Misexpression of basic helix-loop-helix genes in the murine cerebral cortex affects cell fate choices and neuronal survival

Li Cai, Eric M. Morrow and Constance L. Cepko*

Department of Genetics, Howard Hughes Medical Institute, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115, USA

*Author for correspondence (e-mail: cepko@genetics.med.harvard.edu)

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SUMMARY

To investigate the role(s) of basic helix-loop-helix genes (bHLH) in the developing murine cerebral cortex, Mash1, Math2, Math3, Neurogenin1 (Ngn1), Ngn2, NeuroD, NeuroD2 and Id1 were transduced in vivo into the embryonic and postnatal cerebral cortex using retrovirus vectors. The morphology and location of infected cells were analyzed at postnatal stages. The data indicate that a subset of bHLH genes are capable of regulating the choice of neuronal versus glial fate and that, when misexpressed, they can be deleterious to the survival of differentiating neurons, but not glia.

Key words: bHLH, Neuron, Glia, Telencephalon, Cell Fate, Cell Death, Mouse

INTRODUCTION

In the mammalian central nervous system (CNS) the two major classes of cells, neurons and glia, play fundamentally different roles. Neurons are the functional units that receive, process and transmit information through synapses, while glial cells serve as supporting elements, providing structural and metabolic support. In vivo lineage analyses using retroviral vectors (Leber and Sanes, 1995; Turner and Cepko, 1987; Walsh and Cepko, 1992) and experiments using in vitro cultures (Levison and Goldman, 1997; Qian et al., 1997) indicated that both neurons and glia can be generated by common progenitor cells. In the cerebral cortex, some retrovirally infected embryonic progenitors were observed to give rise to both neurons and glia (Walsh and Cepko, 1992), while postnatal progenitors were found to give rise only to glial cells (Levison and Goldman, 1993). This latter finding was predicted from the known birth order of neurons and glia, as neurons have not been found to be produced postnatally using the classic method of 3H-thymidine labeling (Angevine and Sidman, 1961; Bayer and Altman, 1991; Caviness, 1982). The lack of production of neurons by postnatal progenitor cells can be interpreted as a lack of competence to make neurons and/or as a lack of support for neuronal production.

Some insight into the molecular mechanisms that underlie the determination and differentiation of neurons versus glia is beginning to emerge. In Drosophila, glial cells fail to differentiate when the gene, glial cells missing (gem), is mutated; when ectopically expressed, GCM causes many presumptive neurons to be transformed into glia (Hosoya et al., 1995; Jones et al., 1995). In the mammalian CNS the cytokine, ciliary neurotrophic factor, can induce the formation of glia in cultures of embryonic cortical cells (Bonni et al., 1997). The basic helix-loop-helix (bHLH) transcription factors are also known to be important in CNS development, but the precise roles are still being defined. Mash1 mutant mice show a severe loss of progenitors in the subventricular zone (SVZ) of the medial ganglionic eminence (Casarosa et al., 1999; Horton et al., 1999) and Math1 is required for the formation of cerebellar granule cells (Ben-Arie et al., 1997). bHLH genes were found to induce ectopic neurons when introduced into early Xenopus embryos by microinjection (Brown et al., 1998; Ferreiro et al., 1994; Kanekar et al., 1997; Lee et al., 1995; Ma et al., 1996; McCormick et al., 1996; Olson et al., 1998; Sharma et al., 1999; Sommer et al., 1996; Takebayashi et al., 1997). Transient expression of neural bHLH proteins can convert mouse P19 embryonal carcinoma cells into differentiated neurons (Farah et al., 2000). These studies showed that different bHLH genes had differing capacities to elicit ectopic neurons. In the case of Xath3 (Ferreiro et al., 1994), additional neurons were found only in the areas undergoing primary neurogenesis, whereas in the cases of X-NGNR-1 (Ma et al., 1996) and NeuroD (Lee et al., 1995), induced neurons were found in the area of primary neurogenesis as well as in non-neurogenic regions. The types of neurons formed in these assays were not studied in detail, nor was an effect on gliogenesis reported. We and others examined the role of bHLH genes in retinal development. Overexpression of Xath5 (Kanekar et al., 1997), Math5 and Mash1 (Brown et al., 1998) in Xenopus and of NeuroD in rat retina (Morrow et al., 1999) resulted in a severe reduction in Müller glial cells and more subtle alterations in the ratios of neuronal cell types. Mutant mice lacking NeuroD (Morrow et al., 1999) and Mash1 (Tomita et al., 1996) showed an increase in Müller glial cell production. Thus, bHLH genes appear to be involved in the neuron versus glial cell fate choice and in the choice of neuronal subtypes among neurons in the retina.
The cerebral cortex of the mammalian CNS comprises diverse neuronal and glial cell types, and exhibits expression of multiple bHLH genes during development (Anderson et al., 1997; Lee, 1997; Ma et al., 1997). As the roles of bHLH genes in cortical cell fate choice and differentiation have not been explored, we set out to examine this issue. Using a replication-incompetent retroviral vector, we misexpressed bHLH proteins in vivo in the developing mouse cerebral cortex to address the following questions. (1) Is the blockade of gliogenesis observed in the retina a general rule for bHLH activity in the CNS? (2) Do neural progenitor cells at different stages of development in a particular tissue respond to bHLH proteins differently?

