

Neural stem cell lineages are regionally specified, but not committed, within distinct compartments of the developing brain

Seiji Hitoshi^{1,*†}, Vincent Tropepe^{1,*§}, Marc Ekker² and Derek van der Kooy^{1,‡}

¹Department of Anatomy and Cell Biology, University of Toronto, Toronto, Ontario M5S 1A8, Canada

²Ottawa Health Research Institute, Ottawa Hospital and Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario K1Y 4E9, Canada

*These authors contributed equally to this work

†Present address: Department of Neurology, University of Tokyo, Tokyo 113-8655, Japan

§Present address: Whitehead Institute for Biomedical Research, Cambridge, MA 02142, USA

‡Author for correspondence (e-mail: derek.van.der.kooy@utoronto.ca)

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SUMMARY

Regional patterning in the developing mammalian brain is partially regulated by restricted gene expression patterns within the germinal zone, which is composed of stem cells and their progenitor cell progeny. Whether or not neural stem cells, which are considered at the top of the neural lineage hierarchy, are regionally specified remains unknown. Here we show that the cardinal properties of neural stem cells (self-renewal and multipotentiality) are conserved among embryonic cortex, ganglionic eminence and midbrain/hindbrain, but that these different stem cells express separate molecular markers of regional identity *in vitro*, even after passaging. Neural stem cell progeny derived from ganglionic eminence but not from other

regions are specified to respond to local environmental cues to migrate ventrolaterally, when initially deposited on the germinal layer of ganglionic eminence in organotypic slice cultures. Cues exclusively from the ventral forebrain in a 5 day co-culture paradigm could induce both early onset and late onset marker gene expression of regional identity in neural stem cell colonies derived from both the dorsal and ventral forebrain as well as from the midbrain/hindbrain. Thus, neural stem cells and their progeny are regionally specified in the developing brain, but this regional identity can be altered by local inductive cues.

Key words: *Emx1*, *Dlx2*, *En1*, *Dlx5*, Neurosphere, Mouse, Cell fate

INTRODUCTION

The vertebrate brain is divided into specialized regions during development. A prevailing model proposes that regional specialization in the vertebrate brain is broadly established through the formation of transient segment-like structures during development. These structures are called prosomeres in the forebrain, mesomere in the midbrain and rhombomeres in the hindbrain and are defined by morphological, hodological and gene expression criteria (Rubenstein et al., 1994; Lumsden and Krumlauf, 1996; Wullimann and Puelles, 1999).

In mammals, the cerebral cortex and ganglionic eminence (GE) (composed of the lateral and medial ganglionic eminences; LGE and MGE) make up the main dorsal and ventral anatomical derivatives, respectively, of the second most anterior prosomeric segment within the forebrain (Puelles, 1995). Together with distinct proliferative zones, early expression of regulatory genes prefigures compartmental histogenesis in these specialized regions. For instance, members of the *Emx* family of homeobox genes, such as *Emx1* and *Emx2*, are expressed within the cortical primordium (Simeone et al., 1992), while members of the *Dlx* family of homeobox genes, such as *Dlx1* and *Dlx2*, are expressed within

the GE (Bulfone et al., 1993). In these regions, gene expression is correlated with precursor cells within the germinal zone, which is composed of stem cells and their progenitor cell descendants. Thus, non-overlapping patterns of gene expression along the dorsoventral axis may enable proliferating precursor cells within the germinal zone of these compartments to be specified to a restricted dorsal or ventral forebrain cell fate. Indeed, there is evidence for clonal restriction of some (Fishell et al., 1993), but not all (Anderson et al., 1997a) progenitor cell migration within these dorsal and ventral forebrain territories, and this pattern of lineage restriction is also observed between rhombomeres in the developing hindbrain (Fraser et al., 1990).

Regional patterning is regulated by inductive signals from adjacent tissues, such as the prechordal plate and notochord (Lumsden and Krumlauf, 1996). Neural stem cells, which are considered the lineage precursors to all neuronal and glial cells in the mammalian nervous system (Weiss et al., 1996; Gage, 2000), are mitotically active at the onset of forebrain neurogenesis and in the neural plate prior to the overt segregation of dorsal and ventral forebrain compartments (Tropepe et al., 1999; Martens et al., 2000). Hence, neural stem cells together with their progeny are poised to receive inductive

signals that establish a regional identity in vivo. It is of interest to ask whether neural stem cells possess intrinsic regional identity, which, for example, can be maintained in the absence of their in vivo environment, or whether neural stem cells in the developing brain generally lack positional information. The former possibility was suggested by a study showing that neural stem cell colonies derived from cortex and spinal cord of embryonic day (E) 14.5 mice differentially express regional marker genes along the anteroposterior axis (Zappone et al., 2000). In the present study, we investigated whether mouse neural stem cells isolated from different germinal zone regions along the dorsoventral and anteroposterior axes of the brain displayed region-specific characteristics, and whether these characteristics were reversible.

MATERIALS AND METHODS

Dissections

The protocol used to generate neural stem cell colonies in vitro from CD1 (Charles River), green fluorescent protein (GFP) (Hadjantonakis et al., 1998) or *zfdlx4/6lacZ* transgenic mice (Zerucha et al., 2000) at the specified gestational ages has been described previously (Tropepe et al., 1999). Tissues dissected from these regions were transferred to serum-free media (see below) and mechanically dissociated into a cell suspension with a small-bore, fire-polished Pasteur pipette. Brains derived from *zfdlx4/6lacZ* transgenic mice were bisected; one hemisphere was processed for β -galactosidase histochemistry (see below) and the other used for cell cultures. Cell viability was assessed using Trypan Blue exclusion (0.4%; GIBCO) and typically ranged from 80–85%.

Cell culture

Cells from cortex, GE, midbrain/rostral hindbrain (MB/rHB) and caudal hindbrain (cHB) were cultured in a neural stem cell colony-forming assay (Reynolds et al., 1992) to generate sphere colonies. Cells were plated at 10 cells/ μ l in 24-well (0.5 ml/well) uncoated plates (Nunc) in serum-free medium containing 10 ng/ml fibroblast growth factor-2 (FGF2) (human recombinant; Sigma) with 2 μ g/ml heparin (Upstate Biotech). In order to assess self-renewal, primary sphere colonies (selecting mainly floating colonies after 7 days in vitro) were mechanically dissociated and cultured at 2 cells/ μ l in 24-well plates (0.5 ml/well) in serum-free medium containing FGF2 and 20 ng/ml epidermal growth factor (EGF) (mouse submaxillary; Upstate Biotech). Stem cell self-renewal was assessed by identifying new sphere colonies after a further 7 days in vitro.

Immunocytochemistry

Seven days after primary culture, single sphere colonies were transferred to individual wells of a 24-well culture plate (Nunc) previously coated with MATRIGEL basement membrane matrix (0.6 mg/ml in serum-free medium; Becton-Dickinson) in 0.5 ml/well of the medium containing 1% fetal bovine serum (Gibco). Wells were processed 7 days later using immunocytochemistry as described previously (Tropepe et al., 1999). We used anti-MAP-2 mouse monoclonal (IgG) (1:1000; Roche), anti-GFAP rabbit polyclonal (IgG) (1:400; Chemicon), or anti-O4 mouse monoclonal (IgM) (1:40; Roche) antibodies as primary antibodies, then appropriate FITC- or TRITC-conjugated secondary antibodies. Cultures were counter labeled with the nuclear stain Hoechst 33258 (1 μ g/ml; Sigma) and fluorescence was visualized using a Nikon Microphot microscope.

