (D) The proliferation rates for RIP^- (α) and RIP^+ (γ) cells in insulin-free (no IGF-I) conditions (black line) and treated with 500 ng/ml IGF-I (blue and red lines). (E) The differentiation rate for RIP^- (β) cells treated with 500 ng/ml IGF-I. These data are representative of at least three independent experiments. Error bars (except in D and E) represent SDs. The error bars in D and E represent the upper and lower bounds for proliferation (α, γ) and differentiation (β) rates.

ing the 36- and 48-h periods, probably due to limited cell survival in the absence of IGF-I. In cultures treated with IGF-I, the percentage of RIP^- cells that incorporated BrdU also exhibited a decreasing trend in the first three time periods, but to a lesser extent compared with the cells grown without IGF-I (Fig. 3 B, blue columns). This initial decrease in proliferation is most likely due to FGF-2 mitogen withdrawal. The fact that we could already observe the generation of RIP^+ oligodendrocytes at the 24-h time point (Fig. 4 C), coupled with the observation that there was a decrease in BrdU uptake of RIP^- cells, suggests that IGF-I did not have a major proliferative effect on progenitor cells.

These data taken alone may not be sufficient to fully evaluate the effects of IGF-I on proliferation of RIP^- progenitors because if there is also a differentiation effect (conversion of RIP^- to RIP^+ cells), a substantial decrease in the percentage of RIP^- cells that were $BrdU^+$ may be seen. Therefore, to separate the IGF-I effects on proliferation of progenitors from the instructive differentiation of progenitors to oligodendrocytes, we developed a mathematical model (Fig. 4 A). This modeling approach has previously been used to quantify the effects of hippocampal astrocytes on the proliferation and neuronal differentiation of adult neural progenitor cells (Song et al., 2002). To distinguish the proliferation and differentiation rates, the model required that we count the number of RIP^+ cells that were either positive or negative for BrdU at each time point. There was a progressive increase in the overall number of RIP^+ cells over time in the presence of IGF-I (Fig. 4 C). We also stained the cultures at each time point for $Tuj1^+$ neurons and $GFAP^+$ astrocytes to determine if the model should account for the differentiation to alternative cell types; because neuronal differentiation was minimal and astrocyte differentiation was undetectable, these possibilities did not merit inclusion into the model. Interestingly, after 24 h, there was also a significant increase in RIP^+ cells positive for BrdU,

suggesting that committed oligodendrocytes may still be proliferating in the presence of IGF-I (Fig. 3 B, red columns). Because of this finding, we decided to separate the proliferation rate of RIP^- (defined as α) and RIP^+ cells (γ), as well as the differentiation rate (β) of RIP^- to RIP^+ cells. The overall death rate measured by Hoechst dye in live cultures could not be experimentally separated into a RIP^- death rate (δ) and a RIP^+ death rate (ε; Song et al., 2002). Therefore, the model was bounded by the two possibilities that all cell death is from only RIP^- or only RIP^+ cells. We also considered the more probable assumption that the death rates for both RIP^- and RIP^+ cells were equal. Six equations were used to derive values for these rates. The first equation describes the differential gain in RIP^- cells:

$$dN^{RIP-}/dt = (\alpha - \delta)N^{RIP-} - \beta N^{RIP-} \quad (1)$$

where N^{RIP-} is the number of RIP^- cells, α is the proliferation rate of RIP^- cells (per 12-h interval), β is the differentiation rate of RIP^- into RIP^+ cells, and δ is the death rate of RIP^- cells. Similarly for RIP^+ cells,

$$dN^{RIP+}/dt = (\gamma - \varepsilon)N^{RIP+} + \beta N^{RIP-} \quad (2)$$

where N^{RIP+} is the number of RIP^+ cells, γ is a proliferation rate of RIP^+ cells, and ε is the RIP^+ death rate. The sum of (Eq. 1) and (Eq. 2) is the equation defining total cell count:

$$dN^{Total}/dt = (\alpha - \delta)N^{RIP-} + (\gamma - \varepsilon)N^{RIP+} \quad (3)$$

The time-course experiments were performed in 12-h intervals, allowing for measurement of the proliferation and differentiation rates at each 12-h time interval; α_i is the proliferation rate of RIP^- cells at time "i" and so forth. BrdU uptake experiments allow for direct measurements of the number of new cells that were generated within the time interval:

$$\#BrdU_{i+1} = 2\gamma_i N^{RIP+}{}_i + 2\alpha_i N^{RIP-}{}_i \quad (4)$$

where $\#BrdU_{i+1}$ is the number of cells that incorporated BrdU between time "i" and "i+1." Inspection of $BrdU^+$ cells that are negative or positive for RIP gives the equations:

$$\#BrdU_{i+1}{}^{RIP-} = 2\alpha_i N^{RIP-}{}_i - \beta_i[\alpha_i/(1+\alpha_i)]N^{RIP-}{}_i \quad (5)$$