MATERIALS AND METHODS

Animals and retroviral injections
Timed-pregnant Swiss Webster mice and Sprague-Dawley rats were purchased from Taconic (Germantown, NY, USA), and maintained on a 12 hour/12 hour (7:00 am to 7:00 pm) light/dark schedule. The day on which a vaginal plug was detected was designated as embryonic day 0 (E0). By this convention, gestation usually lasts 19 days for mouse and 21 days for rat. Retinal injections were performed as previously described (Morrow et al., 1999). Surgical procedures used for the embryonic injection of the retrovirus vector into the lateral ventricles of fetal mouse brains have been described elsewhere (Cai et al., 1997). For postnatal injection into the SVZ of the cerebral cortex, newborn pups were anesthetized by immersion on ice for 5-10 minutes; 0.5 μl of retroviral stock was injected unilaterally with a 5 μl Hamilton syringe. Coordinates of the injection sites (relative to bregma) were anterior 0.5-0.8 mm and lateral 1.5-2.0 mm, at a depth of 1.5-2.0 mm. Pups were revived by warming prior to being returned to their mother. For embryonic mice, injections were made early (E11/E12) and at mid-gestation stages (E15). For the study of postnatal animals, injection was on postnatal day 1 (P1)/P2 and P6.

Tissue processing and histochemistry
Embryonically injected animals were killed at P7, P14, P21 and P28; postnatally injected animals were killed at P14, P21, P28 and P60. The tissue was processed for human placental alkaline phosphatase (PLAP) and/or β-galactosidase activity as described previously (Cepko et al., http://axon.med.harvard.edu/~cepko/protocol/xgalplapstain.htm). The number, morphology and location of PLAP+ cells in the cerebral cortex were analyzed.

Construction and generation of replication-incompetent retroviral vectors
Blunt-ended fragments encoding bHLH genes were ligated in pLIA (Bao and Cepko, 1997) at the SnaBI site. Coding fragments were as follows: NeuroD2, a PstI fragment from pSK-1.1.1 (McCormick et al., 1996), Neurogenin1/NeuroD3, a PstI-EcoRI fragment from pSK-15A1a (McCormick et al., 1996), Neurogenin2, a Nra-BsoI fragment p30R containing the mouse genomic clone (Sommer et al., 1996), Id1, an EcoRI-EcoNI fragment from EMCSV-Id1 (gift from A. Lassar, unpublished), Mash1, a SacI-SalI fragment from pCla12Nco, Math2, a HindIII-SpeI fragment from pCMV-Math2 (Shimizu et al., 1995), Math3, an Xmal-SphI fragment from pBS-Math3 (Takebayashi et al., 1997), LIA-NeuroD as previously described (Morrow et al., 1999). To generate virus, transfections into a subline of 293T cells (Dr Martine Roussel, St Jude Children’s Research Hospital, Memphis, USA) utilizing calcium phosphate was used (Cepko et al., 1998) and supernatant was collected every 12 hours, starting at 24 hours post-transfection. Stocks were concentrated and titered as described (Cepko et al., 1998).

