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

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SUMMARY

To investigate the role(s) of basic helix-loop-helix genes (bHLH) genes 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 [^{3}H]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 environmental 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* (*gcm*), 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/xgalplap-stain.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 *Sna*BI site. Coding fragments were as follows: *NeuroD2*, a *Pst*I fragment from pSK-1.1.1 (McCormick et al., 1996), *Neurogenin1*/*NeuroD3*, a *Pst*I-EcoRI fragment from pSK-15A1a (McCormick et al., 1996), *Neurogenin2*, a *Nru*I-*Bsa*I fragment p30R containing the mouse genomic clone (Sommer et al., 1996), Id1, an *Eco*RI-*Eco*NI fragment from EMCSV-Id1 (gift from A. Lassar, unpublished); *Mash1*, a *Sac*I-*Sal*I fragment from pCla12Nco, *Math2*, a *Hin*dIII-*Spe*I fragment from pCMV-*Math2* (Shimizu et al., 1995), *Math3*, an *Xma*I-*Ssp*I 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 NeuroTACS™ in situ Apoptosis Detection Kit (R&D Systems) was used to label cells that are undergoing apoptosis. Cryosections of LIA-bHLH 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 retinae. 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 infection 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

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

Day of infection	Retrovirus	Number of clones with glial cell(s) (sum of trials)	Number of clones scored (sum of trials)	% of clones with glia (mean ± s.e.m.)
P0	LIA	89	1278	7.34±0.89*
	NeuroD	0	1533	0*
	NeuroD2/NDRF/KW8	0	750	0
	Math2/Nex1	0	750	0
	Ngn1/*NeuroD*3	0	750	0
	Mash1	0	750	0
	Math3	0	326	0
	Ngn2/Math4A	0	318	0
P7	LIA	95	813	11.51±0.40*
	NeuroD	0	455	0*

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.

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-infected cells (Fig. 1A,B). To confirm that cells with a glial morphology expressed a glial marker, some infected cells were subjected to double immunohistochemical staining with anti-PLAP and an antibody to a glial-specific marker, glial fibrillary acidic protein (GFAP). Confocal microscopy revealed that some of the LIA-*Ngn1* infected cells expressed GFAP (Fig. 2A-C). To confirm that virally infected cells did express the introduced bHLH proteins, infected sections were labeled with anti-PLAP and anti-NeuroD (Fig. 3A-C) or anti-PLAP and anti-MASH1 antibodies (Fig. 3D-F). Detection of double labeled cells indicates that the virally infected cells expressed both the histochemical marker, PLAP (in red, Fig. 3A,C,D,F), and the bHLH proteins (in green, Fig. 3B,C,E,F).