RT-PCR

Total RNA was isolated (RNeasy extraction kit, Qiagen) and 1 μ g of total RNA was used to synthesize cDNA with oligo(dT)_{12–18} primers

and MoMLV reverse transcriptase (Superscript II; Roche) in 25 μ l reaction mixture at 42°C for 1 hour. The PCR reaction mixture (20 μ l) consisted of 1 μ l cDNA, 16 pmol each of 5' and 3' primers, 0.2 mM dNTP, 2 μ l PCR reaction buffer and 0.8 U of Taq polymerase (Promega). cDNA was amplified in a thermal cycler (Perkin-Elmer). For all primer pairs denaturation at 95°C for 30 seconds, annealing at a specified temperature below for 40 seconds and extension at 72°C for 40 seconds was used for 40 cycles. The sense and antisense primers, Mg²⁺ concentration and annealing temperature were used for the following genes. *Emx1*: sense 5'-TGAGAAGAATCACTAC-GTGG-3', antisense 5'-AGGAGACATCAATGTCTCTCC-3', 1.5 mM Mg²⁺, 56°C. *Emx2*: sense 5'-GTCCCAGCTTTTAAAGGCTAGA-3', antisense 5'-CTTTTGCCTTTTGAATTTTCGTTC-3', 1.5 mM Mg²⁺, 56°C. *Dlx2*: sense 5'-ACACCGCCGCGTACACCTCCTA-3', antisense 5'-CTCGCCGCTTTTCCACATCTTCTT-3', 1.2 mM Mg²⁺, 56°C. *Dlx5*: sense 5'-ACAGAGAAGGTTTCAGAAGAC-3', antisense 5'-CTGAGCAAGAGAAAAGTAGCCC-3', 1.2 mM Mg²⁺, 58°C. *En1*: sense 5'-GACAGTGGCGGTGGTAGTG-3', antisense 5'-GAGGAGCCTGGAGGTGGC-3', 1.2 mM Mg²⁺, 58°C. *Hoxb1*: sense 5'-CCGGACCTTCGACTGGATG-3', antisense 5'-GGTCAGAGGC-ATCTCCAGC-3', 1.5 mM Mg²⁺, 58°C. *Otx1*: sense 5'-ACTTCG-CAGTGGGAGCCCTGAA-3', antisense 5'-GCGGCGGTTCTTG-AACCAAA-3', 1.5 mM Mg²⁺, 58°C. To normalize the cDNA amount in the samples, the *GAPDH* gene (glyceraldehyde-3-phosphate dehydrogenase) was used: sense primer 5'-ATCAAAGAAGGAC-TGGCGAG-3', antisense primer 5'-CCACTCGGTTACTGTAGC-CATA-3' and PCR reaction conditions were similar to conditions used for *Emx1* amplification. These pairs of primers for all genes are designed to encompass at least one intron in order to avoid false-positive amplification from contaminated genomic DNA. As negative controls, the reverse transcriptase was omitted at the cDNA synthesis step and the samples then proceeded to the PCR reaction in the same manner as above. These control experiments gave no positive amplification.

For single sphere RT-PCR, total RNA was extracted from single sphere colonies and eluted in 15 μ l of H₂O. One μ l of the RNA preparation was used for quantification and the remainder (containing ~0.1 μ g RNA) was used to synthesize cDNA in a 25 μ l reaction mixture. Aliquots (5 μ l) of each cDNA sample were subject to PCR as described above.

Co-cultures

CD1 embryos at 14.5 days of gestation were isolated and their brains dissected in PBS as described above. E14.5 brains were embedded in a 1.5% solution of low melting point agarose (Sigma), then coronal sections were cut on a vibratome (Vibratome Tissue Sectioning System 100, Pelco International) in ice cold PBS. Approximately two to three 400 μ m thick coronal sections were cut from each brain. The analysis included cortex, GE, septum and preoptic areas rostrally (surrounding the lateral ventricles) and anterior thalamus caudally (surrounding third ventricle). Sections that were more rostral (including only cortex and septum and no or very little GE tissue) were either excluded or used as a control for the absence of GE. Individual sections were placed flat on a Transwell-Col filter insert (Corning) measuring 24 mm in diameter (3.0 μ m pore size) in 6-well plates and incubated in 1.5 ml of serum-free medium (as above) for 1 hour. A similar procedure was used for cortical (dorsal cortex) tissue substrates and midbrain (presumptive tectum) tissue substrates except that the slices were horizontally dissected and placed ventricular zone side up on the filters.

Two to three individual sphere colonies (derived from E14.5 *zfdlx4/6lacZ* transgenic cortical, GE or MB/rHB tissue) were placed on the coronal GE slice or on the tissues dissected from cortex or midbrain with a pipette under an inverted microscope, and co-cultures were incubated for a maximum of 5 days. Co-cultures were then analyzed histochemically for β -gal expression (see below).

Single sphere colonies derived from the cortex, MGE or MB/rHB

tissue of E14.5 GFP-transgenic embryo were placed on the germinal zone of the slice tissue (in the region of the GE) or on the cortical or midbrain explants. Co-cultures were observed using fluorescence microscopy for 5 days in vitro, and then fluorescent portions of the tissues were excised to generate secondary sphere colonies in serum-free medium containing FGF2 and EGF.

β -galactosidase histochemistry

X-gal staining was used to detect the expression of the *lacZ* transgene in single hemispheres of *zfdlx4/6lacZ* transgenic brains, primary *zfdlx4/6lacZ* sphere colonies generated from the cortex, GE or MB/rHB tissues, primary monolayer cultures from these same tissues, or 5-day co-cultures of E14.5 CD1 forebrain and midbrain tissue and *zfdlx4/6lacZ* primary sphere colonies (as above). Tissues were fixed in 0.2% glutaraldehyde, 2 mM MgCl₂, 5 mM EGTA and 100 mM sodium phosphate (pH 7.3), and then stained in 1 mg/ml X-gal, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 0.02% NP-40, 0.01% deoxycholate, 2 mM MgCl₂ and 100 mM sodium phosphate buffer (pH 7.3) at 37°C overnight.

RESULTS

Cardinal features of neural stem cells are conserved between neural compartments

Neural stem cell behavior can be operationally defined (and empirically tested) as the ability to proliferate and produce progenitor cells, self-renewal capacity and neural multilineage potential. Using the colony-forming neurosphere assay, FGF2-responsive neural stem cell colonies were generated from cultures of cortex, GE and MB/rHB (Fig. 1A). Consistent with previous findings (Tropepe et al., 1999), EGF-responsive neural colonies were also generated from all three regions (data not shown), suggesting that distinct FGF2- and EGF-responsive neural stem cell populations are distributed not only along dorsoventral compartments of the forebrain, but also along the anteroposterior axis between the forebrain and hindbrain. However, it is worth noting here that another neural stem cell (the retinal stem cell) shows different behavior in at least one respect, it proliferates to form spheres in the absence of exogenous growth factors (Tropepe et al., 2000).

Single FGF2-responsive primary colonies from cortex, GE and MB/rHB were capable of producing new secondary colonies after 7 days in serum-free medium with FGF2 and EGF (Fig. 1A). The number of new secondary sphere colonies generated from the subcloning of primary colonies can be considered an estimate of the extent to which the initial primary colony-forming stem cell underwent symmetric (expansory) divisions (Reynolds and Weiss, 1996). More secondary sphere colonies were generated from primary colonies derived from MB/rHB than from those derived from cortex or GE ($P < 0.05$). To assess neural stem cell multipotentiality, single FGF2-responsive colonies were cultured for 7 days under differentiation conditions, and were processed for immunocytochemistry to detect neurons (MAP2), astrocytes (GFAP), and oligodendrocytes (O4). Colony-forming stem cells derived from cortex, GE and MB/rHB demonstrated neural multilineage potential (Fig. 1B). Overall, the proportion of neuronal and glial cells that differentiated under these conditions was comparable among the region-specific stem cell colonies, although the percentage of MAP2⁺ neurons observed from the MB/rHB stem cell colonies (6.4%) was less than the percentage of MAP2⁺ neurons derived from GE stem