Immunohistochemistry and TUNEL assay
For immunohistochemistry, cryosections of infected brains were fixed with 4% paraformaldehyde for 20 minutes and blocked in TNB blocking buffer (NEN™ Life Science Products), and processed for immunohistochemistry with anti-GFAP (mouse monoclonal, 1:400, Sigma), anti-NeuN (mouse monoclonal, 1:200, Chemicon); anti-MASH1 (mouse monoclonal, 1:1, and rabbit polyclonal; 1:1000, generous gifts from Dr Jane Johnson); anti-NeuroD (goat polyclonal, 1:100) and anti-PLAP (mouse monoclonal; 1:4000, Sigma; rabbit polyclonal, 1:50, Dako). For TUNEL assay, a Genzyme NeuroTACSTM in situ Apoptosis Detection Kit (R&D Systems) was used to label cells that are undergoing apoptosis. Cryosections of LIA-βHLH infected brains were fixed with 4% paraformaldehyde for 30 minutes. Endogenous peroxidase activity was quenched using hydrogen peroxide, and the manufacturer’s instructions were followed to detect apoptic cells. Signals were amplified by a TSA™-Direct tyramide signal amplification Kit (NEN™ Life Science Products).

RESULTS

Positive regulatory bHLH genes block gliogenesis in the retina
Previous work in the retina had shown that introduction of NeuroD into the postnatal rodent retina resulted in a lack of Müller glia in the infected clones. We wished to investigate whether this was a general property of the bHLH genes that are predicted to positively regulate transcription, some of which are expressed in several CNS locations, including the cerebral cortex. To this end, a series of LIA (Bao and Cepko, 1997) retroviral vectors were made in which each encoded a bHLH gene and the histochemical reporter gene, human placental alkaline phosphatase (PLAP). LIA uses the viral LTR to direct the expression of both genes, with translation of PLAP under the control of an IRES. Each virus was introduced into the P0 rat retina and the resulting clones scored following histochemical detection of PLAP in mature retinai. It is straightforward to identify retinal cells in sections stained for PLAP activity by observing their laminar location and morphology (Morrow et al., 1999; Turner and Cepko, 1987). As shown in Table 1, no clones with Müller glia were observed following transduction of Mash1, Math2, Math3, Ngn1, Ngn2, NeuroD, and NeuroD2. In control clones infected with the LIA virus, however, approximately 7% of clones had at least one Müller glial cell. These data indicate that many, and perhaps all, positive regulatory bHLH genes block gliogenesis in the retina. This includes Ngn1 and Math2, which are not normally expressed in the retina, but which are in the cortex. These genes also include members of the bHLH genes that are different subfamilies of positive regulators (e.g., Math2, NeuroD, Ngn1 and Ngn2 are in the atonal subfamily; Mash1 is in the achaete-scute subfamily).

Positive regulatory bHLH genes do not block gliogenesis in the postnatal cerebral cortex
Previous lineage analyses of the rat cerebral cortex demonstrated that over 99% of the cells labeled by retroviral injection of postnatal progenitors were glial cells (Levison and Goldman, 1993). This progenitor cell pool thus provides an ideal system to begin to test whether the blockade of gliogenesis by bHLH genes as seen in the retina is a general rule throughout the CNS. Control retroviruses LIA or BAG (encoding β-galactosidase) were injected into the area of...
Day of glial cell(s) clones scored with glia acidic protein (GFAP). Confocal microscopy revealed that PLAP and an antibody to a glial-specific marker, glial fibrillary acidic protein (GFAP), subjected to double immunohistochemical staining with anti-PLAP and anti-NeuroD antibodies (Fig. 3A-C). NeuroD expression was observed in a majority of glial cells, including both astrocytes and oligodendrocytes (arrowhead in Fig. 1A). As with the retina, it is straightforward to identify cells as neurons or glia; however, it was sometimes difficult to count each astrocyte as they sometimes appeared in tight clusters of several cells. In these cases, we estimated the number of astrocytes in a group. It was straightforward to count the number of neurons.

Control LIA infected postnatal progenitor cells generated the two types of glial cells, astrocytes (arrowhead in Fig. 1A) and oligodendrocytes (arrow in Fig. 1A,B). A total of 1405 retrovirally labeled cells from nine LIA-infected brains were analyzed. All PLAP+ cells were glia. Their morphology was similar to the retrovirally infected cells described in previous studies (Levison and Goldman, 1993; Walsh and Cepko, 1992).

