Neural precursor differentiation into astrocytes requires signaling through the leukemia inhibitory factor receptor


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ABSTRACT The differentiation of precursor cells into neurons or astrocytes in the developing brain has been thought to be regulated in part by growth factors. We show here that neural precursors isolated from the developing forebrain of mice that are deficient in the gene for the low-affinity leukemia inhibitory factor receptor (LIFR<sup>−/−</sup>) fail to generate astrocytes expressing glial fibrillary acidic protein (GFAP) when cultured in vitro. Precursors from mice heterozygous for the null allele show normal levels of GFAP expression. These findings support the in vivo findings that show extremely low levels of GFAP mRNA in brains of embryonic day 19 LIFR<sup>−/−</sup> mice. In addition, monolayers of neural cells from LIFR<sup>−/−</sup> mice are far less able to support the neuronal differentiation of normal neural precursors than are monolayers from heterozygous or wild-type animals, indicating that endogenous signaling through the LIFR is required for the expression of both functional and phenotypic markers of astrocyte differentiation. LIFR<sup>−/−</sup> precursors are not irreversibly blocked from differentiating into astrocytes: they express GFAP after long-term passaging or stimulation with bone morphogenetic protein-2. These findings strongly implicate the LIF family of cytokines in the regulation of astrocyte differentiation and indeed the LIF-deficient animals showed a significant reduction in the number of GFAP cells in the hippocampus. However, because this reduction is only partial it suggests that LIF may not be the predominant endogenous ligand signaling through the LIFR.

A number of studies indicate that astrocytes and neurons arise from a common precursor in the developing central nervous system (CNS) (1–5) and that the choice of lineage is determined, at least in part, by environmental factors such as growth factors. In vitro studies have identified several factors that promote neuronal development (2, 6), and more recently it has been shown, again in vitro, that leukemia inhibitory factor (LIF) stimulates precursors from the embryonic day 10 (E10) spinal cord to become glial fibrillary acidic protein (GFAP)-positive cells (7). In addition, this study showed that antibodies to the LIF receptor (LIFR) significantly reduced the number of astrocytes that developed in the absence of exogenous growth factors, suggesting that endogenous ligands acting through the LIFR influence astrocyte development. Other ligands that signal through the LIFR complex—a heterodimer composed of LIFR and gp130—such as ciliary neurotrophic factor, also have been shown to promote GFAP expression in CNS precursor populations (8). Thus, the in vitro results strongly suggest that ligands that signal through the LIFR complex may have a role in regulating astrocyte differentiation. We recently reported that E19 embryonic mice with a targeted disruption of the low affinity LIF receptor gene, which appear to have normal CNS development, have a deficiency of GFAP-positive cells (9), supporting the contention raised by the previous in vitro findings. Unfortunately, because these animals die at E19—which is just 2 days after the first appearance of GFAP (10)—it was difficult to determine whether this astrocyte deficiency was caused by general retardation in development or a failure in astrocyte generation caused by lack of signaling through the LIFR. To explore these possibilities further, the properties of precursor cells from the forebrain of LIFR-deficient mice have been examined in vitro. We show here that precursors from the forebrains of mice homozygous for the LIFR null mutation (LIFR<sup>−/−</sup>) fail to generate significant numbers of GFAP-positive cells even after 3 weeks in vitro and also lack the functional property of supporting neuronal differentiation and/or survival. Clonal examination reveals no apparent loss of the number of neural precursors in the LIFR<sup>−/−</sup> mice, and because precursors can be induced to express GFAP with bone morphogenetic protein-2 (BMP-2) the results strongly suggest that endogenous signaling through the LIFR is primarily required for the differentiation of precursor cells into astrocytes in the developing CNS.

**MATERIALS AND METHODS**

**Mice.** A description of the production of mice with a targeted disruption of the LIFR gene has been previously given (9). Mice were bred by mating mice heterozygous for the LIFR established from the founder line that was on the C57BL/6 × 129/J background. Genotyping was carried out as previously described (10). Mice with a targeted disruption in the LIF gene were kindly provided by Colin Stewart (Roche). All of the mice, including the CBA mice, were bred and maintained at The Walter and Eliza Hall Institute of Medical Research.