$$\#BrdU_{i+1}{}^{RIP+} = 2\gamma_i N^{RIP+}{}_i + \beta_i[\alpha_i/(1+\alpha_i)]N^{RIP-}{}_i \quad (6)$$

where the $\beta_i[\alpha_i/(1+\alpha_i)]N^{RIP-}{}_i$ term represents the number of RIP^- cells that may have divided then differentiated within the same 12-h interval [probability of differentiation β_i times the $2\alpha_i/(1+\alpha_i)$ term for probability that the differentiating cell is $BrdU^+$, with a 1/2 correction for the order of events]. Because dead cells cannot be accurately separated into RIP^+ and RIP^- cells, the model is fitted under three possible assumptions: all death is RIP^+ ($\delta = 0$); all death is RIP^- ($\varepsilon = 0$); and the death rates are equal ($\delta = \varepsilon$). The proliferation Eq. 5 and Eq. 6 were solved with the differentiation equation 2 to find values for α_i, β_i, and γ_i.

To ensure that the model for oligodendrocyte differentiation accurately represented the biological system, we compared the values predicted by the model for total cell counts (Eq. 3) and cell differentiation (Eq. 2) to direct measurements at the time points between 12 h and 2 d. No significant differences were found between direct measurements (Fig. 4, B and C, columns) and predicted values (Fig. 4, B and C; lines). The model-derived values indicated that there was no increase in the proliferation rate of RIP^- cells (α) over time, reinforcing the idea that the increased proliferation rate of oligodendrocyte progenitors was not the cause for the net increase in oligodendrocyte numbers (Fig. 4 D, blue line). The model does predict an increase in differentiation rate (β; Fig. 4 E, blue line); together with the lack of change in the proliferation rate (α), this can only be taken to mean an increase in instructive differentiation of RIP^- to RIP^+ cells by IGF-I.

The increase in instructive differentiation by itself is not enough to account for the conversion of RIP^- to RIP^+ cells. The net increase in RIP^+ cells must also include in the explanation an increase in the proliferation rate (γ) of RIP^+ cells (Fig. 4 D, red line). If the γ term was set to zero (no RIP^+ proliferation), the model predicted significantly fewer RIP^+ oligodendrocytes than were observed experimentally. Therefore, the only way to resolve the net increase in oligodendrocyte numbers according to the model is an instructive differentiation from adult multipotent neural progenitors to oligodendrocytes and a subsequent proliferation of committed oligodendrocytes in the presence of IGF-I.

IGF-I–induced oligodendrocyte differentiation is mediated through an inhibition of BMP signaling

In the developing vertebrate telencephalon and spinal cord, BMPs have been shown to act as inhibitory signals for oligodendrocyte fate specification (Gross et al., 1996; Mekki-Dauriac et al., 2002). Additional analyses have shown that oligodendrocyte lineage progression requires an active inhibition of BMP signaling (Mabie et al., 1999; Mehler et al., 2000). To gain insight into the molecular mechanism of IGF-I–induced oligodendrocyte differentiation, we asked whether IGF-I effects are mediated through an inhibition of BMP signaling. First, we tested whether BMPs could inhibit adult neural progenitor cell oligodendrocyte differentiation. Addition of BMP2 in the presence of 500 ng/ml IGF-I resulted in a suppression of oligodendrocyte differentiation, as evidenced by a reduction in RIP^+ cells compared with cultures treated with IGF-I alone (Fig. 5, A–C). Similar results were observed with BMP4 (unpublished data). This inhibition of oligodendrocyte differentiation is dose-dependent; higher concentrations of BMP2 (5–50 ng/ml) resulted in a greater suppression of oligodendrocyte differentiation, whereas partial differentiation could still be observed at lower BMP2 concentrations (0.05–0.5 ng/ml; Fig. 5 C). These results are consistent with the ability of BMPs to repress oligodendrocyte differentiation.

BMP signaling can be inhibited by extracellular proteins, such as Noggin, that bind BMP ligands in a competitive manner, interfering with the ability of ligand binding to cognate cell surface receptors (Balemans and Van Hul, 2002). If the IGF-I induction of oligodendrocyte differentiation is mediated through an inhibition of BMP signaling, the function of BMP antagonists could be involved. To test the involvement of BMP antagonists, we treated neural progenitor