In contrast to the results observed in retina, infection of the cerebral cortex with a LIA-bHLH retrovirus (Mash1, Math2, Math3, NeuroD, NeuroD2, Ngn1 and Ngn2) at P1/P2 resulted in a majority of glial cells, including both astrocytes and oligodendrocytes (Fig. 1C,D). For LIA-Ngn1, an average of 89% of the cells were glial, out of a total of 719 retrovirally labeled cells from five animals. The labeled cells displayed normal glial cell morphology compared to the LIA- or BAG-labeled cells from five animals. The labeled cells displayed 89% of the cells were glial, out of a total of 719 retrovirally labeled cells from nine LIA-infected brains were analyzed. All PLAP+ cells were glia. Their morphology was similar to the retrovirally infected cells described in previous studies (Levison and Goldman, 1993; Walsh and Cepko, 1992).

Table 1. Misexpression of positive regulating bHLH genes blocks glial cell fate in vivo in rat retina

<table>
<thead>
<tr>
<th>Day of infection</th>
<th>Retrovirus</th>
<th>Number of clones with glial cell(s) (sum of trials)</th>
<th>Number of clones scored (sum of trials)</th>
<th>% of clones with glia (mean ± s.e.m.)</th>
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<tr>
<td>P0</td>
<td>LIA</td>
<td>89</td>
<td>1278</td>
<td>7.34±0.89*</td>
</tr>
<tr>
<td></td>
<td>NeuroD</td>
<td>0</td>
<td>1533</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>NeuroD2/NDRF/KW8</td>
<td>0</td>
<td>750</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Math2/Nex1</td>
<td>0</td>
<td>750</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ngn1/NeuroD3</td>
<td>0</td>
<td>750</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mash1</td>
<td>0</td>
<td>750</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Math3</td>
<td>0</td>
<td>326</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ngn2/Math4A</td>
<td>0</td>
<td>318</td>
<td>0</td>
</tr>
<tr>
<td>P7</td>
<td>LIA</td>
<td>95</td>
<td>813</td>
<td>11.51±0.40*</td>
</tr>
<tr>
<td></td>
<td>NeuroD</td>
<td>0</td>
<td>455</td>
<td>0*</td>
</tr>
</tbody>
</table>

Neonatal rat littermates were injected in vivo at P0 with LIA-NeuroD, LIA-NeuroD2, LIA-Ngn1, LIA-Ngn2, LIA-Mash1, LIA-Math2 and LIA-Math3. Mature retinae were harvested at 4-6 weeks of age, and processed for PLAP activity. The cellular composition of clones (clonally related cells forming radial clusters) was scored after reconstruction.

*Data from Morrow et al. (1999).

proliferating cells, the SVZ and the lateral ventricle in the murine forebrain at P1/P2. Injected animals were harvested at P14, P21, P28 or P60. Infected cells were identified on the basis of the PLAP or β-galactosidase histochemical stain and the cell types were determined on the basis of their morphology (Fig. 1A,B). As with the retina, it is straightforward to identify cells as neurons or glia; however, it was sometimes difficult to count each astrocyte as they sometimes appeared in tight clusters of several cells. In these cases, we estimated the number of astrocytes in a group. It was straightforward to count the number of neurons.

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A subset of positive regulatory bHLH genes stimulate neurogenesis in the postnatal cerebral cortex

Infection at P1/P2 with some LIA-bHLH viruses (LIA-Ngn1, LIA-Ngn2 and LIA-Mash1), but not others (Math2, Fig. 1D; NeuroD, Fig. 1C; NeuroD2 and Math3, data not shown) led to the observation of clones with neurons. In LIA-Ngn1 infected animals, about 10% of the labeled cells (72 of 719) had a typical neuronal morphology (Fig. 4). These neurons appeared to be interneurons, i.e. stellate or basket cells, as judged by their dendritic arborization (Fig. 4E-J) and comparison with embryonically generated normal neurons following LIA infections (Fig. 4A-D); no pyramidal or other neuronal cell types were seen. To confirm the neuronal identity of these LIA-Ngn1 infected cells, double immunohistochemical staining with anti-PLAP and anti-NeuN (a neuronal nuclear marker) was performed on LIA-Ngn1 infected tissue sections (Fig. 2D-F). Some of these LIA-Ngn1 infected cells expressed NeuN (Fig. 2D-F), indicating their neuronal identity.
Among the 72 PLAP+ neurons, 84% (61 of 72) were found in layers II/III and 16% (11 of 72) in layer IV (Fig. 4E). No LIA-Ngn1 infected neurons were found in layers V or VI, suggesting that the ability of a bHLH to elicit neuronal fates was limited. To test whether the ability to produce neurons in response to some bHLH genes persisted into a slightly later stage, infections were also carried out at P6. Neuronal cells were again only found in LIA-Ngn1, LIA-Ngn2 and LIA-Mash1 infected brains, but with a slightly lower percentage (approximately 5% of PLAP+ cells, data not shown).