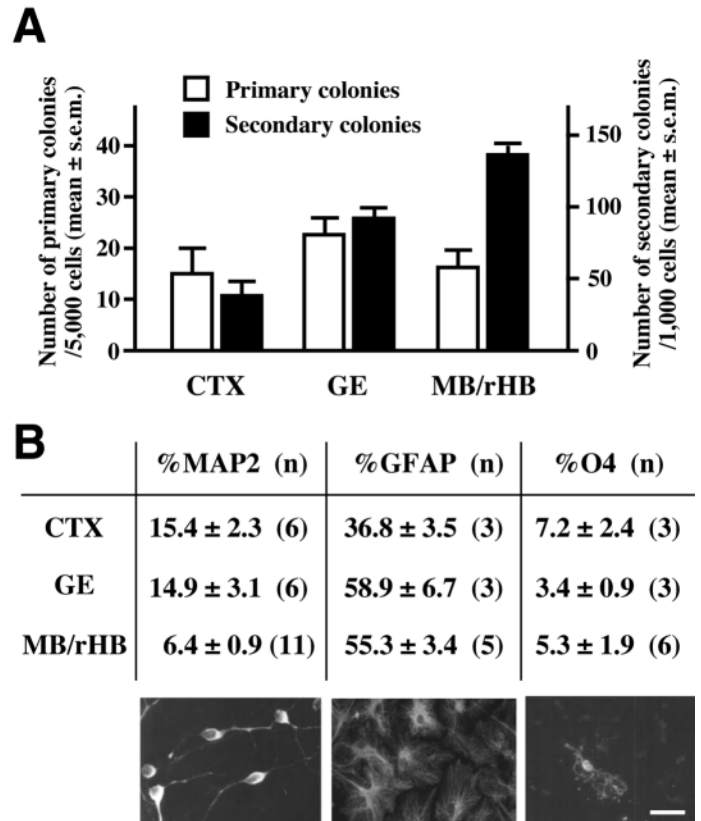


Fig. 1. Neural stem cells isolated from different regions of the E14.5 mouse brain display self-renewal and neuronal/glia multipotentiality characteristics in vitro. (A) Isolation of FGF2-responsive neural stem cells from the cortical (CTX), ganglionic eminence (GE) and midbrain/rostral hindbrain (MB/rHB) germinal zones ($n=12$ embryos per group). The ability to form primary sphere colonies after 7 days of culture was examined. The self-renewal capacity of the original colony-forming neural stem cells, primary CTX, GE and MB/rHB ($n=4$ for each group) was also examined after mechanical dissociation and re-culture (see Materials and Methods for details). The numbers of secondary colonies from each group was quantified after 7 days in culture. (B) Immunolabeling for neurons (anti-MAP2⁺), astrocytes (anti-GFAP⁺) and oligodendrocytes (anti-O4⁺) derived from single neural stem cell colonies cultured for 7 days in serum-free medium and subsequently allowed to differentiate for a further 7 days on an artificial extracellular matrix substrate in medium containing 1% FBS. Scale bar, 20 μ m.

cell colonies (14.9%) or cortical stem cell colonies (15.4%) ($P < 0.05$).

Taken together, these data indicate that the behavior of neural stem cells is generally conserved between dorsal forebrain (cortex), ventral forebrain (GE) and MB/rHB neural compartments. However, some relatively minor differences in self-renewal capacity and neuronal/glia differentiation were evident among neural stem cell progeny from different brain regions (MB/rHB stem cell colonies showed greater expansory self-renewal and less neuronal differentiation than more rostral neural stem cell colonies). These caudorostral differences may be related to the caudorostral gradient in brain development, perhaps reflecting a caudorostral gradient in the expansion of the neural stem cell pool (indeed, more expansory self-renewing divisions may be correlated with a

more advanced stage of neurogenesis) (Martens et al., 2000). We next investigated whether neural stem cells from distinct compartments of the brain displayed differences in molecular regional markers.

Forebrain and midbrain/hindbrain stem cell colonies express unique molecular markers of regional identity

The experiments above showed that neural stem cell population are localized along the dorsoventral and anteroposterior axes (between histogenic compartments) within the brain. To determine if forebrain, midbrain and hindbrain neural stem cell colonies have unique regional identity, we assayed for the expression of region-specific regulatory genes *Emx1*, *Dlx2*, *En1* and *Hoxb1* in isolated forebrain, midbrain and hindbrain stem cell colonies using RT-PCR. During development *Emx1* is expressed in the cortex (Simeone et al., 1992) and *Dlx2* is expressed in the GE (Bulfone et al., 1993). In contrast, *En1* expression is localized to the caudal midbrain and rostral hindbrain region (isthmus) (Davis and Joyner, 1988) and *Hoxb1* expression to cHB (Wilkinson et al., 1989). FGF2-responsive stem cell colonies were generated from the cortical, MGE, MB/rHB and cHB germinal zones from E14.5 embryos and age-matched primary tissue isolates served as positive controls for gene expression. Isolated stem cell colonies demonstrated region-specific gene expression patterns (Fig. 2A); E14.5 cortical colonies express *Emx1*, but not *Dlx2*, *En1* or *Hoxb1*, MGE colonies only express *Dlx2*, MB/rHB colonies only express *En1*, and cHB colonies only express *Hoxb1* gene. The amplification of the *Dlx2* gene observed in the E14.5 cortical tissue could be due to the migration of *Dlx2*-expressing neurons from the ventral forebrain, which occurs starting at E12.5 (Anderson et al., 2001). We also determined the expression of *Otx1*, which is distributed throughout the dorsal and ventral forebrain and midbrain compartments at early stages of neural development (Boncinelli et al., 1993). By E14.5, *Otx1* expression is present in isolated forebrain-derived and MB/rHB-derived colonies, but is not expressed in cHB-derived colonies (Fig. 2A). In contrast to the similar *in vivo* and *in vitro* regional expression patterns of the genes mentioned above, all of the forebrain, midbrain and hindbrain-derived stem cell colonies expressed *Emx2*, although the *Emx2* expression in the primary tissue is restricted to dorsal forebrain (extending more ventrally than *Emx1*), but it is not observed in the midbrain or hindbrain. Thus, the expression of at least one gene (*Emx2*) is differentially regulated *in vivo* and *in vitro*.

These findings suggest that neural stem cells along the dorsoventral as well as anteroposterior axes have intrinsic regional identity that is maintained when isolated *in vitro*. Moreover, this region-specific positional information (in the form of gene expression) is distinct from the region-nonspecific (or relatively comparable) neural stem cell properties of self-renewal and multipotentiality.

Regional identities of forebrain, midbrain and hindbrain stem cell colonies are maintained over passaging

To determine whether neural stem cell colonies can keep their regional identity over an extended period of time *in vitro* (hence independent of local environmental cues), we dissociated sphere colonies in bulk to generate new sphere colonies (a

