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Origin and Molecular Specification of Striatal Interneurons

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The striatum, the largest component of the basal ganglia, contains projection neurons and interneurons. Whereas there is considerable agreement that the lateral ganglionic eminence (LGE) is the origin of striatal projection neurons, less is known about the origin of striatal interneurons. Using focal injections of retrovirus into the ventral telencephalon *in vitro*, we demonstrate that most striatal interneurons tangentially migrate from the medial ganglionic eminence (MGE) or the adjacent preoptic/anterior entopeduncular areas (POa/AEP) and express the NKX2.1 homeodomain protein. Although the majority of striatal interneurons (cholinergic, calretinin $^+$, and parvalbumin $^+$) maintain the expression of NKX2.1 into adulthood, most of the interneurons expressing somatostatin (SOM), neuropeptide Y (NPY), and neural nitric oxide synthase (NOS) appear to downregulate the expression of NKX2.1 as they exit the neuroepithelium. Analysis of striatal development in mice lacking *Nkx2.1* suggests that this gene is required for the specification of nearly all striatal interneurons. Similar analysis of mice lacking the *Mash1* basic helix–loop–helix (bHLH) or both the *Dlx1* and *Dlx2* homeodomain transcription factors demonstrates that these genes are required for the differentiation of striatal interneurons. *Mash1* mutants primarily have a reduction in early-born striatal interneurons, whereas *Dlx1/2* mutants primarily have reduced numbers of late-born striatal interneurons. We also present evidence implicating the *Lhx6* and *Lhx7* LIM-homeobox genes in the development of distinct interneuron subtypes. Finally, we hypothesize that, within the MGE, radially migrating cells generally become projection neurons, whereas tangentially migrating cells mainly form interneurons of the striatum and cerebral cortex.

Key words: NKX2.1; DLX; MASH1; LHX; basal ganglia; interneuron; medial ganglionic eminence; lateral ganglionic eminence; striatum; neuron identity; neuronal specification; telencephalon

The integration of complex information within a neural system frequently requires the coordinated activity of projection neurons and interneurons. In the striatum, the largest component of the basal ganglia, projection neurons comprise 90% of the cells, give rise to its outputs, and receive nearly all of the synapses from extrinsic striatal afferents as well as from the striatal interneurons (for review, see Heimer, 1995; Parent and Hazrati, 1995; Gerfen and Wilson, 1996). Interneurons, on the other hand, comprise only ~10% of striatal cells and are implicated in regulating striatal projection function (for review, see Kawaguchi et al., 1995; Kawaguchi, 1997).

The mechanisms that control the generation of different striatal neuronal subtypes and their assembly into functional circuits are poorly understood. The basal ganglia (striatum and pallidum) derive from both the lateral and the medial ganglionic eminences in the telencephalon (LGE and MGE, respectively). Striatal projection neurons are thought to derive from the LGE (Deacon et al., 1994; Olsson et al., 1995, 1998; Anderson et al., 1997a), and *Dlx1*,*2*,*5*,*6*, *Gsh2*, *Mash1,* and retinoid receptor transcription factors have been implicated in the control of their specification and differentiation (Porteus et al., 1994; Hsieh-Li et al., 1995; Anderson et al., 1997a; Szucsik et al., 1997; Casarosa et al., 1999; Eisenstat et al., 1999; Toresson et al., 1999). For instance, the *Dlx* genes are homeodomain transcription factors that are expressed in overlapping patterns during the differentiation of basal telencephalic neurons (Anderson et al., 1997a; Eisenstat et al., 1999). Mice lacking *Dlx1* and *Dlx2* have a block in the differentiation of late-born neurons in the basal telencephalon, which affects to a large number of projection neurons of the striatum as well as interneurons of the cerebral cortex and olfactory bulb (Anderson et al., 1997a,b; Bulfone et al., 1998). In addition, *Mash1* encodes a basic helix–loop–helix transcription factor that is required for the development of early-born neurons in the basal telencephalon and some cortical interneurons (Casarosa et al., 1999).

Unlike striatal projection neurons, the origin of striatal interneurons and the genetic control of their development are poorly understood. Striatal interneurons comprise four major classes: (1) cholinergic neurons; (2) GABAergic neurons containing calretinin (CR); (3) GABAergic neurons containing parvalbumin (PV); and (4) GABAergic neurons containing somatostatin (SOM), neuropeptide Y (NPY), and neuronal nitric oxide synthase (NOS) (for review, see Kawaguchi et al., 1995) (but also see Figueredo-Cardenas et al., 1996). Previous transplantation studies in the rat have suggested that SOM $^+$ interneurons mainly derive from the LGE, whereas cholinergic interneurons may derive from the MGE (Olsson et al., 1998). The origin of the GABAergic interneurons containing PV or CR has not been elucidated. Recent studies have found that there are migrations of cells from the MGE into the LGE (Sussel et al., 1999; Wichterle et al., 1999), raising the possibility that some striatal cells are derived from the MGE. Furthermore, the expression pattern of the *Nkx2.1* homeobox gene suggests that at least some of these migrating cells may express this gene. Although it is known that *Nkx2.1* is required for the specification of MGE derivatives (Sussel et al., 1999), the analysis of striatal interneurons in this mutant has not been described.

In this work we describe our studies that investigated the origin of the four major types of striatal interneurons, and that demonstrates the essential role of the *Nkx2.1, Dlx1, Dlx2,* and *Mash1* transcription factors in controlling their development. In addition, we also provide evidence that two different LIM-homeodomain transcription factors, *Lhx6* and *Lhx7*, might control the develop-

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ment of distinct subtypes of striatal interneurons. *In toto*, these results begin to elucidate the pathway of transcription factors that control specification and differentiation of striatal interneurons.

MATERIALS AND METHODS

Animals. C57BL/J6 mice were used for the organotypic slice culture experiments and for immunohistochemical colocalization studies. In addition, mouse mutant strains with null alleles of *Nkx2.1* (a gift of S. Kimura, National Cancer Institute, Bethesda, MD), *Mash1* (Guillemot et al., 1993), and *Dlx-1* and *Dlx-2* (Qiu et al., 1997) were used for the immunohistochemical localization of striatal interneurons. These mutant strains were maintained by backcrossing to C57BL/J6 mice for at least 10 generations. For staging of the embryos, midday of the vaginal plug was considered as embryonic day 0.5 (E0.5). Mouse colonies were maintained at University of California, San Francisco in accordance with National Institutes of Health and UCSF guidelines.

Retrovirus preparation. The production of Moloney murine leukemia virus/vesicular stomatitis virus G (MMLV/VSV-G) pseudotyped retrovirus has been described in detail previously (Ory et al., 1996). Briefly, a replication-defective retroviral construct (ΔU3nlxLacZ) encoding a nuclear-localizing β-galactosidase (β-gal) under the control of the human cytomegalovirus (HCMV) enhancer–promoter was cotransfected into a 293-derived packaging cell line. Viral supernatants were harvested and concentrated by ultracentrifugation. Viral pellets were resuspended in PBS, and aliquots of virus were stored at −80°C.

Organotypic slice cultures. Organotypic slice cultures of embryonic mouse telencephalon were prepared as previously described (Anderson et al., 1997a). Briefly, embryos (E12.5–E16.5) were removed by cesarean section and decapitated. Brains were removed in ice-cold Krebs' buffer, pH 7.4, containing (in mM) 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 11 glucose, and 25 $NaHCO_3$. Then the brains were embedded in 4% low melt point agarose (FMC Bioproducts, Rockland, MA), and 250-μm-thick coronal sections were cut on a vibratome and collected in ice-cold Krebs' buffer. The sections subsequently were transferred to sterile ice-cold Krebs' buffer (filtered Krebs' containing 10 mM HEPES, penicillin, streptomycin, and gentamycin). After 15 min the sections were transferred to polycarbonate culture membranes (13 mm diameter, 8 μm pore size; Corning Costar, Cambridge, MA) in organ tissue dishes containing 1 ml of medium with serum (Gibco MEM with glutamine, 10% fetal calf serum, penicillin, and streptomycin). Subsequently, they were incubated for 1 hr in a sterile incubator (at 37°C in 5% CO_2), after which the medium was change to Neurobasal/B-27 (Life Technologies, Gaithersburg, MD). Injections were performed immediately after this step. Retroviruses were pressure-injected focally onto the LGE or MGE by a pneumatic PicoPump (Narishige, Tokyo, Japan) through a glass micropipette. After incubation for various times the slices were fixed in 4% paraformaldehyde (PFA) in 0.1 M PBS for 1 hr at 4°C and processed for β-gal histochemistry or β-gal/NKX2.1 double immunohistochemistry as described below. In control experiments 2 μg/μl of cytochalasin-D (Sigma, St. Louis, MO) was added to the culture medium 12 hr after the injections to inhibit cell migration.

β-Gal staining. β-Gal histochemistry was performed in free-floating sections at 37°C overnight in a solution containing 0.005% Na-deoxycholate, 0.01% Nonidet P40, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, and 0.8 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in 10 mM Tris–HCl buffer (TB), pH 7.3. Then the sections were rinsed in TB, fixed for 1 hr in 4% PFA, dehydrated, and cleared in xylene. For β-gal/NKX2.1 double immunohistochemistry the organotypic slice cultures were fixed, cryoprotected in 30% sucrose in PBS, cut into 20 μm sections, and mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Then the sections were preincubated in 5% normal goat serum (NGS), 1% bovine serum albumin (BSA), and 0.2% Triton X-100 (TX) in PBS for 30 min at room temperature; subsequently, they were incubated overnight at 4°C in a cocktail of primary antisera diluted 1:1000 with 2% NGS and 0.2% TX in PBS. Mouse anti-β-gal (Promega, Madison, WI) and rabbit anti-NKX2.1 (Biopat Immunotechnologies, Caserta, Italy) antisera were used. Sections were rinsed in PBS and incubated for 1 hr in a cocktail of Alexa 488 goat anti-mouse and Alexa 594 goat anti-rabbit secondary antisera (Molecular Probes, Eugene, OR) diluted 1:200 in the same solution as the primary antisera. Washings were performed in PBS, and the sections were coverslipped with Prolong Antifade mounting medium (Molecular Probes). In all cases, NKX2.1 immunoreactivity was used to demarcate the mantle region of the MGE and the LGE.

