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The transcription factor neurogenin 2 restricts cell migration from the cortex to the striatum

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SUMMARY

The dorsal and ventral domains of the telencephalon are delineated by a unique boundary structure that restricts the migration of dorsal and ventral cells to a different extent. While many cells invade the dorsal cortex from the ventral ganglionic eminence (GE), hardly any cortical cells cross the boundary into the GE. Several molecules have been implicated in the regulation of ventral to dorsal cell migration, but so far nothing is known about the molecular mechanisms restricting cortical cell migration in vivo. Here we show that in the absence of the transcription factor neurogenin 2, cells from the cortex migrate into the GE in vitro and in vivo as detected in transgenic mice containing a *lacZ* gene in the neurogenin 2 locus. In contrast, the migration of cells from the GE is not affected. Molecular and cellular analysis of the cortico-striatal boundary revealed that neurogenin 2 regulates the fasciculation of the cortico-striatal boundary which may explain the non cell-autonomous nature of the migration defect as detected by in vitro transplantation. Taken together, these results show that distinct cues located in the cortico-striatal boundary restrict cells in the dorsal and ventral telencephalon.

Key words: Cortico-striatal boundary, *Pax6*, *Mash1*, *Dlx*, *SFRP2*, Mouse

INTRODUCTION

Regionalisation of the brain occurs during development by formation of distinct neighboring compartments that develop as independent units. This process involves molecular and cellular events, including differential gene expression and the restriction of cell migration between neighboring domains. The sequence of events has been best examined in the hindbrain, a region of the CNS that arises from 7 segmental units known as rhombomeres (Lumsden and Krumlauf, 1996). In the hindbrain, transcription factors are differentially expressed in neighboring rhombomeres, resulting in compartmental differences in cell surface properties that restrict cell mixing. For example, the transcription factor Krox20 regulates the EphA receptor tyrosine kinase EphA4 in rhombomeres 3 and 5 and signaling between ephrin receptors and their ligands (expressed in even-numbered rhombomeres) then mediates the segregation of cells at rhombomere boundaries (Xu and Wilkinson, 1997; Mellitzer et al., 1999; Xu et al., 1999), such that very few cells cross between adjacent rhombomeres (Fraser et al., 1990; Birgbauer and Fraser, 1994).

Migrational restriction has also been observed in other parts of the developing CNS, but the mechanisms that regulate cell migration elsewhere in the brain are less well understood (Figdor and Stern, 1993; Fishell et al., 1993; Anderson et al., 1997; Tamamaki et al., 1997). In particular, it is not known how the asymmetry of cell migration between dorsal and ventral regions in the telencephalon is regulated. Here, ventral cells migrate into dorsal regions to a much larger extent than dorsal cells into ventral territories (Fishell et al., 1993; Anderson et al., 1997; Tamamaki et al., 1997; Chapouton et al., 1999; Lavdas et al., 1999; Wichterle et al., 1999; Anderson et al., 2001). A dorsoventral boundary forms at approximately embryonic day (E) 12/13 in the murine telencephalon, separating the dorsally located cortex from the ventrally located ganglionic eminence (GE). Adhesive differences precede the formation of the mechanical boundary formed by a radial glial fascicle (Stoykova et al., 1997; Inoue et al., 2001), similar to the development of boundaries between rhombomeres (Guthrie and Lumsden, 1992; Wizenmann and Lumsden, 1997). The telencephalic radial glial fascicle is often referred to as the cortico-striatal boundary, since it separates the cortex from the lateral part of the GE (LGE) that will later give rise to the striatum (Olsson et al., 1995). Cells in the cortico-striatal boundary express some, but not all, genes characteristic for the dorsal telencephalon and this region has therefore been named ‘ventral pallium’ (Puelles et al., 2000). However, boundary cells do not express genes characteristic for the ventral telencephalon and the radial glia fascicle forms just above this sharp border of gene expression. The molecular events underlying the biased cell movements across this boundary, with more migration from the GE into the cortex, than from the cortex to the GE, remain largely unknown (Fishell, 1993; Anderson et al., 1997; Chapouton et al., 1999; Neyt et al., 1997; Tamamaki et al., 1997; Lavdas et al., 1999; Wichterle et al., 1999).

Recently it has been demonstrated that the cadherins restrict ventral to dorsal and dorsal to ventral cell movements at early developmental stages prior to boundary formation (Inoue et al., 2001), whereas diffusible factors of unknown nature seem to restrict the movements of cortical cells at later stages of development (Neyt et al., 1997). Furthermore, in the absence of Pax6, a paired-homeodomain transcription factor that is normally expressed in the cortex, cell migration from the GE into the cortex is strongly enhanced, whereas migration from the cortex into the GE is affected much less (Chapouton et al., 1999). Patterning of the telencephalon is, however, severely distorted in the absence of Pax6 (Stoykova et al., 2000). For example, the expression domains of the basic helix-loop-helix (bHLH) transcription factors *neurogenin 1* (*ngn1*; also known as *Neurod3*), *ngn2* (also known as *Atoh4*; *atonal homolog 4*) and *Mash1* (*Ascl1*) are completely changed (Stoykova et al., 2000; Toresson et al., 2000). In the developing telencephalon, *Mash1* and *ngn2* are expressed in a defined dorsoventral pattern, with *Mash1* expressed at high levels in the developing GE and *ngn1/2* restricted to the developing cortex (Gradwohl et al., 1996; Ma et al., 1997), consistent with a role for these genes in specifying region-specific phenotypes, such as the migratory behavior of neurons (Gradwohl et al., 1996; Ma et al., 1997; Casarosa et al., 1999; Fode et al., 2000). In the absence of *ngn2*, *Mash1* is upregulated in the cortex while most other ventral transcription factors do not expand into the cortex (Fode et al., 2000). Therefore in this study we have examined whether the loss of *ngn2* and acquisition of *Mash1* allows the spread of cortical cells into ventral regions.

MATERIALS AND METHODS

Animals

Wild-type ($ngn2^{WT/WT}$), heterozygous ($ngn2^{WT/lacZ}$) and homozygous mutant ($ngn2^{lacZ/lacZ}$) mouse embryos were obtained by heterozygous crosses and were genotyped as described previously (Fode et al., 2000). For some control experiments an EGFP knock-in mouse ($ngn2^{GFP}$; Gerard Gradwohl and F. G., unpublished data) was crossed to a $ngn2^{lacZ}$ mouse to obtain null mutants with only one *lacZ* copy. For in vitro transplantation experiments, mice expressing the EGFP (enhanced green fluorescent protein) transgene under the actin promoter (Okabe et al., 1997) were used. The day of vaginal plug was considered as embryonic day 0 (E0).