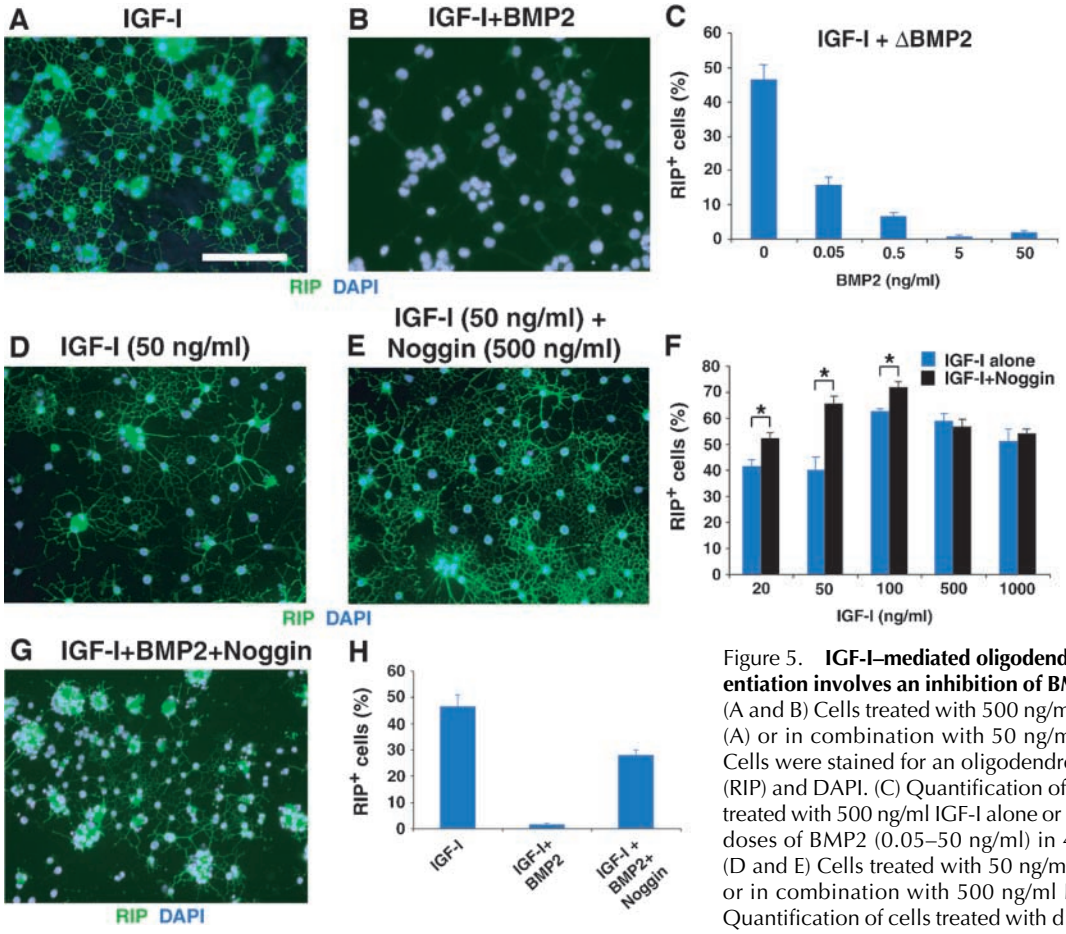

Figure 5. **IGF-I–mediated oligodendrocyte differentiation involves an inhibition of BMP signaling.** (A and B) Cells treated with 500 ng/ml IGF-I alone (A) or in combination with 50 ng/ml BMP2 (B). Cells were stained for an oligodendrocyte marker (RIP) and DAPI. (C) Quantification of RIP^+ or cells treated with 500 ng/ml IGF-I alone or with different doses of BMP2 (0.05–50 ng/ml) in 4-d cultures. (D and E) Cells treated with 50 ng/ml IGF-I alone or in combination with 500 ng/ml Noggin. (F) Quantification of cells treated with different doses of IGF-I (20–1,000 ng/ml) alone (blue columns) or in conjunction with 500 ng/ml Noggin (black columns). Significant differences are indicated with an asterisk ($P < 0.05$, *t* test). (G) Cells treated with a combination of IGF-I, BMP2, and Noggin. (H) Quantification of RIP^+ cells in IGF-I alone, IGF-I + BMP2, and IGF-I + BMP2 + Noggin. For G and H, concentrations are as follows: IGF-I, 500 ng/ml; BMP2, 50 ng/ml; Noggin, 500 ng/ml. Bar, 50 μm. All error bars represent SDs.

cells with different concentrations of IGF-I in conjunction with exogenous Noggin and compared the extent of oligodendrocyte differentiation to cultures treated with IGF-I alone (Fig. 5, D–F). When 500 ng/ml Noggin was added to cultures treated with 20–100 ng/ml IGF-I, there was a significant increase in the percentage of oligodendrocytes compared with cultures treated with IGF-I alone (Fig. 5 F; $P < 0.05$, *t* test). Addition of 500 ng/ml Noggin together with highest concentrations of IGF-I (500–1,000 ng/ml) did not result in a significant change in the percentage of RIP^+ oligodendrocytes compared with IGF-I alone (Fig. 5 F), presumably due to saturating effects of IGF-I. Next, we wanted to rule out if Noggin by itself had any effects on oligodendrocyte differentiation and/or enhanced survival. Neural progenitor cells treated with 500 ng/ml Noggin alone in insulin-free media did not display any enhanced differentiation or survival effects. Finally, addition of 500 ng/ml Noggin together with 50 ng/ml BMP2 and 500 ng/ml IGF-I efficiently reversed the inhibitory effects of BMP signaling on oligodendrocyte differentiation (Fig. 5, G and H).