Immunohistochemistry single labeling. E18.5 embryos, removed by caesarean section, and neonates were anesthetized by cooling and perfused with 4% PFA. Brains were removed and post-fixed for 3 hr, cryoprotected in 30% sucrose in PBS, and cut frozen in the transverse plane on a sliding microtome at 40–50 μm. Then free-floating sections were preincubated in 5% normal serum of the species in which the secondary antibody was raised, 1% BSA, and 0.3% TX in PBS for 1 hr at room temperature; subsequently, they were incubated with the primary antisera for 36 hr at 4°C in 2% normal serum and 0.3% TX in PBS. The following antibodies were used: rabbit anti-CB (Swant, Bellinzona, Switzerland), diluted 1:5000; rabbit anti-CR (Chemicon, Temecula, CA), diluted 1:5000; goat anti-ChAT (Chemicon), diluted 1:100; rabbit anti-NPY (Incstar, Stillwater, MN), diluted 1:3000; rat anti-SOM (Chemicon), diluted 1:250; and rabbit anti-NOS (Zymed, San Francisco, CA), diluted 1:1000. Sections then were incubated in biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA), diluted 1:200, and processed by the ABC histochemical method (Vector Laboratories). The sections were mounted onto Superfrost Plus slides (Fisher Scientific), dried, dehydrated, and coverslipped with Permount (Fisher Scientific). In each experiment the sections from homozygous mutants and their wild-type or heterozygous littermates were processed together. Primary antiserum omission controls and normal mouse, rabbit, and goat serum controls were used to confirm further the specificity of the immunohistochemical labeling. Labeled cells were plotted by using a system for image analysis (Openlab Improvision, UK). For counting the numbers of striatal interneurons in *Nkx2.1* and *Mash1* mutants, we analyzed two defined striatal levels in three independent experiments. Wild-type and mutant striatal sections were matched by using external anatomical references. At the rostral striatal level, equivalent cortical and septal levels were used as landmarks. At the caudal striatal level, equivalent regions of the hippocampus and preoptic area were used as landmarks. The mean striatal area ($mm^2 \pm$ SEM) at the rostral section plane is 0.95 ± 0.12 for the wild-type and 1.1 ± 0.16 and 0.86 ± 0.09 for the *Nkx2.1* and *Mash1* mutants, respectively. At the caudal section plane the mean striatal area ($mm^2 \pm$ SEM) is 0.79 ± 0.08 in wild-type mice and 0.85 ± 0.12 and 0.72 ± 0.11 in *Nkx2.1* and *Mash1* mutant mice, respectively. Because the area surface of the striatum in *Dlx1/2* mutant mice is relatively constant throughout the rostrocaudal extend of the nucleus, a single level was analyzed in three independent experiments. The cortex and septum were used as anatomical landmarks to match the rostrocaudal level from both wild-type and mutant sections. The mean striatal area ($mm^2 \pm$ SEM) is 0.81 ± 0.07 for the wild-type and 0.23 ± 0.03 for the *Dlx1/2* mutant. The mean diameter (μm ± SEM) of striatal neurons is 9.22 ± 0.89 in wild-type and 9.32 ± 0.58, 9.36 ± 0.68, and 9.29 ± 0.56 in *Nkx2.1*, *Mash1,* and *Dlx1/2* mutant mice, respectively.

Immunohistochemistry double labeling. Mice 3–4 weeks old were anesthetized with an overdose of chloral hydrate and perfused transcardially with 20 ml of 0.9% NaCl, followed by 60 ml of 4% PFA in PBS. The brains were removed, post-fixed for 2 hr at 4°C, cryoprotected, and sectioned frozen in the transverse plane on a sliding microtome at 30–40 μm. Free-floating sections were preincubated in 5% normal serum of the species in which the secondary antibody was raised, 1% BSA, and 0.4% TX in PBS for 1 hr at room temperature; subsequently, they were incubated in a cocktail of primary antisera for 36 hr at 4°C. The cocktail always includes a rabbit anti-NKX2.1 antiserum, diluted 1:1000 in 2% normal serum and 0.4% TX in PBS, and one of the following antisera: mouse anti-CB (Sigma), diluted 1:1000; mouse anti-DARPP-32, diluted 1:15,000; mouse anti-CR (Chemicon), diluted 1:1000; goat anti-ChAT (Chemicon), diluted 1:100; sheep anti-NPY (Chemicon), diluted 1:1000; or rat anti-SOM (Chemicon), diluted 1:250. Then the sections were rinsed in PBS and incubated for 2 hr in a cocktail of secondary antibodies diluted 1:200 in the same solution as the primary antisera. Alexa 488 goat anti-mouse and Alexa 594 goat anti-rabbit were used for NKX2.1 and CB, DARPP-32, CR, NPY, or SOM double labeling, whereas Alexa 488 goat anti-rabbit and Alexa 594 donkey anti-goat (Molecular Probes) were used for NKX2.1 and ChAT double labeling. After being rinsed in PBS, the sections were stained with 50 μg/ml of Hoechst 33342 (Molecular Probes), mounted, and coverslipped with Prolong Antifade mounting medium. Analysis of colocalization of NKX2.1 with striatal markers was performed in three defined striatal sectors (dorsomedial, dorsolateral, and ventral striatum; 0.5 mm^2 each) in two sections at defined rostrocaudal levels of the striatum in a total of three mice. The percentage of double-labeled cells (e.g., NKX2.1-ChAT double-labeled/total ChAT neurons at the three rostrocaudal levels) is averaged for the three mice. The mean number (± SEM) of neurons counted for each marker per animal is as follows: CB, $n = 327 \pm 7.3$; DARPP-32, $n = 380.3 \pm 5.8$; ChAT, $n = 148.7 \pm 11.6$; PV, $n = 65.3 \pm 6.9$; CR, $n = 42 \pm 4.7$; NPY, $n = 187 \pm 13.3$; and SOM, $n = 194.3 \pm 10.2$.

NADPH-diaphorase (NADPHd) staining. NADPHd histochemistry was performed in free-floating sections at 37°C as previously described (Marín et al., 1998). Briefly, E18.5 embryos and neonates were anesthetized by cooling and were perfused with 4% PFA. Brains were removed and post-fixed for 3 hr, cryoprotected in 30% sucrose in PBS, and cut frozen in the transverse plane on a sliding microtome at 40 μm. Then the free-floating sections were preincubated in a medium containing 1 mM β-NADPH (Sigma), 0.8 mM nitroblue tetrazolium (Sigma), and 0.06% TX in PBS at 37°C for 1–2 hr. After incubation the sections were rinsed thoroughly in PBS, mounted, and, after drying overnight, were coverslipped.

5-Bromo-2′-deoxyuridine (BrdU) labeling. Pregnant females were injected intraperitoneally with 40 mg/kg of BrdU (Sigma) and killed 30 min later, or gestation was continued to term. Embryos were removed by caesarean section and decapitated; their brains were removed and fixed in 4% PFA in PBS overnight at 4°C. Neonates were perfused as described above. In all cases the brains were cryoprotected in 30% sucrose, cut into 10 μm sections, and mounted onto Superfrost Plus slides. Then the sections were preincubated in 5% NGS, 1% BSA, and 0.2% TX in PBS for 30 min at room temperature; subsequently, they were incubated overnight at 4°C in a rabbit antiserum against NKX2.1 diluted 1:500 in 2% NGS and 0.2% TX in PBS. After being rinsed in PBS, the sections were post-fixed in 4% PFA in PBS for 20 min and subsequently were incubated with 2N HCl in PBS

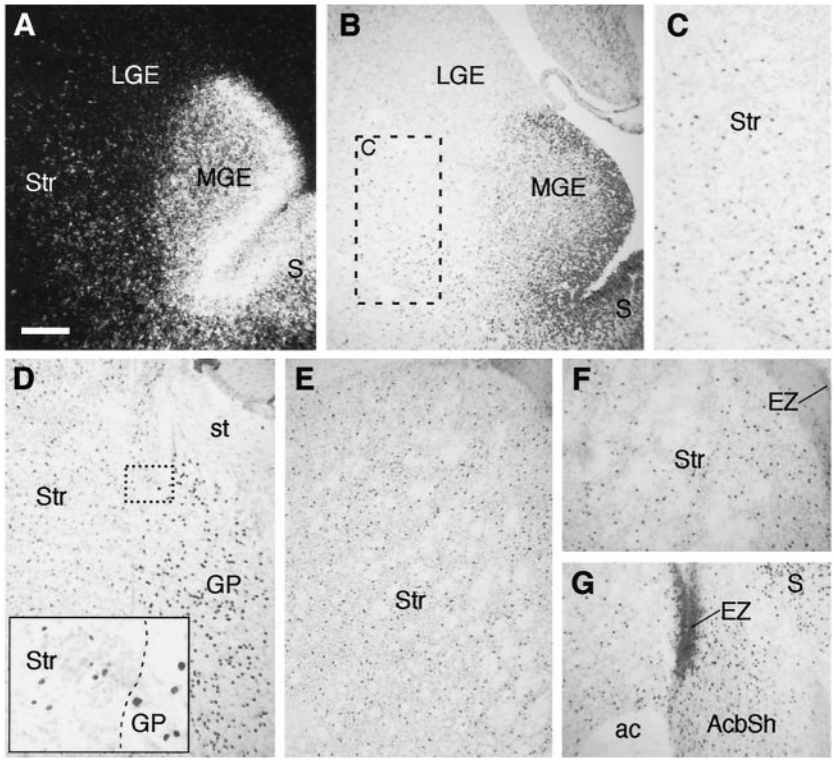

Figure 1. Developmental expression of *Nkx2.1* RNA and protein in the basal telencephalon. *A*, RNA *in situ* hybridization analysis of *Nkx2.1* expression in a coronal section of the basal telencephalon at E14.5. *B*, NKX2.1 protein distribution in an adjacent section to that shown in *A*. *C*, A higher magnification image of the *outlined boxed region* in *B*. *D–G*, Expression of NKX2.1 in coronal sections through the telencephalon of the mouse at P18. *ac*, Anterior commissure; *AcbSh*, shell of the nucleus accumbens; *EZ*, ependymal zone; *GP*, globus pallidus; *LGE,* lateral ganglionic eminence; *MGE,* medial ganglionic eminence; *S*, septum; *st*, stria terminalis; *Str*, striatum. Scale bar: *A, B, F, G*, 200 μm; *C*, 100 μm; *D, E,* 300 μm.

for 20 min at 37°C. Then the sections were rinsed in PBS and incubated 2 hr in a mouse anti-BrdU antibody (Chemicon) diluted 1:200 as before. After being rinsed in PBS, the sections were incubated in a cocktail of Alexa 488 goat anti-mouse and Alexa 594 goat anti-rabbit secondary antibodies diluted 1:200 in the same solution as the primary antisera. Washings were performed in PBS, and the sections were stained with Hoechst and coverslipped as above.

In situ *RNA hybridization. In situ* hybridization experiments were performed by using ^{35}S riboprobes on 10 μm frozen sections as described previously (Bulfone et al., 1993). The cDNA probes used in this study were *Nkx2.1* (Shimamura et al., 1995), *Lhx6* and *Lhx7* (kindly provided by V. Pachnis, National Institute for Medical Research, London, UK), and *ENK* (kindly provided by C. Gerfen, National Institute of Mental Health, Bethesda, MD).

RESULTS

NKX2.1 expression in the basal telencephalon

Nkx2.1 expression is first detectable in the mouse basal telencephalon at the 11 somite stage, at approximately E8.75 (Shimamura et al., 1995). Between E10.5 and E11.5 *Nkx2.1* is strongly expressed in several regions within the basal telencephalon/medial ganglionic eminence (MGE), part of the septum, anterior entopeduncular area (AEP), and preoptic area (POa) (Price et al., 1992; Kohtz et al., 1998; Sussel et al., 1999) (see also Fig. 10*A*). In these structures *Nkx2.1* is expressed in both proliferative and postmitotic cells (Sussel et al., 1999). Conversely, *Nkx2.1* expression is not found at these stages in cells of the lateral ganglionic eminence (LGE), a proliferative zone that is thought to give rise to the striatum (Deacon et al., 1994; Anderson et al., 1997a). From E13.5 the immature basal telencephalic nuclei that express *Nkx2.1* are clearly recognizable and include the globus pallidus, ventral pallidum, entopeduncular nucleus, substantia innominata/basal magnocellular region, anterior part of the bed nucleus of the stria terminalis, and part of the septum (Puelles et al., 1999; Sussel et al., 1999).