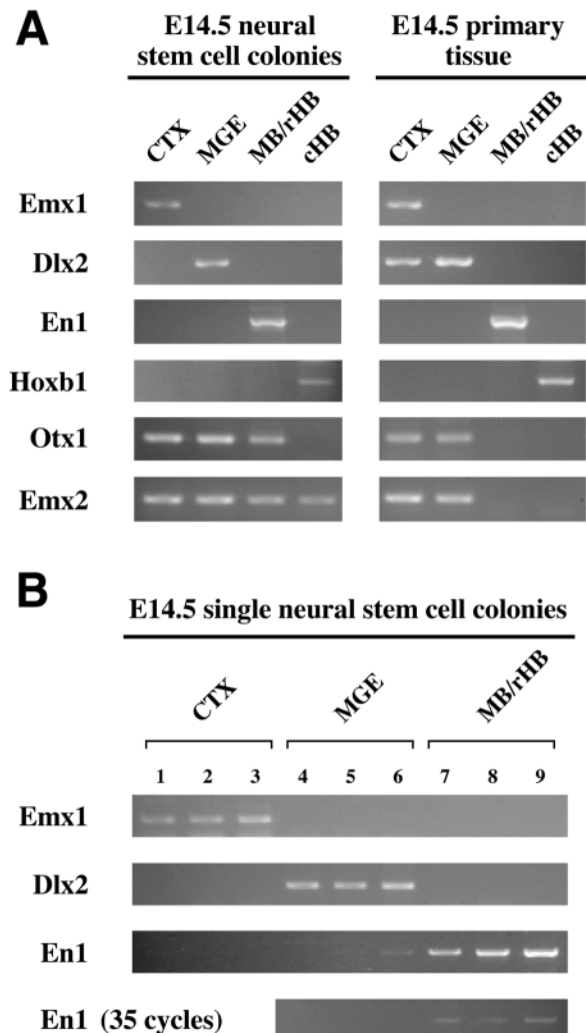


Fig. 2. Isolated E14.5 neural stem cell colonies express region-specific genes. (A) Gene expression analysis was determined using RT-PCR. RNA was isolated from neural stem cell colonies derived from cortical (CTX), medial ganglionic eminence (MGE), midbrain/rostral hindbrain (MB/rHB) or caudal hindbrain (cHB) germinal zone dissections, and generated after 7 days of culture. Primers were designed to detect *Emx1* (229 bp), *Dlx2* (310 bp), *En1* (567 bp), *Hoxb1* (307 bp), *Otx1* (347 bp) and *Emx2* (151 bp). To normalize for the amount of cDNA present in the sample, the cDNA for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (401 bp) was amplified and band intensity was comparable to the band intensities from sphere colonies and tissue (not shown). Data are representative of at least 3 separate experiments. (B) Single sphere RT-PCR analyses of E14.5 primary sphere colonies were performed and representative examples (lanes 1-9) are shown. Some of MGE-derived sphere colonies (lane 6 in this figure) as well as all of the MB/rHB colonies expressed *En1* as revealed by 40 cycles of PCR amplification. However, the expression of the *En1* gene in the MGE sphere colonies was relatively weaker than that in the MB/rHB colonies; the expression in the single MGE colonies was below the level of detection after 35 PCR cycles.

passaging procedure), and then examined the regional gene expression patterns in the newly generated colonies. Overall, RT-PCR results from sphere colonies passaged twice using this method were similar to those from primary sphere colonies

Table 1. Gene expression in neural stem cell sphere colonies derived from different E14.5 brain regions

	<i>Emx1</i>	<i>Dlx2</i>	<i>En1</i>
Neurosphere colonies after passaging twice*			
Cortex	7/7	3/7	0/7
MGE	0/7	7/7	3/7
MB/rHB	0/7	0/7	7/7
Single sphere colonies from primary neurosphere assay†			
Cortex	20/20 (100%)‡	5/20 (25%)	2/20 (10%)
MGE	1/20 (5%)	20/20 (100%)‡	8/20 (40%)
MB/rHB	1/18 (5.6%)	2/18 (11.1%)	17/18 (94.4%)‡

*Neurosphere colonies in bulk were passaged twice and then cultured in serum-free medium containing FGF2 and EGF for 7 days. Resultant neurosphere colonies were subject to RT-PCR to examine the regional identity of gene expression. Frequencies of positive expression from seven independent experiments are shown.

†Single sphere colonies were subject to single sphere RT-PCR.

‡The clonal stem cell colonies from this region show more frequent expression of this specific gene than the clonal stem cell colonies from other regions ($P < 0.05$, χ^2 test). MGE, medial ganglionic eminence; MB/rHB, midbrain/rostral hindbrain.

(Table 1). However, expression of *Dlx2* was detected in passaged cortical sphere colonies (3 out of 7 independent experiments), and expression of *En1* was detected in passaged MGE sphere colonies (3 out of the 7 independent experiments). We did not detect any expression of *Emx1*, *Dlx2* or *En1* in passaged cHB neurosphere colonies, but weak expression of the *Hoxb1* (1 out of 3 independent experiments) was detected (data not shown). The gene expression profiles of cortical, MGE and MB/rHB colonies passaged 6 times (i.e. 50 days in vitro) were the same as those displayed by cortical, MGE and MB/rHB colonies passaged twice (data not shown), including some occasional non region-specific expression of marker genes (for example, a minority of both the twice and 6 times passaged MGE colonies demonstrated some *En1* expression).

The differences in marker gene expression profiles between primary and passaged sphere colonies derived from cortex and MGE might be explained in at least two ways. First, sphere colonies, partially, may lose their restricted regional identities to express a broader range of marker genes upon passaging. Second, the primary colonies may contain a minor subpopulation of colonies that already express more than one of the marker genes, and that are incidentally expanded to detectable levels by passaging. To address this issue, we performed a single sphere RT-PCR analysis on primary cortical, MGE and MB/rHB colonies. The results support the second possibility (Table 1, Fig. 2B); all of the primary cortical colonies expressed *Emx1* and 25% of them express both *Emx1* and *Dlx2*; all of the primary MGE colonies expressed *Dlx2* and 40% of them express both *Dlx2* and *En1*. The apparent discrepancies between the gene profiles of primary colonies in bulk (for example, lack of *En1* expression in MGE sphere colonies, Fig. 2A) and single primary sphere colonies (for example, *En1* expression in a minority of the single MGE colonies, Table 1) might be explained as follows. The expression of *Dlx2* in the single primary cortical colonies and the expression of *En1* in the single primary MGE colonies was relatively weak (undetectable after 35 cycles but positive by 40 cycles; Fig. 2B), as compared to the expression of *Dlx2* in the single primary MGE colonies or the expression of *En1* in the single primary MB/rHB colonies (positive after 35

cycles). Alternatively, given that we selected relatively large colonies (~0.4 mm in diameter) to obtain enough RNA for RT-PCR analysis of single sphere colonies, neural stem cells that give rise to larger sphere colonies may have been more likely to express more than one regional marker gene. In either case, the small amounts of mRNA from non regionally specific genes would be diluted out in the RNA preparation from the bulk sphere colonies. Thus, although stem cell regional identity is maintained after repeated passaging, these results indicate that a small proportion of stem cells express markers of dorsal and ventral forebrain, or of anterior and posterior brain regions.

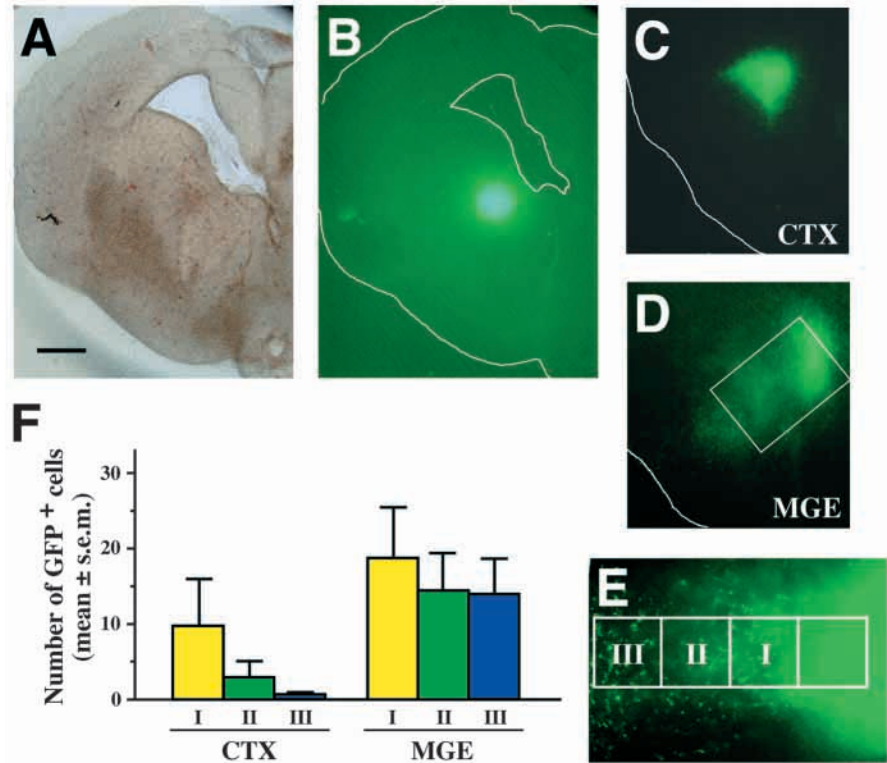
These results imply that the regional phenotypes of neural stem cells may not be demarcated rigidly at the boundaries of the brain regions, but instead may be specified in a more graded fashion with stem cells near the boundary expressing the regional identities of both compartments. However, much of the regional identity of neural stem cells appears to be maintained over passaging for weeks in vitro. Indeed, we examined primary colonies derived from the adult striatum (an LGE derivative) and their descendants, which were passaged 36 times (more than 8 months), for marker gene expression and observed that both primary colonies as well as colonies that had been bulk passaged 36 times expressed *Dlx2*, but were negative for *Emx1* and *En1*.