X-gal histochemistry and in situ hybridization

Telencephalic slices were cut frontally (300 μm thick with a tissue chopper (McIllwain) or 100 μm thick with a vibratome (Campden), fixed in 0.5% glutaraldehyde and stained following standard protocols. For the combination with in situ hybridization, telencephali were fixed in 4% paraformaldehyde (PFA) for 1.5 hours at 4°C, cryoprotected and 12 μm sections were cut using a cryostat and stained as described by Houzelstein and Tajbakshs (Houzelstein and Tajbakshs, 1999). Digoxigenin-labeled RNA probes were made by in vitro transcription using the NTP labeling mix from Roche and T3, T7 or SP6 polymerase from Stratagene. The following RNA probes were used: *lacZ* (A. Stoykova, F. Cecconi, MPI of Biophysical Chemistry, Göttingen, Germany), *ngn2* (Gradwohl et al., 1996), *Math2* (Bartholomä and Nave, 1994), *Pax6* (Stoykova et al., 1996), *R-cadherin* (*Cdh4*) (Mastunami and Takeichi, 1995), *cadherin 6* and *11* (*Cdh6, Cdh11*) (Inoue et al., 2001) (M.Takeichi, University of Kyoto, Japan); *cadherin 8* (*Cdh8*) (Korematsu and Redies, 1997); *Slit1, Slit2, Slit3*, *Robo1*, *Robo2* (Y. Rao, Washington University School of Medicine, St. Louis, MI, USA; A. Chédotal, Hopital de la Salpetriere, Paris, France), *Tenascin C* (Götz et al., 1997), *Wnt7b* (A. McMahon, Harvard University, Cambridge, MA, USA), SFRP2 (Kim et al., 2001), *Dlx1*, *Dlx5*, *Mash1* (Casarosa et al., 1999; Toresson et al., 2000), *ephrin A5* and *EphA5* (L. Lindemann, FMI, Basel, Switzerland), *ephrin B1, B2, B3* (R. Klein, MPI of Neurobiology, Martinsried, Germany), *EphA3* (P. C. own construction). In situ hybridizations were performed as described previously (Cau et al., 1997).

Immunohistochemistry

Brains were fixed and sectioned as described above. Sections were incubated in primary antisera over night at 4°C and fluorescently tagged secondary antisera for 45 minutes at room temperature as described by Hartfuss et al. (Hartfuss et al., 2001). The RC2 antiserum was used 1:500 (mouse IgM, P. Leprince, University of Liège, Belgium), antiserum against BLBP 1:5000 (polyclonal rabbit, N. Heintz, Rockefeller University, New York, USA) and the monoclonal 9-4 antiserum 1:10 (T. Hirata, Kyoto University, Japan). Sections were analyzed using a Zeiss Axiophot or Leitz confocal microscope.

Migration assay and transplantations

300 μm thick slices of E14 telencephali were cut with a tissue chopper, injected focally with EGFP adenovirus and cultured in millicell-CM inserts (Millipore) in DMEM with 10% FCS (Chapouton et al., 1999). Photos of the slices were taken 24 hours and 45 hours after injection. Slices were prepared from individual embryos that were genotyped thereafter. Migration analysis was performed blind and the genotype was revealed only after the number of migrating cells had been analyzed. Data is expressed as ± s.e.m. In transplantation experiments a small piece (about 300×300 μm) of cortex from the EGFP mice (Okabe et al., 1997) was laid onto a host slice (from $ngn2^{lacZ}$-crosses) and cultured for 24 hours. After fixation in 4% PFA slices were examined using the confocal microscope.

RESULTS

Ectopic cells in the GE of homozygous $ngn2^{lacZ}$ mice

To examine a possible role for *neurogenin 2* (*ngn2*) in the restriction of cortical cell migration, we used X-gal histochemistry to visualize the distribution of β-galactosidase-positive cells in mice heterozygous ($ngn2^{WT}/ngn2^{lacZ}$) and homozygous ($ngn2^{lacZ}/ngn2^{lacZ}$) for a knock-in allele of *ngn2* in which coding sequences had been replaced by the *lacZ* gene (Fode et al., 2000). Mice with only one mutant allele have previously been reported to show no phenotype (Fode et al., 2000). Consistent with this, the pattern of β-galactosidase staining in the telencephalon of $ngn2^{WT}/ngn2^{lacZ}$ mice at E14 closely resembled the expression pattern of *ngn2* with a strong signal in the cortex and a sharp limit at the border to the unstained GE (Fig. 1A). As previously reported (Fode et al., 2000), X-gal staining was present not only in the ventricular zone (VZ) of the cortex, where *ngn2*-expressing precursor cells are located (Gradwohl et al., 1996), but also in postmitotic neurons of the cortical plate that do not normally express *ngn2*. The persistence of β-galactosidase activity in comparison to the endogenous protein is best explained by the relatively long half life of the enzyme [see also Nieto et al. (Nieto et al., 2001)].

A similar pattern of X-gal staining was detected in homozygous mutant littermates ($ngn2^{lacZ}/ngn2^{lacZ}$), with a sharp border between the β-galactosidase-positive cortex and the negative GE. However, in contrast with the heterozygous

situation, a large number of ectopic X-gal-positive cells were scattered in the GE of *ngn2*^{lacZ}/*ngn2*^{lacZ} mice (Fig. 1B; 50±19 cells per section, *n*=10), with most ectopic X-gal-positive cells located in the rostral half of the telencephalon, and few in the caudal half. A further difference observed between heterozygous and homozygous embryos was the intensity of X-gal staining. In heterozygous mice (Fig. 1A), β-galactosidase activity was weaker in the cortical plate (CP) than in the ventricular zone (VZ), whereas it was equally strong throughout the cortex of homozygous mutants (Fig. 1B). We next examined whether ectopic cells were also observed in homozygous *ngn2* mutants carrying only one *lacZ* allele. Heterozygous *ngn2*^{WT}/*ngn2*^{lacZ} and *ngn2*^{WT}/*ngn2*^{GFP} mice were crossed and generated *ngn2*^{GFP}/*ngn2*^{lacZ} homozygous mutant embryos that contained similar numbers of ectopic cells in the GE as observed in *ngn2*^{lacZ}/*ngn2*^{lacZ} mice (Fig. 1D). Thus, the appearance of ectopic X-gal-positive cells in the GE and increased signal in the CP is not due to the increased copy number of *lacZ* in homozygous embryos, but to the loss of *ngn2* function.

If the ectopic cells in the GE were due to migrational spread from the cortex, one might expect their accumulation over time. We therefore analyzed *ngn2*^{lacZ} mice three days later, at E17, and observed twice as many ectopic cells in the GE (95±19 cells per section, *n*=10) than at E14 (50±19 cells per section, *n*=10). Interestingly, cells had spread deeper into the GE at E17 than at E14 and ectopic cells were also located in the ventricular zone of the GE at E17 where no cells were detected at E14 (Fig. 1F). Indeed, an additional hint for an unusual cell migration from the cortex into the GE in the *ngn2*^{lacZ}/*ngn2*^{lacZ} mice is that many β-galactosidase-positive cells in GE exhibit the morphology of migrating cells, with an elongated cell body and a leading process (Fig. 1G).

Dorsoventral cell migration from the cortex into the GE in homozygous *ngn2*^{lacZ} mice

To directly examine if the loss of *ngn2* function affects cell migration, we used a previously established assay involving the focal injection of an EGFP-expressing adenovirus into telencephalic slices (Chapouton et al., 1999). Cortical slices (300 μm) from E14 wild-type and *ngn2* mutant littermates were infected close to the cortico-striatal border with an EGFP-adenovirus as depicted in Fig. 2. Note that the sulcus between the cortex and GE allows the identification of the dorsoventral boundary for more than 2 days in vitro (Chapouton et al., 1999). As observed previously (Chapouton et al., 1999), few cells infected in the cortex of E14 wild-type mice crossed the boundary into the GE, 18 or 45 hours post-infection (Fig. 2A), with ectopic cells found in the GE of only 17% of slices and a mean number of 0.3 cells crossing the border per slice (Table 1). In contrast, many labeled cells were observed to migrate within the cortex, confirming that infected cells retained their migratory capacity (Fig. 2A).