Here we extended previous analyses by examining the expression of NKX2.1 protein in detail. In the telencephalon NKX2.1 expression appeared to be restricted primarily to the cell nucleus (Fig. 1). From E14.5, NKX2.1 expression is found in scattered cells of the striatum (Fig. 1*A–C*), despite the fact that it is not expressed in the proliferative zones of the LGE at the time when striatal neurons are born (mostly between E11 and E16 in the mouse; see references in Semba et al., 1988; Sadikot and Sasseville, 1997). Thus BrdU injections 30 min before death at different developmental stages (E12.5–E16.5) double-label NKX2.1$^+$ cells in the MGE, but not in the LGE (data not shown).

As development proceeds, NKX2.1 is maintained in a subpopulation of striatal cells. In the postnatal brain NKX2.1$^+$ cells are found not only in the pallidal components of the basal ganglia (Fig. 1*D*) but also in scattered cells within the dorsal and ventral subdivisions of the striatum (Fig. 1*D–G*). Immunoreactivity for NKX2.1, however, is always stronger in the nuclei of pallidal than of striatal neurons. In summary, NKX2.1-expressing cells are present in the striatum, despite evidence that progenitor cells of this region (LGE) do not express this protein.

NKX2.1 striatal cells migrate from the MGE and POa/AEP

Because the proliferative zone of the striatum (LGE) does not express NKX2.1 before E16.5, we wondered whether this population of striatal cells arrives via a tangential migration from the MGE. Our previous study, using DiI labeling of MGE cells, suggested that there is a tangential migration from the MGE to the LGE (Sussel et al., 1999). However, that study did not resolve several important questions, including the origin of the progenitors that give rise to the migrating cells and their eventual phenotype. In addition, it is also possible that striatal NKX2.1$^+$ cells derive from the LGE, but, in contrast to the cells originated in the MGE, they start to express NKX2.1 only when they become postmitotic. To distinguish between these possibilities, we studied the origin of striatal NKX2.1$^+$ cells by infecting telencephalic slices with replication-incompetent retroviruses encoding the marker β-galactosidase (see Materials and Methods). These viruses can integrate only into mitotically active cells, and thus this method enables us to follow the movements of progenitor cells at different times after their infection.

Retroviral vectors were injected into either the VZ/SVZ of the LGE or the MGE in coronal slices from the telencephalon of E12.5–E16.5 mice (Fig. 2*A*). After 20 hr the infected cells were always located within a radius of 100–400 μm from the injection site ($n = 9$ of 9; Fig. 2*B–D*), suggesting that retroviral particles do

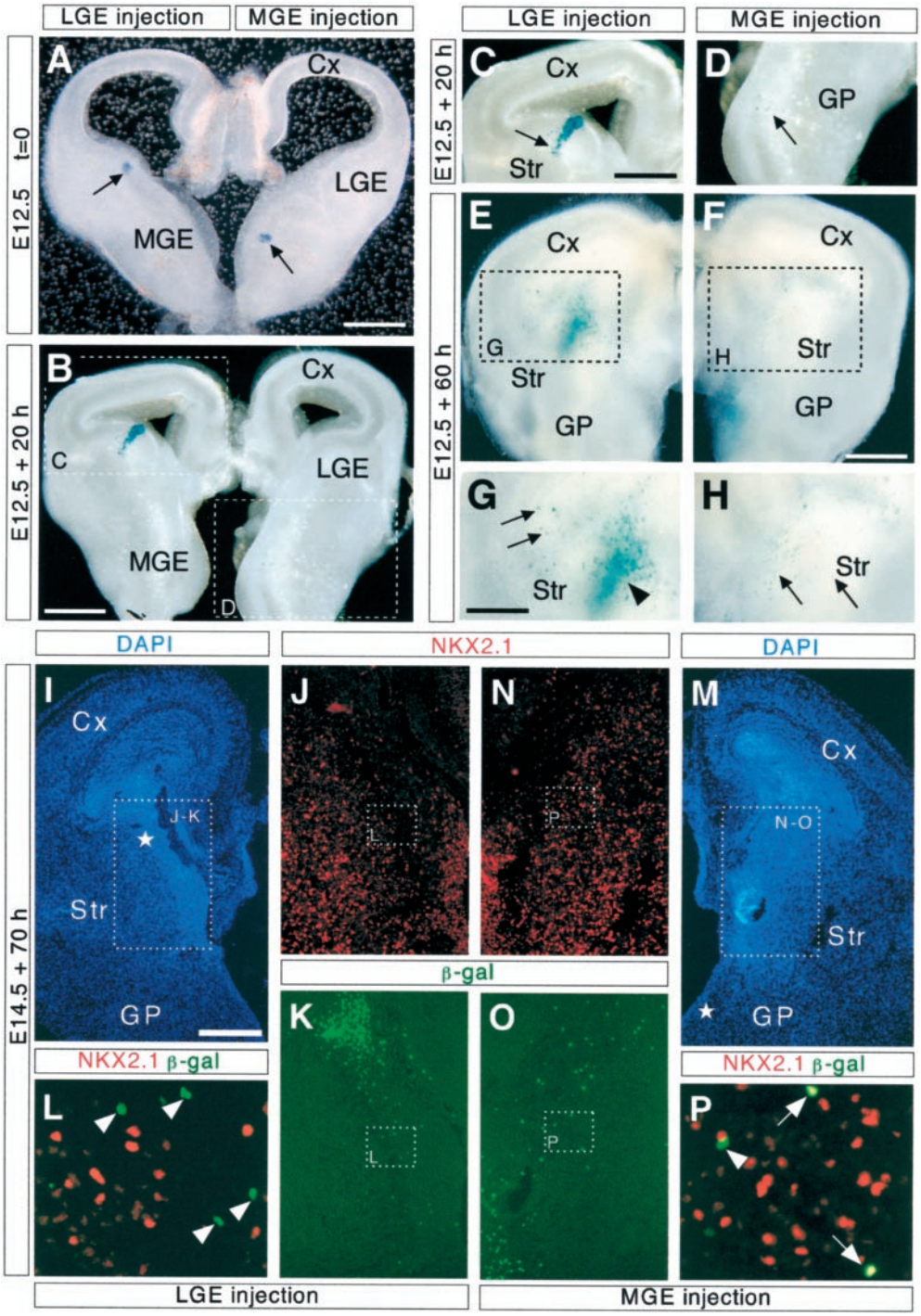

Figure 2. NKX2.1$^+$ cells migrate from the MGE into the striatum in telencephalic slice cultures. *A*, Retroviral injections into the LGE (*left side*, *arrow*) and MGE (*right side, arrow*) in a 250 μm coronal slice through the telencephalon of an E12.5 mouse. *B*, Migration of β-gal$^+$ cells 20 hr after retroviral injection in the LGE (*left side*) and the MGE (*right side*). *C*, *D*, Higher magnification images of the *outlined boxed regions* in *B*. *E*, *F*, Migration of β-gal$^+$ cells 60 hr after retroviral injection in the LGE (*E*) or the MGE (*F*). *G*, *H*, Higher magnification images of the *outlined boxed regions* in *E* and *F*, respectively. Note that whereas both radially (*arrowhead*) and tangentially (*arrows*) migrating cells are observed after injection in the LGE (*E, G*), most migrating cells migrate radially after MGE injection at this age (*F, H*). *I–P*, A large number of β-gal$^+$ cells migrating from the MGE to the striatum are NKX2.1$^+$ (*N–P*), whereas none of the cells is NKX2.1$^+$ after retroviral injection in the LGE (*I–L*). *I, M*, DAPI-stained sections (10 μm) obtained from a 250 μm coronal slide through the telencephalon of an E14.5 mouse after retroviral injection into the LGE (*star*, *I*) or the MGE (*star*, *M*) and 70 hr in culture. *J, K, N, O*, Higher magnification images of the *outlined boxed regions* in *I* and *M*, respectively. *L*, *P*, Higher magnification images of the *outlined boxed regions* in *J, K, N,* and *O*, respectively. *Arrows* point to double-labeled cells, whereas *arrowheads* show single β-gal$^+$ cells. *Cx*, Cortex; *GP*, globus pallidus; *LGE,* lateral ganglionic eminence; *MGE,* medial ganglionic eminence; *Str*, striatum. Scale bars: *A*, *B*, *E*, *F*, 400 μm; *C, D, G, H*, 300 μm; *I, M*, 200 μm; *J, K, N, O*, 100 μm; *L, P*, 40 μm.

not diffuse passively between different proliferative zones. By 60 hr the cells were detected outside the proliferative zones, and their number and distance from the injection site increased with time (Fig. 2*E–H*). Blocking cell migration by the addition of cytochalasin-D (0.5–1 mg/ml) resulted in no β-gal$^+$ cells outside the injection site (n = 8; data not shown).

When retroviral particles were injected into the MGE, β-gal$^+$ cells were observed to migrate into the mantle of the striatum at all stages that were examined (E12.5, E13.5, E14.5, and E16.5; n = 10 of 10; Fig. 2*F,H,K*; data not shown). Usually, between 50 and 100 cells were labeled per experiment. Approximately 50% of the striatal β-gal$^+$ cells derived from the MGE were also positive for NKX2.1 (Fig. 2*I–L*). Moreover, viral injections into the adjacent POa/AEP (ventral to the MGE) at E12.5 also double-labeled NKX2.1 cells in the striatal mantle (n = 3 of 5; data not shown). In contrast, none of the β-gal$^+$ cells derived from the LGE expressed NKX2.1 at any of the stages that were examined (n = 14 of 14; Fig. 2*M–P*). Taken together, these results suggest that striatal cells expressing NKX2.1 derive primarily from the MGE, although some NKX2.1 cells also originate in the adjacent POa/AEP.

NKX2.1 is expressed in most striatal interneurons

Because the mammalian striatum contains several cell types with different functional roles (Kawaguchi et al., 1995; Kawaguchi, 1997), we analyzed the expression of NKX2.1 in the distinct subtypes of striatal cells. We performed double-labeling experiments during the third postnatal week in the mouse, when the neurochemical characteristics of the striatum begin to resemble those of adults (Liu and Graybiel, 1992; Schlösser et al., 1999).