**Detection of GFAP mRNA.** Total RNA from E19 brains was purified by the acid phenol method and quantitated by denaturing agarose gel electrophoresis and ethidium bromide staining. cDNA was synthesized from 25 μg of total RNA using Superscript A II reverse transcriptase (Life Technologies/GIBCO/BRL) according to the manufacturer’s instructions. GFAP primers were 5′-AGTTACAGGAGGCACCTT-GCT-3′ and 5′-TAGCTCCAGCAGCCTGTTGAA-3′. Actin primers were 5′-CTGAAGTACCCCATTGAACATGGC-3′ and 5′-CAGAGCAGTAATCTCCTTCTGCAT-3′. For GFAP, 35 cycles of PCR were performed, and the reaction product was detected by Southern blotting with a probe derived from the above primers. For beta-actin, 25 cycles of

Abbreviations: GFAP, glial fibrillary acidic protein; LIF, leukemia inhibitory factor; LIFR, low-affinity LIF receptor; CNS, central nervous system; E, embryonic day; BMP-2, bone morphogenetic protein.

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PCR were performed, and reaction products were visualized by ethidium bromide staining.

**Neuroepithelial Cell Culture and Growth Factors.** Neuroepithelial cells were prepared from E10, E12, and E14 forebrains as previously described (11, 12). Cells were cultured in MonoMed medium (CSL, Victoria, Australia) containing 10% (vol/vol) fetal bovine serum. The recombinant growth factors FGF-2 and FGF-1 (PeproTech, Rocky Hill, NJ) were used at 50 ng/ml; recombinant mouse LIF (Amrad, Melbourne, Australia) was used at 1 ng/ml; recombinant BMP-2 (PeproTech) was used at 10 ng/ml. The heparan sulfate proteoglycan, HSPG-1, was prepared from E12 CBA mouse forebrains as previously described (13).

**Clonal Assay.** Single-cell suspensions of neuroepithelial cells were prepared and cloned into wells of HLA plates in medium containing FGF-2 as previously described (2). Wells were examined the day after plating to ensure that each well contained no more than a single cell. To promote neuronal differentiation, cells were cloned into medium containing FGF-1 and heparan sulfate proteoglycan (HSPG-1) (3, 13).

**Immunostaining.** Cultures were fixed in absolute methanol at −20°C for 20 min and then extensively washed with PBS before the application of antibody. The cultures then were incubated for 1 hr at ambient temperature with rabbit anti-150 kDa neurofilament antibody (Chemicon) used at a dilution of 1:400 or rabbit anti-GFAP antibody (DAKO) used at a dilution of 1:50. Immunoperoxidase staining was performed by using a biotinylated goat anti-rabbit IgG followed by avidin and horseradish peroxidase solutions supplied in the Elite Vectastain Kit (Vector Laboratories).

**Counting of GFAP-Positive Cells in Hippocampus of LIFR-Deficient Mice.** Adult mice of approximately 6 weeks of age were anesthetized with chloral hydrate (400 mg/kg) and killed by transcardiac perfusion with 4% buffered paraformaldehyde. Serial coronal sections of the brain were cut at 40 µm on a CO2 freezing microtome. Every third section was immunostained for GFAP. The number of astrocytes was counted in the stratum moleculare of the dentate gyrus from three separate experiments, each with three replicates. PCR were performed, and reaction products were visualized by ethidium bromide staining.

**RESULTS**

**LIFR-Deficient Mice Express Extremely Low Levels of GFAP mRNA.** Previously, we had shown by immunohistochemistry that LIFR−/− mice appeared to have few, if any, GFAP-expressing cells at the time of their death—around E19 (9). To explore this observation further we examined the levels of GFAP mRNA expressed in the brains of these animals at E19. It was found by PCR that although there were significant levels in heterozygotes, there was little detectable expression in the mice homozygous for the null mutation (Fig. 1).

**Failure of Neuroepithelial Cells from LIFR−/− Mice to Express GFAP and Support Neuronal Production in Vitro.** Because GFAP expression is not found until around E17 in the brains of normal animals (10), the low levels of GFAP mRNA at E19 could have been related to a developmental delay in GFAP expression and not to an innate failure of precursor differentiation. To examine this further, neuroepithelial cells from the forebrains of E12 littermates were cultured in vitro for periods from 10 to 20 days and examined by immunohistochemistry for GFAP expression. It was found that cell cultures from LIFR+/+ and LIFR+/− embryos both contained large numbers of GFAP-positive cells, whereas cultures from the LIFR−/− embryos, although their morphology appeared indistinguishable from wild-type cultures by phase microscopy, contained <0.01% of positive cells (Fig. 2).