EGFP-adenovirus infection of slices from the telencephalon of *ngn2* homozygous mutant embryos revealed an increased capacity for mutant cells to migrate from the cortex into the GE. In the example shown in Fig. 2B, four infected cortical cells migrated into the GE 45 hours after infection. A quantification of this effect in several embryos indicated that migration from the cortex into the GE was doubled in *ngn2* homozygous mutants compared to wild-type littermates (Table 1). However, as the total number of cortical cells infected and migrating into the GE was low in these experiments, the significance of the differences was difficult to evaluate. To overcome this problem we increased the number of infected cells and thus the probability of observing cells crossing the boundary by placing several injections on the cortical side of the boundary. In these high-density infections, 51% of wild-type slices had cells crossing the boundary; a number which was further increased to 71% in slices obtained from homozygous mutant littermates. Moreover, the mean number of cells crossing the cortico-striatal boundary increased 1.8 fold in *ngn2* mutant compared to wild-type slices. In contrast, the migration distance into the GE was comparable (wild type: 315±39 μm; *ngn2*$^{-/-}$: 371±45 μm respectively). Thus, as suggested by the low-density viral infections, there is a significant increase in the dorsoventral migration of cortical cells in *ngn2* mutant slices.

Table 1. Adenovirus injections into the cortex

	Single injections in the cortex		Several injections in the cortex	
	% of slices with cells in the GE	Mean number of cells in the GE	% of slices with cells in the GE	Mean number of cells in the GE
ngn2$^{+/+}$	17%, *n*=29	0.3±0.1	51%, *n*=39	1.4±0.4
ngn2$^{-/-}$	33%, *n*=30	0.8±0.4	72%, *n*=19	2.3±0.6
Normalized to WT	×1.9	×2.7	×1.4	×1.6

We also examined whether the boundary in the *ngn2* mutant telencephalon is more permeable to cells from the GE, by injecting EGFP-adenovirus in the GE of E14 wild-type and *ngn2*$^{-/-}$ telencephalic slices. As previously observed, GE cells migrate into the cortex much more frequently than cortical cells migrate into the GE (Tamamaki et al., 1997; Anderson et al., 1997; Chapouton et al., 1999; Lavdas et al., 1999; Wichterle et al., 1999; Anderson et al., 2001). In most of the injected slices (79%), cells from the GE had migrated into the cortex 2 days after labeling and no difference was detected in telencephalic slices from homozygous *ngn2* mutants (GE cells migrated into the cortex in 89% of slices, see Table 2). Thus, the absence of *ngn2* does not affect cell migration from the GE into the cortex, but only from the cortex into the GE.

Cellular and molecular changes at the boundary between cortex and GE in homozygous *ngn2*^{lacZ} mice

Because cortical cells in *ngn2*-deficient brains are able to cross the boundary into the GE, we tested whether this boundary is affected in the mutant. A prominent radial glial fascicle labeled by RC2 and BLBP antisera delineates the cortex and the GE from E12 onwards (Edwards et al., 1990; Stoykova et al., 1997; Hartfuss et al., 2001). While the characteristic fasciculation of RC2- and BLBP-immunoreactive radial glial fibers was present, it was less tight in the homozygous *ngn2* mutant telencephalon compared to wild-type littermates (Fig. 3A-D). This was seen throughout the rostrocaudal extent of the boundary. Similarly, immunostaining of the 9-4c1c9 antigen, a marker for boundary radial glial cells, also revealed a certain defasciculation of radial glia fibers at the boundary in the homozygous *ngn2* mutant telencephalon (Fig. 3E,F).

We also examined whether molecular changes in border

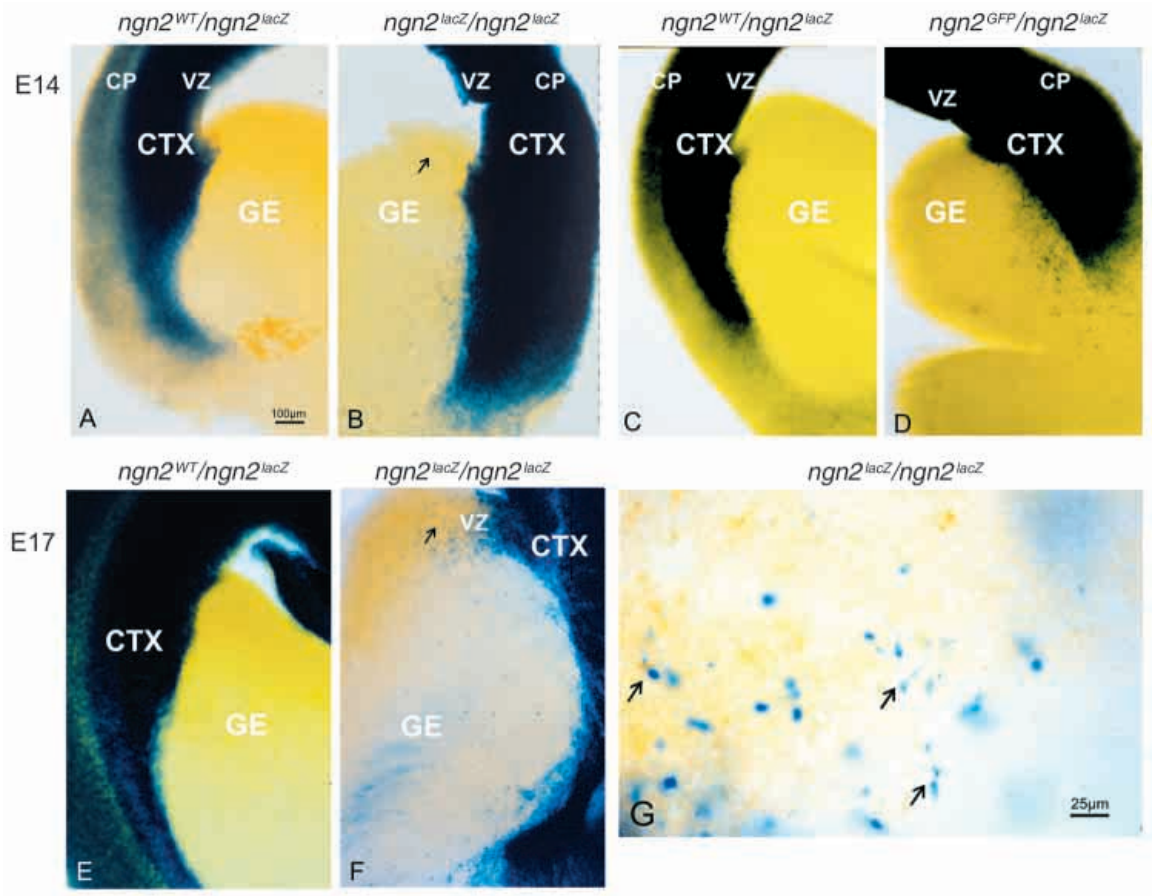

Fig. 1. Ectopic β-galactosidase-containing cells in the ganglionic eminence of *ngn2*^{lacZ}/*ngn2*^{lacZ} mice. Sections of the telencephalon from *ngn2*^{lacZKI} mice at E14 (A-D,G) and E17 (E,F) showing the expression pattern of β-galactosidase in blue. (A) *ngn2*^{WT}/*ngn2*^{lacZ} heterozygous, and (B) *ngn2*^{lacZKI}/*ngn2*^{lacZKI} homozygous littermates. The heterozygous mutant reveals a sharp boundary of X-gal staining between the positive cortex (CTX) and the negative ganglionic eminence (GE), whereas the homozygous mutant contains many β-galactosidase-positive cells scattered in the GE. The arrow in B indicates the absence of ectopic cells in the ventricular zone (VZ) of the GE, in contrast to later stages (E17; F). CP indicates the cortical plate containing postmitotic neurons. (C) *ngn2*^{WT}/*ngn2*^{lacZ} heterozygous, and (D) *ngn2*^{GFP}/*ngn2*^{lacZ} homozygous mutant littermates. In the homozygous mutant carrying only one *lacZ* allele (D), a similar number of ectopic cells are present in the GE, as in the homozygote (B). (E) *ngn2*^{WT}/*ngn2*^{lacZ} heterozygous, and (F) *ngn2*^{lacZ}/*ngn2*^{lacZ} homozygous littermates at E17. The number of ectopic cells in the GE has increased compared to E14. The arrow in F indicates ectopic cells in the ventricular zone of the GE. (G) Higher magnification of the ectopic cells in the GE of *ngn2*^{lacZ}/*ngn2*^{lacZ} homozygous mutants at E14. The arrows indicate cells with morphologies characteristic of migratory neurons. Scale bar in A-F, 100 µm; in G, 25 µm.