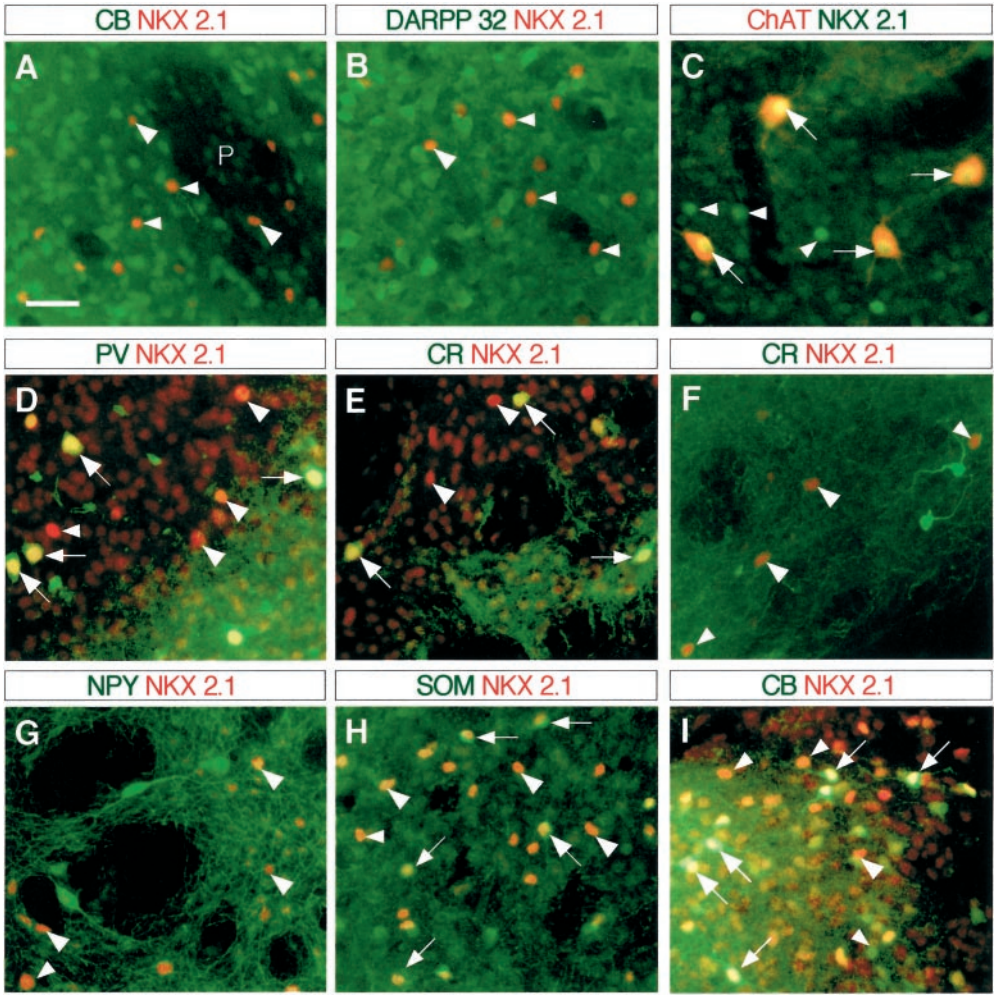

Figure 3. NKX2.1 is expressed in most striatal interneurons (*C–I*), but not in striatal projection neurons (*A, B*). Shown is colocalization of NKX2.1 and CB (*A, I*), DARPP32 (*B*), ChAT (*C*), PV (*D*), CR (*E, F*), NPY (*G*), or SOM (*H*) in the striatum of P18 mice. *Arrows* show double-labeled cells, whereas *arrowheads* indicate cells expressing only NKX2.1. *P*, Patch. Scale bar for *A–I*, 40 μm.

First, we used CB immunohistochemistry to identify striatal projection neurons. CB is a calcium-binding protein that is expressed predominantly in medium-sized spiny neurons of the striatal matrix (Gerfen, 1992). Double-labeling experiments revealed that the medium-sized CB^+ neurons of the striatum do not express NKX2.1 (Fig. 3*A*). In addition, we analyzed the expression of NKX2.1 in striatal neurons containing DARPP-32. DARPP-32 is a D1 receptor-associated protein found in striatal projection neurons, including both substance P and enkephalin-containing projection neurons in the patch and matrix compartments, and is virtually absent from striatal interneurons (Anderson and Reiner, 1991). No NKX2.1 was detected in DARPP-32^+ striatal neurons (Fig. 3*B*).

Double labeling revealed that all cholinergic striatal interneurons, as identified by the presence of the synthetic enzyme ChAT, are labeled for NKX2.1 also (Fig. 3*C*). In fact, all $ChAT^+$ neurons in the telencephalon are labeled with NKX2.1 (data not shown). Like the cholinergic neurons, all PV^+ striatal interneurons were found to contain NKX2.1 (Fig. 3*D*). In addition, the vast majority of CR^+ striatal interneurons also was labeled for NKX2.1 (94.2 ± 1.2%; Fig. 3*E*), although a small proportion of the CR^+ neurons in the striatum does not contain NKX2.1 at this age (Fig. 3*F*).

In contrast to the cholinergic, PV^+, or CR^+ interneurons, ~90% of striatal interneurons expressing SOM, NPY, and NOS do not express NKX2.1 at postnatal day 18 (P18) (*arrowheads* in Fig. 3*G,H*). We found that only ~10% of the SOM^+ or NPY^+ cells were immunolabeled for NKX2.1 (13.4 ± 1.4% for SOM; 11.3 ± 1.7% for NPY; *arrows* in Fig. 3*H*; data not shown). Most of these cells were located in the ventral striatum, although a few double-labeled cells also were found in the dorsal striatum.

In addition to its expression in projection neurons of the matrix, CB also is found in a small subpopulation of striatal NOS interneurons (Bennett and Bolam, 1993; Kubota and Kawaguchi, 1993). These CB^+ neurons are larger and more intensely stained than the striatal projection neurons and are distributed in both the patch and the matrix compartments (Kiyama et al., 1990; Bennett and Bolam, 1993; Kubota and Kawaguchi, 1993). In our double-labeling experiments we found that a small number of CB^+ neurons contained NKX2.1 (*arrows* in Fig. 3*I*). As in the case of the NPY/SOM/NOS cells immunolabeled for NKX2.1, the CB^+ neurons containing NKX2.1 were located primarily in the ventral striatum, suggesting that they actually represent a subpopulation of the striatal interneurons containing SOM, NPY, and NOS.

To evaluate the relationship between NKX2.1 localization and interneuronal phenotype in the striatum further, we performed similar double-labeling experiments at earlier times of development. At P0 the number of SOM^+, NPY^+, or NOS^+ neurons that also contain NKX2.1 is substantially higher than at P18, in particular at rostral striatal levels (data not shown). Nevertheless, most SOM^+ or NPY^+ striatal neurons do not contain NKX2.1 at this age.

The results from these double-labeling experiments (Fig. 3) combined with the cell migration assay (see Fig. 2) support the notion that striatal cholinergic ($ChAT^+$), PV^+, and CR^+ interneurons derive from the MGE and that NKX2.1 might play an important role in their development. In addition, at least a subpopulation of the NPY/SOM/NOS striatal interneurons also may derive from the MGE. It is not clear, however, whether all striatal interneurons containing SOM, NPY, and NOS may derive from the MGE but downregulate the expression of NKX2.1 as they progressively differentiate. To clarify this problem, we analyzed the distribution of striatal interneurons in *Nkx2.1* mutant mice.

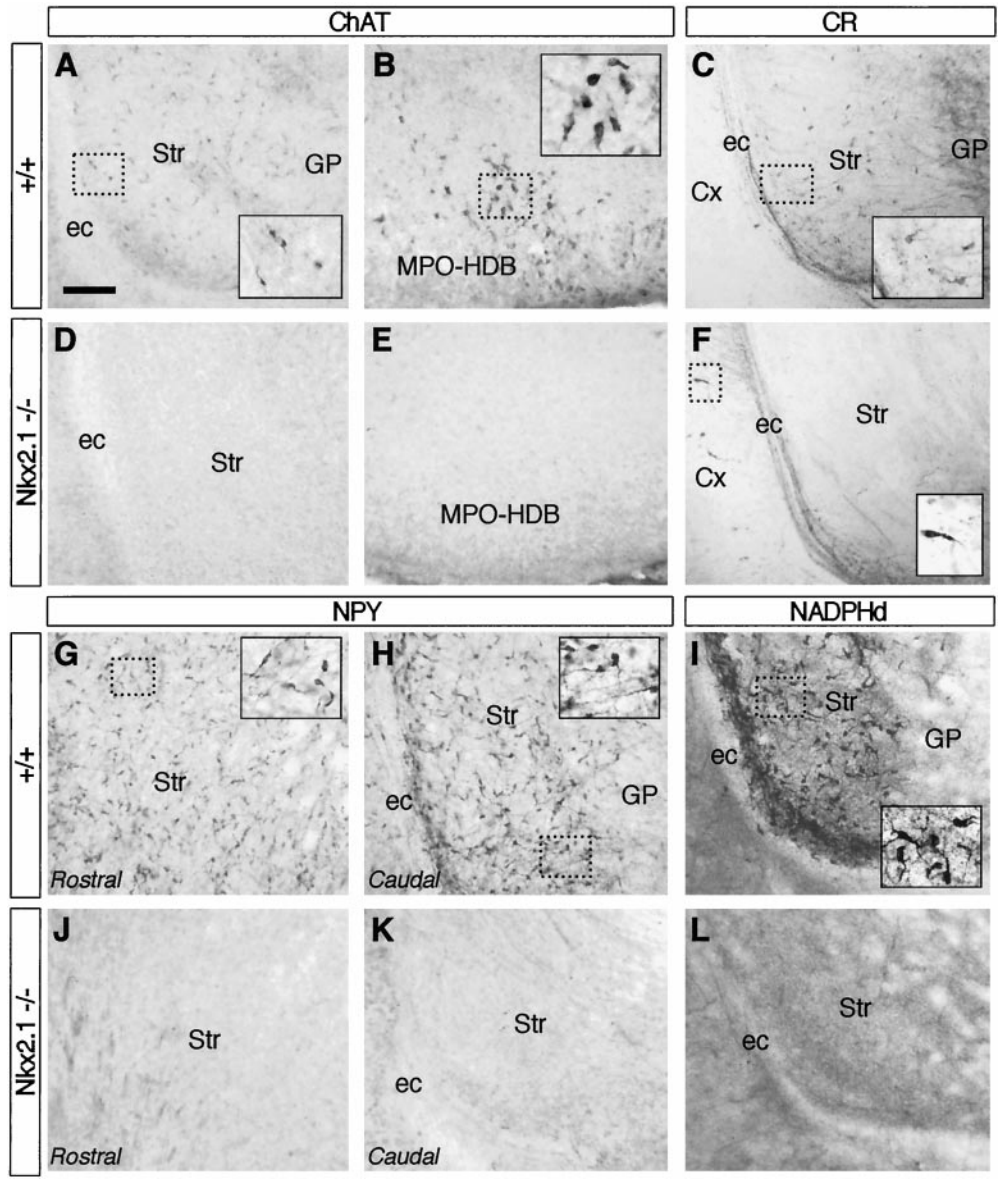

Figure 4. Loss of striatal interneurons in *Nkx2.1* mutant mice. *A–C, G–I*, Coronal sections through the telencephalon of E18.5 wild-type fetuses showing ChAT (*A, B*), CR (*C*), and NPY immunoreactivity (*G, H*) and showing NADPHd staining (*I*) in the rostral striatum (*G*), caudal striatum (*A, C, H, I*), and basal telencephalon (*B*). *D–F, J–L*, Coronal sections through the telencephalon of E18.5 *Nkx2.1*$^{-/-}$ fetuses showing loss of ChAT (*D, E*), CR (*F*), and NPY immunoreactivity (*J, K*), and showing NADPHd staining (*L*) in the rostral striatum (*J*), caudal striatum (*D, F, K, L*), and basal telencephalon (*E*). *Insets* in *A–C* and *F–I* show higher magnification images of neurons from the *outlined boxed regions*. *Cx*, Cortex; *ec*, external capsule; *GP*, globus pallidus; *MPO-HDB*, medial preoptic region-horizontal limb of the diagonal band; *Str*, striatum. Scale bar for *A–L*, 100 μm.