To determine whether the lack of GFAP expression in LIFR−/− precursors fully reflected a failure in astrocyte development, an assay to assess astrocyte function was performed. Previously, it has been shown that astrocytes promote neuronal differentiation and/or survival in a number of systems (14, 15); thus, the ability of established monolayers to support the neuronal differentiation and survival of E14 forebrain cells from CBA mice was tested. The assay was carried out on monolayers obtained from E12 littermates’ forebrains, which had been passaged at least three times to ensure that there were few residual neurofilament-positive neurons present on the monolayers (<2 per monolayer). There was no significant difference found between the number of

**Fig. 1.** Low levels of GFAP mRNA in LIFR−/− brain. mRNA was prepared from the brain, including forebrain, midbrain, and hindbrain of E19 littermates, and the presence of GFAP mRNA was determined by PCR.

**Fig. 2.** Failure of LIFR−/− neural cells to express GFAP. Neuroepithelial cells from E12 forebrain were plated in vitro at a density of 2.5 × 10⁴ per 200 mm² into multwell plates (Falcon 3047) and cultured for 20 days in the presence of serum. Cultures then were stained by immunoperoxidase for the presence of GFAP. The LIFR+/+ cultures contained large numbers of GFAP-positive cells (A and C) whereas the LIFR−/− cultures (B and C) contained few if any (<2 cells per well in every case). The data shown in C are the mean and SEM obtained from three separate experiments, each with three replicates.
Monolayers of cells were obtained from E12 forebrain cells and grown for either 20 or 60 days in vitro before being tested for their ability to support the generation of neurons from freshly isolated E14 CBA forebrain cells. Data represent the mean ± SEM of six replicates for each value.

*Statistical significance of \( P < 0.001 \) using Student’s \( t \) test.

neurons produced on the LIFR\(^{+/+}\) or LIFR\(^{+/-}\) monolayers, however, there were approximately 10-fold fewer neurons found on the LIFR\(^{-/-}\) monolayers (Table 1).

**Precursors Express GFAP After Long-Term Passage or Stimulation with BMP-2.** To determine whether the astrocyte precursor population in the LIFR\(^{-/-}\) forebrain was permanently blocked from differentiating, or was indeed present, forebrain cultures were stimulated with BMP-2, a member of the transforming growth factor \( \beta \) family previously shown to stimulate GFAP expression in astrocytes (16). Under these conditions cultures from E12 LIFR\(^{-/-}\) mice were found to contain a significant percentage of GFAP-positive cells after 10 days in vitro, which was not significantly different from that found in LIFR\(^{+/+}\) or LIFR\(^{+/-}\) cultures (Table 2). However, it did not approach the level of GFAP expression found in LIF-stimulated LIFR\(^{+/-}\) cultures where over 90% of cells expressed GFAP after 10 days in vitro (Table 2). It also was found that after >6 passages in vitro (>5 weeks in vitro) there were significant numbers of GFAP cells appearing in LIFR\(^{-/-}\) cultures (data not shown). In addition, when tested for their ability to support neuron generation and/or survival, long-term cultured cells from the LIFR\(^{-/-}\) monolayers performed as well as the LIFR\(^{+/+}\) monolayers (Table 1).

**Clonal Examination of Precursor Levels in the LIFR Mutant.** To determine whether the decrease in astrocyte generation in the absence of the LIFR could be accounted for by a decrease in the overall number of precursors cells found in the developing forebrain, neuroepithelial cells from E10 forebrain were plated out at clonal density as previously described (2) in developing forebrain, neuroepithelial cells from E10 forebrain and the total number of the presence of FGF-2—which we have shown previously to be required for this signal (17). This finding raises the possibility that although GFAP expression is directly regulated by LIFR other astrocytic characteristics may not be. This study shows that one of the best-described functions of astrocytes—ability to promote neuron differentiation and survival—also is greatly reduced in LIFR\(^{-/-}\) precursor populations.