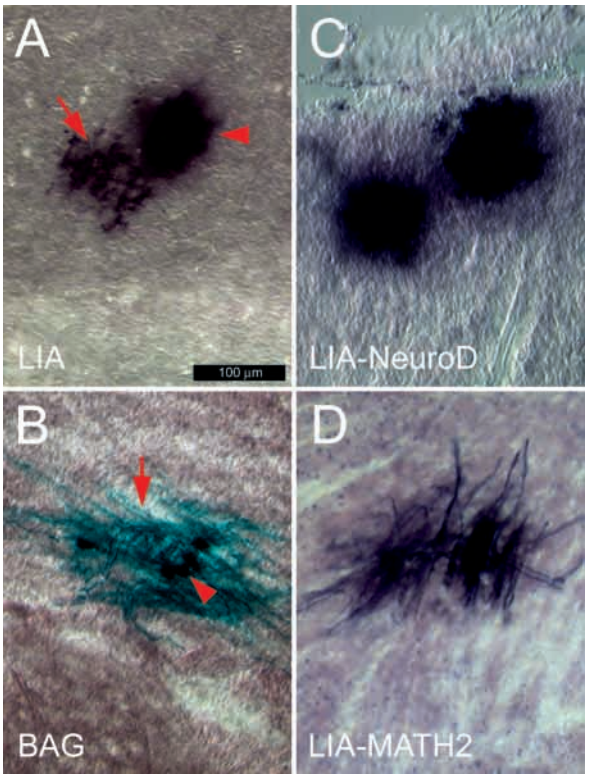

Fig. 1. Misexpression of bHLH genes did not block gliogenesis in the postnatal cerebral cortex. Retroviruses were injected into the SVZ/VZ at P1/P2 in mouse. Brain tissue was harvested and processed for PLAP/β-galactosidase activity at P14, P21, P28 and P60. (A) Control retrovirus LIA labeled an oligodendrocyte (arrow) and an astrocyte (arrowhead) in the grey matter of the deep layers of the cerebral cortex at P21. (B) Control retrovirus BAG labeled white matter oligodendrocytes at P14; an $Xgal^+$ myelinating sheath wrapping the axons of neurons is shown (arrow) and the nuclei of the oligodendrocytes are obvious (arrowhead). (C) LIA-*NeuroD* labeled astrocytes in the grey matter of the superficial layers of the cerebral cortex at P21. (D) LIA-*Math2* labeled white matter oligodendrocytes at P14. Compared to the LIA-infected astrocyte in A or BAG labeled oligodendrocytes in B, LIA-*NeuroD* or LIA-*Math2* labeled glial cells showed no morphological differences. Bar, 100 μm.

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 maker) 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.

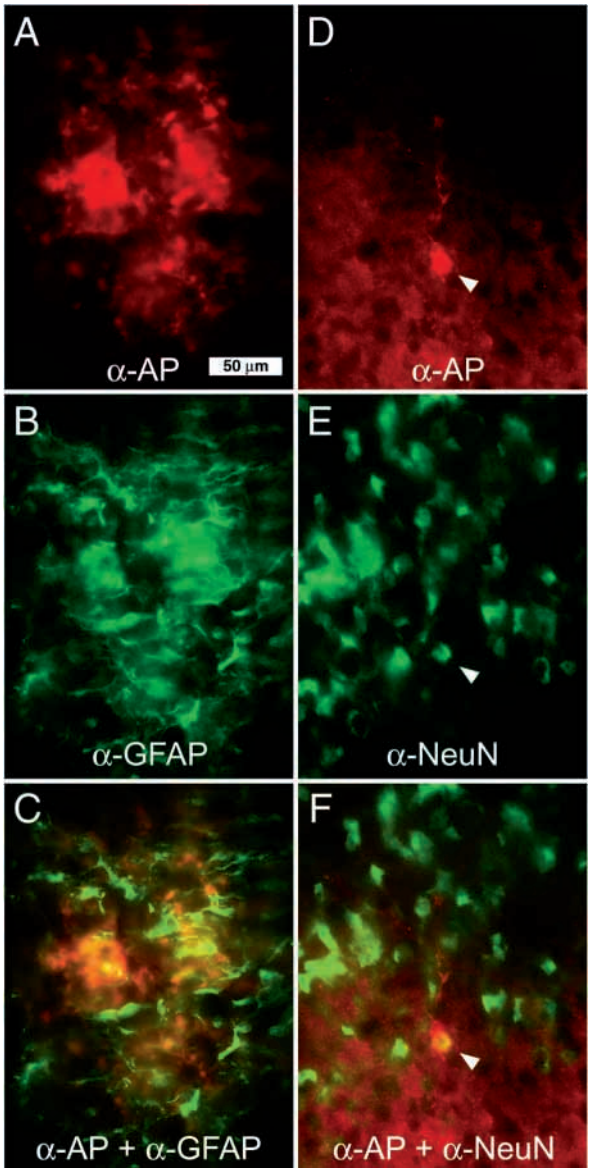

Fig. 2. Immunohistochemical identification of infected glia and neurons. LIA-*Ngn1* was injected at P1 and infected brains were harvested at P21. (A-C) Double immunohistochemical staining with anti-PLAP (red in A) and anti-GFAP (green in B). Double labeled glial cells are shown in yellow (C). (D-F) double immunohistochemical staining with anti-PLAP (red in D) and anti-NeuN (nuclear staining in green in E); a double labeled neuron is shown in yellow (F). Bar, 50 μm.