If IGF-I induction of oligodendrocyte differentiation involves the inhibition of BMP signaling, a change in the expression of BMP antagonists upon IGF-I treatment might be observed. In addition to extracellular antagonists of BMP signaling, such as Noggin, intracellular proteins that can interfere with downstream BMP receptor signaling, called inhibitory Smads (Smad6, Smad7), have also been identified (Christian and Nakayama, 1999). Therefore, we performed Q-PCR analysis of cultures treated with 500 ng/ml IGF-I for 24 h and examined the relative fold change of Noggin, Smad6, and Smad7 (Fig. 6; all values were normalized to expression levels under FGF-2 conditions). Expression of Noggin and Smad6 increased by at least four- and sixfold, respectively, after IGF-I treatment. There was only a slight increase in Smad7 levels with IGF-I treatment (~1.2-fold). In each case, if IGF-I–treated cultures included 20 ng/ml FGF-2, the up-regulation of BMP antagonists was not observed, suggesting that FGF-2 can suppress the IGF-I–mediated up-regulation of Noggin and Smad6. Together, these results suggest that IGF-I–instructive effects on oligodendrocyte differentiation are mediated, at least in part, through an inhibition of BMP signaling.

Effects of IGF-I on oligodendrocytes in vivo

Our findings raised the question of whether IGF-I also has effects on oligodendrocyte differentiation in vivo in the region where adult hippocampal neural progenitor cells normally reside. Therefore, we used adeno-associated virus

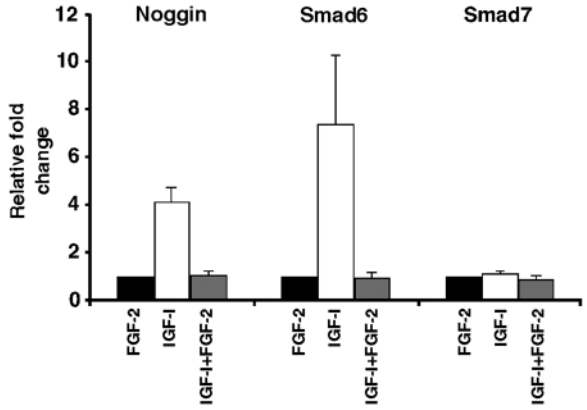

Figure 6. **IGF-I induction of oligodendrocyte differentiation is associated with an up-regulation of BMP antagonists Noggin, Smad6, and Smad7.** Q-PCR analysis and quantification of relative fold change (normalized to expression levels under FGF-2 conditions) of Noggin, Smad6, and Smad7. RNA was harvested from 24-h cultures treated with 20 ng/ml FGF-2, 500 ng/ml IGF-I, or both. All error bars represent SDs.

(AAV) to overexpress IGF-I in the adult rat hippocampus. To evaluate effects on the endogenous oligodendrocyte population due to IGF-I overexpression in vivo, we used the oligodendrocyte marker RIP. We focused our analyses on the hilar region of the dentate gyrus because there is a higher concentration of oligodendrocytes in this region. An increase of RIP staining in the hilus of rAAV-IGF-I–infected animals compared with rAAV-β-gal–infected controls was apparent (Fig. 7, A and E). Quantification of RIP immunofluorescence in the hilus of rAAV-β-gal–infected control animals resulted in an average pixel intensity of 45.49 ± 2.01 ($n = 3$ rats), compared with an average pixel intensity of 99.95 ± 18.27 ($n = 4$ rats) in rAAV-IGF-I–infected animals ($P < 0.05$, t test). Similar results were seen in at least three adjacent fields of the same section.

A similar trend was observed using MBP immunofluorescence as a marker for mature oligodendrocytes. The extent of MBP staining appeared more intense in the hilus of IGF-I–infected animals compared with control animals (Fig. 7, B and F). The average pixel intensity of MBP immunofluorescence in controls was 30.8 ± 6.84 ($n = 3$ rats) compared with an average pixel intensity of 53.53 ± 5.72 ($n = 4$ rats) in rAAV-IGF-I–infected animals ($P < 0.05$, t test). There was no observable difference in GFAP staining between control and IGF-I–infected animals, suggesting that there is not a general increase in all glial cell lineages in animals overexpressing IGF-I (Fig. 7, C and G).

Because we observed an increase in overall staining for various oligodendrocyte markers (RIP and MBP) after IGF-I overexpression in the adult hippocampus, we next determined if there was also a change in the number of oligodendrocytes. For quantification of oligodendrocytes, sections were labeled for the π isoform of GST (GST-π). GST-π has been determined in many experiments to label both immature and mature myelinating oligodendrocytes (Tansey and Cammer, 1991; Mason et al., 2001), and facilitates cell counting due to its predominant localization in oligodendrocyte cell bodies as well as in a few of the processes. The average number of GST-π–positive cells per section was 8.08 ± 2.43 ($n = 3$ rats) for rAAV-β-gal control animals and 29.14 ± 5.04 ($n = 4$ rats) for rAAV-IGF-I animals, which is a threefold difference (Fig. 7, I–K; $P < 0.001$, t test). These results support that IGF-I is a regulator of oligodendrocyte differentiation in vivo as well as in vitro.