Nkx2.1 mutants are defective in striatal interneurons

In mice deficient for *Nkx2.1*, pallidal structures are not detectable and the cerebral cortex has reduced numbers of interneurons (Sussel et al., 1999). Because homozygous mutants die immediately after birth, we analyzed the expression of interneuronal markers in the striatum at E18.5. Although at this age several markers are either not cell type-specific (e.g., CB) (Liu and Graybiel, 1992) or are not expressed (PV; Schlösser et al., 1999), we were able to determine that cholinergic (ChAT$^+$), CR$^+$, and SOM/NPY/NOS$^+$ interneurons are either eliminated or severely reduced in the *Nkx2.1* mutants. ChAT$^+$ interneurons were not found at any rostrocaudal level in the striatum (compare Fig. 4*A,D* with 5*A*) nor in any region of the telencephalon, including the magnocellular nucleus, horizontal and vertical limbs of the diagonal band of Broca, medial septum, or the medial preoptic region (Fig. 4*B,E*; data not shown). CR$^+$ striatal interneurons were reduced severely at caudal striatal levels (approximately sixfold reduction; Figs. 4*C,F,* 5*A*) although some CR$^+$ cells were present at rostral striatal levels (Fig. 5*A*). Finally, SOM/NPY/NOS-immunoreactive cells are missing almost completely from the *Nkx2.1* mutant striatum (compare Figs. 4*G,H, J,K* and 5*A*); when present, there would be two or three immunoreactive, abnormally large, and brightly stained cells in the ventral striatum (data not shown). In addition, neurons expressing NADPH-diaphorase (NADPHd), which is a marker of the SOM/NPY/NOS$^+$ interneurons, were not found in the mutant striatum (see Fig. 4*I,L*). Thus, at E18.5 the *Nkx2.1* mutants appear to lack most striatal interneurons.

Striatal interneurons are reduced in *Mash1* mutants

Mash1 is a basic helix–loop–helix transcription factor gene that is required for the development of subsets of neurons in the peripheral nervous system and CNS (Guillemot et al., 1993; Sommer et al., 1995; Cau et al., 1997; Hirsch et al., 1998; Casarosa et al., 1999). In the telencephalon *Mash1* is expressed in the proliferative zones of the LGE and the MGE (Lo et al., 1991; Guillemot and Joyner, 1993; Porteus et al., 1994; Torii et al., 1999) (see also Fig. 10*B*). Mice lacking *Mash1* have a very small MGE (Casarosa et al., 1999; Horton et al., 1999). Thus, if the MGE is the source of most striatal interneurons, we hypothesized that there would be a reduction in their number in *Mash1* mutants.

We analyzed the expression of striatal interneuronal subtype markers at P0, because *Mash1* homozygous mutant mice die within hours after birth. *Mash1* mutants have a severe reduction of NKX2.1$^+$ striatal neurons (approximately threefold reduction; Figs. 5*B*, 6*A,D*). In line with these results, the number of cholinergic neurons in the striatum was reduced severely in *Mash1* mutants (approximately sixfold reduction; Figs. 5*B*, 6*B,E*), as it was in other basal telencephalic regions (~10-fold reduction; Figs. 5*B,*

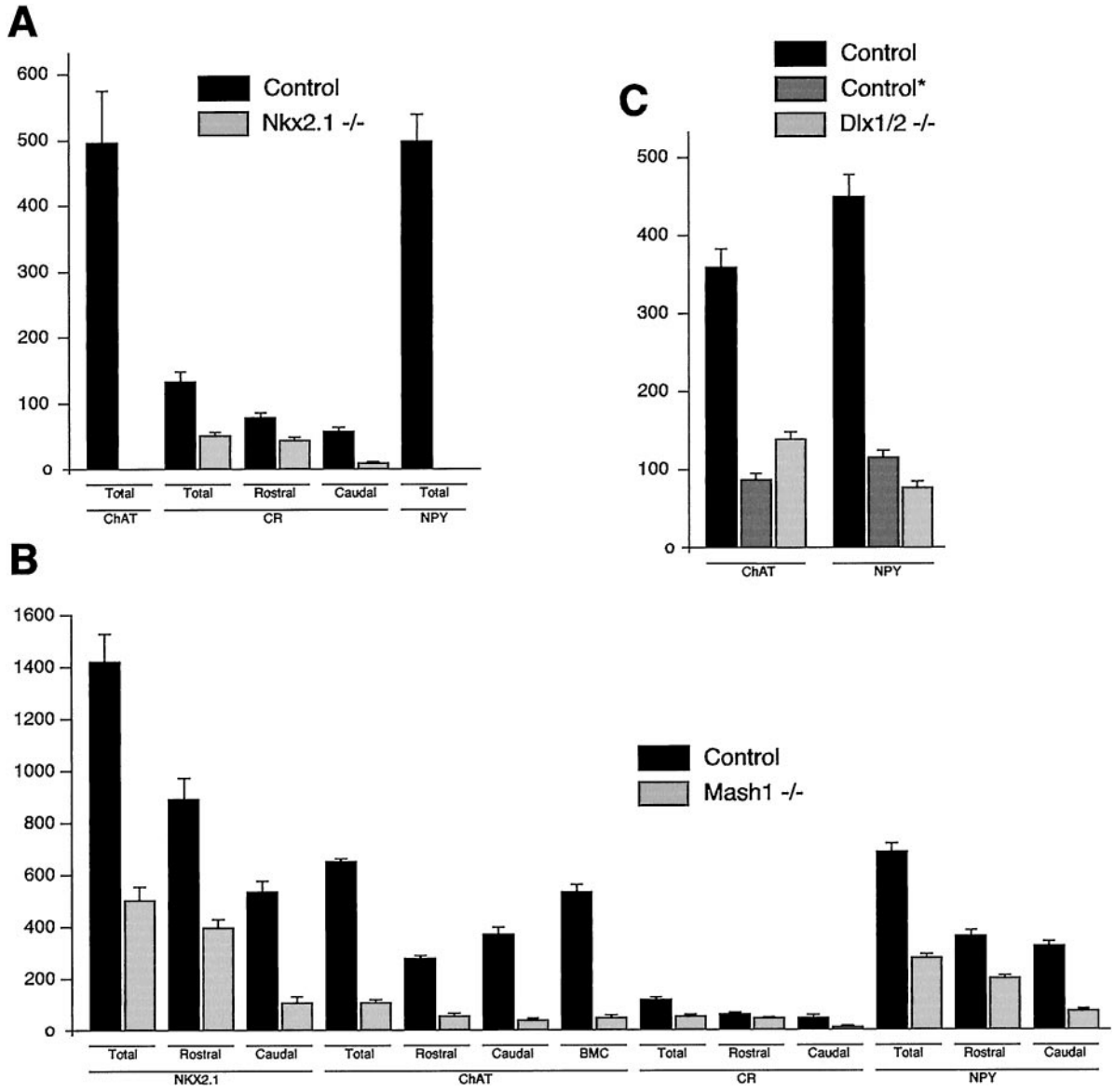

Figure 5. Reduction of different striatal interneuron subtypes in E18.5 *Nkx2.1*$^{-/-}$ fetuses (*A*) and P0 *Mash1*$^{-/-}$ (*B*) and *Dlx1/2*$^{-/-}$ (*C*) newborns. Histograms show averages ± SEM of the numbers of specific types of striatal interneurons. In *A* and *B* the cells were counted in two defined sections of the striatum (rostral and caudal) in three independent experiments in each case. *Total* represents the collective consideration of both rostral and caudal sections. In *C* the cells were counted in a single striatal level in three independent experiments. In *Control** we have taken into account the fourfold reduction in striatal surface area in the *Dlx1/2* mutants (see Materials and Methods) by dividing the number of striatal interneurons in the wild-type striatum by four. *BMC*, Basal magnocellular complex.

6*C,F*; data not shown). In addition, CR-immunoreactive cells are missing almost completely from the caudal striatum in *Mash1* mutants, although a few CR cells are present in the rostral striatum (Figs. 5*B,* 6*G,J*). Finally, the number of striatal SOM/NPY/NOS/NADPHd^{+} interneurons also was reduced in *Mash1* mutants (Fig. 5*H,I,K,L*; data not shown). The reduction of NPY/SOM/NOS/NADPHd, as estimated via the number of NPY^{+} neurons, was more prominent at caudal (~4.5-fold reduction; Figs. 5*B*, 6*H,K*) than at rostral levels (approximately twofold reduction; Figs. 5*B,* 6*I,L*). These results suggest that the number of neurons that adopt an interneuronal phenotype in the striatum is proportional to the number of *Nkx2.1* neurons that become postmitotic in the ventral telencephalon.

The results from the colocalization studies, together with the data of the expression of striatal interneuronal markers in *Nkx2.1* and *Mash1* mutants, strongly suggest that most striatal interneurons derive from the MGE/*Nkx2.1* region. Furthermore, most ChAT, CR, and PV striatal interneurons maintain the expression of NKX2.1 into adulthood, whereas most NPY/SOM/NOS interneurons do not. These results suggest that most NPY/SOM/NOS striatal interneurons derive from the MGE/*Nkx2.1* region, but they downregulate the expression of NKX2.1 shortly after leaving the proliferative zone of the MGE. As described in the next section, the analysis of striatal interneurons in *Dlx1/2* double mutants supports this hypothesis.

Dlx1/2 mutants provide evidence that striatal NPY^{+}/NKX2.1^{-} interneurons are derived from the MGE

Dlx-1 and *Dlx-2* are homeobox transcription factor genes that are expressed in the proliferative zones of the LGE and MGE (Bulfone et al., 1993; Eisenstat et al., 1999) (see also Fig. 10*C*). Mice homozygous for a deletion of both genes (*Dlx1/2* mutants) have a time-dependent block in striatal differentiation, resulting in the accumulation of late-born LGE neurons within the proliferative zone (Anderson et al., 1997a). In addition, a similar defect may occur in the MGE, as judged by the expression of NKX2.1 at P0. In the telencephalon of control mice the NKX2.1^{+} cells are located primarily in part of the septum, globus pallidus, ventral