cells had occurred in *ngn2* mutants. Interestingly, a molecular marker of the boundary region, the soluble frizzled-related protein 2 (SFRP2) (Kim et al., 2001) also showed a broader expression domain in the *ngn2*$^{-/-}$ telencephalon compared to wild-type littermates (Fig. 3G,H). In wild-type embryos, *SFRP2* is most strongly expressed in the VZ of the ventral pallium, the border region extending from the GE to the cortico-striatal sulcus (arrow in Fig. 3G). In the *ngn2*$^{-/-}$ telencephalon, however, the *SFRP2* expression domain spreads above the sulcus into the cortex (Fig. 3H). This enlargement of SFRP2 expression in *ngn2* mutants was observed at all rostrocaudal levels of the cortex. In contrast, expression of *Wnt7b*, the *SFRP2* ligand, was not affected in the lateral cortex of *ngn2* mutants (data not shown, analyzed at E14 and 17). Similarly, we did not see any changes in the expression patterns of Slit1, 2, 3 or Robo1, 2 (data not shown), secreted molecules and their receptors that were previously suggested to play a role in tangential cell migration in the telencephalon (Zhu et al., 1999). In particular Slit2 has a prominent expression domain along the boundary between the cortex and GE at E16, a pattern that is not affected in *ngn2* mutant mice (not shown). Cadherin-mediated cell adhesion has also been implicated in restricting the intermingling of cells from the cortex and the GE (Götz et al., 1996; Inoue et al., 2001), but no changes were observed in the expression of R-cadherin, cadherin 6, 8 and 11 in the *ngn2*$^{-/-}$ telencephalon (data not shown). Likewise, the expression patterns of ephrins (ephrin A5, B1, B2 and B3) and Eph receptors (EphA5 and A3) were unaffected in *ngn2*$^{-/-}$

Table 2. Single adenovirus injections into the ganglionic eminence

	% of slices with cells in the cortex	Mean number of cells in the cortex
ngn2$^{+/+}$	79%, *n*=19	5.5
ngn2$^{-/-}$	89%, *n*=9	6
Normalized to WT	×1.1	×1.1

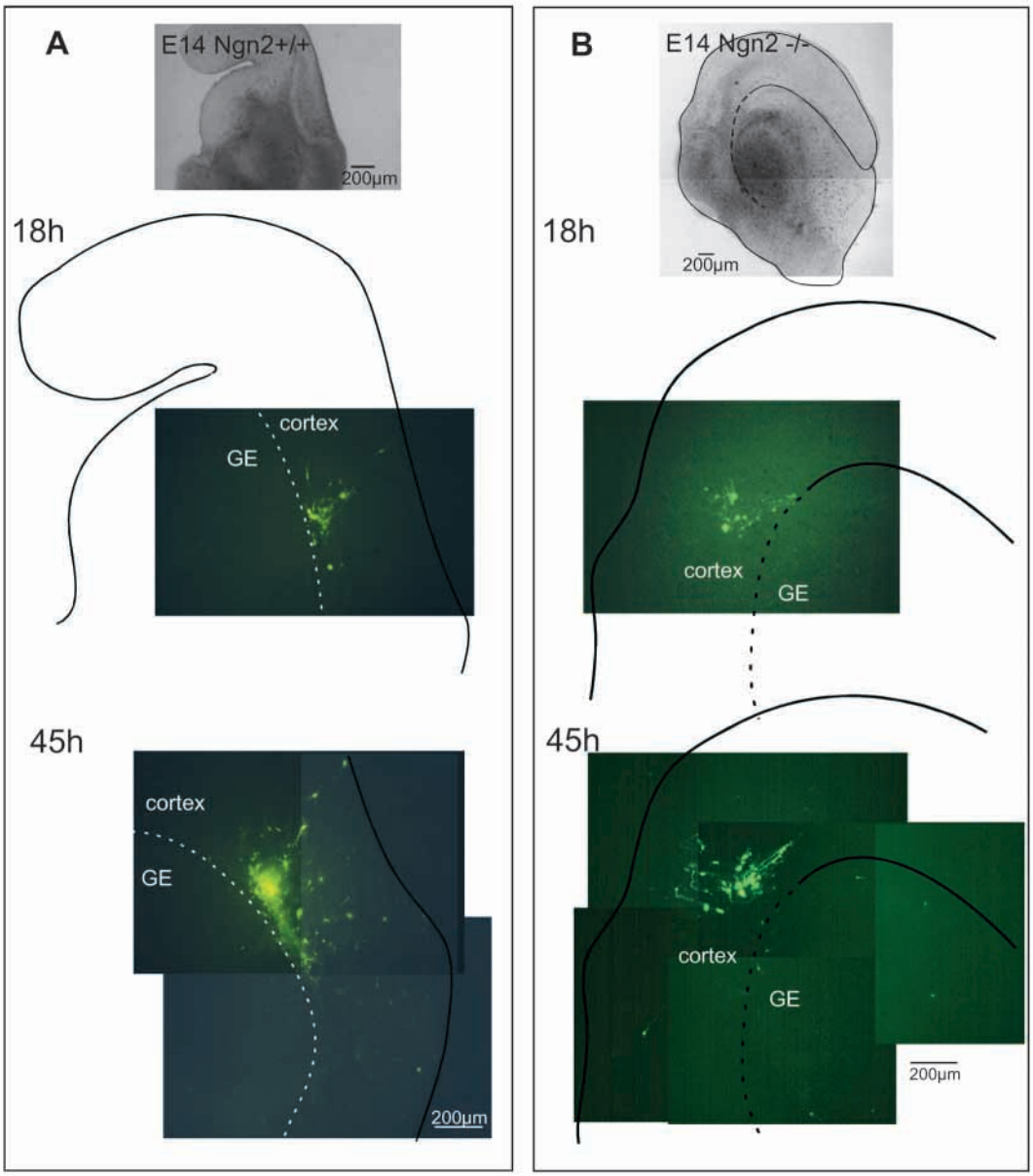

Fig. 2. Cortico-striatal cell migration is increased in *ngn2*^{lacZ}/*ngn2*^{lacZ} *mice*. EGFP adenovirus was injected focally in the cortex of a telencephalic slice from wild-type (A) and a homozygous *ngn2* mutant littermate (B) at E14. Micrographs were taken 18 hours and 45 hours after the virus injection as indicated in the figure. The phase contrast micrograph and fluorescence micrograph after 18 hours depict the position of the injection site in the cortex and the dashed line indicates the cortico-striatal boundary. Note that cells spread further away from the injection site after 45 hours. No cortical cells have migrated into the GE in the wild-type telencephalic slice, while 4 cortical cells have entered the GE of a *ngn2*$^{-/-}$ mouse. Scale bars, 200 μm.

telencephalon (data not shown). Thus, no defects were observed in the expression of cell surface and signaling molecules known to regulate cell adhesion and migration in the developing telencephalon of *ngn2*$^{-/-}$ mice, except a broadening of the boundary between the cortex and the GE at cellular and molecular level.