The neuron versus glia ratio is not significantly altered by the introduction of positive regulatory bHLH genes into embryonic cortical progenitors

In order to determine if cortical progenitor cells from an earlier age might respond differently from postnatal progenitor cells to the bHLH genes, infections were also carried out at E11/E12 and E15. Infected tissue was examined at P7, P14, P21 and P28. Consistent with previous studies (Reid et al., 1995; Walsh and Cepko, 1992), LIA labeled various types of neurons and glia, including pyramidal neurons (arrowhead in Figs 4A,
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bHLH affects neuronal cell fate and survival

A,B), stellate interneurons (arrows in Figs 4A, 5A,B), and oligodendrocytes and astrocytes (arrows in Fig. 4D). Among the PLAP+ cells, an average of 81% (1669 neurons of a total of 2071 infected cells from nine animals) had a neuronal cell morphology and 19% had a glial cell morphology. Control LIA infected cells were similar to those seen in Golgi stained preparations (Cajal, 1995; Werner et al., 1985), and the laminar positions of the PLAP+ cells were as predicted by birthdating studies carried out in mice; that is, they were found in all six layers of the cerebral cortex. When LIA-Ngn1 was introduced into the E12 lateral ventricles, the same overall distribution of cells was seen similar to those seen in Golgi stained preparations (Cajal, 1995; Werner et al., 1985), and the laminar positions of the PLAP+ cells were as predicted by birthdating studies carried out in mice; that is, they were found in all six layers of the cerebral cortex. When LIA-Ngn1 was introduced into the E12 lateral ventricles, the same overall distribution of cells was seen as when LIA was introduced, if the tissue was examined at P7 (Fig. 5). When LIA-Ngn1 was injected at E15, LIA-Ngn1 labeled neurons were only found in the more superficial layers (Fig. 5D,E), as predicted by birthdating analysis. Ngn1 thus does not alter the overall neuronal or glial production by embryonic cortical progenitor cells. Among the LIA-Ngn1, LIA-Ngn2 and LIA-Mash1 infected neuronal population, however, there was an alteration in the ratios of the subtypes of neurons (data not shown).

**Misexpression of positive regulatory bHLH genes induces neuronal degeneration**

When animals (n=6) infected with LIA-Ngn1 at P1/P2 were examined at P60, neuronal cells were no longer found. Since Ngn1 is normally only expressed between E8.5 and E15.5, it seemed possible that sustained expression of Ngn1 might induce neuronal degeneration. Since few neurons were produced following infection of postnatal tissue, it was difficult to address the fate of infected neurons using postnatal infections. We thus

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**Fig. 4.** Transduction of Ngn1 into postnatal cortex led to clones containing neurons and glia. (A–D) PLAP+ cells infected with control retrovirus LIA at P21 from an E12 injection (for comparison with embryonically infected normal neurons with neurons induced by bHLH genes in the postnatal experiments). (A) A pyramidal neuron (arrowhead) in layer V of the cerebral cortex and two interneurons (arrows) in layers IV and V, respectively. (B) An interneuron in layer III. (C) An interneuron in layer V. (D) An interneuron in layer IV (arrowhead) and two astrocytes in layer IV (arrows). (E–J) PLAP+ cells infected with LIA-Ngn1 at P1/P2 injection and harvested at P21. (E) Three interneurons (arrows) in layer II/III and one in layer IV and a cluster of LIA-Ngn1 labeled glial cells in layer I (arrowhead). (F) A higher magnification of the neuron shown in E. (G) Camera lucida drawing of the neuron shown in F. (H,I) LIA-Ngn1 labeled neuron in layer III. (J) Camera lucida drawing of the neuron shown in I. (K) Histogram showing the frequency of neurons in clones of LIA and LIA-Ngn1 infected cells. The x axis represents the control LIA (1405 PLAP+ cells from nine animals) and LIA-Ngn1 groups (719 PLAP+ cells from five animals) and the y axis represents the percentage of PLAP+ cells that were neurons. Bars, 100 μm (E); 100 μm (A–D, F–G); 50 μm (H–J).
turned to embryonic infections to investigate whether sustained expression of positive regulatory bHLH genes in neurons might induce death. LIA-Ngn1 was injected into the lateral ventricles at E12 or E15 and the tissue analyzed at P7, P14, P21 and P28. At P14, neurons infected with a LIA-Ngn1 virus appeared to be undergoing degeneration (Fig. 5E,H). Dendrites and axons of the PLAP+ neurons were found fragmented (arrow in Fig. 5E). Neuronal cell bodies were irregular and condensed compared to those of LIA-infected cells, which had a round or pyramidal shape. By P21, virtually no retrovirally labeled cells with a neuronal morphology could be found, and only glial cells remained (Fig. 5I). This result was observed following transduction of each of the bHLH genes (Mash1, Math2, Math3, Ngn1, Ngn2, NeuroD and NeuroD2) that we have examined.