Neural stem cell colonies show regionally specified migratory behavior in slice co-culture

Although the above experiments showed that forebrain, midbrain and hindbrain neural stem cell colonies maintain unique regional marker gene expression patterns, it remains unknown whether or not they (neural stem cells and their progeny in the colonies) behave differently when adapted to new environments. Progenitor cells derived from GE neural stem cells (especially from the MGE) are known to undergo robust radial and then tangential migration as they differentiate into striatal and cortical interneurons during development in vivo and in vitro (Anderson et al., 1997a; Wichterle et al., 1999; Anderson et al., 2001). We reasoned that if MGE neural stem cells and their progeny have been specified to acquire the potential to respond to local environmental cues for migration, then the progenitor cells in stem cell sphere colonies from MGE but not from other regions may respond to specific cues provided by the GE tissue. We examined this hypothesis using a sphere colony/slice co-culture system. Cortical, MGE or MB/rHB stem cell colonies were generated from GFP transgenic mice and placed on the surface of GE germinal zone tissue from E14.5 forebrain coronal slices (Fig. 3A,B). Progenitor cells from the MGE sphere colonies showed robust migration lateroventrally within 48 hours (Fig. 3D), but those from cortical (Fig. 3C) or MB/rHB (data not shown) sphere colonies did not. The migration distances from the original spots where sphere colonies were placed were quantified 48 hours after co-culturing (Fig. 3F). More progenitor cells from the MGE sphere colonies migrated longer distances than those from the cortical sphere colonies ($t = 2.53$, $P < 0.05$, for the most distant bin – #III). MB/rHB sphere colonies showed the same, relatively limited migration patterns as cortical sphere colonies.

These observations are consistent with the idea that MGE neural stem cells and their progeny in sphere colonies may be

Fig. 3. Neural stem cell colonies from E14.5 medial ganglionic eminence germinal zone show a unique migratory potential. Isolated neural stem cell colonies derived from cortical (CTX) or medial ganglionic eminence (MGE) germinal zone of GFP transgenic mice were deposited on CD1 coronal slices and observed under phase contrast (A) and fluorescent (B) microscopy. After 48 hours co-culturing, cells migrating out of sphere colonies derived from CTX (C) or MGE (D) were observed using fluorescence microscopy. (E) Higher magnification image of the boxed area in D is shown and the numbers of cells that had migrated into the three 0.25 mm × 0.25 mm areas indicated (I-III) were counted. Distance migrated: I, 0.25-0.5 mm; II, 0.5-0.75 mm; III, 0.75-1.0 mm. (F) The number of cells in zones I, II or III (derived from CTX ($n=9$) or MGE ($n=12$) sphere colonies after 48 hours co-culturing with CD1 coronal slice) were quantified. Scale bar, 0.5 mm (A-D).



specified to respond to local ventral forebrain environmental cues which induce migration.

Specific cues from the ventral forebrain induce the expression of early onset, ventral-specific gene in both forebrain and MB/rHB stem cells

Neural stem cells self-renew to generate new stem cell sphere colonies after mechanical dissociation in serum-free medium. We hypothesized that stem cells in the sphere colonies that derived from forebrain or MB/rHB tissue of GFP transgenic mice and were placed on the GE of CD1 slices would maintain their “stemness” after 5 days of in vitro slice co-culture. To test this, we excised the largest fluorescent portion of each sphere/GE slice co-culture under the fluorescence microscope, dissociated it mechanically, and then plated the cells at 10-50 cells/ μ l in serum-free medium with FGF2 and EGF. New GFP-positive sphere colonies were observed after 7 days in vitro

Table 2. Gene expression in secondary neural stem cell sphere colonies derived from sphere/slice co-cultures

Donor region	<i>Emx1</i>	<i>Dlx2</i>	<i>En1</i>
Cortex	1/9 (11.1%)	8/9 (88.9%)	1/9 (11.1%)
MGE	0/9 (0%)	9/9 (100%)	2/9 (22.2%)
MB/rHB	0/9 (11.1%)	9/9 (100%)	3/9 (33.3%)

Primary neurosphere colonies from the GFP mouse brain were placed on the ganglionic eminence in the CD1 host coronal slices for 5 days, and then the fluorescent-positive portion of each slice co-culture was excised, dissociated, and cultured in serum-free media containing FGF2 and EGF for 7 days. Resultant clonal GFP+ secondary neurosphere colonies were subject to single sphere RT-PCR to examine the regional identity of gene expression. Frequencies of positive expression from three independent experiments are shown. MGE, medial ganglionic eminence; MB/rHB, midbrain/rostral hindbrain.

from the co-cultures of the cortical, GE or MB/rHB GFP neurospheres and CD1 coronal slices. We tested whether the GFP-positive neural stem cell colonies maintained their donor regional identities, or alternatively whether they acquire a ventral forebrain (the GE host in the slice culture) regional identity by analyzing the expression of *Dlx2* in the newly generated GFP-positive secondary colonies (Table 2). New colonies derived from co-cultures of GFP-positive cortical stem cell colonies expressed only *Dlx2* (8/9, 88.9%), but were negative for their original regional identity marker *Emx1*. One secondary GFP-positive sphere colony expressed only *Emx1* (1/9, 11.1%). In contrast, new colonies derived from co-cultures of GFP-positive MB/rHB stem cell colonies expressed only *Dlx2* (6/9, 66.7%), or both *Dlx2* and *En1* (3/9, 33.3%). New colonies derived from co-cultures of GFP-positive GE stem cell colonies retained expression of *Dlx2* (9/9, 100%). These findings suggest that specific cues from the ventral forebrain induce neural stem cells in cortical or MB/rHB colonies to acquire ventral forebrain identities and to suppress their original regional identities. Thus, even the early regional identities of neural stem cells are not irreversible and can be altered by local inductive cues.

Relatively late markers of ventral forebrain regional identity are not expressed by isolated ganglionic eminence stem cell colonies

Clonal neural stem cell-derived colonies are composed of undifferentiated cells. The expression of *Dlx2* in GE-derived stem cell colonies is consistent with the fact that in vivo, *Dlx2* is localized to the germinal zone of the ventral forebrain where neural stem cells and their early progenitor cell progeny reside (Eisenstat et al., 1999). As the progenitor cells migrate away from the germinal zone into the overlying mantle zone (MZ)

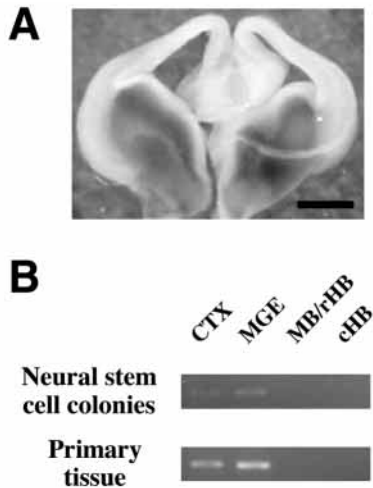


Fig. 4. The expression of a ganglionic eminence-specific late marker gene in vivo and in neural stem cell colonies. (A) A coronal slice (400 μm thick) of E14.5 *zfdlx4/dlx6lacZ* transgenic mice brain was subject to X-gal staining, and showed robust transgene expression in subventricular zone and mantle zone, but not in ventricular zone, of the ganglionic eminence. Bar, 1.0 mm. (B) The expression of the endogenous *Dlx5* gene was determined using RT-PCR. RNA was isolated from E14.5 primary neural stem cell colonies derived from cortical (CTX), medial ganglionic eminence (MGE), midbrain/rostral hindbrain (MB/rHB) or caudal hindbrain (cHB) germinal zone dissections, and generated after 7 days in culture. Positive amplification of 438 bp bands was demonstrated in the neural stem cell colonies from CTX and MGE, as well as in the corresponding primary tissues.

