Differential Origins of Neocortical Projection and Local Circuit Neurons: Role of Dlx Genes in Neocortical Interneuronogenesis

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Herein we review the evidence that neocortical projection neurons and interneurons are derived from distinct regions within the telencephalon. While neocortical projection neurons are derived from the ventricular zone of the neocortex, neocortical interneurons appear to be derived from the germinal zone of the basal ganglia. These interneurons follow a tangential migratory pathway from the ganglionic eminences to the cortex. Interneurons of the olfactory bulb follow a distinct tangential migration from the basal ganglia. The *Dlx* homeobox genes, which are essential for basal ganglia differentiation, are also required for the development of neocortical and olfactory bulb interneurons. Furthermore, evidence is presented that retroviral-mediated expression of DLX2 in neocortical cells can induce GABAergic interneuron differentiation.

Introduction

There are two general classes of neocortical neurons, the excitatory pyramidal neurons, which typically send their axons to distant targets, and the inhibitory (GABAergic) interneurons, which form only local synaptic connections. The inhibitory neurons comprise 15–30% of all neocortical neurons (Parnavelas *et al.*, 1977; Hendry *et al.*, 1987; Meinecke and Peters, 1987). Despite their smaller number, the inhibitory neurons play a vital role in modulating neocortical functions. Recent evidence suggests that cortical projection neurons and interneurons are derived from distinct proliferative zones. Most differentiating neocortical neurons migrate from the ventricular zone (VZ) towards the pial surface along the processes of radial glia (Rakic, 1972; Misson *et al.*, 1991, O'Rourke *et al.*, 1992; Tan and Breen, 1993; Tan *et al.*, 1998). However, as described below, there is a substantial amount of tangential migration within the nascent neocortex (Walsh and Cepko, 1988; O'Rourke *et al.*, 1992; Mione *et al.*, 1997), much of it originating from the subcortical telencephalon (De Carlos *et al.*, 1996; Anderson *et al.*, 1997b; Tamamaki *et al.*, 1997). This paper will review the emerging evidence that cortical projection neurons originate from the cortical VZ, whereas the majority of cortical interneurons are born in the basal ganglia anlage (Anderson *et al.*, 1997b). In addition, evidence will be presented that suggests that the *Dlx-2* gene can support the expression of a GABA-ergic phenotype in neocortical cells.

Cortical and Subcortical Origins of Cortical Neurons: Radial Migration of Projection Neurons and Tangential Migration of Interneurons

Until the late 1980s, experimental evidence supported a model in which most newborn cortical neurons migrate radially from the VZ to the overlying mantle zone (MZ) (Rakic, 1988), although the potential for tangential migrations was recognized (for example see Karten, 1969; Fig. 1 of Boulder Committee, 1970). This radial relationship between progenitor cells and mature neurons formed the foundation for the Protomap model of neocortical regionalization (Rakic, 1988). Subsequently, experiments using lineage analysis with retroviral vectors (Price and Thurlow, 1988; Walsh and Cepko, 1988; Austin and Cepko, 1990) and vital dyes (O'Rourke *et al.*, 1992; Fishell *et al.*, 1993) demonstrated substantial tangential migrations within the neocortex. While most lineage analyses supported the predominance of radial migration (Luskin *et al.*, 1988; Tan and Breen, 1993; Kornack and Rakic, 1995; Tan *et al.*, 1998), the dispersion of many clonally related cells with respect to their site of origin called into question whether neocortical regional specification could indeed be regulated by positional information within its VZ.

Beyond describing migration patterns of clonally related cells, lineage studies have also explored the relationship between migration patterns and cell phenotype. Clones of glia tend to be more dispersed than neuronal clones (Luskin *et al.*, 1988). More recently, experiments using retroviral labelling or chimeric embryos provided evidence that radially migrating cells give rise primarily to projection neurons and tangentially migrating cells give rise to interneurons (Mione *et al.*, 1997; Tan *et al.*, 1998). However, these studies did not address the origin of the tangentially migrating cells.

Tangentially Migrating Interneuron Precursors in the Neocortex may Originate in the Basal Telencephalon

Recent investigations of neuronal migration suggested that cells migrate from the lateral ganglionic eminence (LGE), the primordium of the striatum (Deacon *et al.*, 1994; Olsson *et al.*, 1995), into the neocortex via the SVZ and/or mantle zone. Focal tracer injections have been made into the LGE of rat embryos (De Carlos *et al.*, 1996). Although most of the cell migration from the LGE appeared to be radially directed towards the striatal mantle and the primary olfactory cortex, a few cells migrated tangentially into the neocortex. We found a robust cell migration from the mouse basal telencephalon to the cortex using slice cultures; this migration is eliminated when basal ganglia differentiation is blocked in mice lacking the *Dlx1* and *Dlx2* homeobox genes (Anderson *et al.*, 1997b). Likewise, it has been demonstrated (Tamamaki *et al.*, 1997) that migration from the LGE into the cortex using both cultures of rostral telencephalon and in-utero injections into E16 rat embryos. Neurotrophin-4 has been applied to E16 forebrain slice cultures (Brunstrom *et al.*, 1997) and heterotopias have been found to develop in the neocortical marginal zone; these authors provided evidence for an LGE origin of the cells in the ectopia (Brunstrum *et al.*, 1998).