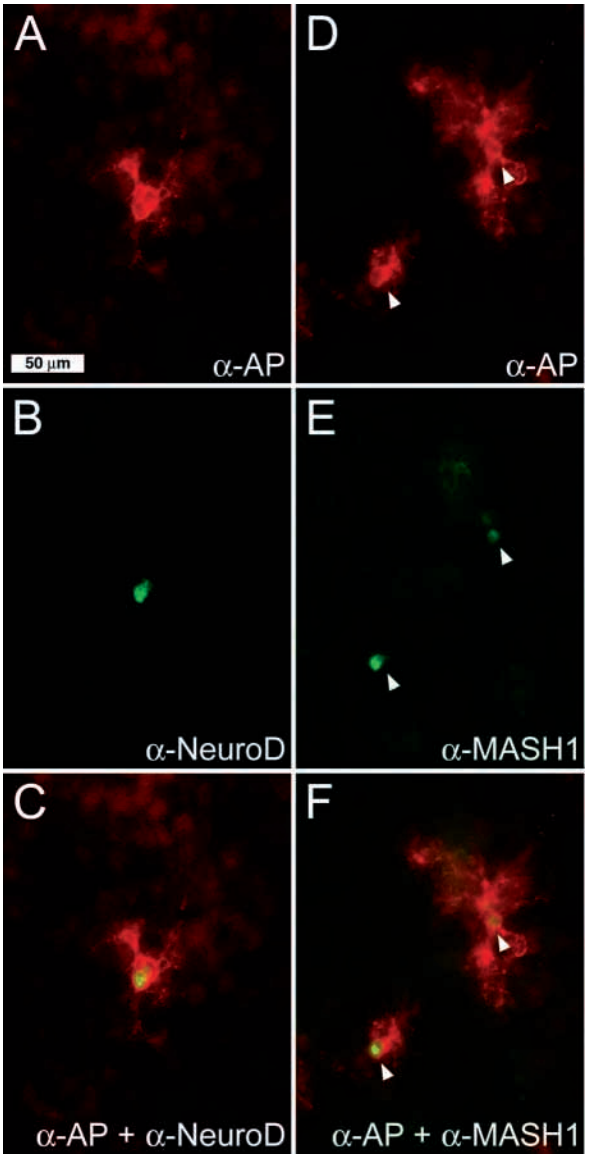

Fig. 3. Cells infected with bHLH retroviral vectors express both the transduced bHLH protein and the retroviral marker protein, PLAP. Retroviruses were injected either postnatally at P1 (A-C) or embryonically at E12 (D-F). Double immunohistochemical staining of LIA-*NeuroD* (A-C) or LIA-*Mash1* (D-F) infected brain sections at P7 with anti-PLAP and anti-*NeuroD* or anti-*Mash1* antibodies. Anti-PLAP stained the processes of one (A) or two (D) developing glial cells (arrowheads) shown in red and anti-*NeuroD* (B) or anti-*Mash1* (E) stained the nuclei in green; (C,F) double labeling. Bar, 50 μm.