they begin to express other genes of the *Dlx* family, such as *Dlx5* and in more mature cells *Dlx6* (Eisenstat et al., 1999). To test if neural stem cell colonies express relatively late markers of regional identity, we utilized *zfdlx4/dlx6lacZ*-transgenic mice (Zerucha et al., 2000). Transgene expression in the E14.5 ventral forebrain resembles the expression of endogenous *Dlx5* and *Dlx6*, but is thought to more closely mimic endogenous *Dlx5* expression (Zerucha et al., 2000). We confirmed that the *zfdlx4/dlx6*-enhancer-driven *lacZ* expression is exclusively confined to a majority of cells in the subventricular zone (SVZ) and MZ of the ventral forebrain, including the GE and septum, but not the ventricular zone (VZ) (Fig. 4A) (Zerucha et al., 2000). Thus, expression of this specific reporter construct acted as a molecular marker for precursor cells within GE stem cell colonies that progressed through to later stages of differentiation, essentially recapitulating the in vivo differentiation sequence. However, neural stem cell colonies isolated from the GE (as well as cortical and MB/rHB colonies) did not express the transgene when assayed histochemically. These findings stand in clear contrast to the RT-PCR results, which showed apparently weak amplification of the endogenous *Dlx5* gene from cortical and GE colonies (Fig. 4B). The expression of the *Dlx5* transgene in these colonies may be too weak to be detected histochemically, or alternatively and more likely, the promoter region that drives the *lacZ* reporter gene may contain a late ventral forebrain-specific promoter region but may not contain a region that drives minimal baseline expression of the gene.

Thus, cells within GE-derived stem cell colonies do not

express a transgene (at least high enough for histochemical analysis) that is restricted normally to more mature striatal cells. There are two possible explanations for these results. First, cells within a neural stem cell colony may require further differentiation in order to express late regional markers. Second, the expression of this specific transgene (and other late markers of differentiation) in stem cell progeny may depend not on the degree of differentiation, but rather on regional cues provided by the cells within the ventral forebrain environment. To distinguish between these two possibilities, we tested whether further differentiation of neural colony-derived progenitor cells was sufficient for transgene expression by culturing stem cell colonies in conditions that promoted neuronal and glial differentiation. After 7 days of differentiation, cells were assayed for transgene expression histochemically. Approximately 200 cells were randomly sampled for each differentiated sphere colony. β -gal-positive cells were extremely rare (<0.3% averaged over all of the sphere colonies assessed) in differentiated GE stem cell colonies ($n=152$ colonies). The majority of differentiated GE sphere colonies did not contain β -gal-expressing cells. Furthermore, no transgene-expressing cells were observed from cortical colonies ($n=36$ colonies) or MB/rHB colonies ($n=23$). Thus, differentiation alone is not sufficient for transgene expression (a later marker of regional identity) in stem cell-derived neuronal and glial progeny, even though it is sufficient, for example, to induce neurotransmitter-specific neuronal differentiation – a late event in neuronal maturation (Daadi and Weiss, 1999). These data suggest that although the expression of early markers of regional identity may be intrinsic to isolated neural stem cells (and inherited by their early progeny) the expression of late regional markers in these progeny may require the induction and/or maintenance by specific regional cues provided by other cells.

Specific cues from the ventral forebrain induce the expression of a late onset, ventral-specific transgene in both forebrain and midbrain/hindbrain stem cell colonies

To determine if late markers of regional identity can be induced by local cues we used two versions of a co-culture paradigm. In the first experiment, cortical or GE stem cell colony-derived cells from *zfdlx4/dlx6lacZ*-transgenic mice were dissociated and co-cultured with host CD1 cells from a primary (non-sphere forming) GE dissection (1:1 ratio) at relatively high cell densities (50 cells/ μl each) in similar differentiation conditions as above. Marker gene expression was not induced in colony cells derived from either the neocortex or the GE ($n=6$ -12 co-cultures per group). However, given that these co-culture conditions do not compensate for the extensive contact-mediated cellular interactions that may be necessary for regional specification, we employed a more sensitive sphere/slice co-culture paradigm (Fig. 5A), where tissues remained relatively intact and viable over a 5-day period. After 5 days of co-culturing, transgene expression was detected histochemically in 82% of the co-cultured sphere colonies (Table 3) in the germinal zone region of the GE (lateral to the ventricular lumen) and of the septum (medial to the ventricular lumen) (Fig. 5B,C). Some distortion of the slice morphology is evident after 5 days, and β -gal-positive cells were often observed very close to the midline between the hemispheres.

Very few X-gal-positive cells were found to have migrated out from the colonies into the host slice after 5 days in vitro even when examined under higher magnification. The probable explanation for this is that single cells did not express the transgene strongly enough to be detected histochemically, but accumulations of X-gal-positive cells were visible. In contrast to the GE coronal slices, horizontal slice cultures of cortex or midbrain (or rostral coronal slices that do not contain GE tissue) were not adequate substrates for inducing transgene expression in GE stem cell colonies (Fig. 5D,E). Thus, the GE acts as a specific inductive cue for the expression of a relatively late molecular marker of regional identity in isolated ventral forebrain stem cell lineages.

In order to define more precisely the source of the inductive signal, we dissected away the VZ tissue (which included some SVZ) from the remaining SVZ/MZ of the GE in 400 μm E14.5 coronal slices and cultured the separate VZ tissues on the same filter situated at a distance from the remaining brain section (Fig. 5F). We reasoned that since the progeny of neural stem cells (derived from the germinal zone) within the clonally derived colonies did not express the transgene in isolation, the source of the inductive signal in the co-culture paradigm likely arose from the GE MZ, where transgene expression is observed in vivo. Transgene expression from GE-derived stem cell colonies was not observed when colonies were co-cultured with isolated VZ tissue (Fig. 5G). In contrast, robust transgene expression was induced in co-cultured GE-derived stem cell colonies when placed in the SVZ/MZ of the ventral forebrain (Fig. 5H). These findings suggest that the once neural stem cell progeny (born in the germinal zone) migrate into the overlying MZ in the ventral forebrain, local cues regulate the expression of different sets of regionally restricted genes in a cell nonautonomous manner.

We next sought to determine if dorsal forebrain and MB/rHB stem cell lineages, which rarely express early or late markers for ventral forebrain identity in vivo, but instead are specified to their region of origin, could be induced to express the ventral forebrain *zfdlx4/dlx6lacZ* transgene marker. Cortical and MB/rHB-derived stem cell colonies were similarly co-cultured on CD1 GE (coronal slices) or on CD1 cortical or midbrain primordia (horizontal slices) under the same culture conditions. After 5 days, both cortical (85%) and MB/rHB (75%) colonies (Table 3) had strong transgene expression in an analogous pattern when exposed to the ventral forebrain environment. In contrast, no transgene expression was observed when these colonies were co-cultured with either cortical or midbrain substrates. These data indicate that local cues from ventral forebrain tissue can specifically induce the expression of a relatively late-expressing ventral forebrain gene in isolated neural stem cell lineages from both the dorsal forebrain and the