What types of cells migrate from the basal telencephalon to the cerebral cortex? Several lines of evidence suggest that one or more type of GABAergic interneuron contribute to this tangential migration. First, GABA immunoreactive cells can be found in the lateral striatal mantle and the intermediate zone of the neocortex at early stages of telencephalic development

(mouse E12.5). Many of these cells have a morphology and orientation that suggests they may be migrating from the LGE into the neocortex (Van Eden *et al.*, 1989; Del Rio *et al.*, 1992; DeDiego *et al.*, 1994). Second, as described above lineage analyses support the concept that radially oriented neocortical clones tend to contain projection neurons and are clonally distinct from tangentially dispersed clones which tend to contain interneurons (Mione *et al.*, 1997; Tan *et al.*, 1998). Third, Brunstrom *et al.* observed that many cells in the NT4-induced marginal zone ectopia were GABAergic (Brunstrom *et al.*, 1998). Finally, we provided three lines of evidence that some cells migrating from the basal telencephalon into the neocortex were GABAergic (Anderson *et al.*, 1997b). We found that many tangentially migrating cells from the basal telencephalon express GABA and/or calbindin. Surgically separating the basal ganglia from the cortex greatly decreased the number of cortical GABA-ergic, Dlx1-positive and calbindin-positive cells; *Dlx1/Dlx2* mutant mice, which lack this tangential migration, have a four-fold reduction in neocortical GABAergic and calbindin-positive cells on the day of birth (when these animals die) (Anderson *et al.*, 1997b).

The MGE is a Major Source of Tangentially Migrating GABAergic and Calbindin-positive Cells that Migrate from the Basal Telencephalon into the Cortex

Although evidence suggests that many interneurons in the maturing neocortex are derived from the basal telencephalon, their precise origin has not been demonstrated. There are several histologically and morphologically distinct primordia within the basal telencephalon, including the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE), septum (SE) and caudal ganglionic eminence (CGE). These are believed to give rise to the striatum, pallidum, septum and amygdala, respectively (Deacon *et al.*, 1994; Olsson *et al.*, 1995). There is now evidence for the genetic basis underlying the regional differences in these primordia. For instance, while normal cell migration out of the LGE and MGE requires *Dlx1* and *Dlx2* (Anderson *et al.*, 1997b and unpublished data), only the MGE requires the *Nkx2.1* homeobox gene (Sussel *et al.*, unpublished data).

Based in part upon the analysis of the *Nkx2.1* and *Dlx1/Dlx2* mutant mice, different subcortical primordia appear to produce interneurons which migrate tangentially along distinct pathways. Mice that have a mutation of the *Nkx2.1* homeobox gene lack MGE specification, whereas the LGE specification and differentiation appears to be normal (Sussel *et al.*, unpublished data). At E18.5 these mutants lack most calbindin-expressing cells in the cortex, and have about a two-fold reduction in cortical GABAergic interneurons. These results suggest that the MGE is an important source of subcortical cells that migrate into the neocortex. This hypothesis is substantiated using DiI labelling of the MGE in slice cultures, which reveals migrations from the MGE through the LGE and then into the cortex (Lavdas *et al.*, 1999) (S. Anderson *et al.*, unpublished data; Sussel *et al.*, unpublished data).

By comparing the neocortical phenotypes of the *Nkx2.1* mutants (which lack migration from the MGE) and the *Dlx1/Dlx2* mutants (which lack migration from both MGE and LGE), the subcortical origins of neocortical cells can be examined. Whereas *Dlx1/Dlx2* and *Nkx2.1* mutants have a similar reduction in neocortical calbindin-positive cells, fewer GABAergic neurons are detectable in the *Dlx1/Dlx2* (~75% of wild type) than the *Nkx2.1* mutants (~50% of wild type) (Anderson *et al.*, 1997b) (Sussel *et al.*, unpublished data). Additionally, in the *Nkx2.1* mutants DLX2 immunoreactivity is greatly reduced in the superficial portion of the neocortex, but is only reduced ~50% in the intermediate zone and deeper layers of the cortical plate. We are currently investigating whether the residual GABAergic and DLX-positive cortical neurons in the *Nkx2.1* mutants migrate from the LGE. If so, it implies that cortical cells migrating from the MGE and LGE may follow distinct pathways, with the marginal zone being primarily utilized by the MGE-derived cells.

There is additional evidence for distinct origins and intra-telencephalic migration pathways of subcortical cells. There is a well-described tangential migration of precursors of GABAergic interneurons from the basal telencephalon into the olfactory bulb, known as the rostral migratory stream (Hinds, 1968; Luskin, 1993; Lois and Alvarez-Buylla, 1994). Interneurons derived from this pathway do not differentiate in the *Dlx1/Dlx2* mutants, e.g. there is no detectable GABA in the olfactory bulb (Anderson *et al.*, 1997a; Bulfone *et al.*, 1998), whereas their differentiation appears to be normal in the *Nkx2.1* mutants (Sussel *et al.*, unpublished data). Thus, the LGE and perhaps septum are required for this rostral interneuron migration, whereas the MGE may have a primary role in the dorsal tangential migration.

Potential Guidance Mechanisms for Tangential Migration into the Neocortex

Essentially nothing is known about the mechanisms that guide the tangentially migrating cells from the MGE and LGE into the cerebral cortex and then into their final positions within the cerebral cortex. On the other hand, some mechanisms have been elucidated for the rostral migratory stream. These cells appear to crawl over each other in long chains within the SVZ (Lois *et al.*, 1996). At postnatal ages, this 'chain migration' takes place in channels created by specialized cells (Lois *et al.*, 1996). Sialylated NCAM is required to support the migration (Tomasiewicz *et al.*, 1993; Hu *et al.*, 1996). There is no evidence that these cells are migrating along the fibres of axon tracts or of radial glial cells. Chemotrophic factors may play a role in guiding this migration, as the septum appears to produce a chemorepellent factor for these migrating cells (Hu and Rutishauser, 1996), although no chemoattractant has been identified.