Non cell-autonomous effects on cell migration in *ngn2*$^{-/-}$ telencephalon

If these alterations in the cortico-striatal boundary were significant, wild-type cells should also be affected in their migration across a *ngn2*$^{-/-}$ telencephalic border. To test this idea, we used an in vitro transplantation approach and transplanted small pieces of cortex or GE from 'green mice', a transgenic line that expresses EGFP ubiquitously in all cells (Okabe et al., 1997), onto slices of *ngn2*$^{-/-}$ or wild-type telencephali. We first verified that the normal migrational properties at the corticostriatal boundary were maintained in this transplantation assay by putting small pieces of 'green' GE (E14) onto a slice of the GE from a wild-type telencephalon (E14). A mean of 96.2±3.2 green cells per slice (n=4) migrated from the GE into the cortex during 1 day in vitro. In contrast, when pieces of green mouse cortex of comparable size were transplanted onto the cortex of slices from E14 wild-type telencephalon, only very few green cells crossed into the GE (5.5±1.2 green cells in the GE/slice, n=42). This experimental

Table 3. GFP-cortex transplants

	Transplant area (mm^2)	Mean number of migrating cells in the cortex	Mean number of migrating cells in the GE	GE cells/ total number of cells
WT	0.364	266±62	3±1.2	1.1%
ngn2$^{-/-}$	0.406	424±57	16±4.9	3.7%
Normalized to WT	×1.1	×1.6	×5.3	×3.4

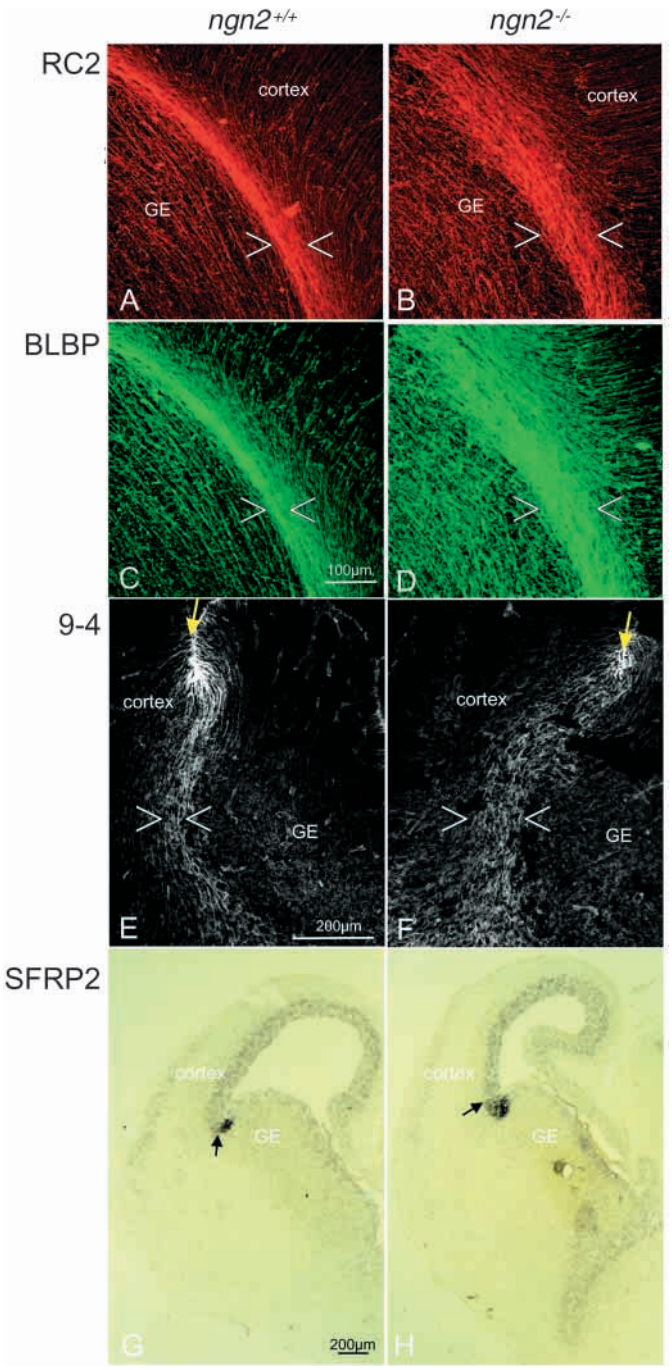

Fig. 3. Broadening of the cortico-striatal boundary in *ngn2*^{lacZ}/*ngn2*^{lacZ} mice. Cortico-striatal boundary markers as indicated in the panel were detected in sections of telencephali at E16 (A-D) or E14 (E-H) in wild-type (A,C,E,G) and *ngn2* homozygous mutant littermates (B,D,F,H). (A-F) Immunostainings for radial glia fibers. Note that the width of the radial glial fascicle (indicated by carets) in A-F and the territory of SFRP2 expression (indicated by arrows) (G,H) are enlarged in the absence of Ngn2. The yellow arrows in E and F indicate the cortico-striatal sulcus.

paradigm thus respects the typical asymmetrical restriction at the cortico-striatal boundary.

When we transplanted pieces of green fluorescent cortex onto the cortex of slices from *ngn2*$^{-/-}$ telencephalon (Fig. 4A) we observed a clear increase in the number of cortical cells crossing into the GE compared to transplants placed on slices from wild-type littermates. While on a wild-type cortex a mean number of 2.9±1 (n=12) green cortical cells had entered the GE after 1 day in vitro, more than five times as many cells (16.3±5; n=12) had crossed the boundary on a *ngn2*$^{-/-}$ substratum. Thus, wild-type cortical cells spread to a larger extent into the GE on a *ngn2*$^{-/-}$ versus a wild-type substratum (Table 3). Two possible mechanisms could lead to this result: first, migration is enhanced on the *ngn2*$^{-/-}$ telencephalon, or, second, the mutant boundary is more permissive for cortical cells to enter the GE. Indeed, a higher number of cells migrated out of transplants placed on *ngn2*$^{-/-}$ telencephalic slices compared to wild-type slices (1.6-fold increase; Fig. 4B; Table 3), suggesting that the mutant cortex is a more permissive substratum for migration than the wild-type cortex. Nevertheless, taking this difference into account by normalizing the proportion of cells entering the GE to the total number of migrating cells, there were still more cells crossing the boundary on a mutant substratum (3.7%±0.7%) than on a wild-type substratum (1.2%±0.5%; Fig. 4C; 3 fold increase on *ngn2*$^{-/-}$ substratum; Table 3). This suggests that the boundary between the cortex and the GE has become more permissive for cortical cells in the absence of *ngn2* and that the loss of *ngn2* is not required in the migrating cells since wild-type cells also react to the substratum changes of *ngn2*$^{-/-}$ slices.