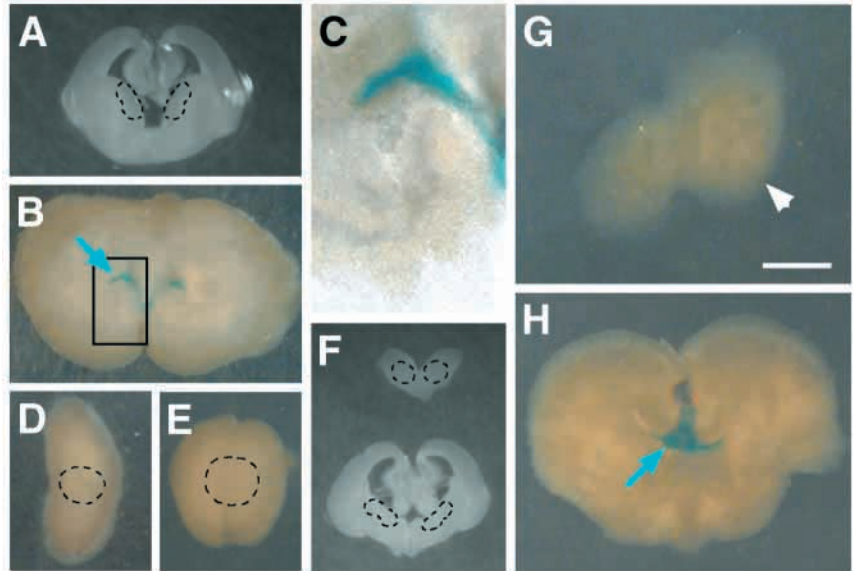


Fig. 5. Ganglionic eminence-specific late marker gene expression in both forebrain and midbrain/hindbrain neural stem cell colonies. Neural stem cell colonies derived from E14.5 *zfdlx4/dlx6lacZ* transgenic mice were co-cultured with E14.5 CD1 tissues. (A,B) Sphere colonies derived from cortex, ganglionic eminence (GE) or midbrain/rostral hindbrain (MB/rHB) were placed (A, dotted areas) on the GE of host in the CD1 coronal slices. After 5 days in vitro (B), although some distortion of the tissue was seen, robust transgene expression (blue arrow pointing to blue staining) could be observed only when sphere colonies were placed in the region of the ventral forebrain. (C) Higher magnification of the boxed area in B showing transgene expression. (D,E) Transgene expression was not induced in cortical, GE and MB/rHB colonies (originally located in dotted areas) when placed on the germinal layer of CD1 cortical tissue (D) or midbrain tissue (E) after 5 days in vitro. (F-H) Neurosphere colonies were placed on the isolated VZ (F, dotted areas on top slice) from E14.5 CD1 coronal slice and on the remaining GE in the slice (F, dotted areas on bottom slice). Transgene expression was not observed in the neural stem cell colonies (0/4 co-cultures) on isolated VZ tissue (G, arrowhead), but was expressed (4/4 co-cultures) in the neural stem cell colonies on the remaining GE tissue, composed of subventricular zone and mantle zone (H, blue arrow pointing to blue staining). Data are representative of at least 3 separate experiments in each case. Scale bar, 1.74 mm (A), 1.42 mm (B), 0.39 mm (C), 12.1 mm (D,E), 2.2 mm (F), or 1.2 mm (G,H).

more posterior MB/rHB. Thus, neural stem cell lineage specification along the dorsoventral and anteroposterior axes is not irreversible and region-specific gene expression, especially later markers of regional identity, depend on inductive signals provided by the local environment.

DISCUSSION

Histogenesis of distinct forebrain, midbrain and hindbrain compartments is regulated by the generation of neuronal and glial progenitor cells from neural stem cells localized to the germinal zone. In principle, the pattern of regional histogenesis can occur in two ways. First, common (region independent) neural stem cells may divide to produce progenitor cells, which are then induced to manifest a region-specific pattern of gene expression and morphology according to local cues (whether soluble or contact mediated). In this case, neural stem cells are not intrinsically specified and their progeny subsequently acquire their regional identity. Some regional identities seem

Table 3. Transgene expression in the colonies from *zfdlx4/dlx6lacZ* transgenic mice co-cultured on CD1 tissues

Donor region	X-gal positive host tissue		
	GE	Cortex	MB
GE	9/11 (82%)	0/12 (0%)	0/6 (0%)
Cortex	11/13 (85%)	0/8 (0%)	0/6 (0%)
MB/rHB	3/4 (75%)	0/4 (0%)	0/2 (0%)

Primary neurosphere colonies from the *zfdlx4/dlx6lacZ* transgenic mouse brain were placed on the CD1 host slices or tissues for 5 days, and then subjected to X-gal staining. Frequencies of positive staining from at least three independent experiments are shown. GE, ganglionic eminence; MB/rHB, midbrain/rostral hindbrain.

to be endowed to neural progenitor cells through this mechanism (Eagleson et al., 1997). Second, neural stem cells may harbor a region-specific identity (established prior to the onset of neurogenesis) that is inherited by their progenitor cell progeny. Consistent with this latter possibility, our data demonstrate that neural stem cell-derived colonies in vitro (formed clonally from single neural stem cells) express molecular markers of regional identity, and maintain these regional expression pattern with repeated passaging in vitro. Although these findings do not allow us to conclude unequivocally that the very few neural stem cells themselves within each colony express regional markers, the fact each colony is clonally derived from a single stem cell suggests that regionalization is intrinsic to the colony-forming stem cells within E14.5 forebrain and midbrain/hindbrain compartments.

Previous reports demonstrated that the early expression of transcription factors in the anterior neural plate (E8.5) already defines a regionalization pattern that persists after the onset of neurogenesis (Shimamura et al., 1995). Furthermore, when precursor cells derived from the early neural tube (E9.5-E10.5) are isolated in vitro, they maintain their regional specification (Nakagawa et al., 1996). Our present results demonstrate that the E14.5 embryonic forebrain neural stem cells (from dorsal or ventral compartments) isolated from their in vivo environment generate clonal colonies that express forebrain-specific regional markers (*Emx1* or *Dlx2*), whereas neural stem cells isolated from the MB/rHB instead express a midbrain/rostral hindbrain-specific regional marker (*En1*), and those isolated from cHB express a caudal hindbrain-specific marker (*Hoxb1*). In addition, the *Otx1* expression that is normally restricted to the forebrain and midbrain at E14.5 in vivo (Boncinelli et al., 1993), was observed in cortical, GE, and MB/rHB neural stem cell colonies but not in cHB colonies. These data reveal that neural stem cells in the E14.5 mammalian brain manifest a regional identity along the anteroposterior axis during development. In a recent study, Zappone et al. (Zappone et al., 2000) demonstrated that mouse E14.5 cortical neural stem cell colonies expressed a telencephalic-restricted *Sox2* transgene or the *Otx1* gene, but that these genes were not expressed by spinal cord-derived neural stem cell colonies. Thus, neural stem cell regionalization may be regulated throughout the entire developing CNS. Moreover, neural stem cell regionalization during development is not restricted to the anteroposterior axis. We show that even within the forebrain, most of the neural stem cell colonies derived from the dorsal compartment (cortex) express *Emx1*, but not *Dlx2*, and most of the neural stem cell

colonies derived from the ventral compartment (GE) express *Dlx2*, but not *Emx1*. Thus, neural stem cells maintain a distinct dorsoventral identity within the forebrain, suggesting that neural stem cell regionalization can be regulated within distinct histogenic compartments rather than between broad CNS domains only. Recent advances in gene microarray technology may permit the description of more genes differentially expressed in neural stem cells from distinct regions [for example, see Geschwind et al. (Geschwind et al., 2001)].