Discussion

In this paper, we show that IGF-I, IGF-II, and insulin could all independently stimulate the differentiation of adult multipotent neural progenitor cells into oligodendrocytes. Modeling analysis reveals that IGF-I provides an instructive (rather than a selective) regulation in oligodendroglial fate choice. IGF-I–treated cultures also demonstrate an increase in the proliferation of committed oligodendrocytes. Moreover, the finding that IGF-I overexpression in the adult hippocampus leads to an increase in oligodendrocyte markers supports IGF-I effects in vitro. To our knowledge, this is the first example of a single factor that can induce the robust differentiation of multipotent neural progenitor cells into oligodendrocytes.

To address whether IGF-I effects are instructive and/or selective in nature, we used both experimental and modeling approaches. First, we addressed the question of whether IGF-I might have selective effects on cell survival. Because the frequency of cell death is low in IGF-I–treated cultures and there is no significant difference in the percentage of cells that are dying/dead at each of the time points analyzed, it is unlikely that the net increase in oligodendrocytes by IGF-I comes from the selective survival of committed oligodendrocyte progenitors that then go on to differentiate. Although there is massive cell death in the absence of IGF-I, this cell death applies widely across all types of neural progenitor cells. Treatment of cells with the broad caspase inhibitor Q-VD-OPh further reinforced the finding that IGF-I is not merely acting on cell survival. The addition of Q-VD-OPh is enough for the cells to survive, and not to proliferate or differentiate into definitive oligodendrocytes in short-term cultures; only upon addition of IGF-I is there a massive increase in oligodendrocyte differentiation.

Next, we addressed the question of selective effects on cell proliferation by IGF-I. The BrdU analyses indicate that there is not an increase in proliferation at time points where oligodendrocyte differentiation is already occurring, suggesting that a selective proliferation of progenitors does not appear to make a major contribution to the net oligodendrocyte increase. To address the possibility that a robust differentiation effect by IGF-I might mask a proliferation effect, a model was used to separate the independent contributions of IGF-I on proliferation and differentiation. The model-derived data showed an increase in the rate of oligodendrocyte differentiation and not in the proliferation rate of RIP^- progenitors. The model also showed an increase in the proliferation rate of committed oligodendrocytes in IGF-I–treated cultures. Therefore, the simplest interpretation of our data is that IGF-I acts to control the fate choice of multipotent adult neural progenitor cells to an oligodendroglial lineage, and that the net increase in oligodendrocytes by IGF-I is due to an in-