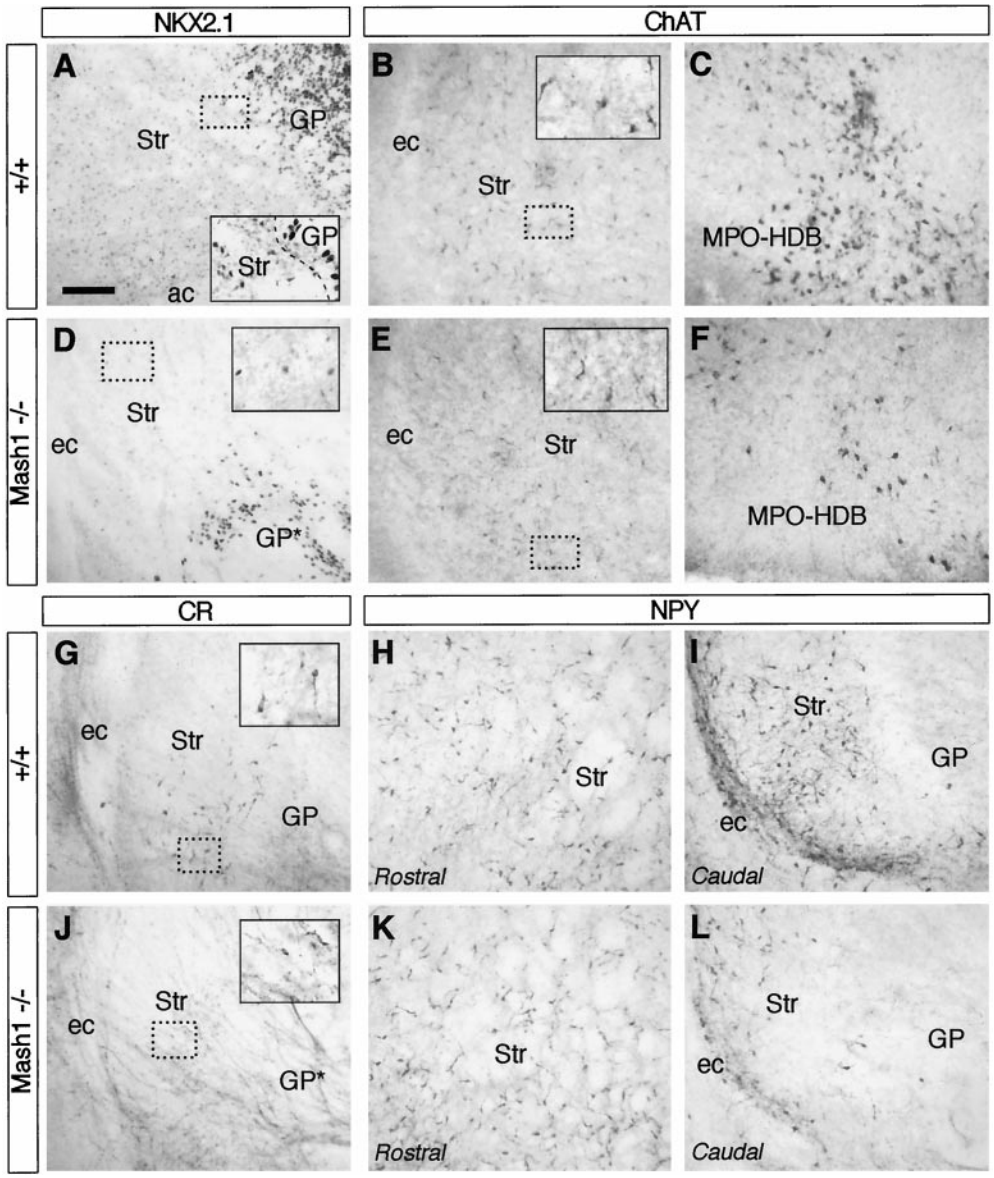

Figure 6. Reduction of striatal interneurons in *Mash1* mutants. *A–C, G–I*, Coronal sections through the telencephalon of P0 wild-type pups showing NKX2.1 (*A*), ChAT (*B, C*), CR (*G*), and NPY immunoreactivity (*H, I*) in the rostral striatum (*H*), caudal striatum (*A, B, G–I*), and basal telencephalon (*C*). *D–F, J–L*, Coronal sections through the telencephalon of P0 *Mash1*$^{-/-}$ pups showing reduction of NKX2.1 (*D*), ChAT (*E, F*), CR (*J*), and NPY immunoreactivity (*K, L*) in the rostral striatum (*K*), caudal striatum (*D, E, J, L*), and basal telencephalon (*F*). *Insets* in *A, B, D, E, G, J* show higher magnification images of neurons from the *outlined boxed regions*. *Cx*, Cortex; *ec*, external capsule; *GP*, globus pallidus; *GP**, mutant GP; *MPO-HDB*, medial preoptic region-horizontal limb of the diagonal band; *Str*, striatum. Scale bar for *A–L*, 100 μm.

pallidum, substantia innominata, and the bed nucleus of the stria terminalis (Fig. 7*A*). The striatum also contains scattered NKX2.1$^+$ cells at all rostrocaudal levels at this age (Fig. 7*A*). At P0 only a small number of NKX2.1$^+$ cells are located in the proliferative region of the ventral telencephalon (Fig. 7*A*), except for the ventral aspect of the lateral ventricle at rostral telencephalic levels, which continues to contain NKX2.1 in the postnatal telencephalon (see Fig. 1*G*). In contrast, *Dlx1/2* mutants have a massive accumulation of NKX2.1$^+$ cells in the periventricular region of the mutant MGE (MGE*). In this area NKX2.1 expression is found both in the proliferative zone (SVZ*) and in large ectopic accumulations of nonproliferating densely packed cells (Fig. 7*D*). Moreover, NKX2.1$^+$ cells spread dorsally through the LGE SVZ, expanding as far as the pallial–subpallial boundary (data not shown).

An analysis of the migration properties of MGE-derived cells was assessed by means of BrdU birthdating. Thus, pregnant animals received a single injection of BrdU at E10.5, E11.5, E12.5, E13.5,and E15.5, and the location of the BrdU-labeled cells was analyzed at birth. In the ventral telencephalon of wild-type mice, E10.5 injections primarily labeled cells in regions superficial to the striatum and pallidum (Fig. 7*B*); E11.5 and E12.5 injections labeled cells in the pallidum (data not shown), whereas injections between E11.5 and E15.5 labeled cells throughout the striatum (Fig. 7*B*; data not shown). In the *Dlx1/2* mutants the early injections (E10.5 and E11.5) labeled cells in the striatum, pallidum, and regions superficial to the basal ganglia (Fig. 7*E*; data not shown); in contrast, most of the mutant cells labeled by either the E12.5 or E13.5 BrdU pulses remained within the mutant proliferative zone (Fig. 7*F*; data not shown). Of note, a large number of the cells that accumulate in the mutant periventricular region are NKX2.1$^+$ (*star* in Fig. 7*H*; also compare 7*A* and *D*).

These data indicate that, like in the LGE, *Dlx1/2* mutants have a time-dependent block in MGE differentiation, resulting in the accumulation of late-born MGE neurons within the proliferative zone as well as in periventricular neuronal ectopias (*stars* in Fig. 7*D,G,I,J*). Consistent with this conclusion is our observation that the number of striatal cholinergic interneurons, which are among the earliest-born cells of the striatum (Semba et al., 1988; Phelps et al., 1989), was less reduced in the *Dlx1/2* mutants (~2.5-fold reduction; Figs. 5*C*, 8*A,C*) than that of later-born striatal NPY/SOM/NOS interneurons (Semba et al., 1988) (approximately sixfold reduction; Figs. 5*C*, 8*B,D*). When the reduced surface area of the *Dlx1/2* mutant striatum is taken into account, the density of striatal cholinergic interneurons is increased in *Dlx1/2* mutants, whereas the density of striatal NPY/SOM/NOS interneurons is reduced (see Fig. 5*C*).

Analysis at P0 shows that most of the cells that accumulated in the MGE SVZ* in the *Dlx1/2* mutants express NPY, SOM, NOS,

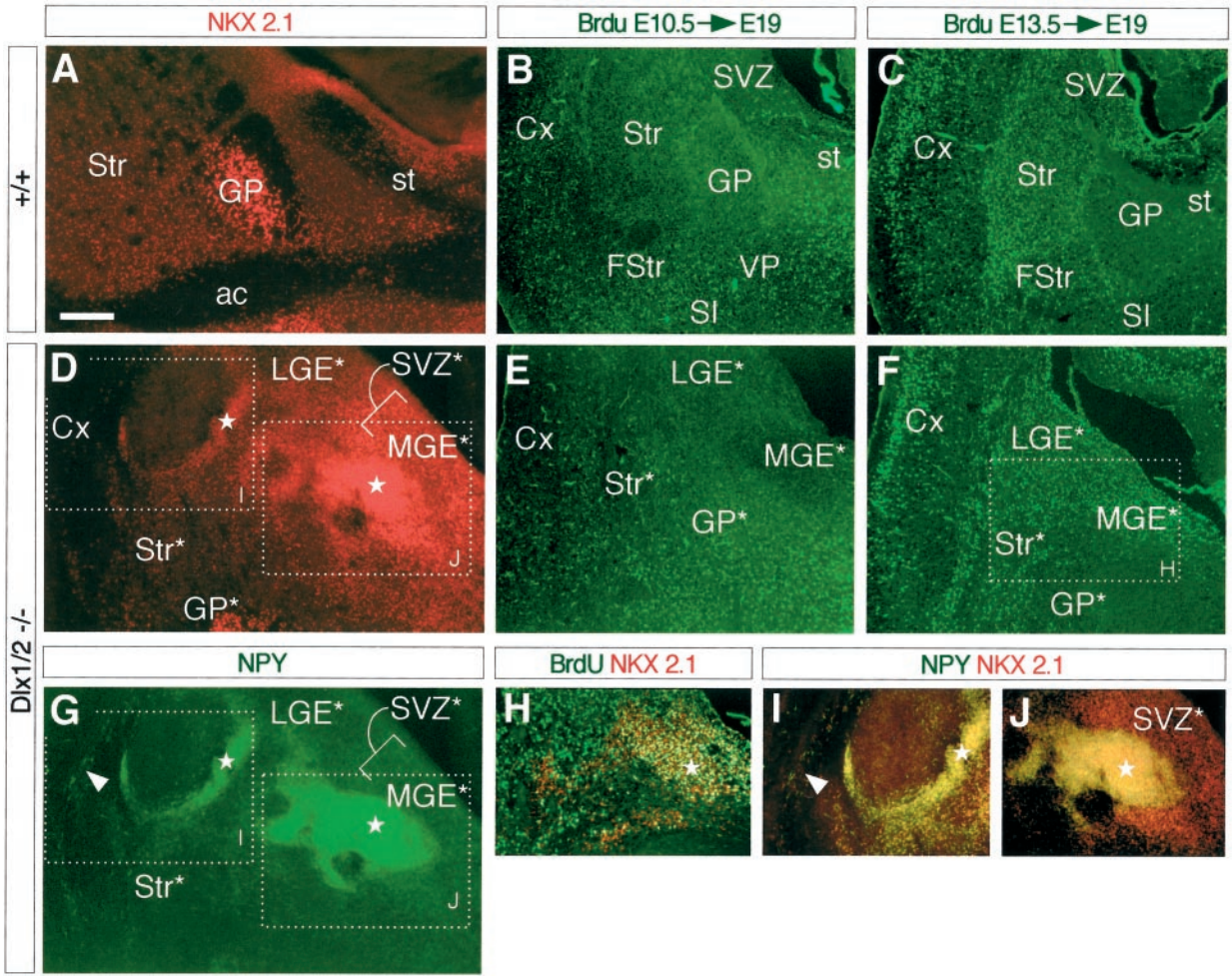

Figure 7. NPY colocalizes with NKX2.1 in the MGE proliferative zone of *Dlx1/2* double mutants. *A, D, G*, Coronal sections through the caudal striatum of P0 pups showing NKX2.1 (*A, D*) and NPY immunoreactivity (*G*) in wild-type (*A*) and *Dlx1/2* double mutants (*D, G*). Periventricular neuronal ectopias express both NKX2.1 and NPY in *Dlx1/2* double mutants (*stars* in *D, G, I, J*). *I, J*, Higher magnification images of neuronal ectopias from the *outlined boxed regions*. The basal telencephalon of *Dlx1/2* mutants also contains cells that stain only for NPY (*arrowhead* in *G, I*) or NKX2.1 (*SVZ** in *D, J*). *B, C, E, F*, Pregnant females received a single injection of BrdU at E10.5 (*B, E*) or E13.5 (*C, F*), and the location of labeled cells was analyzed at E19. Note that although the E10.5 injection results in a similar pattern of labeled cells in wild-type and *Dlx1/2* mutant embryos, most of the cells labeled by the E13.5 injection in the mutant do not migrate to the mantle but remain within the periventricular region. The cells that accumulate in the mutant *MGE** are positive for NKX2.1 (*H*, higher magnification of the region *outlined* in the *boxed region* in *F*). *ac,* Anterior commissure; *Cx*, cortex; *ec*, external capsule; *FStr*, fundus striatum; *GP*, globus pallidus; *GP**, mutant GP; *LGE*, lateral ganglionic eminence; *LGE**, mutant LGE; *MGE*, medial ganglionic eminence; *MGE**, mutant MGE; *SI*, substantia innominata; *Str*, striatum; *Str**, mutant striatum; *SVZ*, subventricular zone; *SVZ**, mutant SVZ; *VP*, ventral pallidum. Scale bar: *A, D, G–J*, 150 μm; *B, C, E, F*, 200 μm.

and NKX2.1 (Fig. 7*G,I,J*; data not shown). In fact, double-labeling experiments demonstrated that NPY and SOM are coexpressed with NKX2.1 in this region (Fig. 7*D,G,I,J*; data not shown). Therefore, in line with the previous experiments, the analysis of NPY/SOM/NOS striatal neurons in *Dlx1/2* mutants suggests that this subtype of striatal interneurons derives primarily from NKX2.1$^+$ progenitors.