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,

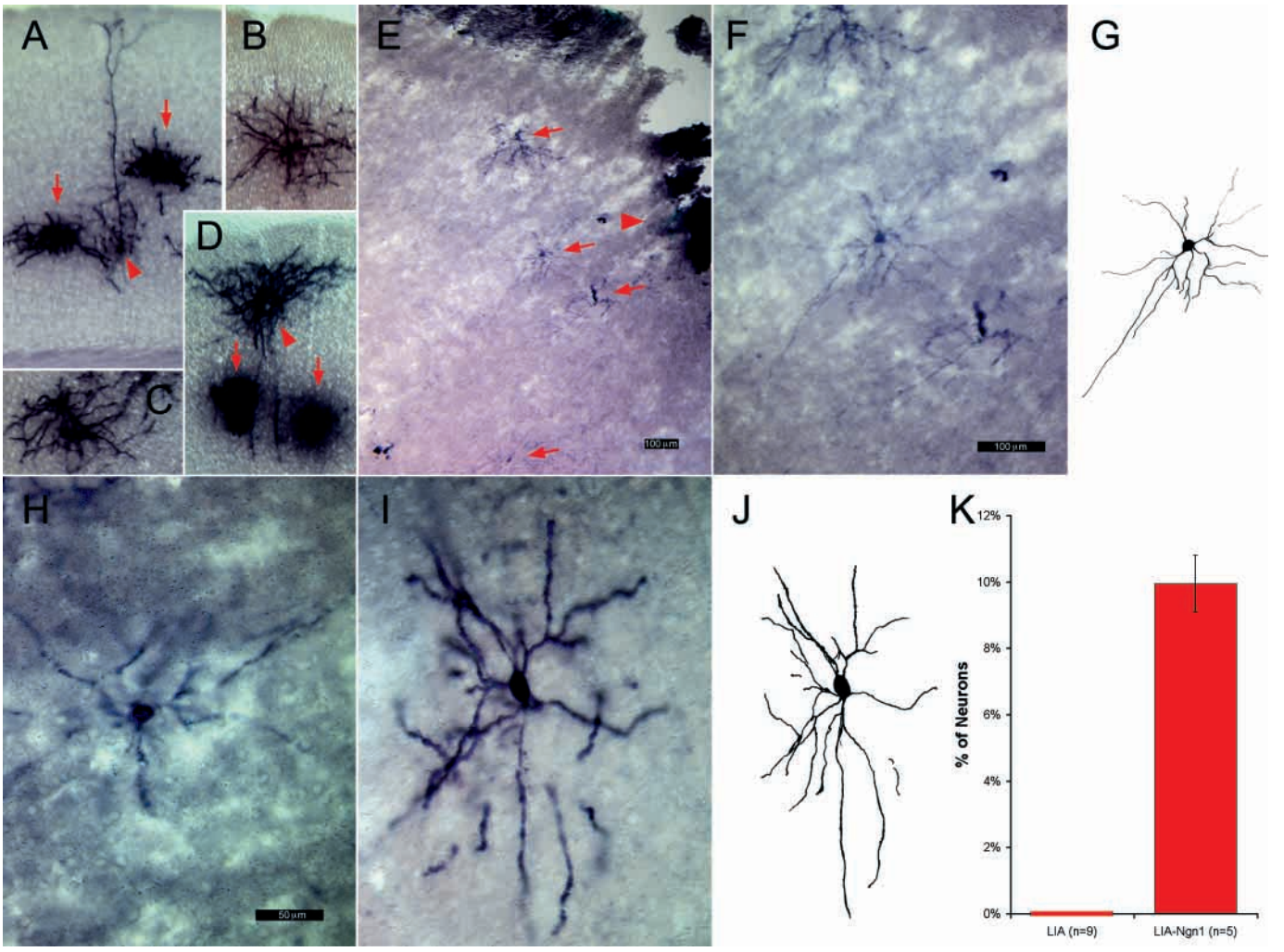

Fig. 4. Transduction of *Ngn1* into postnatal cortex led to clones containing neurons and glia. (A-D) PLAP$^+$ cells infected with control retrovirus LIA at P21 from an E12 injection (for comparison with embryonically infected normal neurons with neurons induced by bHLH genes in the postnatal experiments). (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 II (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 in the middle of 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).

5A,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 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

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. 5F,I). 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$^+$ nuceli 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).