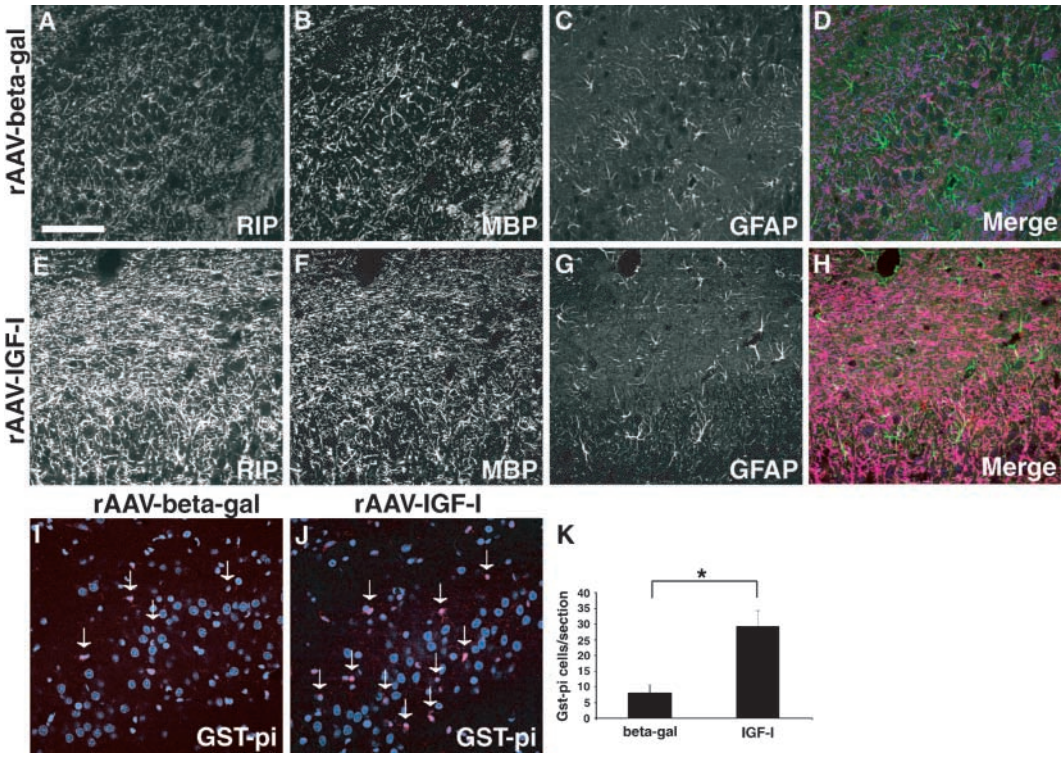

Figure 7. **IGF-I overexpression in the hilus promotes oligodendrocyte differentiation in vivo.** (A–J) Representative images of brain sections focusing in on the hilar region in animals injected with rAAV-β-gal controls (A–D and I) and rAAV-IGF-I (E–H and J). Sections were triple labeled with antibodies to oligodendrocyte markers RIP (A and E) and MBP (B and F), and an astrocyte marker GFAP (C and G). Merged images are shown in D and H; RIP is in red, MBP is in blue, GFAP is in green. (I and J) Representative sections stained with the oligodendrocyte marker GST-π in red and DAPI to visualize cell nuclei. White arrows indicate cells that are GST-π–positive. (K) The average number of cells in the hilus (in three adjacent fields distal to the injection site) per section in which GST-π was detected in each animal group (rAAV-β-gal animals, $n = 3$; rAAV-IGF-I animals, $n = 4$) is plotted. The asterisk indicates that values are significantly different between control and IGF-I–overexpressed animals ($P < 0.001$, t test), and error bars represent SDs. Bar, 100 μm.

structive differentiation and an additional proliferation of committed oligodendrocytes.

Recently, it has been reported that IGF-I can stimulate neurogenesis in the dentate gyrus (Aberg et al., 2000), as well as increase the proliferation and neuronal differentiation of EGF-responsive multipotent neural stem cells derived from E14 mouse striatum (Arsenijevic and Weiss, 1998; Arsenijevic et al., 2001). Although it is not clear whether oligodendrocyte differentiation was examined in these experiments, the possibility still remains that IGF-I promotes the differentiation of multipotent neural progenitor cells to both neuronal and oligodendrocyte lineages. In fact, our work shows that, in IGF-I–treated cultures, although the majority of the cells differentiate into oligodendrocytes, a small number of cells can differentiate into neurons. Because small numbers of cells differentiate into neurons, it was difficult to determine if the effects of IGF-I on neuronal differentiation were instructive or selective in nature, or whether a population of lineage-restricted neuronal progenitors exists that could survive and differentiate in the presence of IGF-I. Because we did not observe an increase in astrocyte differentiation with IGF-I treatment in vitro, or any apparent effects on the existing astrocyte population in vivo, it would appear that IGF-I effects on neural progenitor cells are restricted, at least in part, to oligodendrocyte and neuronal lineages. The possibility remains that IGF-I could have instructive effects on astroglial fate commitment from a multipotent progenitor cell population in vivo.

We hypothesized that oligodendrocyte differentiation of multipotent adult neural progenitor cells might use similar mechanisms as oligodendrocyte progenitors derived from the embryonic brain and spinal cord, and that IGF-I induction of oligodendrocyte differentiation might involve an inhibition of BMP signaling. Our data suggest that BMPs can repress IGF-I–induced oligodendrocyte differentiation from adult neural progenitor cells. Furthermore, addition of Noggin together with lower concentrations of IGF-I results in a greater percentage of cells that differentiate into oligodendrocytes compared with IGF-I alone, suggesting that IGF-I induction of oligodendrocyte differentiation involves the activation of BMP antagonists such as Noggin. This finding, together with the observation that there is an up-regulation of Noggin, Smad6, and Smad7 with IGF-I treatment, further reinforces the role of IGF-I in the inhibition of BMP signaling to promote oligodendrocyte differentiation. A proposed model is shown in Fig. 8. BMP signaling has been shown to alter the fate of neural progenitor cells by stimulating astroglial differentiation while inhibiting neuronal and oligodendroglial differentiation (Fig. 8 A; Mehler et al., 1997; Mabie et al., 1999; Nakashima et al., 2001). IGFs (IGF-I and -II) and/or insulin