The expression of LIM homeodomain genes *Lhx6* and *Lhx7* is differentially affected in *Dlx1/2* double mutants

Whereas expression of *Nkx2.1* in the basal telencephalon is required for the formation of most striatal and cortical interneurons (Sussel et al., 1999; present study), nothing is known about the factors that control the differentiation of distinct interneuron subtypes in the telencephalon. In the spinal cord a combinatorial code of homeodomain (LIM, *Nkx*, *Pax*) transcription factors controls the identity of different types of ventral neurons (see Tanabe and Jessell, 1996; Briscoe et al., 1999). In the basal ganglia the *LIM* proteins Lhx6 and Lhx7 are expressed in the developing MGE (Grigoriou et al., 1998; Lavdas et al., 1999; Sussel et al., 1999; O. Marín and J. L. Rubenstein, unpublished observations) as well as in subsets of neurons in the striatum (Fig. 9*A,B,G,H*).

To define the relationship between transcription factor expression and neuronal identity in the striatum, we analyzed the expression of *Lhx6* and *Lhx7* in the striatum of *Nkx2.1* mutants at E18.5. In these mutants the cells expressing *Lhx6* or *Lhx7* were not found at any rostrocaudal level in the striatum (Fig. 9*D,E*). Because *Nkx2.1* is not expressed in striatal projection neurons (see Fig. 3*A,B*) and it apparently is not required for their proper differentiation (Fig. 9*C,F*) (Sussel et al., 1999), these results suggest that *Lhx6* and *Lhx7* expression most likely is confined to local circuit neurons in the striatum.

Next, we examined *Lhx6* and *Lhx7* expression in *Dlx1/2* mutants. At birth, *Lhx6*$^+$ cells were found in reduced numbers in the mutant striatum, whereas *Lhx7* expression was approximately normal or even increased (Fig. 9*G,H,J,K*). Moreover, both NPY/SOM/NOS and *Lhx6* were expressed abnormally in the periventricular region (see Figs. 7*G,J*, 9*K*), suggesting that this transcription factor might be involved in the development of NPY/SOM/NOS interneurons. The expression of *Lhx7* in *Dlx1/2* mutants suggests, on the other

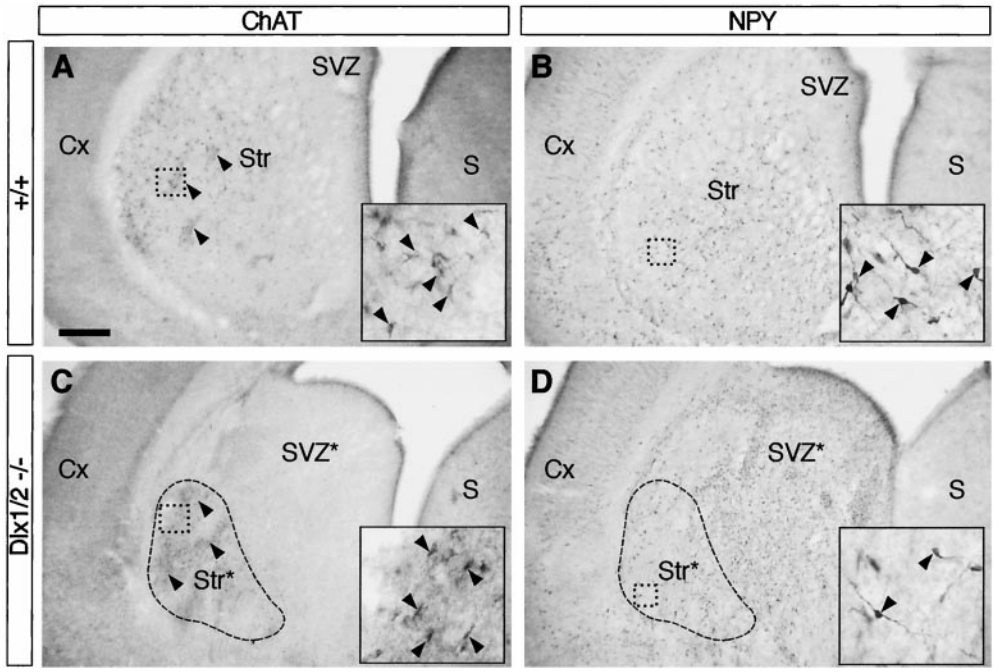

Figure 8. Reduction of striatal interneurons in *Dlx1/2* double mutants. *A, B,* Coronal sections showing ChAT (*A*) and NPY immunoreactivity (*B*) in the striatum of P0 wild-type embryos. *C, D,* Coronal sections showing reduction of ChAT (*C*) and NPY immunoreactivity (*D*) in the striatum of P0 *Dlx1/2* mutant embryos. The *dashed line* outlines the approximate perimeter of the striatal mantle (based in part on being PCNA-negative; data not shown) in *Dlx1/2* mutants. *Insets* in *A–D* show higher magnification images of neurons from the *outlined boxed regions*. Note that ChAT$^+$ interneurons are grouped mainly in striatal patches in both wild-type and mutant embryos (*arrowheads* in *A, C*). *Cx*, Cortex; *S*, septum; *Str*, striatum; *Str**, mutant striatum; *SVZ*, subventricular zone; *SVZ**, mutant SVZ. Scale bar for *A–D*, 250 μm.

hand, that this transcription factor is involved in the development of a different subset of interneurons (e.g., the ChAT$^+$ neurons, which are not present in the periventricular region of *Dlx1/2* mutants) (see Fig. 7*A,C*).

DISCUSSION

In this study we provide evidence that the vast majority of striatal interneurons migrates tangentially from progenitor zones in the MGE and POa/AEP to the postmitotic zone of the LGE. *Nkx2.1,* which is expressed in progenitor zones of the MGE and POa/AEP and in most mature striatal interneurons, is required for the development of nearly all striatal interneurons. In contrast, *Mash1* and *Dlx1/2*, which also are expressed in the precursor cells of this region, regulate the development of early- versus late-born interneurons, respectively. Finally, our analysis implicates distinct LIM homeobox genes (*Lhx6* and *Lhx7*) in the generation of specific subtypes of striatal interneurons.

Most striatal interneurons are derived from NKX2.1$^+$ precursors in the MGE and POa/AEP and tangentially migrate into the striatum

Recent DiI labeling and transplantation experiments have revealed a robust tangential migration from the MGE to the LGE (Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 1999). Here we show, using retroviral cell tracing (see Fig. 2), that cells emanating from the MGE and the adjacent POa/AEP migrate to the developing striatum, where they differentiate into local circuit neurons. Furthermore, this analysis reveals that most striatal cells derived from the MGE/POa/AEP express *Nkx2.1*, whereas none of the LGE-derived cells do.

What subtypes of striatal interneurons derive from the MGE/POa/AEP? In the adult striatum all cholinergic and PV$^+$ and nearly all CR$^+$ interneurons coexpress NKX2.1. In contrast, only ~10% of the NPY/SOM/NOS/NADPHd interneurons contain NKX2.1. These static histochemical analyses suggest that all cholinergic and PV$^+$, most CR$^+$, and some NPY/SOM/NOS/NADPHd striatal interneurons are derived from the MGE/POa/AEP, whereas the LGE is the source of the majority of the NPY/SOM/NOS/NADPHd and some CR$^+$ striatal interneurons. Of note, striatal NPY/SOM/NOS/NADPHd interneurons do not develop in *Nkx2.1* mutants, raising the possibility that some striatal interneurons might derive from regions other than the MGE (e.g., LGE) but require substances produced from a normal MGE to differentiate and/or survive.

An alternative possibility is, however, that most striatal interneurons actually derive from the MGE/POa/AEP, but some of them (expressing NPY/SOM/NOS/NADPHd) downregulate the expression of *Nkx2.1* after leaving the proliferative zone. Three independent lines of evidence support this hypothesis: (1) In the absence of *Nkx2.1* the striatum does not contain cholinergic or NPY/SOM/NOS/NADPHd interneurons (see Fig. 4); (2) NKX2.1 and NPY/SOM are coexpressed in partially differentiated cells in the proliferative zone of *Dlx1/2* mutants (see Fig. 7; also discussed below); and (3) paleo-, neo-, and archicortical interneurons derived from the MGE do not express *Nkx2.1*, suggesting that they downregulate its expression after leaving the proliferative zone (Sussel et al., 1999; S. Anderson, O. Marín, and J. L. Rubenstein, unpublished observations). In summary, we suggest that nearly all striatal interneurons are derived from the MGE/POa/AEP region. The only exception may be a few CR$^+$ interneurons for which the origin is not known.

Our data differ from some of the conclusions of an earlier study that used transplantation experiments to investigate the origins of rat cholinergic and SOM$^+$ striatal interneurons (Olsson et al., 1998). In agreement with our results, striatal cholinergic interneurons were found mainly in grafts derived from the early MGE (E12.5 in the rat; Olsson et al., 1998). In contrast, Olsson and colleagues (1998) suggest that striatal SOM$^+$ interneurons are generated from both the LGE and MGE. However, because SOM$^+$ striatal interneurons are born as early as E12 in the rat (Semba et al., 1988), it is possible that in the experiments described by Olsson et al. (1998) the transplanted LGE already had MGE-derived cells within it.