Aside from its dependence on the *Dlx1/2* genes, it is unknown whether the basal ganglia-neocortex migration has any of the characteristics of the rostral migratory stream. Our preliminary results using explants of wild-type neocortex, LGE, MGE or lamina terminalis, grown in collagen gels, have failed to demonstrate any chemorepulsive or attractive activity for migrating cells. We observe robust cell migration into the collagen from MGE or LGE explants whether or not explants from the neocortex, MGE, or lamina terminalis were placed nearby (Anderson and Rubenstein, unpublished data). It should be noted that a negative result from such experiments by no means rules out the possibility that some chemotrophic activity for these regions exists *in vivo*. We have also examined whether cortical interneuronogenesis is affected in mice that have greatly reduced expression of netrin. Netrin is an extracellular protein expressed at high levels in the prenatal basal ganglia (Metin *et al.*, 1997) that has chemoattractant and chemorepellent properties for axon growth and cell migrations (Kennedy *et al.*, 1994; Serafini *et al.*, 1996). In newborn netrin –/– mice we did not detect a reduction in cortical interneurons, suggesting that the basal ganglia to neocortex migration was not affected (data

not shown). We made the same observations in mice lacking one of the netrin receptors (DCC) (data not shown).

Axons could also provide a mechanism for the guidance of tangentially migrating neurons (Rakic, 1985). Evidence for tangential migration along axons has been reported in the chick forebrain (Gray *et al.*, 1990; Golden *et al.*, 1997), as well as by LHRH containing cells in the basal telencephalon (Yoshida *et al.*, 1995). Within the intermediate zone of the dorsal neocortex, only a few tangentially migrating cells have been shown to make specialized contacts with axons (O'Rourke *et al.*, 1995). However, it is unclear whether tangentially migrating cells from the basal telencephalon interact with cortical axons as they first encounter them in the developing internal capsule. In the mouse, corticofugal axons appear in the SVZ and the mantle zone of the LGE at approximately embryonic day 12.5 (E12.5) (Sheth *et al.*, 1998); the same age at which we first detect subcortical cells entering the neocortex near the striatopallial angle. E12.5 is also the age at which GABA, calbindin and DLX1 or DLX2 immunoreactive cells first appear in the intermediate zone of the lateral neocortex (Del Rio *et al.*, 1992; Anderson *et al.*, 1997b) (S. Anderson and J.L.R. Rubenstein, unpublished data). At E13.5, calbindin expressing, tangentially oriented cells in the neocortical intermediate zone appear to be in close apposition with corticofugal fibers at the striatopallial angle (Metin and Godement, 1996). In light of other evidence that these calbindin immunoreactive cells migrate from the basal forebrain (Anderson *et al.*, 1997b), these findings are suggestive of an interaction between corticofugal axons and the tangentially migrating cells (Metin, 1998). The semaphorin family of secreted guidance molecules (Kolodkin *et al.*, 1993; Puschel *et al.*, 1995), which can mediate both attractive and repulsive cortical axon growth responses (Bagnard *et al.*, 1998), could be a reasonable candidate for mediating tangential neuronal migration along corticofugal axons. It should also be noted that in the region of the lateral neocortex some radial glial fibers follow a course that is roughly parallel to the pial surface (Misson *et al.*, 1991) (see Fig. 1), and thus could potentially support the initial guidance of tangential migration into the neocortex.

Dlx Genes may be Required for Specification of GABAergic Cells in the Neocortex

At the earliest stages of differentiation within the telencephalon there is a remarkable segregation of GABAergic and non-GABAergic cells. GABAergic cells are found in the basal telencephalon, the same region where the *Dlx* genes are expressed. In the *Dlx1/2* mutants, GABA expression in the basal telencephalon is not blocked. Thus, many of the cells that accumulate within the *Dlx1/2* mutant striatal SVZ express GABA and its synthesizing enzyme GAD67 despite their failure to migrate into the mantle zone, i.e. the striatum, olfactory bulb, and neocortex (S. Anderson and J.L.R. Rubenstein, unpublished data). This shows that *Dlx1* and *Dlx2* are not required to express glutamic acid decarboxylase (GAD) in the LGE and MGE. We consider it likely that there are genes which share some functions with the *Dlx1* and *Dlx2*, which can take the place of *Dlx1* and *Dlx2* in controlling the GABAergic specification in the basal ganglia.

On the other hand, several lines of evidence suggest that *Dlx1* and *Dlx2* are required for the development of many neocortical GABAergic neurons. Around E12.5, DLX1, DLX2 and GABA immunoreactive cells appear to spread from the LGE into the adjacent cortex coincidently. GABA and GAD67 immunoreactivity is greatly reduced in the neocortex of *Dlx1/2* mutants (Anderson *et al.*, 1997b). This is particularly striking when comparing GAD67 expression in the marginal zones of the neocortex and the paleocortex [Fig. 4 in (Anderson *et al.*, 1997b)]. DLX1 and DLX2 expression, which is present in many marginal zone cells of the wild-type neocortex, also has an abrupt boundary in layer 1 at this same position (Fig. 2). Furthermore, we have observed expression of DLX1 in a subset cortical GABAergic cells at E13.5 (Anderson *et al.*, 1997b), and at P0 (Fig. 2), and have evidence that DLX1 and DLX2 are expressed in the cells that are tangentially migrating from the basal telencephalon to the neocortex.

The requirement of *Dlx1* and *Dlx2* for the development of neocortical GABAergic cells, as well as the co-expression of DLX1 in GABAergic cortical interneurons, suggests that DLX1 and/or DLX2 may directly regulate the GABA phenotype in the neocortex. To test this hypothesis, we constructed a retroviral vector that expresses DLX2 and infected primary cultures of neocortical cells from *Dlx1/2* mutants. The results of these experiments are described in this paper.

Materials and Methods

Virus Production

The LZRSpBMN (Kinsella and Nolan, 1996) vector was used to generate a replication incompetent retrovirus encoding full-length mouse DLX2 protein ($LZRS^{pBMN-Dlx2}$). A *Eco*RI–*Not*I fragment, encoding amino acids 1–332 of mouse Dlx2, was cloned into the corresponding sites of $LZRS^{pBMN}$. The retroviral vector $LZRS^{pBMN-LacZ}$ encoding for *E. coli* β-galactosidase (a gift of G. Nolan, Stanford, CA, USA) was used to produce control virus. Titers of approximately 10^5–10^6 gene transducing units/ml were obtained 48 h after calcium phosphate-mediated transfection into Phoenix ecotropic producer cells (Grignani *et al.*, 1998). Cells infected with the $LZRSp^{BMN-LacZ}$ virus were assayed using X-gal immunohistochemistry. Cells infected with the $LZRSp^{BMN-Dlx2}$ virus were assayed using anti-DLX2 immunohistochemistry (Porteus *et al.*, 1994).