Fate change of ectopic cells in the GE of *ngn2*^{lacZ} mice

The results shown above strongly support the interpretation that the X-gal-positive cells detected in the GE of *ngn2*^{lacZ} mice are indeed cortical cells having crossed the cortico-striatal border. Do these cells maintain their cortical identity in a GE environment or do they acquire the identity of GE cells? We therefore examined the expression of several transcription factors specific for dorsal or ventral regions in the developing telencephalon. First, we analyzed whether X-gal-positive cells still express *ngn2* transcripts (using a *neurogenin 2* riboprobe, which hybridizes to a region still present in the mutated gene) or the *lacZ* gene at ectopic positions. In the cortex, as expected, most of the X-gal-positive cells express the *lacZ* mRNA (Fig. 5A). In contrast, the X-gal-positive cells in the GE did not contain detectable levels of *lacZ* (Fig. 5A) or *ngn2* transcripts (data not shown). Thus, expression from the *ngn2* locus seems to be down regulated after cortical cells have crossed the boundary into the GE. Interestingly, the same was observed for expression of the transcription factor Pax6, which is expressed in many cortical precursors, but only at very low levels in the GE. Indeed, most β-galactosidase-positive cells in the cortex express *Pax6*, while most ectopic cells in the GE did not. Interestingly, the few X-gal-positive cells that retained *Pax6* transcripts were still located close to the boundary (data not shown). We also examined the expression of *Math2*, a bHLH transcription factor expressed in postmitotic neurons of the cortex, but not the GE (Bartholomä and Nave, 1994; Fode et al., 2000) and found that β-galactosidase-containing cells in the GE of *ngn2*^{lacZ}/*ngn2*^{lacZ} did not express *Math2* (Fig. 5B). Similar results were also obtained with the riboprobe for *R-cadherin* that is contained in both neurons and precursors in the cerebral cortex, but not in the GE (data not shown). Thus, cortical cells entering the GE lose their dorsal identity, raising

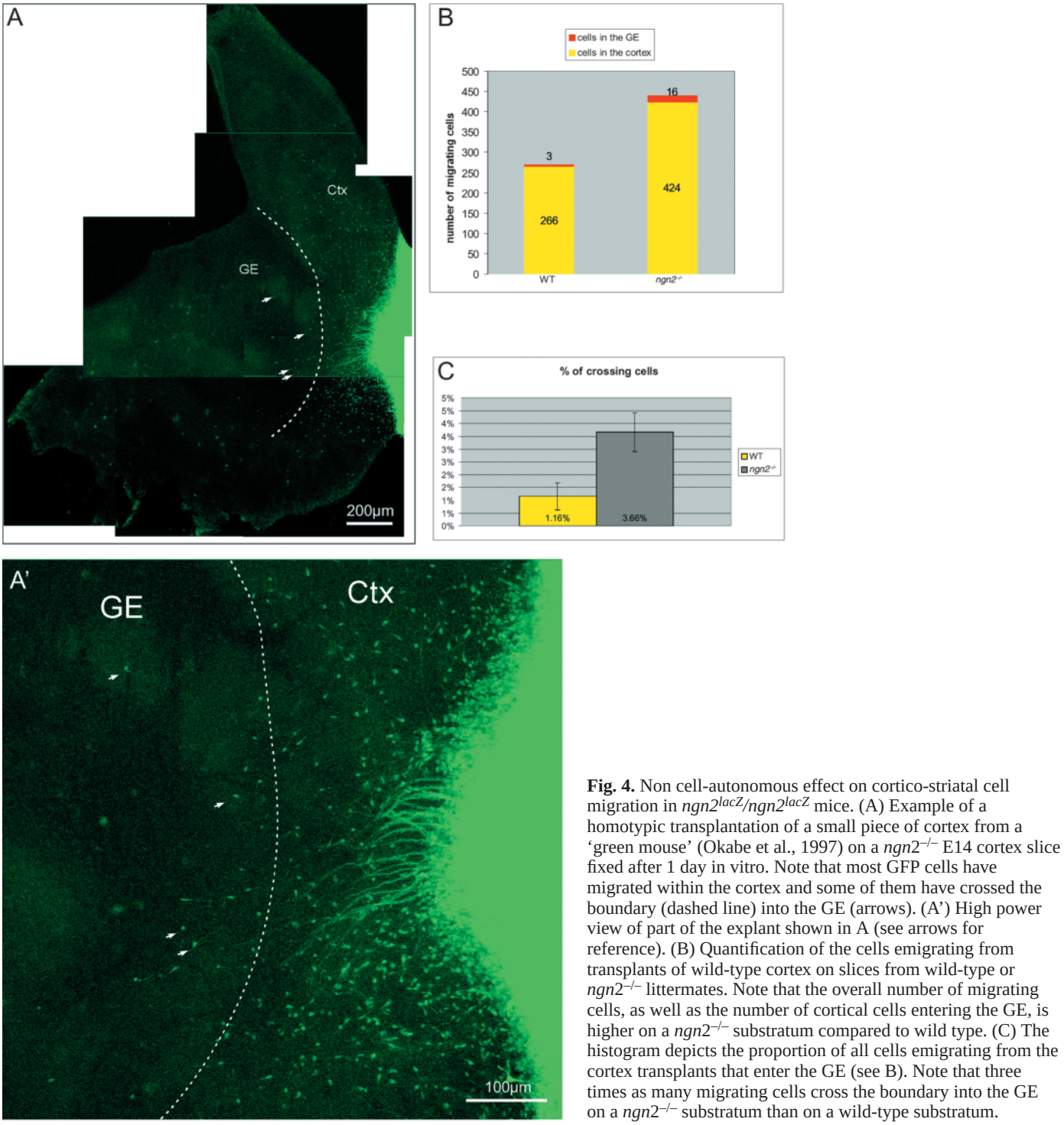

Fig. 4. Non cell-autonomous effect on cortico-striatal cell migration in *ngn2*^{lacZ}/*ngn2*^{lacZ} mice. (A) Example of a homotypic transplantation of a small piece of cortex from a 'green mouse' (Okabe et al., 1997) on a *ngn2*$^{-/-}$ E14 cortex slice fixed after 1 day in vitro. Note that most GFP cells have migrated within the cortex and some of them have crossed the boundary (dashed line) into the GE (arrows). (A') High power view of part of the explant shown in A (see arrows for reference). (B) Quantification of the cells emigrating from transplants of wild-type cortex on slices from wild-type or *ngn2*$^{-/-}$ littermates. Note that the overall number of migrating cells, as well as the number of cortical cells entering the GE, is higher on a *ngn2*$^{-/-}$ substratum compared to wild type. (C) The histogram depicts the proportion of all cells emigrating from the cortex transplants that enter the GE (see B). Note that three times as many migrating cells cross the boundary into the GE on a *ngn2*$^{-/-}$ substratum than on a wild-type substratum.

the question of whether they instead acquire a ventral identity in their new environment.

Mash1 is a bHLH transcription factor expressed in the GE, but only at low levels in the cortex (Casarosa et al., 1999; Fode et al., 2000). However, in the absence of *ngn2*, *Mash1* expression is up regulated in the cortex (Fode et al., 2000). In the cortex of *ngn2*^{lacZ}/*ngn2*^{WT} mice most β-galactosidase-positive cells in the cortex did not contain *Mash1* transcripts, suggesting that *ngn2* and *Mash1* are expressed in different populations of cortical precursors (Nieto et al., 2001). In the cortex of *ngn2*^{lacZ}/*ngn2*^{lacZ} mice, however, most β-galactosidase-positive cells also expressed *Mash1*, consistent with the notion that *Mash1* functionally replaces *ngn2* and thereby misspecifies cortical precursors (Fig. 5C). Also, most of the ectopic β-galactosidase-positive cells in the GE expressed *Mash1* (Fig. 5C). Although the endogenous expression pattern of *Mash1* in the GE is restricted to the VZ and SVZ (Bulfone et al., 1993), ectopic X-gal-positive cells

outside the VZ were clearly *Mash1*-positive. Since, however, cortical cells also express *Mash1* in the absence of *ngn2*, we examined other ventral markers, such as *Dlx5* that are not expressed by the β-galactosidase-positive cells in the lateral cortex abutting the LGE (Fig. 5D). Interestingly, all ectopic β-galactosidase-positive cells in the GE contained *Dlx5* transcripts suggesting that they start to express *Dlx5* when they enter the GE (Fig. 5D). Taken together, these results suggest that *ngn2*-deficient cells migrating from the cortex into the GE lose their cortical identity and acquire the identity of their host region.