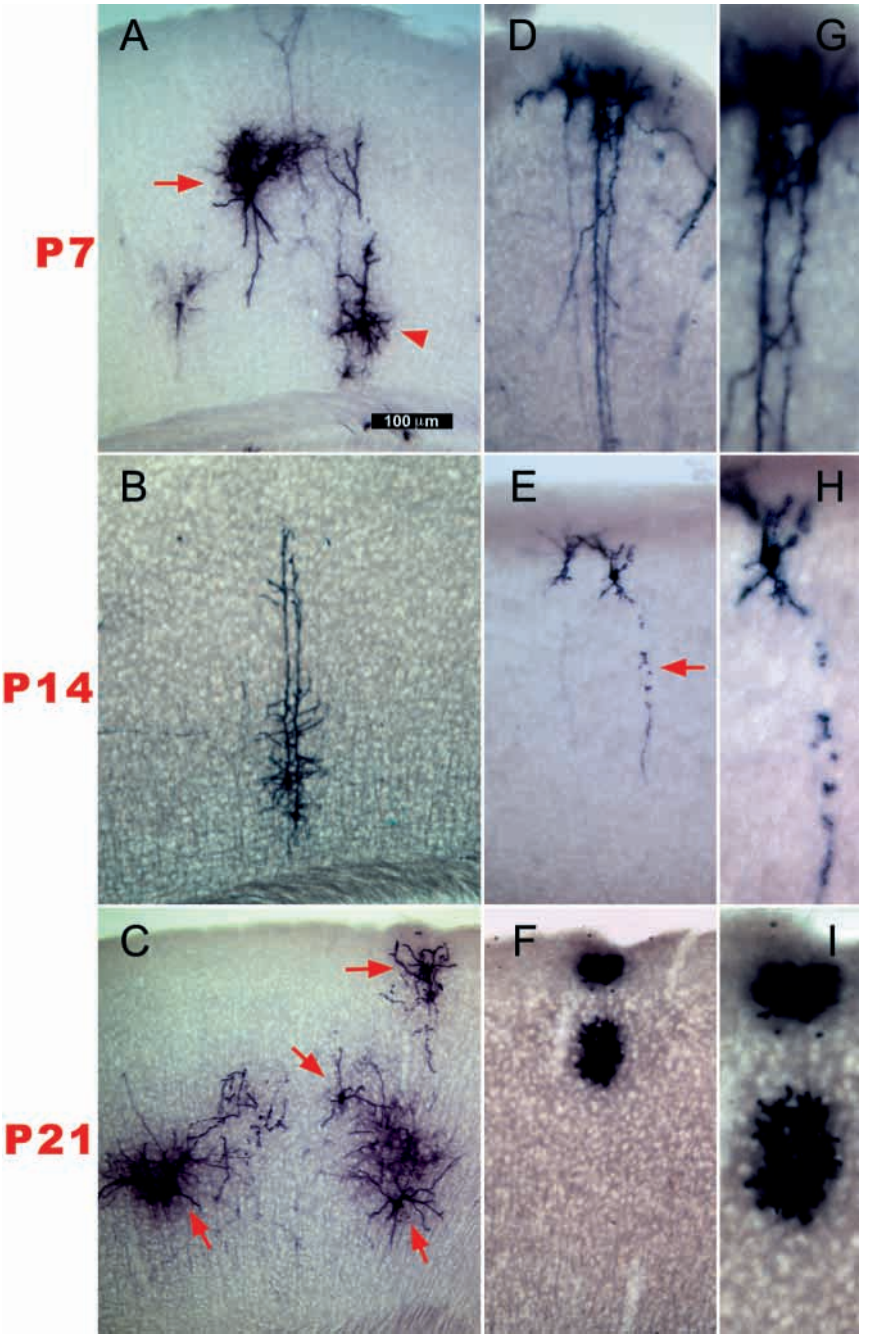

Fig. 5. Transduction of positive regulatory bHLH genes induces neuronal degeneration. PLAP$^+$ cells at P7 (A,D,G), P14 (B,E,H) and P21 (C,F,I) with control retrovirus LIA injection at E12 (A-C) and LIA-*Ngn1* at E15 (D-I). In LIA infected animals, PLAP$^+$ neurons were observed with normal morphology at all harvest times (A-C). (A) A stellate neuron (arrow) in layers III and a pyramidal neuron (arrowhead) in layer V at P7. (B) Two pyramidal neurons in layer V/VI with normal spiny dendrites at P14. (C) Four interneurons with stellate/basket cell morphology (arrows) distributed in layers II to V at P21. In LIA-*Ngn1* infected animals, PLAP$^+$ neurons appeared to be undergoing progressive degeneration (D-E). (D) A cluster of pyramidal neurons with relatively normal axons and dendrites at P7. (E) A pyramidal neuron appeared to be undergoing degeneration with a fragmented axon (arrow) at P14. (F) A cluster of glial cells at P21. PLAP$^+$ neurons were not observed at P21 or a later stage. (G,H and I) are higher magnifications of (D,E and F), respectively. Bar, 100 µm (A-F); 50 µm (G-I).

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

LIA				LIA-MASH1			
Animal	AP$^+$ cells	TUNEL$^+$ cells	%	Animal	AP$^+$ cells	TUNEL$^+$ cells	%
		Neurons			Neurons		
1	254	8	3.15	A	188	112	59.57
2	267	15	5.62	B	179	87	48.60
3	179	7	3.91	C	206	121	58.74
4	211	5	2.37	D	214	79	36.92
Subtotal	911	35	3.76	Subtotal	787	399	50.96
		Glia			Glia		
1	185	11	5.95	A	155	11	7.10
2	221	7	3.17	B	198	5	2.53
3	179	4	2.23	C	281	17	6.05
4	169	9	5.33	D	167	12	7.19
Subtotal	754	31	4.17	Subtotal	801	45	5.71
		Unclassified cells			Unclassified cells		
1	53	4	7.55	A	81	7	8.64
2	66	4	6.06	B	101	9	8.91
3	54	7	12.96	C	87	3	3.45
4	86	3	3.49	D	115	6	5.22
Subtotal	259	18	7.51	Subtotal	384	25	6.55

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.