The expression of *Emx1*, *Dlx2* or *En1* is clearly restricted in vivo to the cortex, ventral forebrain or midbrain/hindbrain junction (isthmus), respectively, as revealed by in situ hybridization analyses (Simeone et al., 1992; Bulfone et al., 1993; Davis and Joyner, 1988). However, some spilling over of regional marker gene expression was occasionally observed in single neurosphere colonies derived from those regions. A minority of the single sphere colonies (clonally derived from single neural stem cells) from the cortex expressed *Dlx2* or *En1* in addition to *Emx1*, a minority of those from the GE expressed *Emx1* or *En1* in addition to *Dlx2*, and a minority of those from the MB/rHB expressed *Emx1* or *Dlx2* in addition to *En1*. Contamination of cells from other regions during dissection is formally possible, although great care was taken to avoid such contamination. Indeed, the fact that some of single sphere colonies express more than one regional marker gene makes this possibility unlikely. An alternative explanation is that stem cell migration occurs between compartments in vivo. Migration of neuronal progenitors from GE germinal zone to the cortex has been reported (Anderson et al., 2001), although whether or not neural stem cells also migrate is unknown. Indeed, there is no evidence of migration from the cortex to the GE nor intermingling between the forebrain and midbrain/hindbrain has been observed. The most parsimonious interpretation for our data is that a given regional identity is specified by local inductive cues in a graded fashion and that neural stem cells are particularly sensitive to these cues. Cortical neural stem cells, for example, may respond to weak ventral forebrain inductive cues (*Sonic hedgehog* or other factors are candidates, see below), which cross the cortex/GE boundary, to make them express *Dlx2*, whereas their later progenitors may lose responsiveness to the cues and downregulate *Dlx2* expression. In any case, the progeny of neural stem cells need further specification by inductive and repressive cues during differentiation to refine their regional identities. *Emx2* is expressed in a graded fashion in vivo, with the highest expression in the posteromedial region and lowest expression in the anterolateral region of the dorsal forebrain. Although only very weak *Emx2* gene expression is detected in midbrain or hindbrain by in situ hybridization (Gulisano et al., 1996; Bishop et al., 2000) and by RT-PCR (this study), *Emx2* expression can be observed in neural stem cell colonies derived from the forebrain, MB/rHB and cHB (this study), as well as from the spinal cord (Zappone et al., 2000). Thus, neural stem cells, which reside in regions outside the forebrain in vivo, may receive repressive signals during later differentiation that decrease *Emx2* gene expression.

Clonal neural stem cell colonies derived from the cortex, GE or MB/rHB were specified regionally not only in terms of marker gene expression, but also by their potentials to respond to local migratory cues. The striatum and pallidum of the adult brain derive from LGE and MGE of the developing

telencephalon, respectively. While most of the projection neurons in the striatum are thought to derive from the LGE (Anderson et al., 1997b), substantial numbers of the striatal interneurons derive from the MGE (Marín et al., 2000). In addition some, if not all, of the cortical interneurons are provided by MGE precursor cells (Lavdas et al., 1999). The MGE-derived neurons migrate into the LGE to become the striatal interneurons or follow a radial then tangential migratory pathway to cortex to become the cortical interneurons. The precursor cells derived from the MGE (but not from the LGE or cortex) showed extensive migratory ability in vivo and in vitro, suggesting that the MGE precursor cells have a unique and widespread migratory potential (Wichterle et al., 1999; Lavdas et al., 1999). This migratory ability was posited to be cell autonomous after transplantation analyses using *Dlx1/2* mutant mice (Anderson et al., 2001). In the present study, we demonstrated that the clonal progeny of MGE neural stem cells showed robust migration, migrating greater distances than those of cortical or MB/rHB stem cells. These results imply that the MGE neural stem cells themselves are specified to produce progeny that respond to the local migratory cues of the developing ventral forebrain.

The expression of different *Dlx* genes in the ventral forebrain is correlated with the sequence of cellular differentiation (Liu et al., 1997; Eisenstat et al., 1999). *Dlx1* and *Dlx2* are expressed in progenitor cells primarily within the VZ and SVZ of the developing GE. In the process of differentiation and migration out of the germinal zone, progenitor cells begin to express first *Dlx5* and then *Dlx6* once they reside in the MZ and differentiate into mature projection neurons and interneurons. Although *Dlx2* is expressed in GE-derived neural stem cell colonies, extremely few colony cells express the *zfdlx4/dlx6lacZ* transgene, which normally mimics the endogenous expression of *Dlx5* and *Dlx6* in vivo (Zerucha et al., 2000), after an additional 7 days in differentiation conditions in vitro. Our findings suggest that a late regional identity may require further region-specific inductive cues that are not present within neural stem cell colonies.

The nature of the specific ventral forebrain inductive signal on neural stem cell colonies is not known. *Sonic hedgehog* (*Shh*) has been shown to be critical for inducing a ventral forebrain regional identity (Chiang et al., 1996; Nakagawa et al., 1996; Shimamura and Rubenstein, 1997; Kohtz et al., 1998). However, Kohtz et al. (Kohtz et al., 1998) demonstrated that *Dlx2* induction by *Shh* was limited by an early period of competence in telencephalic explants, which was lost after E12.5 in rat (~E10.5 in mouse). We demonstrated that the *zfdlx4/dlx6lacZ* transgene (which mimics *Dlx5* and *Dlx6* in vivo) could be induced in GE, cortical or MB/rHB neural stem cell colonies by mouse ventral forebrain tissue at E14.5. One possibility is that this transgene expression (or late regional markers in general) is regulated independently of early regional marker gene expression (e.g. *Dlx2*). However, our data showing that neural stem cells derived initially from the cortex and MB/rHB could be induced to express *Dlx2* after co-culture with the GE SVZ/MZ suggests that ventral forebrain cues can induce both early *Dlx2* and late *Dlx5/6* expression. Thus, upregulation of *Dlx1* and *Dlx2* may be required prior to transgene expression in all of the co-cultured neural stem cell colonies. If this model is correct, then one prediction is that late regional gene expression would be attenuated in forebrain

or MB/rHB neural stem cell colonies derived from *Dlx1/Dlx2* double knockouts (Anderson et al., 1997b) and exposed to ventral forebrain cues in vitro.

Several studies have demonstrated that forebrain precursors (primarily progenitor cells rather than stem cells) can be transplanted ectopically to integrate into ectopic tissues and differentiate into neurons and glia in vivo (Brüstle et al., 1995; Campbell et al., 1995; Fishell, 1995; Na et al., 1998; Herrera et al., 1999). However, the forebrain precursors were shown to maintain their original regional marker gene expression after transplanted into the midbrain (Na et al., 1998), and their abilities to survive, migrate and differentiate into neurons and glia in the ectopic tissues are limited (Herrera et al., 1999). Consistent with these observations, the present study demonstrated that the precursor cells within neural stem cell colonies derived from distinct compartments of the brain maintain their original regional marker gene expression and differentially respond to local environmental cues to migrate. However, cortical and MB/rHB neural stem cell colonies recovered from sphere colony/slice co-cultures changed their regional identities to express *Dlx2* instead of their original regional homeobox gene expression pattern. Thus, neural stem cells may have more plasticity than their progeny. Indeed, our data suggest that neural stem cell lineages may be specified but not committed to any one regional compartmental identity.

Neural stem cells and their progeny cultured in vitro (as stem cell colonies or monolayers) also can be transplanted from one brain region and survive and differentiate into neurons and glia in another host brain region (Fricker et al., 1999; Shihabuddin et al., 2000; Nishino et al., 2000). Neural precursor cells produced by neural stem cells in vitro may inherit the identities of the original brain compartment from which the neural stem cells were derived. Indeed, the migration and full differentiation of the transplanted progenitor neurons derived from neural stem cells in vitro are limited (Takahashi et al., 1998), and neural precursor cells derived from neural stem cells from different brain regions in vitro have distinct abilities to produce region-specific neurotransmitters after transplantation (Nishino et al., 2000). Given that neural stem cells have the potential for regional fate plasticity in response to local environmental factors, the use of such factors prior to transplantation could facilitate the adaptation of neural stem cell progeny to the host tissue, in order to differentiate into region-specific neurons and glia.

In conclusion, these findings reveal that neural stem cell lineages from different compartments in the developing mammalian brain behave in a remarkably similar manner with respect to self-renewal and neuronal/glial multipotentiality, but have distinct spatial identities. Among histogenic compartments (along the dorsoventral or anteroposterior axes), isolated neural stem cell lineages have a region-specific pattern of gene expression and migratory potentials. Nevertheless, changes in the regional identity of neural stem cells can be induced by local cues in a cell nonautonomous manner. Thus, regional specification of neural stem cell lineages between dorsal and ventral or anterior and posterior histogenic compartments may not be irreversible. The fates of neural stem cell lineages (although specified) are not committed to specific compartments, but rather some regional phenotypic plasticity is inherent to all neural stem cell lineages.

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