Ectopic Expression In Vitro

The effects of retrovirally driven expression of *Dlx2* were studied in primary cultures of cortex derived from *Dlx1/2* –/– E15.5 embryos and their wildtype (wt) littermates. Briefly, embryos resulting from mating between *Dlx1/2* –/–heterozygous parents were examined for the presence of cleft palate (which is 100% penetrant in *Dlx1/2* –/– mice; Qiu *et al.*, 1997). The genotype of each embryo was then confirmed using PCR amplification of genomic DNA as described previously (Anderson *et al.*, 1997a). Each brain was processed separately, the pial membrane was removed and neocortical regions corresponding to the presumptive motor and somatosensory cortex were dissected out. Cells were dissociated in 0.01% trypsin and plated on polyornithin/fibronectin-coated coverslips at a density of 2×10^5 cells/ml. Two hours after plating, cells were infected with 100 µl of retroviral suspension, containing approximately 10^4 infectious viral particles. Cells were cultured for 5 days in neurobasal medium (Gibco) containing 10 ng/ml recombinant bFGF.

Sister cultures from wild-type and *Dlx1/2* –/– cortices that were infected with a control virus or with $LZRS^{pBMN-Dlx2}$ virus were analysed for cell proliferation, neuronal differentiation and GABA expression. The number of proliferating cells was evaluated following exposure to 10^{-5} M BrdU during the last 12 h *in vitro* as previously described (Cavanagh *et al.*, 1997). Differentiating neurons identified by their immunoreactivity with a mouse monoclonal Map2 antiserum (diluted 1:500; Boehringer). GABA expressing cells were revealed using a rabbit polyclonal antiserum (diluted 1:2000; Sigma). The results represent the mean percentage ± SEM of positive cells from three different experiments, in which two coverslips per experimental condition and staining procedure were evaluated. A minimum of 500 cells per coverslipped culture was counted.

RT-PCR

A minimum of 5×10^4 wild type or *Dlx1/2* –/– cells infected with the *LacZ* or the *Dlx2* retrovirus were harvested in 0.5 ml Triazol (Gibco). Total RNA was extracted following the manufacturer's instructions.