The loss of neuron-promoting function raises a paradox because it appears that neuronal generation is normal in the LIFR mice and, as we have shown here, the number of precursor clones that give rise to neurons is not diminished in these animals. Closer examination of the CNS of E18–19 LIFR\(^{-/-}\) mice shows, however, that there is a degree of disruption of the neuropil in areas where early astrocyte development occurs, predominantly in the brainstem and spinal cord with many neurons showing vacuolation and pyknotic nuclei (9). This finding suggests that neurons are

Table 1. Neuronal support provided by LIFR\(^{-/-}\) neural cells

<table>
<thead>
<tr>
<th>LIFR genotype</th>
<th>Numbers of neurofilament positive cells, days in vitro</th>
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<tbody>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td>(-/-)</td>
<td>45 ± 15*</td>
</tr>
<tr>
<td>(+/-)</td>
<td>420 ± 26</td>
</tr>
<tr>
<td>(+/-)</td>
<td>467 ± 34</td>
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</tbody>
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*Statistical significance of \( P < 0.001 \) using Student’s \( t \) test.

A total of 10\(^3\) wells were examined for each condition, and the data represent mean and SEM obtained from three experiments.

Table 3. Clonal analysis of LIFR\(^{-/-}\) forebrain precursors

<table>
<thead>
<tr>
<th>LIFR genotype</th>
<th>Total clones(^*)</th>
<th>Neuronal clones(^†)</th>
</tr>
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<tbody>
<tr>
<td>(+/-)</td>
<td>54.3 ± 10.4</td>
<td>29.4 ± 5.4</td>
</tr>
<tr>
<td>(+/-)</td>
<td>48.2 ± 9.5</td>
<td>32.8 ± 4.9</td>
</tr>
<tr>
<td>(-/-)</td>
<td>45.7 ± 6.3</td>
<td>28.3 ± 8.0</td>
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\( \times \)Number of clones generated in the presence of FGF-2.

\( \times \)Number of clones with one or more neurons generated in the presence of FGF-1 and HSPG-1.

Reduced from 15.2 ± 12.2 per clone in the LIFR\(^{+/+}\) forebrains to 3.1 ± 1.5 per clone in the LIFR\(^{-/-}\) forebrains.

**LIF-Deficient Animals Also Have Fewer GFAP-Positive Astrocytes.** Although mice that are LIF gene-deficient show no overt signs of damage or deficiencies in the nervous system, we undertook a detailed count of GFAP numbers in the hippocampus of these mice and found a significant decrease in the number of GFAP-positive cells compared with wild-type controls, particularly in the area of the dentate gyrus. The percentage of cells expressing GFAP in a defined area of the dentate gyrus was reduced from 30.4% ± 3.1 in wild type to 19.8% ± 2.6 in the LIF-deficient animals. Seven animals were examined for each genotype, and the results were expressed as the SEM and the difference was significant, \( P < 0.01 \) using a two-tailed \( t \) test.

**DISCUSSION**

This study provides evidence in support of the concept that signaling through the LIFR is one of the most important regulatory steps in vivo controlling the differentiation of neural precursor cells into astrocytes. Previously, in vitro studies had implicated the LIFR ligands, LIF and ciliary neurotrophic factor, as stimulators of astrocyte differentiation (7, 8), but because molecules such as BMP-2 (16) and interferon \( \gamma \) (17), also stimulate the appearance of GFAP astrocytes in vivo, the in vivo importance of LIFR stimulation was unclear. The only previous in vitro experiment that suggested that signaling through the LIFR was a key endogenous mechanism was the demonstration that antibodies that blocked the LIFR function also significantly reduced the number of astrocytes appearing in cultures devoid of exogenous growth factors (7).

The present study also shows that signaling through the LIFR is required for the generation of functional astrocytes not just for the expression of GFAP. This is an important point, because it recently has been shown that one of the downstream signaling pathways activated by signaling through LIFR—the JAK-STAT pathway—can directly activate the GFAP gene. It has been shown that STAT 3 can directly bind to a consensus site in the promoter region of the GFAP gene (17). Thus, the regulation of GFAP expression can be regulated directly through the LIFR complex—both LIFR and gp130 appear to be required for this signal (17). This finding raises the possibility that although GFAP expression is directly regulated by LIFR other astrocytic characteristics may not be. This study shows that one of the best-described functions of astrocytes—ability to promote neuron differentiation and survival—also is greatly reduced in LIFR\(^{-/-}\) precursor populations.