DISCUSSION

This is the first report identifying a gene required for the restriction of cortical cells in their territory. The absence of *neurogenin 2* in the dorsal region of the developing telencephalon, results in a leakage of dorsally derived cortical cells into the ventral region of the GE. This conclusion is supported by in vivo tracing of cortical cells expressing *lacZ* in the *neurogenin 2* locus and by the observation of cell migration in slice preparations in vitro. We could further show that the loss of neurogenin 2 leads to subtle changes in the boundary region between the cortex and the GE which are also recognized by wild-type cortical cells. Since migration of cells from the GE is not affected, we conclude that neurogenin 2 is required for boundary features recognized specifically by cortical cells. Thus, these data show that distinct molecules regulate boundary properties responsible for restricting dorsal and ventral cells. This is an important step forward in unravelling the molecular signals that endow the telencephalic boundary with its unique feature of asymmetry, allowing a larger number of ventral cells to cross in the dorsal territories than dorsal cells to migrate ventrally.

Distinct migrational restriction of cells from the cortex and GE

Migration of cells from the GE into the cortex has recently gained a lot of attention, since it is thought that these cells are the source of most interneurons in the cortex (Anderson et al., 1997; Anderson et al., 2001; Pleasure et al., 2001). Analysis of mouse mutants and in vitro experiments have revealed several molecules that are required for, or act positively on, this ventrodorsal cell migration [*Dlx1* and *2* (Anderson et al., 1997); *Nkx2.1* (Sussel et al., 1999); *Mash1* (Casarosa et al., 1999); *Slit* (Zhu et al., 1999); *HGF* (Powell et al., 2001)]. In contrast, the strict confinement of cortical cells in their territory is much less understood. There are two general possibilities of how such a selective restriction of cells on the two different sides of the boundary could be achieved. Either, dorsal and ventral cells react differently to the same inhibitory cues in the boundary, or the boundary region contains distinct cues restricting selectively dorsal or ventral cells. Our data favor the latter possibility for the following reasons.

The loss of Ngn2 affects only the migration of cortical cells, but not of GE cells. This is concluded from our migration analysis using focal injections of EGFP adenovirus into the GE or the cortex in telencephalic slices. In *ngn2*$^{-/-}$ slices more cortical cells migrated into the GE than in wild-type slices, while no difference was detected in the migration of GE cells on wild-type or *ngn2*$^{-/-}$ slices. The number of cortical cells entering the GE in *ngn2*$^{-/-}$ mice was relatively small, but seemingly accumulates with time. Within 3 days the number of ectopic *lacZ*-positive cells in the GE of *ngn2*$^{-/-}$ mice doubled. Moreover, ectopic cells were found at more ventral positions than at earlier stages, suggesting that they continue to migrate during this period. Consistent with the formation of the boundary at E12/13, we observed the earliest ectopic cells around this time in the *ngn2*$^{-/-}$ mice (data not shown), suggesting that the number of ectopic cells observed at E14 is already the result of about 2 days accumulated migration. Thus, a relatively small leakage of cells from the cortex across the boundary may result in a considerable number of ectopic cells during development.

Ngn2 is expressed in the cortex, but not in the GE (Gradwohl et al., 1996; Ma et al., 1997; Fode et al., 2000). The loss-of-function condition of Ngn2 could therefore result in either cell autonomous defects of cortical cells, e.g. by regulation of receptor molecules on the migrating cells, or in non cell-autonomous defects of properties of the migration substratum in the cortex and/or the boundary region. We have two sets of evidence that favor the latter interpretation, but do not exclude the former. First, the radial glial fascicle forming the cortico-striatal boundary is less fasciculated in the *ngn2*$^{-/-}$ telencephalon compared to wild type, and expression of SFRP2, the molecular marker of this boundary region, is expanded. While these changes are very subtle, they are clearly non cell-autonomous and relevant for migrating cells as demonstrated in our in vitro transplantation experiments. Thus, Ngn2 regulates specific features of the cortico-striatal boundary required for the tight restriction of cortical cells. Cells from the GE, however, do not recognize these changes since their migration into the cortex is not affected. Thus, the molecules regulated by Ngn2 in the boundary affect only the restriction of cortical, but not striatal cells. This feature obviously distinguishes the telencephalic border from rhombomere boundaries where the same molecules are responsible to restrict cells on both sides of the boundary (Mellitzer et al., 1999; Xu et al., 1999). A prominent boundary in the diencephalon, the zona limitans also shows unique features (Zeltser et al., 2001), suggesting that boundary structures are highly specialized in different brain regions.

Comparison of tangential migration in *Pax6* and *ngn2* mutant mice

Our previous analysis of cell migration in the *Pax6* mutant mice *Small eye* (Chapouton et al., 1999) showed a prominent increase in ventrodorsal and a weaker effect on dorsoventral cell migration. The latter is comparable in its extent to the effect seen in the *ngn2*-deficient mice analyzed in this study. Indeed, *ngn2* is also down regulated in the cortex and spinal cord of *Pax6* mutant mice (Stoykova et al., 2000; Scardigli et al., 2001), consistent with a similar effect on cortical cell migration in both mutants. In addition, the loss of Pax6 function affects the restriction of cells from the GE, while *ngn2* does not. Thus, molecular changes present in the *Pax6*$^{-/-}$ telencephalon, but absent in the *ngn2*$^{-/-}$ mice should specifically affect GE cell migration, while changes present in both mutants are likely involved in the defects in cortical migration.