To confirm that sustained expression of bHLH proteins induces neuronal cell death, double-labeling experiments were performed to detect in situ apoptosis and PLAP antigen (Fig. 6, Table 2). Animals were injected with LIA (n=4) or LIA-Mash1 (n=4) at E12 and brain tissue was harvested at P4. The majority of the infected cells showed typical neuronal (Fig. 6A-D) or glial cell (Fig. 6E) morphology, although many of the neurons appeared to be undergoing degeneration, as shown in Fig. 5. TUNEL+ nuclei were detected in 3.8% of LIA infected neurons, and 51% of LIA-Mash1 infected neurons (Fig. 6C,D,F, Table 2); however, LIA-Mash1 infected glial cells did not show a significant change in the percentage of apoptotic cells compared to LIA infected glial cells (Fig. 6E,F, Table 2).

Misexpression of the HLH protein, Id1, blocks neurogenesis

The negative regulatory gene, Id1, was introduced into progenitor cells in the VZ at E12, and PLAP+ cells were examined at E16 (data not shown), P7 and P21. For all three stages analyzed, LIA-Id1 labeled cells were found to be exclusively glial cells (Fig. 7A,C). No labeled cells with neuronal morphology were observed even when the tissue was examined at E16 (data not shown). The number of glial cells in these brains was quite high, higher than would have been expected if the infection had resulted in the usual production of neurons that subsequently died. LIA-Id1 also was injected into the cerebral cortex at P1/P2 and the infected brains were examined at P14 and P28. LIA-Id1 labeled cells were again found to be exclusively glial cells, with typical glial cell morphologies (Fig. 7B,D).
DISCUSSION

We have conducted a series of experiments that reveal that bHLH genes can have different effects on the neuron versus glial cell-fate choice in different regions of the CNS. Our results also point to differences among progenitor cells, and/or among extrinsic cues that contribute to the behavior of progenitor cells, in their response to bHLH genes.

We began our experiments in order to test whether most or all bHLH genes are capable of blocking gliogenesis in the retina. It was not clear a priori how similarly the different bHLH genes would perform in one CNS area, particularly those that are normally not expressed in the retina. We found that all positive regulatory bHLH genes tested were able to block gliogenesis in the retina, including those that were not expressed in the retina and including bHLH genes from different subfamilies. We then wanted to test whether this was a general rule throughout the vertebrate CNS, as very little is known concerning the choice of neuronal versus glial fate in the CNS. Even fundamental questions, such as whether there is a hierarchy of decisions that determine, for example, whether a cell first decides to be a neuron or a glial cell, and then which type of neuron or glial cell, is unknown. Work in *Drosophila* suggests that a common mechanism might operate throughout the CNS (Hosoya et al., 1995; Jones et al., 1995). Loss-of-function mutations in the nuclear protein, *glial cell missing* (GCM), were found to lead to loss of glia throughout the *Drosophila* CNS. Conversely, when overexpressed, *gcm* caused cell-fate transformations from neuron to glia (Hosoya et al., 1995; Jones et al., 1995). Interestingly, the excess neurons formed in the loss of function scenario, or the excess glia formed in the gain of function scenario, were not 'generic' neurons or glia. At each location where excess neurons or glia were present, the appropriate type of neuron or glial cell was found. There might then be a common mechanism for the neuron versus glial cell-fate decision that is working in concert with positional information, and/or cell-cell interactions, that direct the precise cell-fate decisions. The fact that all positive

<table>
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<tr>
<th>Animal</th>
<th>LIA</th>
<th>LIA-MASH1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP+ cells</td>
<td>188</td>
<td>112</td>
</tr>
<tr>
<td>TUNEL+ cells</td>
<td>206</td>
<td>121</td>
</tr>
<tr>
<td>%</td>
<td>79</td>
<td>36.92</td>
</tr>
</tbody>
</table>

Two sets of four pregnant mice were injected at E12 with LIA (1-4) and LIA-Mash1 (A-D), respectively. Infected brain tissue was harvested at P4 and processed for PLAP and TUNEL assay.