The loss of neuron-promoting function raises a paradox because it appears that neuronal generation is normal in the LIFR mice and, as we have shown here, the number of precursor clones that give rise to neurons is not diminished in these animals. Closer examination of the CNS of E18–19 LIFR\(^{-/-}\) mice shows, however, that there is a degree of disruption of the neuropil in areas where early astrocyte development occurs, predominantly in the brainstem and spinal cord with many neurons showing vacuolation and pyknotic nuclei (9). This finding suggests that neurons are
undergoing degeneration in these animals because of a lack of functional astrocytes. In addition, we found that the number of neurons generated per clone was significantly reduced in the LIFR<sup>-/-</sup> mice, supporting the idea that they lacked trophic factors produced by astrocytes. Also of interest is the finding that LIFR<sup>-/-</sup> mice show a significant decrease in the number of motor neurons. Because these are the first neurons born in the spinal cord such a loss may be associated with a subsequent lack of trophic support by surrounding glia. Preliminary studies from our laboratory show that the loss in LIF<sup>-/-</sup> mice does indeed occur after motor neuron formation consistent with a lack of trophic support provided by surrounding cells (P.F.B. and R. Dutton, unpublished observations).

The demonstration that precursor populations are extant in LIFR<sup>-/-</sup> mice and can be stimulated to differentiate into GFAP-positive cells through a different ligand-activated pathway with BMP-2 provides support for the concept that the primary function of signaling through the LIFR is not to promote precursor survival but to stimulate astrocyte differentiation. Previous studies have shown that LIF had no affect on mature astrocyte survival or proliferation, re-enforcing the idea that the action of LIF is primarily focused on promoting astrocyte differentiation (7). The finding that there is no decrease in the total number of neural clones generated from the LIFR<sup>-/-</sup> mouse forebrain precursors with FGF-2 also strongly suggests that LIFR signaling is not essential for the maintenance of precursor cells. Previous studies have shown that FGF-2-stimulated forebrain precursors have the ability to generate two types of clones: clones that contain both neurons and glia, or clones restricted to astrocytes (2, 12). Because the frequency of neuron-containing clones generated with FGF-1 and heparan sulfate proteoglycan (HSPG-1)—we previously have shown that these clones are produced by bipotential precursors (3)—also is unaltered in the LIFR<sup>-/-</sup> population, it suggests that there is no change in the relative frequency of either the bipotential or astrocyte-restricted clones in these animals.

The question arises as to whether signaling through the LIFR instructs a precursor to become committed to the astrocyte pathway. Several pieces of evidence support such an hypothesis: first, it has been shown that in the presence of LIF>80% of precursors become GFAP-positive in vitro (ref. 7 and Table 2); second, that STAT-3, which is directly activated by LIFR signaling, can bind to the promoter region of the GFAP gene and regulate its expression; and third, that stimulation with LIF or ciliary neurotrophic factor can significantly inhibit neuronal differentiation (ref. 17; P.F.B., unpublished observations). The last result suggests that precursors with a propensity to become neurons can be redirected down another lineage by signaling through the LIFR. The only way to answer this question unequivocally, however, is to directly monitor a cohort of precursors and to determine the fate of all of their progeny and show that precursors that can become neurons under one condition become astrocytes when stimulated through the LIFR.

We have shown in this study that LIF-deficient animals also have fewer GFAP-positive cells in one area examined, the hippocampus. This finding suggests that LIF may be an important ligand in this process, however, it suggests that there are other ligands that either act in concert with or are capable of substituting for LIF. Also, other ligand-receptor pathways may replace LIF at later stages of development. The finding that long-term cultures from LIFR mice ultimately do start to express GFAP and are functionally active supports this idea as do recent experiments in which portions of LIFR<sup>-/-</sup> brains were transplanted to a syngeneic recipient and shown to contain GFAP cells several weeks after transplantation (P.F.B. and A.R. Harvey, unpublished observations).

In addition to its role in astrocyte differentiation, signaling through the LIFR has been shown in vitro to regulate several aspects of neuronal differentiation in both the CNS (18) and peripheral nervous system (19, 20). To date, however, the only observable deficit in this lineage found in LIFR<sup>-/-</sup> mice is in the motor neuron pool. Whether further close examination will reveal subtle changes in these systems is unclear, but from this study it would seem that the major function of signaling through the LIFR during early neural development of the CNS is to regulate the production of astrocytes.

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