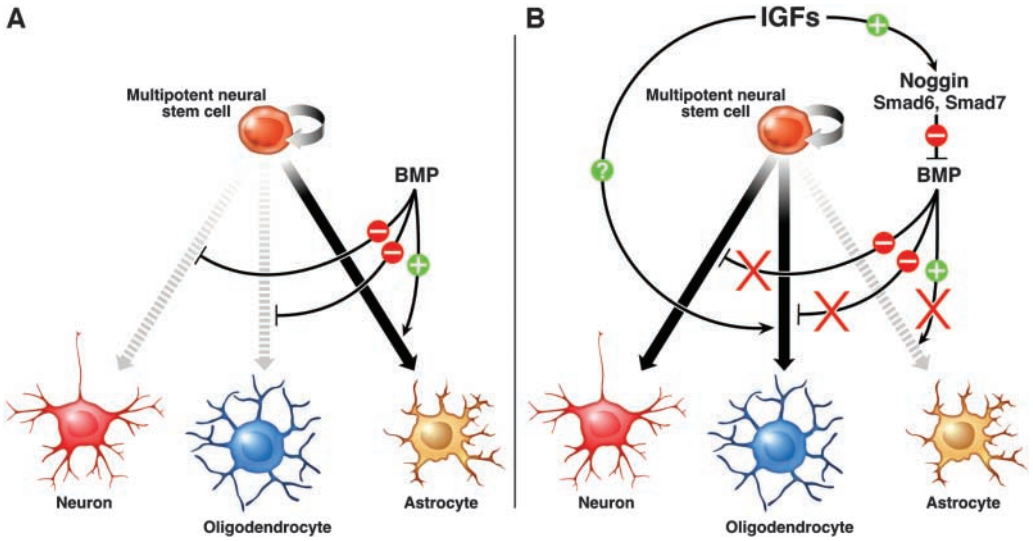

Figure 8. **Proposed model for the role of IGFs in multipotent neural progenitor cell fate specification: oligodendroglial and neuronal fate commitment at the expense of astroglial fates?** (A) BMP signaling has been shown to stimulate astroglial differentiation and inhibit neuronal and oligodendroglial differentiation. (B) Activation of the IGF-I receptor on multipotent neural progenitor cells by IGFs leads to the up-regulation of Noggin, Smad6, and Smad7. Because Noggin, Smad6, and Smad7 inhibit BMP signaling, the net effects of IGF signaling are a block in astrocyte differentiation and an induction of neuronal and oligodendroglial differentiation. Alternatively, IGF-instructive effects on oligodendrocyte differentiation could occur in a Noggin/Smad6, Smad7-independent pathway.

activate IGF-I receptors located on multipotent neural progenitor cells, which leads to the up-regulation of BMP antagonists such as Noggin, Smad6, and Smad7. Because Noggin, Smad6, and Smad7 inhibit BMP signaling, the net effects of IGF signaling are a decrease or absence of astrocyte differentiation and an increase in neuronal and oligodendroglial differentiation (Fig. 8 B). Future analyses are needed to determine whether all of the IGF-instructive effects on oligodendrocyte differentiation occur through an inhibition of BMP signaling, or if IGFs can directly promote oligodendroglial lineage commitment in a Noggin/Smad6, Smad7-independent manner.

Although IGF-I overexpression in vivo mediates an increase in oligodendrocyte markers, a finding that supports IGF-I effects on neural progenitors in vitro, there are several other possibilities that should be taken into consideration. The increase in the number of GST-π–positive cells could be due to a general increase in cell number with IGF-I overexpression in vivo; in fact, previous reports have indicated that IGF-I transgenic mice have a general increase in cell number that affects oligodendrocytes and nonoligodendrocytes equally, and that a change in the percentage of oligodendrocytes was not found (Carson et al., 1993). Further analyses are needed to determine if IGF-I affects the existing oligodendrocyte population (maturation, survival) and/or the multipotent stem or progenitor cell population (proliferation, fate choice). The differentiation of multipotent adult neural progenitor cells likely depends on a complex interplay of signals found in the local CNS environment. Our results suggest that IGFs may represent a general class of molecules that instruct multipotent neural progenitor cells to differentiate into oligodendrocytes. Future work will be directed at characterizing the mechanism of adult oligodendrocyte differentiation and the nature of an oligodendrocyte niche in different CNS environments.

Materials and methods

Cell culture and in vitro differentiation analysis

The neural progenitor cells isolated from the hippocampus of adult female Fischer 344 rats used in this work have been characterized previously (Gage et al., 1995; Palmer et al., 1997). The whole brain–derived neural stem cells from P10 ICR mice were isolated and cultured according to the methods as described, with slight modifications. Cells were cultured as described previously (Ray et al., 1993; Gage et al., 1995). Cells between passages 10 and 20 were used for in vitro differentiation analyses. To induce differentiation, cells were plated into 4-well chamber slides at a density of 55,000–75,000 cells/well and were allowed to proliferate in insulin-containing N2-supplemented (Invitrogen) DME:Ham's F12 (Omega Scientific) medium containing 20 ng/ml FGF-2 (PeproTech, Inc.) for 24 h. FGF-2 was then withdrawn and cells were subsequently treated with differentiation media. Differentiation conditions were either N2 medium with 1 μM RA (Sigma-Aldrich) and 1% FBS (Omega Scientific) for 4 d (mixed); 1 μM RA and 5 μM forskolin (Sigma-Aldrich) for 4 d (neuronal), or 50 ng/ml leukemia inhibitory factor (CHEMICON International, Inc.) and 50 ng/ml BMP2 (R&D Systems) for 6 d (astrocytic; Nakashima et al., 1999). For IGF induction experiments, cells were trypsinized, washed with 1× PBS, and plated into insulin-free N2 medium. Either 500 ng/ml human recombinant IGF-I or IGF-II (R&D Systems) or 500 ng/ml insulin (Sigma-Aldrich) was added for 4 d, except when indicated otherwise. In some cultures, 2.5 μM BrdU (Sigma-Aldrich) was added to label dividing cells, and 2 μM Q-VD-OPh (Enzyme Systems Products) was added to prevent apoptosis. Recombinant mouse Noggin (R&D Systems) was added in some cultures to a final concentration of 500 ng/ml.