%, TUNEL+ cells as a percentage of AP+ cells.

Data from this table is also presented in a histogram in Fig. 6F.

Table 2. TUNEL+ cells in LIA and LIA-Mash1 infected brain tissue

![Fig. 6. TUNEL assay of infected cells in the cerebral cortex. Infected brain tissue was double stained using the TUNEL assay and immunohistochemistry for PLAP. (A,B) Examples of LIA infected neurons. Anti-PLAP staining showed typical neuronal morphology; no TUNEL signal was detected. (C,D) Examples of LIA-Mash1 infected neurons. An infected pyramidal neuron (C) and a bipolar neuron (D) were detected with anti-PLAP antibody; the cytoplasm and dendritic arborization or processes are in red. The TUNEL signal was detected in the nucleus, as shown in green. (E) A cluster of three glial cells were detected with anti-PLAP antibody; no TUNEL signal was detected. (F) Quantitation of TUNEL+ cells in infected tissue at P4 (means ± s.d.). Empty bars and red bars represent control LIA cells and LIA-Mash1-infected cells, respectively. Cells that were not clearly neuronal or glial due to ambiguous morphology are also indicated. This quantitation is also presented in Table 2. Bar, 50 μm.
1000 PLAP+ cells were scored from a total of 4-6 different animals. For each experimental group, more than Id1 neurogenesis. (A,C) E12 and (B,D) P1 injection of LIA-Id1 100 protein, Bar, 100 µm. However, misexpression of the negative regulatory HLH gliogenesis in the embryonic or postnatal cerebral cortex. Several positive regulatory bHLH genes did not block (Brown et al., 1998; Kanekar et al., 1997). In mouse led to production of excess Müller glia and bipolar cells. The overexpression of NeuroD favors formation of amacrine and rod cells over bipolar cells (Morrow et al., 1999). The overexpression of NeuroD most likely reflects the normal role for this protein, as targeted mutation of this gene in the mouse led to production of excess Müller glia and bipolar cells. In Xenopus, misexpressed bHLH genes also gave a blockade of gliogenesis and differing effects on neuronal composition (Brown et al., 1998; Kanekar et al., 1997).

In contrast to the results in the retina, misexpression of several positive regulatory bHLH genes did not block gliogenesis in the embryonic or postnatal cerebral cortex. However, misexpression of the negative regulatory HLH protein, Id1, did lead to a complete lack of neurons among the labeled cells. We interpret this result as a block of neurogenesis. It is unlikely that the lack of neurons was due to neuronal death as the number of glial cells following infection with LIA-Id1 was far greater than the number normally found following infection with a comparable titer of a LIA or BAG control viruses (current work; Reid et al., 1995; Walsh and Cepko, 1992). From the Id1 result, it appears that bHLH genes can play a role in the neuron versus glial fate decision. Perhaps one or more positive regulatory bHLH genes that we did not misexpress are the critical bHLH genes in the cerebral cortex that are negatively impacted by expression of Id1. Since the complete collection of bHLH genes expressed at different times in the development of cerebral cortex is undoubtedly not yet known, we could not test all such genes. However, we did test genes (Mash1, Math2, Math3, Ngn1, Ngn2, NeuroD, and NeuroD2) that are expressed in the embryonic cerebral cortex. As shown in the misexpression assay, all of these genes can block gliogenesis in retinal tissue. The fact that none of the bHLH genes tested blocked gliogenesis in the cerebral cortex but did in the retina suggests that there is more specific and/or complex regulation of the neuron versus glial cell fate decision in different areas of the CNS.