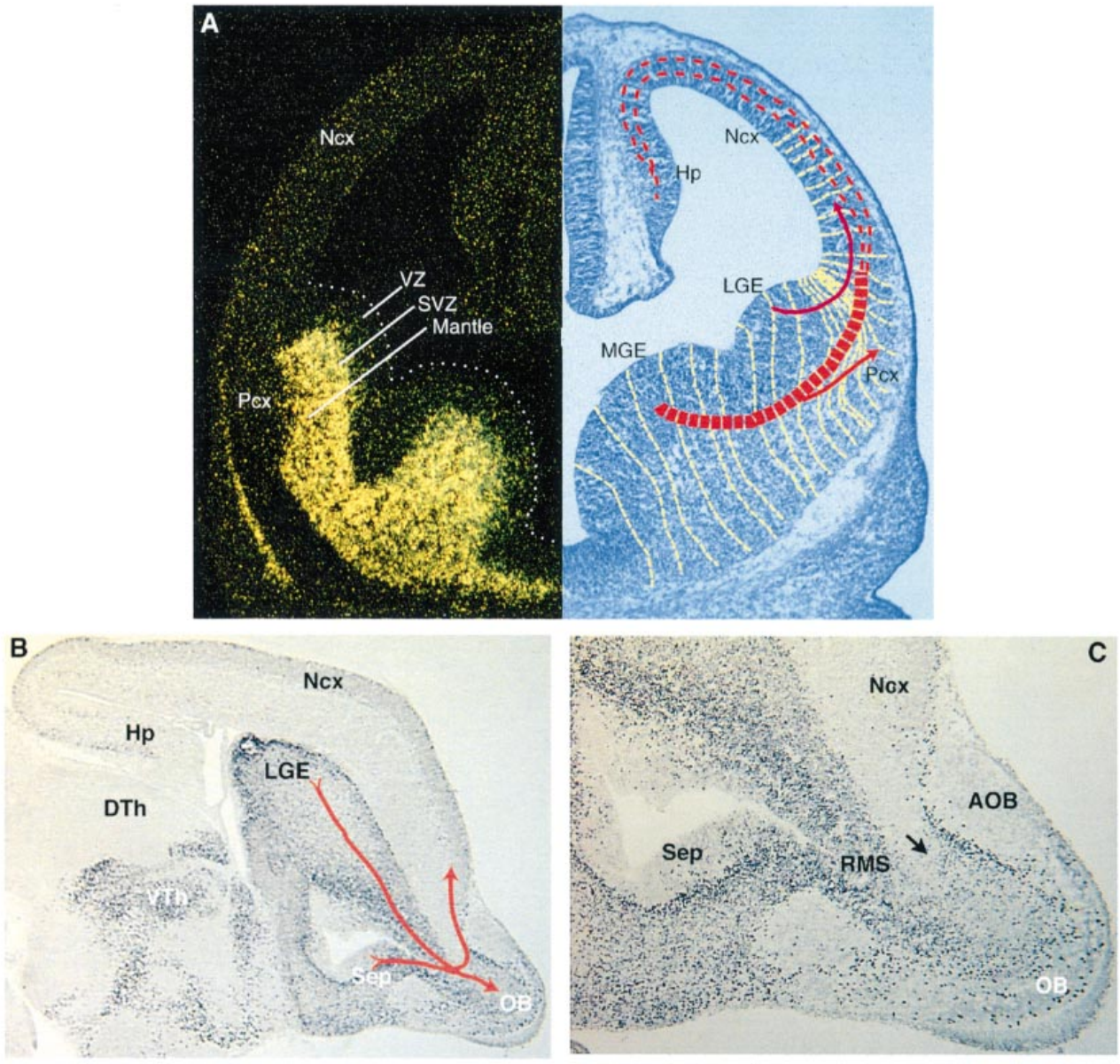


Figure 1. The lateral (*A*) and medial (*B* and *C*) routes of subcortical to neocortical migration. *A* shows a nissl stain of a coronal E12.5 section on the right, with radial glial fibers shown in yellow. The pattern of radial glial processes extending from the striatopallial angle and the 'pallial' portion of the LGE was derived from observations of DiI labeling in slices, and from other studies (Misson *et al.*, 1991; De Carlos *et al.*, 1996). The left side of *A* shows Dlx5 expression in a serial section to that on the right. Note that the expression domain of Dlx5 in the LGE demarcates the boundary, within the SVZ and mantle, between the striatal (basal) LGE and the neocortical (pallial) portion of the LGE. Abbreviations: Ncx, neocortex; Pcx, paleocortex; Hp, hippocampus. *B* and *C* show DLX-1 immunoreactivity in a sagittal section from E16.5 mouse embryos. The red lines in *B* indicate the hypothetical migration path of cells from the LGE, septal, and retrobulbar region into the rostral neocortex. Later in gestation and postnatally, cells migrate along this pathway into the olfactory bulb (the rostral migatory stream). The arrow in *C* shows the DLX-1 positive cells that may be moving dorsally from the retrobulbar region into the neocortex. GABA immunoreactivity labels a similar distribution of cells (Meyer *et al.*, 1998). Abbreviations: AOB, accessory olfactory bulb; DTh, dorsal thalamus; OB, olfactory bulb; Sep, septal area; RMS, rostral migratory stream; VTh, ventral thalamus.

The RNA was electrophoresed on a 1.5% agarose gel to assay for degradation. For each RT-PCR reaction, 200 ng of RNA was reverse transcribed using 2 units of AMV-RT (Promega) in the presence of 8 units of RNase inhibitor, 5 nmol of dNTPs and 10 pmol of one of the following sets of primers:

mouse GAD67:

(sense): AAGGCATGGCGGCTGTGCCCAAAC

(antisense): ACCACCCCAGGCAGCATCCACATG

corresponding to nucleotides 817–841 (sense) and nucleotides 1108–1131 (antisense) of a GAD67 cDNA; GenBank accession number Y12257.

mouse Dlx2:

(sense): GGCACCAGTTCGTCTCCGGTCAA

(antisense): CGCCGAAGTCCCAGGATGCTG

corresponding to nucleotides 2985–3007 (sense) and nucleotides 4205–4225 (antisense) of genomic Dlx2; GenBank accession number U51002.

mouse GAPDH:

(sense): GTGGCAAAGTGGAGATTGTTGCC
(antisense): GATGATGACCCGTTTGGCTCC
corresponding to nucleotides 114–136 (sense) and nucleotides 382–404 (antisense) of GAPDH cDNA; GenBank accession number M32599.

Reverse transcription was carried out at 42°C for 45 min. After adding 1 unit of Taq polymerase (Perkin-Elmer-Cetus, Emeryville, CA, USA), mixtures were subjected to the following thermal cycles: 97°C for 2 min, 1 cycle; 97°C for 1 min, 60°C for 1 min, 72°C for 45 s, 20 cycles for GADPH; 25 cycles for Dlx2 and 30 cycles for GAD67. As a positive control, a PCR reaction was carried out with 10 ng of Dlx2, GAPDH or GAD67 cDNAs as templates. Different amounts (1 μg, 500 ng, 250 ng and 125 ng) of total RNA extracted from E15.5 mouse forebrain were used as an indication that the amount of DNA amplified was a function of the starting amount of RNA. Twenty microliters of each individual PCR reaction was electrophoresed on a 1.5% agarose gel; the DNA fragments were stained with ethidium bromide. The predicted sizes of the PCR products were: 314 bp (GAD67); 365 bp (Dlx2); 280 bp (GAPDH). Genomic DNA amplification, which sometimes occurs because of contamination, could be easily differentiated from cDNA amplification by the size of the PCR products. In fact, for each primer pair, the sense and antisense primers were positioned on two different exons. In addition, no PCR amplification was obtained when AMV-RT was omitted from the reaction.

Results

Infection with Recombinant Retroviruses Encoding Dlx2 Leads to DLX2 Expression in Cortical Cells Derived from Dlx1/2 Mutants

Neocortical primary cultures from E15.5 wild-type and *Dlx1/2* –/– mice appeared indistinguishable in morphology and density. Cells were infected with recombinant retroviruses within 2 h of being placed in culture and grown for 5 days. Infection with either the LZRSp$^{BMN-LacZ}$ or LZRSp$^{BMN-Dlx2}$ virus, did not affect the growth or survival of the cells. DLX2 immunoreactive cells were readily detected in non-infected or LZRSp$^{BMN-LacZ}$ virus-infected wild-type cortical cultures (roughly 20% of the cells were DLX2 positive; Fig. 3*a*). Cultures derived from *Dlx1/2* –/– cortices had no DLX2-immunoreactive cells whether or not they were infected with LZRSp$^{BMN-LacZ}$ (Fig. 3*b*). However, upon infection with LZRSp$^{BMN-Dlx2}$, DLX2 immunoreactive cells were found in the *Dlx1/2* –/– cultures (roughly 5% of the cells; Fig. 3*c*). These DLX2-expressing cells were often observed in clusters of two to eight cells.