Immunocytochemistry and in vitro quantification

Cells were fixed with 4% PFA, followed by immunocytochemical staining as described previously (Palmer et al., 1999). Labeled cells were visualized using an upright microscope (model E800; Nikon) or an inverted microscope (model E600; Nikon) and a CCD camera (Spot RT; Diagnostic Instruments). DAPI was used to identify individual cells. Quantification of cell phenotypes was with StereoInvestigator (MicroBrightfield Inc.); for each marker, 500–1,000 cells were sampled systematically from standardized fields at 40×. For quantification of live/dead cells, images were taken of cultures live stained with 1 μg/ml propidium iodide (Molecular Probes, Inc.) and 1 μg/ml Hoescht 33342 (Sigma-Aldrich) at 10×, and cells were counted using Image-Pro (Media Cybernetics). Primary antibodies were used as follows: rabbit anti-Tuj1 (1:7,500; Covance), mouse anti-RIP (1:25–1:75; Developmental Studies Hybridoma Bank, Iowa City, IA); guinea pig anti-GFAP (1:500–1:2,500; Advanced Immunochemical, Inc.); rabbit

anti-MBP (1:500; CHEMICON International, Inc.), mouse anti-O4 (1:2; hybridoma supernatant from cells provided by O. Boegler, University of California, San Diego, San Diego, CA), rat anti-BrdU (1:400; Accurate). Secondary antibodies were all purchased from Jackson ImmunoResearch Laboratories and used at 1:250 dilution. The detection of BrdU in cultured cells required treatment in 2N HCL at 37°C for 30 min (Palmer et al., 1999). All experiments were independently replicated at least three times.

IGF-I overexpression in vivo and quantification

cDNA encoding the human IGF-I gene was cloned into a recombinant AAV vector and the virus was prepared as described previously (Kaspar et al., 2002). Expression of IGF-I and β-gal (control AAV) was first confirmed in human embryonic kidney (HEK-293) cells by RT-PCR and Western analysis and subsequently in the hippocampus by RT-PCR analysis (unpublished data). Recombinant AAV-IGF-I (rAAV-IGF-I) or AAV-β-gal (rAAV-β-gal) was stereotaxically injected into the hippocampus (anteroposterior axis, −4.0 mm; mediolateral axis, +2.0 mm; dorsoventral axis, −3.0 from skull, with nose bar at 3.3 mm up) of anesthetized female Fischer-344 rats (150–175 g; $n = 6$). After 4 wk to allow for virus processing and transgene expression, animals were killed. Animals were perfused with 4% PFA and the brains were excised, stored in fixative overnight, and transferred to 30% sucrose. 40-μm coronal sections were cut on a sliding microtome, and sections were processed for standard immunohistochemical staining as described previously (Gage et al., 1995). Sections were triple labeled with mouse anti-RIP (1:50), rabbit anti-MBP (1:500), and guinea pig anti-GFAP (1:1,000). In some cases, sections were labeled with mouse anti-GST-π (1:100; BD Biosciences). Images were acquired using a confocal microscope (Radiance 2100; Bio-Rad Laboratories) and an inverted microscope (model TE2000; Nikon) equipped with a 40× NA 1.3 Plan Fluor objective lens. Images were post processed using Adobe PhotoShop®.

All comparative analyses were focused on the hilus of the injected side on matched hippocampal sections. For RIP or MBP staining, immunofluorescence average pixel intensity of equivalent-sized fields (four from each animal) in control and experimental groups was determined from digital images using Adobe Photoshop®. To quantify the number of oligodendrocytes, sections were immunostained with GST-π. Only immunolabeled cells with a clear DAPI-positive nucleus were scored. For quantification of GST-π cells, area counts of the hilus within the dentate gyrus of the hippocampus were performed. In each section, three adjacent fields were sampled. Cells in the uppermost focal plane were ignored, and we focused through the thickness of the section to avoid errors caused by oversampling. The results are expressed as the average number of cells per section.

Quantitative real-time PCR analysis

Total RNA was isolated from cell cultures using RNeasy columns (QIAGEN). For real-time quantitative PCR, reactions were performed essentially as described previously (Zhao et al., 2001). The relative amount of the tested message was normalized to the level of the internal control message, GAPDH. Furthermore, independent experiments were performed with a different internal control message, β-actin, and showed similar results. Primer sequences are available upon request.

Statistics

Results were analyzed for statistical significance using *t* test or by ANOVA, and all error bars (except in Fig. 3, D and E) are expressed as SDs. Post-hoc analysis was done using Bonferroni corrected planned comparison.

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