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

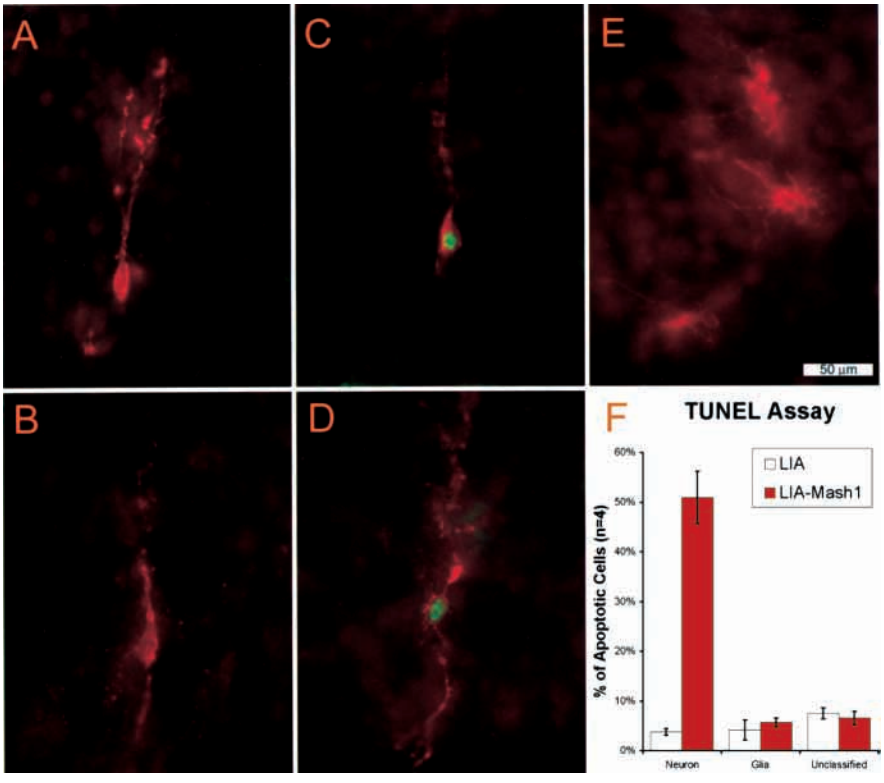

Fig. 6. TUNEL assay of infected cells in the cerebral cortex. Infected brain tissue was double stained using the TUNEL assay and immunohistochemistry for PLAP. (A,B) Examples of LIA infected neurons. Anti-PLAP staining showed typical neuronal morphology; no TUNEL signal was detected. (C,D) Examples of 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.

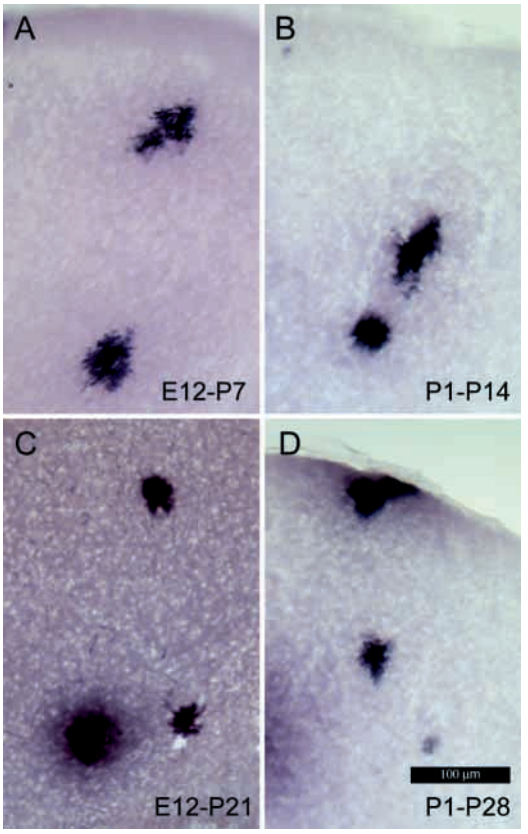

Fig. 7. Misexpression of a bHLH negative regulator, *Id1*, blocked neurogenesis. (A,C) E12 and (B,D) P1 injection of LIA-*Id1* retrovirus; no neurons were observed at any of the four stages P7 (A), P14 (B), P21 (C) or P28 (D). LIA-*Id1* infected glial cells had normal morphology (A-D). For each experimental group, more than 1000 $PLAP^+$ cells were scored from a total of 4-6 different animals. Bar, 100 μm.

regulatory bHLH genes that we expressed in the retina blocked gliogenesis was reminiscent of this global role of GCM in controlling gliogenesis. Even positive regulatory bHLH genes that are not normally expressed in the retina were able to suppress retinal gliogenesis. However, despite the fact that they had a common activity in this role, they gave subtly different results when the neuronal composition was examined. For example, in rats and mice, *NeuroD* favored 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 neural 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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