Effects of Ectopic Dlx2 Expression on Proliferation, Differentiation and GABA Expression in Wild Type and Dlx1/2–/– Cortical Cells

The number of proliferating cells in cortical cultures derived from wild-type and *Dlx1/2* –/– mice was estimated based upon the number of BrdU$^+$ cells after a 12 h exposure to the thymidine analogue. Approximately 73 ± 13% cells were labeled with BrdU in *Dlx1/2* –/– cultures, as compared to 76 ± 8% in cultures from wild-type littermates. Infection of *Dlx1/2* –/– cultures with LZRSp$^{BMN-Dlx2}$ did not affect the percentage of proliferating cells (72 ± 8%).

Next, we studied the effect of infection with LZRSp$^{BMN-Dlx2}$ on differentiation (based on expression of the neuronal marker, MAP2) in the wild-type and *Dlx1/2* mutant neocortical cultures. We found no significant difference between various cultures (34 ± 6% of the cells were MAP2 immunoreactive in each case). While this general marker of cortical differentiation was not affected by the *Dlx1/2* mutation, there was a marked reduction of GABA$^+$ cells in cortical cultures from *Dlx1/2* –/– mice (Fig. 3*e*) compared with cultures from wild type mice (Fig. 3*d*). In all, 19 ± 5% of the wild-type cortical cells were GABA$^+$, whereas only 2 ± 2% cells were GABA$^+$ in *Dlx1/2* –/– cortical cells. Upon infection with LZRSp$^{BMN-Dlx2}$, the number of GABA$^+$ cells in *Dlx1/2* –/– cultures increased roughly five-fold, to 11 ± 3% (Fig. 3*f*).

The induction of *Dlx2* and *GAD* expression via infection with the LZRSp$^{BMN-Dlx2}$ virus was assayed by RT-PCR. Using primers specific for *GAD67* and *Dlx2*, we observed DNA fragments of the expected size from wild type (Fig. 3*g*, lane 3), but not from *Dlx1/2* –/– cortical cultures (lane 4). Upon infection of *Dlx1/2* –/– cortical cultures with LZRSp$^{BMN-Dlx2}$, we could now detect expression of both *Dlx2* (lane 5), and *GAD67* (lane 5) RNA.

Discussion

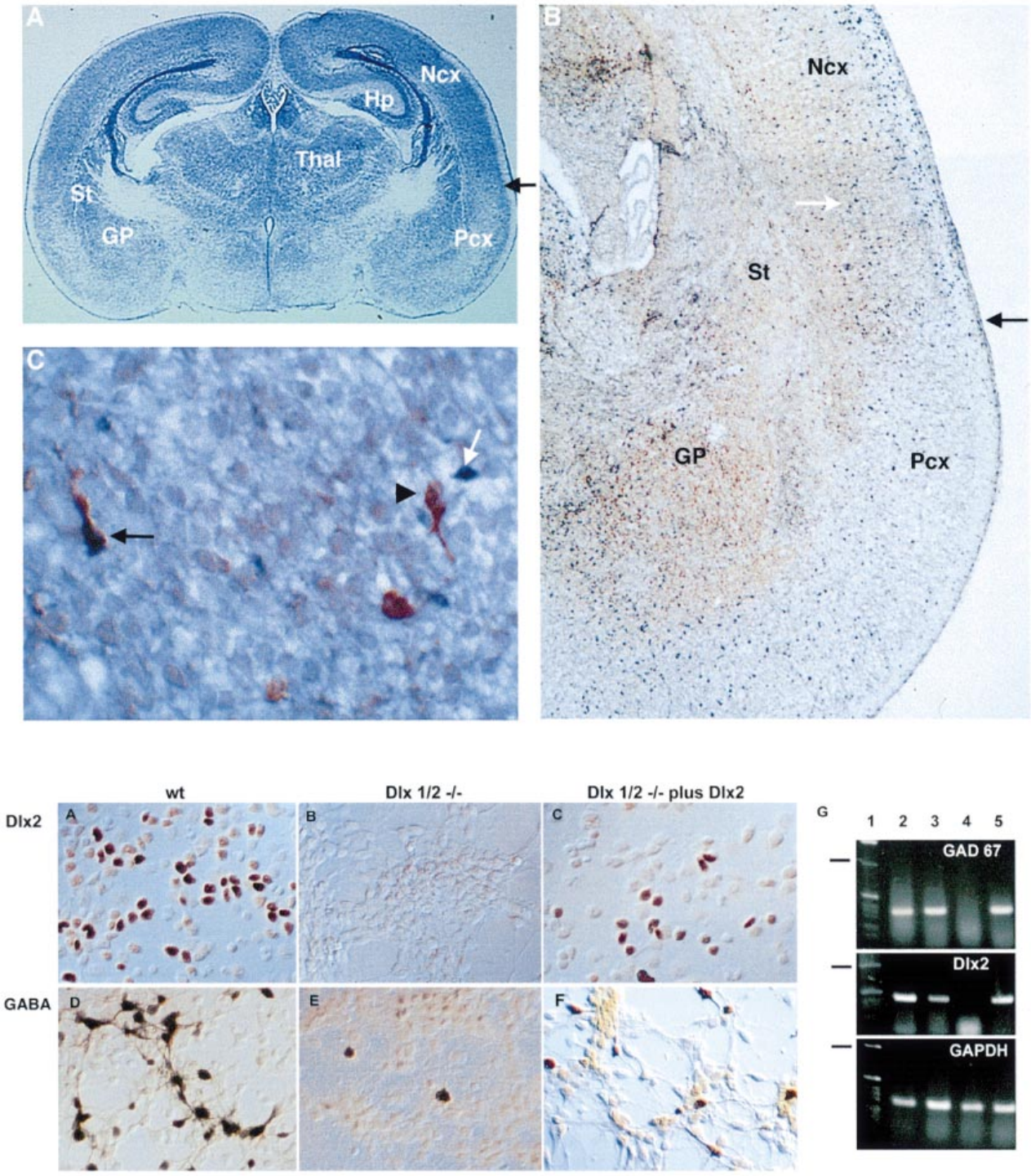
Lateral and Medial Subcortical-to-cortical Migrations

Herein we have reviewed the evidence that most neocortical interneurons, as observed at birth, arrive to the cortex via tangential migration from the basal telencephalon. There appear to be at least two major sources for these migrating cells: the MGE and the LGE. We suggest that there are at least two distinct ventral-to-dorsal pathways by which subcortical cells reach the cerebral cortex. One is a lateral pathway, in which cells begin their migration in the region of the MGE, pass through the LGE and then enter paleocortex, neocortex and archicortex (Fig. 1*a*). This pathway is greatly reduced or eliminated in the *Nkx2.1* mutant mice (Sussel *et al.*, unpublished data); we are presently exploring whether there is a late lateral migration from the LGE that is independent of the MGE.