Indeed, the *Pax6* mutant mice exhibit more pronounced

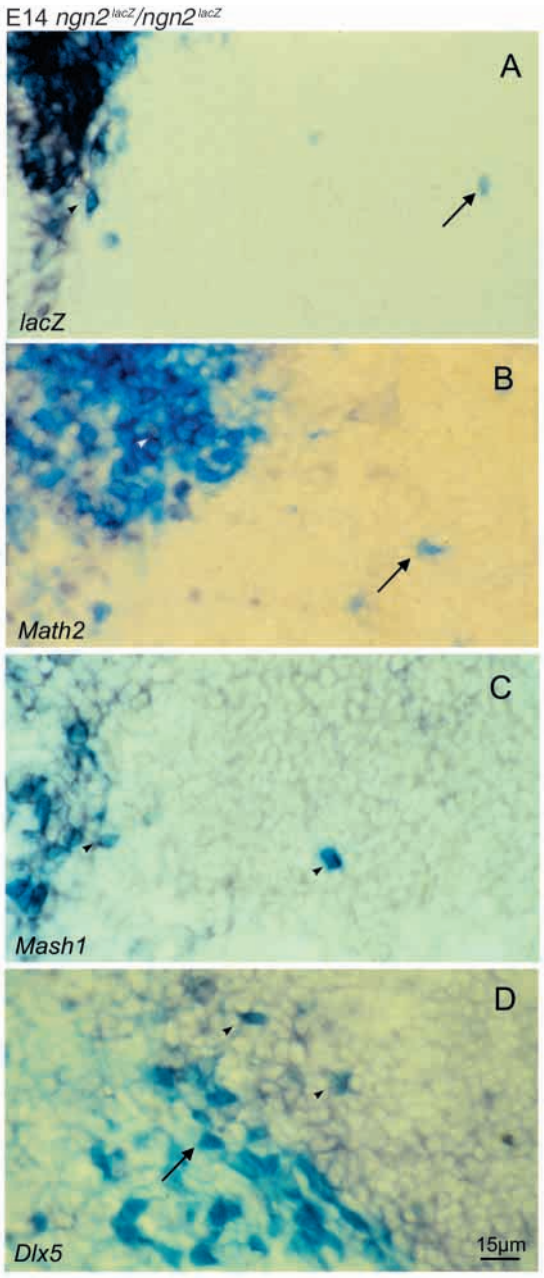

Fig. 5. Cortical cells entering the GE in *ngn2*^{lacZ}/*ngn2*^{lacZ} mice acquire a ventral identity. (A-D) The cortico-striatal border in sections of the telencephalon from E14 *ngn2*^{lacZ}/*ngn2*^{lacZ} mice stained for β-gal (blue) and hybridized with the probes indicated in the panel (purple ring). Sections are oriented with the cortex to the left and the GE to the right. Double-positive cells are indicated by arrowheads, β-galactosidase-positive cells negative for the respective transcripts are marked by an arrow. Note that ectopic cells lose expression of cortical genes (*lacZ* in the *ngn2* locus in A and *Math2* in B), while they maintain (C) or acquire (D) expression of ventral genes such as *Mash1* or *Dlx5*, respectively.

changes within the cortex and the boundary than *ngn2*$^{-/-}$ mice. For example, the entire boundary structures are lost at the cellular and molecular (TN-C, SFRP2) level in the *Pax6* mutant *Small eye* (Stoykova et al., 1997; Chapouton et al., 1999; Kim et al., 2001). Furthermore, *R-cadherin*, *Wnt7b*, *Slit2* and *ephrin B2* expression are down-regulated in the cortex of the *Pax6* mutant (P. C. and M. G., unpublished observations) (Stoykova et al., 1997; Kim et al., 2001), while these molecules are not affected in the *ngn2*$^{-/-}$ telencephalon, with the exception of the broader SFRP2 expression domain. Besides cell surface or extracellular signaling molecules, the expression of transcription factors changes dramatically in the *Pax6* mutant cortex. In particular, gene expression normally restricted to the ventral telencephalon, such as *Dlx1*, *Mash1* and *Gsh2*, spreads into the cortex in the *Pax6* mutants (Stoykova et al., 1996; Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001). Obviously this ventralization might endow cortical cells with ventral cell surface properties allowing the mixing of cortical with striatal cells (Stoykova et al., 1997; Chapouton et al., 1999). Similarly, *Mash1*, *Dlx1*, *Dlx5* and *GAD67* are up regulated ectopically in the cortex of *ngn2*$^{-/-}$ mice (Fode et al., 2000). However, *GAD67*, *Dlx1* and *Dlx5* are ectopically expressed only in medial cortical regions and hence could not affect cells close to the cortico-striatal boundary (Fode et al., 2000). In contrast, *Mash1* is expressed throughout the cortex in precursor cells and hence could mediate cortical cells acquiring ventral cell surface identities allowing them to mix with GE cells. To test this hypothesis, we examined β-gal-positive cells in *ngn2;Mash1* double mutants (Fode et al., 2000; Nieto et al., 2001). Ectopic β-gal-positive cells in the GE also occurred in *ngn2*$^{-/-}$ and *ngn2;Mash1* mutants, suggesting that the ectopic expression of *Mash1* in the cortex is not required for the spread of cortical cells into the GE in the absence of Ngn*2*. Thus, the loss of Ngn2 is sufficient to cause a leakage of cortical cells into the GE, consistent with the suggestion that Pax6 might regulate the cues responsible for the restriction of cortical cells in their territory via Ngn2.

Fate change of *ngn2* mutant cortical cells in the GE

The tight delineation of adjacent brain regions avoids the leakage of cells from one territory into the other. Such a leakage would result in a foreign cell population in the adjacent brain region with the danger of these cells differentiating into neurons with the wrong physiological properties. An example is the cortex of *Pax6* mutant mice, which contains a significantly higher number of GABAergic cells due to the increased invasion by this cell type from ventral positions (Chapouton et al., 1999). The tight balance between excitatory neurons that originate mostly in the cortex and inhibitory GABAergic neurons of ventral origin is crucial for the appropriate functioning of the cortex (Roberts et al., 1995; Götz, 2001). Since there is always a small amount of leakage at boundaries between brain regions (Birgbauer and Fraser, 1994; Chapouton et al., 1999; Hamasaki et al., 2001), an additional mechanism has to re-adjust the fate of the few cells entering the neighboring region. Gurdon (Gurdon, 1988) coined the term ‘community effect’ to describe the influence that cellular majorities have on the fate of cellular minorities [see also Götz (Götz, 1995)]. Indeed, cells from odd-numbered rhombomeres change their fate when they enter even-numbered rhombomeres (Xu et al., 1995), as do transplanted cells (Fishell, 1995; Campbell et al., 1995; Olsson et al., 1997) [however see Na et al. (Na et al., 1998)]. Unfortunately, few of these transplantation studies examined whether regulation of region-specific transcription factors is a prerequisite for such fate changes (Na et al., 1998).

The ectopic cells originating in the cortex and entering the GE in the *ngn2*$^{-/-}$ telencephalon therefore provided a unique opportunity to examine the regulation of transcription factors characteristic of the dorsal or ventral regions in the telencephalon. This analysis clearly revealed that β-gal-positive cells still have a dorsal identity in the cortex but change to a ventral identity after entering the GE. β-gal-positive cells expressed *ngn2-lacZ*, *Pax6*, *R-cadherin*, *Math2* and *Mash1* in the *ngn2*$^{-/-}$ cortex. When they entered the GE, however, they down-regulated expression of most of these genes, characteristic of the cortex, and acquired instead expression of *Dlx5*, characteristic of the ventral telencephalon. Taken together, these data seem to argue against the possibility that dorsal cells acquire a ventral identity already in the cortex allowing them to enter the GE. Rather, β-gal-positive cells within the cortex express dorsal and no ventral genes (except *Mash1*) while they turn off dorsal genes and acquire a ventral phenotype only after they have migrated for some distance into the GE. In order to reveal the identity of molecules essential in mediating the fate change of ectopic cortical cells, *ngn2*$^{-/-}$ mice could be crossed with mice deficient for key patterning genes in the GE.

These results also suggest a further level of asymmetry between dorsal and ventral cells in the developing telencephalon. While most cells entering the cortex from the GE seem to maintain their specification as ventral cells and continue to express *Lhx6*, *Dlx1/5* and *GAD67* (Anderson et al., 1997; Anderson et al., 2001; Lavdas et al., 1999; Pleasure et al., 2001), cortical cells entering into the GE seem to change their fate. Interestingly, Hamasaki et al. (Hamasaki et al., 2001) detected a population of cells, generated at early stages in the piriform cortex, migrating into the developing striatum. This population is eliminated by cell death at later stages, a second mechanism eliminating inappropriate types of neurons. Thus, not only migrational restriction, but also the mechanisms instructing fate changes seem to act asymmetrically on ventral and dorsal cells in the developing telencephalon.

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