Consistent with the idea that a positive regulatory bHLH gene promotes neurogenesis over gliogenesis (Anderson et al., 1997; Farah et al., 2000; Kageyama et al., 1997; Lee, 1997), and consistent with their expression pattern in the embryonic VZ of the cerebral cortex when neurons are normally produced (Fode et al., 2000; Gradwohl et al., 1996; Ma et al., 1997), misexpression of Ngn1, Ngn2 and Mash1 in P1/P2 cortical progenitor cells resulted in the production of neurons. These bHLH genes can induce neurons with a typical interneuron morphology (Fig. 2). The majority of these neurons were located in the more superficial cortical layers (II/III). This is of interest since these layers are the latest to be generated during the late embryonic period. Thus, perhaps similarly to the case of overexpression of GCM in Drosophila, the extra neurons produced may be appropriate for their site and time of genesis.

One interpretation of the production of neurons by postnatal progenitor cells is that postnatal cortical progenitor cells are multipotent with respect to their ability to make both neurons and glia. Alternatively, these cells are not multipotent, but a subset of these cells can respond to the transduced bHLH gene by becoming multipotent. The first interpretation is supported by in vitro culture experiments using postnatal tissue, which showed the production of both neurons and glia (Levison and Goldman, 1997; Qian et al., 1997). Nonetheless, Levison and Goldman (1993) showed in their lineage study of the postnatal cerebral cortex that <1% of the cells produced after infection with control retroviruses were neurons. It seems likely then that at least a subset of postnatal progenitors can reveal their competence to make neurons when a positive regulatory bHLH gene is introduced and that, during normal postnatal development, extrinsic cues instruct these multipotent SVZ progenitors to make only glia.

Not all positive regulatory bHLH genes induced the formation of neurons by postnatal progenitor cells. This activity was shown by Ngn1, Ngn2 and Mash1, but not by NeuroD, NeuroD2, Math2 and Math3. The genes with this activity are the bHLH genes that are expressed in early progenitors of the VZ, between E8.5 and E15.5, whereas the ones that do not have this activity are expressed in later stage cells that appear to be differentiating (see review in Lee, 1997). Despite the fact that the genes with the activity are normally expressed early, when deep layer pyramidal neurons are being produced, however, only late neuronal cell types, the superficial interneurons, were
produced following misexpression postnatally. Postnatal progenitors might be restricted to make only such cells, or the environment of the postnatal cortex might be directing the choice of the type of neuron produced. Taken together, our findings indicate that both embryonic and postnatal progenitor cells make the neuronal cell types appropriate to the age of infection when a bHLH gene is introduced.

When the positive regulatory bHLH genes were misexpressed in the retina, the retinal neurons appeared identical to control infected neurons, and did not show any difficulties with survival. We were thus surprised to see that sustained expression of positive regulatory bHLH genes in the cerebral cortex led to a loss of labeled neurons. Following infection of embryonic progenitors, the loss of PLAP\(^{+}\) neurons occurred between P4 and P21, the period when neurons are forming synaptic connections (Dori et al., 1996; Dori and Parnavelas, 1989; Markram et al., 1997). There are at least three possible explanations for this result. First, sustained expression of a particular bHLH protein may disturb neuronal development/survival. Second, neurons that are perhaps generated in excess due to the introduction of a bHLH gene may unable to access appropriate neurotrophins, synaptic targets, etc. and third, it is possible that infected neurons lost expression of PLAP (Halliday and Cepko, 1992). We favor the interpretation of neuronal death for three reasons. First, neurons infected with the control LIA retrovirus, which uses the same transcription regulatory sequences as the LIA-bHLH virus, maintained their expression. Second, during the period of neuronal loss, the PLAP\(^{+}\) neurons exhibited the classical morphological hallmarks of degenerating neurons (Fig. 5E,H). Fragmented processes and abnormally shaped cell bodies have been taken as evidence of degeneration axotomy in multiple systems (Jessell, 1991). Third, TUNEL\(^{+}\) cells, indicative of apoptotic death, were frequently observed among the LIA-bHLH infected neuronal cells, but not the glial cells. Finally, a recent publication by Isaka et al. (1999) showed that overexpression of Math1 (a bHLH gene) in transgenic mice led to neuronal cell loss even at embryonic time periods. The reasons for death following viral transduction are not known at present, but may result from the sustained expression of genes that are normally downregulated during or following differentiation. It is important to take note of this finding when considering strategies to direct cell-fate choices among engrafted neural stem cells. Induction of neuronal fate, particularly neuronal fates appropriate to an engraftment site, by the introduction of genes that induce neuronal fates, would be a reasonable strategy. However, for such a strategy to yield long lived neurons, it may be necessary to use an inducible gene expression system such that the gene used for neuronal induction can be turned off.

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