The second is a medial pathway, in which cells begin their migration in the region of the LGE and septum, and then move to the base of the olfactory bulb. Based upon immunohisto-

Figure 2. DLX1 (blue–black) and GABA (brown) immunoreactivity at P0. *A* shows a low power view of an 8 μm coronal section; the box indicates the region shown in *B*, and the black arrows in *A* and *B* show the region of transition from neocortex to paleocortex. Note the presence of many DLX1 immunoreactive cells in layer I of the neocortex (Ncx), whereas far fewer cells are detected in the paleocortex (Pcx). In the *Dlx1/2* mutants GAD67 and GABA immunoreactivity is dramatically reduced in layer I of the neocortex, but are normal in layer 1 of the paleocortex (Anderson *et al.*, 1997b). *C* shows cells in a deep region of the cortical plate in lateral neocortex (white arrow in *B*). Note the co-labeling for GABA and DLX1 in a cell (black arrow), and two cells that reacted for either GABA (black arrowhead) or DLX1 (white arrow). Less than 10% of GABA immunoreactive cells also express detectable levels of DLX1 in this preparation. It is possible that Dlx1 (and Dlx2) expression is down-regulated as differentiation proceeds. Abbreviations: GP, globus pallidus; Hp, hippocampus; Ncx, neocortex; Pcx, paleocortex; St, striatum; Thal, thalamus.

Figure 3. *A–F* Immunohistochemical analysis of cortical cultures from wild type (wt) (*A*, *D*) and *Dlx1/2* –/– (*B*, *C*, *E*, *F*) mouse forebrain, infected with a control virus (*A*, *B*, *D*, *E*) or with a LZRS$^{pBMN-Dlx2}$ virus (*C*, *F*). *A–C* Dlx2 immunoreactivity; *D–F* GABA immunoreactivity. *A* Approximately 30% of the cortical cells grown in the presence of bFGF for 5 days show Dlx2 immunoreactive nuclei. *B* No Dlx2 immunoreactivity could be detected in cultures derived from *Dlx1/2* –/– mice. *C* Upon infection with LZRS$^{pBMN-Dlx2}$ virus, Dlx2 is expressed in infected cells. *D* Over 20% of the cells in the cultures derived from wild type cortices are GABA immunoreactive. *E* Very few GABA$^+$ cells are found in cultures derived from *Dlx1/2* –/– mice. *F* Infection of *Dlx1/2* –/– cells with a recombinant retrovirus encoding for Dlx2 induces an increase in the number of GABA$^+$ cells. Calibration bar: 20 μm. *G* RT-PCR analysis of GAD67, Dlx2 and GAPDH expression in wild type and *Dlx1/2* –/– cells. Lane 1: DNA ladder, 1 kb band is marked. Lane 2: cDNA for GAD67, Dlx2 or GAPDH. Lane 3: wt cultures infected with a control virus. Lane 4: *Dlx1/2* –/– cultures infected with a control virus. Lane 5: *Dlx1/2* –/– cultures infected with LZRS$^{pBMN-Dlx2}$ virus.



chemical studies of GABA expression in both humans and rodents, some of these cells may migrate into the cortical marginal zone and then move along its rostro-caudal dimension (Fig. 1*b*) (Gadisseux *et al.*, 1992, Meyer *et al.*, 1998, Lavdas *et al.*, 1999). The migration into the olfactory bulb becomes the rostral migratory stream late in gestation and postnatally (Hinds, 1968; Luskin, 1993). We suggest that both the medial and lateral pathways are disrupted in mice carrying the *Dlx1/2* mutation (Anderson *et al.*, 1997b; Bulfone *et al.*, 1998).

It is interesting that the lateral and medial migratory pathways probably correspond to the well-known axon pathways for the lateral forebrain bundle (internal capsule) and medial forebrain bundle. Perhaps the cells and axons utilize the same molecular signals for following these pathways. In addition, there is a migration in the opposite direction of LHRH+ cells, that originate in the olfactory placode, and then migrate to the preoptic area and hypothalamus (Schwanzel-Fukuda and Pfaff, 1989; Wray *et al.*, 1989). These cells appear to migrate along

transient extensions of axons from the vomeronasal organ that course along the medial ventral telencephalon to the lamina terminalis (Yoshida *et al.*, 1995, 1999). Thus, the medial cell migration from basal telencephalon to neocortex may follow portions of the same path.

Heterogeneity of Migrating Cell Types

At this point, we have identified several molecular markers of the migrating subcortical cells. Some or all of the cells derived from the MGE initially express *Nkx2.1*, *Lhx6,7*, *Dlx1,2,5,6*, calbindin and GABA. The $Nkx2.1^+$ and $Lhx7^+$ cells appear to stop migrating within the nascent striatum, where, at least in the case of the $Nkx2.1^+$ cells, they may give rise to striatal cholinergic interneurons (Sussel *et al.*, unpublished data, Olsson *et al.*, 1998), whereas cells expressing the other markers may continue their migration into the cerebral cortex. Cells in the medial migratory pathway do not express *Nkx2.1*, *Lhx6*, or *Lhx7*, whereas they do express the other markers, such as PBX1 and RU49 (Redmond *et al.*, 1996; Yang *et al.*, 1996).

Although interneuron precursors appear to migrate into the neocortex from the subcortical telencephalon, it is unclear whether other cortical cell types may also have a subcortical origin. For example, we have not ruled out the possibility that there are cortical projection neurons that are derived from the basal ganglia. Karten has postulated that during evolution of the mammalian cortex there is a migration of projection neurons from specific nuclear subdivisions of the dorsal ventricular ridge to laminar arrangement in the neocortex (Karten, 1969, 1997). However, since the dorsal ventricular ridge is a cortical structure (Fernandez *et al.*, 1998) (L. Puelles *et al.*, unpublished data), and our studies are of subcortical migrations, our findings do not address this hypothesis. In addition, analysis of the neocortex of the *Dlx1/2* mutants, which have essentially no detectable basal telencephalic to neocortical migration in slices, does not reveal a reduction of cortical plate thickness or abnormalities in neocortical lamination.

Neocortical glial cells or their progenitors may also have subcortical origins. In the spinal cord, oligodendrocyte precursors are specified in ventral positions, and then migrate dorsally (Miller, 1996). Expression of oligodendrocyte markers (e.g. DM20) in ventral locations in the forebrain have led to similar hypotheses (Timsit *et al.*, 1995; Spassky *et al.*, 1998). Furthermore, it is known that the cortical SVZ is an active site for generation of oligodendrocytes (Levison and Goldman, 1993; Luskin *et al.*, 1994). Thus, perhaps the cortical SVZ is seeded by precursors from subcortical sites. Transplantation studies in chick embryos also support this hypothesis (Salvador Martinez, personal communication), as does the finding that the neocortical SVZ is reduced by about 50% in the *Dlx1/2* mutants (Anderson and Rubenstein, unpublished data).

A major caveat of the *Dlx1/2* and *Nkx2.1* mutants is that these animals die on the day of birth. Therefore, we do not know how the reductions in cortical interneurons at P0 reflect development of definitive cortical interneurons and other cell types. We are presently attempting to solve this problem by systematically studying the co-expression of *Dlx* genes and GABA at various postnatal stages in rodents and ferrets (S. Anderson *et al.*, unpublished data). Evidence suggests that subcortical to neocortical migration is not limited to rodents. A similar migration occurs in ferret slices (S. Anderson *et al.*, unpublished data), and preliminary analysis of DLX2 expression in prenatal human tissue also appears to reveal a stream of cells migrating from the LGE into the neocortex (Kletnic *et al.*, unpublished data).

Genetic Control of Interneuron Specification

Several transcription factors are now candidates for regulating the development of cortical interneurons. *Dlx1/2* mutant mice lack most GABAergic cortical cells and virtually all olfactory bulb GABAergic neurons (Anderson *et al.*, 1997a, 1997b; Bulfone *et al.*, 1998). However, these defects may be due to a block in the migration of these cells rather than their specification and differentiation. While we have observed a block in the lateral migratory pathway (Anderson *et al.*, 1997b), we have not yet determined whether migration is affected in the medial pathway.

Herein we have provided the first evidence that the DLX2 protein can regulate differentiation of GABAergic cortical neurons (Fig. 3). By infecting E15.5 neocortical cells from *Dlx1/2* mutants, we found a roughly five-fold increase in GABAergic cells. At this point, we do not know for certain whether all of the induced GABAergic cells are expressing DLX-2 from the virus, or whether some of the $GABA^+$ cells in the mutant cultures were induced by factors secreted from the infected cells. Furthermore, it will be important to determine where DLX2 sits in the hierarchy of GABAergic cell development (specification, differentiation and/or maintenance of GABA phenotype). For instance, our results suggest that DLX proteins can regulate the expression of glutamic acid decarboxylase (GAD), but further studies are needed to confirm a direct regulation of the *GAD* gene by DLX2.

In addition, the factors that induce expression of the *Dlx* genes are just beginning to be identified. There is evidence that sonic hedgehog (SHH) can induce *Dlx* expression (Kohtz *et al.*, 1998). SHH is very likely to be one of the primary inductive regulators of a variety of cell types such as cholinergic motor neurons, midbrain dopaminergic neurons and hindbrain serotonergic neurons (Echelard *et al.*, 1993; Hynes *et al.*, 1995; Ericson *et al.*, 1997; Ye *et al.*, 1998). Thus, it will interesting to determine whether SHH is required for the generation of GABAergic cells, particularly because these cells form in the basal telencephalon of the *Nkx2.1* mutants despite the almost complete lack of telencephalic SHH expression (Sussel *et al.*, unpublished data).

Implications for Human Neuropsychiatric Disorders

Defects in interneuron development and function are implicated in several common brain disorders including the epilepsies and schizophrenias. In the epilepsies, insufficient inhibitory activity predisposes to uncontrolled excitatory discharges. A subset of epilepsies are associated with cortical histological abnormalities, that often have ectopic collections of neurons, which are thought to arise from neuronal migration defects (Flint and Kriegstein, 1997). It will be interesting to determine whether there are specific epilepsy syndromes that are caused by disruption of development and/or function of the cells that participate in the subcortical-to-cortical migrations.

The neurobiological bases of the schizophrenias are unknown. Most patients have some symptomatic improvement with medications that inhibit dopamine receptors. Perhaps the most potent of these medications are those that preferentially affect the D4 dopamine receptor (D4R) (e.g. clozapine). D4R is expressed in neocortical interneurons (Mrzljak *et al.*, 1996), a brain region that is functionally and possibly histologically abnormal in many schizophrenic patients (Lewis and Anderson, 1995; Goldman-Rakic and Selemon 1997; Raedler *et al.*, 1998). In

addition, there is histochemical evidence that neocortical interneurons may be abnormal in subsets of schizophrenic patients (Akbarian *et al.*, 1995; Benes *et al.*, 1996; Woo *et al.*, 1998). We thus suggest that insights into the aetiologies of important human diseases may be garnered by the molecular investigation of the development and function of subcortical cells that migrate to the cerebral cortex.

Notes

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