Dlx1, *Dlx2,* and *Mash1* regulate the generation of early- and late-born striatal interneurons

Dlx1, Dlx2, and *Mash1* are expressed in progenitor cells of the LGE and MGE (Porteus et al., 1994; Liu et al., 1997; Eisenstat et al., 1999; Torii et al., 1999). *Dlx1/2* double mutants previously were shown to have a time-dependent block in the differentiation of radially migrating striatal projection neurons and tangentially migrating cortical local circuit neurons (Anderson et al., 1997a,b). *Mash1* mutants have a complementary defect that affects the generation of early-born neurons in the ventral telencephalon (Casa-

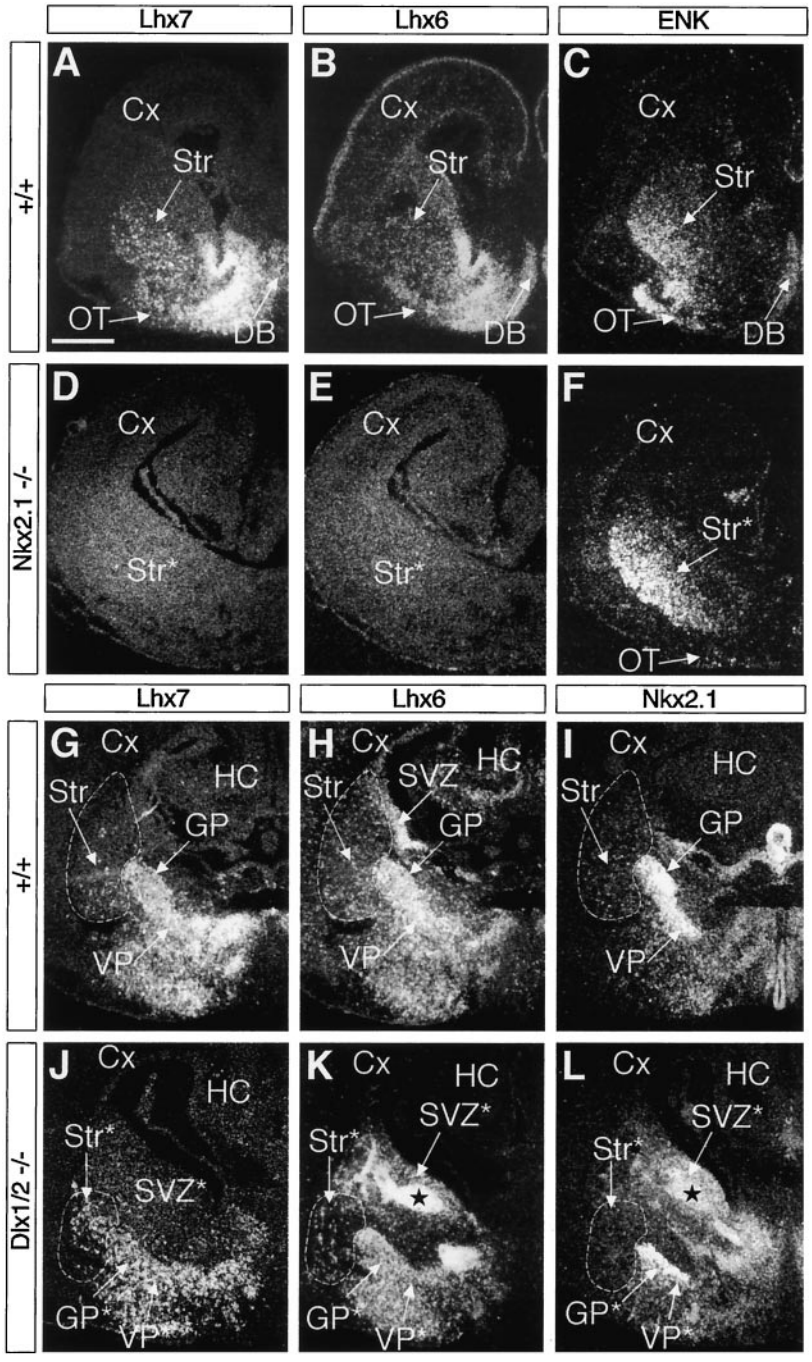

Figure 9. Expression of the LIM homeodomain genes *Lhx6* and *Lhx7* in the striatum of *Nkx2.1* mutants and *Dlx1/2* double mutants. *A–C, G–I*, Serial coronal sections through the telencephalon of an E18.5 wild-type fetus showing *Lhx7* (*A*), *Lhx6* (*B*), and *ENK* (*C*) expression in the striatum, and of a P0 pup showing *Lhx7* (*G*), *Lhx6* (*H*), and *Nkx2.1* (*I*) expression in the basal telencephalon. *D–F*, Serial coronal sections from an E18.5 *Nkx2.1* mutant showing loss of *Lhx7* (*D*) and *Lhx6* (*E*) expression and normal *ENK* (*F*) expression in the expanded striatum. *J–L*, Serial coronal sections from a P0 *Dlx1/2* mutant showing normal *Lhx7* (*J*) expression and reduced *Lhx6* (*K*) and *Nkx2.1* (*L*) expression in the striatum. *G–L*, The *dashed line* outlines the approximate perimeter of the striatal mantle. Note that *Lhx6* expression accumulates in periventricular neuronal ectopias that also express *Nkx2.1* (*stars* in *K, L*), but not *Lhx7* (*J*). *J–L*, The *dashed line* approximates the extension of the striatal mantle in *Dlx1/2* mutants. *Cx*, Cortex; *DB,* diagonal band; *HC*, hippocampus; *OT*, olfactory tubercle; *GP*, globus pallidus; *GP**, mutant GP; *Str*, striatum; *Str**, mutant striatum; *SVZ*, subventricular zone; *SVZ**, mutant SVZ; *VP*, ventral pallidum; *VP**, mutant VP. Scale bar for *A–L*, 500 μm.

rosa et al., 1999). Here we show that these mutants have similar time-dependent defects in the generation of the MGE-derived striatal interneurons (see Figs. 5–8). In addition, the *Dlx1/2* mutants accumulate periventricular ectopia of partially differentiated neurons deep to the subventricular zone (see Fig. 7). As noted above, these ectopia contain cells that are $NKX2.1^+/NPY^+$, implying that *Dlx1/2* are required (directly or indirectly) to enable these striatal interneurons to repress *Nkx2.1* expression. We suggest that *Mash1* and *Dlx1/2* regulate the balance between early versus late differentiation in the basal telencephalon (K. Yun and J. L. Rubenstein, unpublished observations), whereas *Nkx2.1* regulates regional and cell-type specification in this region (Fig. 10).

Specification of neuronal identity in the MGE/POa/AEP

Fate maps of the early telencephalon support the hypothesis that derivatives of the MGE/POa/AEP include the dorsal pallidum, ventral pallidum, and basal magnocellular complex (Rubenstein et al., 1998; I. Cobos, K. Shimamura, J. L. Rubenstein, L. Puelles, and S. Martínez, unpublished observations). In the present study we have shown that this $NKX2.1^+$ region gives rise to neurons that express either acetylcholine or GABA. It is unknown what controls the switch between these two phenotypes. In addition, each of these two cell types gives rise to cells that are either radially migrating projection neurons or local circuit neurons that migrate tangentially to the striatum and cerebral cortex (Fig. 10*D*) (Anderson et al., 1997b; Lavdas et al., 1999; Sussel et al., 1999; present study).

Thus, the basal telencephalon contains two main types of cholinergic neurons, the local circuit neurons of the striatum and the projection neurons of the basal forebrain system, including the nucleus basalis magnocellularis, diagonal band, and medial septum (Mesulam et al., 1983a,b; 1984). Although the cholinergic neurons of the striatum and basal forebrain system are located within different nuclei and are known to have very different functions,

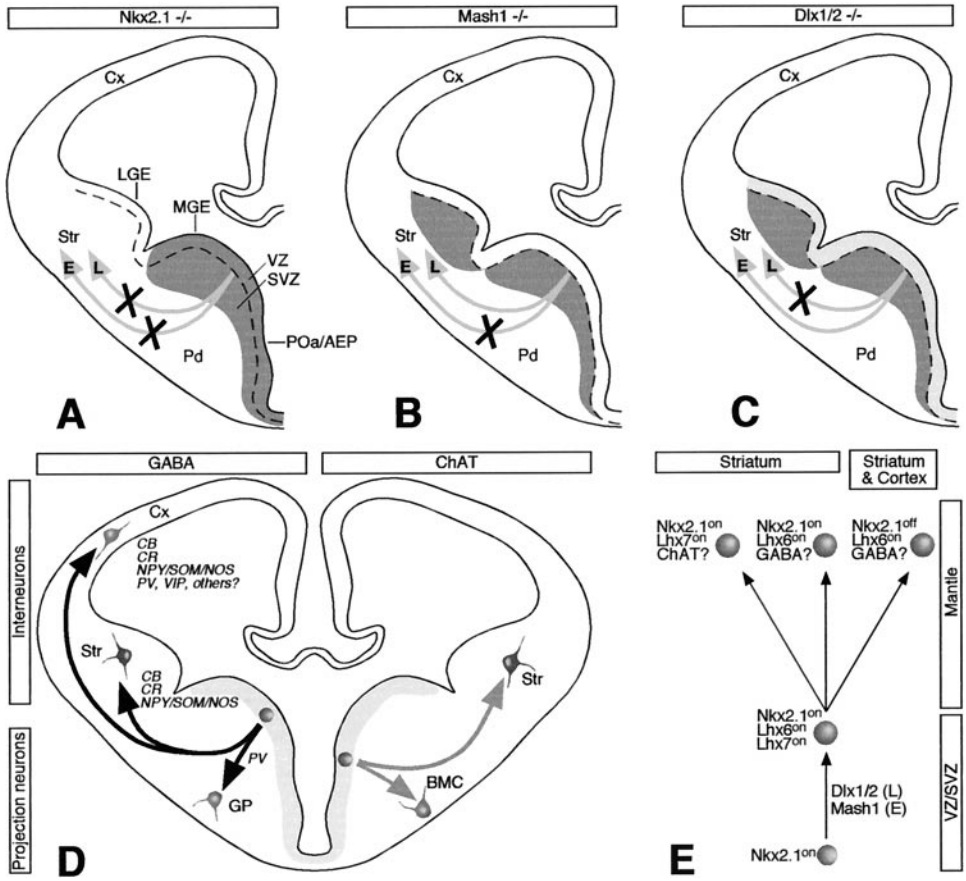

Figure 10. Schemas illustrating the origin and migration of neurons derived from the NKX2.1$^+$ proliferative region in the basal telencephalon and the genes that are involved in their specification and differentiation. *A–C,* These drawings are based on a coronal section of a mouse E12.5 right telencephalic hemisphere. The *light gray arrow-tipped lines* indicate early (*E*) and late (*L*) tangential migrations that originate in the NKX2.1$^+$ progenitor zone (*dark gray*) of the MGE and POA/AEP. A *dashed line* indicates the limit between the VZ and SVZ. *A*, Both tangential migrations are lost (indicated by *black Xs*) in the *Nkx2.1* mutant mouse because of a ventral-to-dorsal respecification of the progenitor zone (Sussel et al.; 1999). *B*, The *Mash1* mutation preferentially blocks the early (*E*) tangential migration. MASH1 expression is in a subset of VZ cells (*light gray*) and in most SVZ cells (*dark gray*). *C*, The *Dlx1/2* mutation preferentially blocks the late (*L*) tangential migration. DLX1 and DLX2 expression is in a subset of VZ cells (*light gray*) and in most SVZ cells (*dark gray*). *D*, Schema of a coronal section through an E12.5 mouse telencephalon. The NKX2.1$^+$ VZ is indicated in *light gray*. Migrations of GABAergic cells from the NKX2.1$^+$ progenitor zone are indicated on the *left*. A radial migration produces the GABAergic projection neurons of the globus pallidus (*GP*), whereas tangential migrations produce GABAergic interneurons of the striatum (this study) and cerebral cortex (Anderson et al., 1999; Sussel et al., 1999; Anderson, Marín, and Rubenstein, unpublished observations). Although the origin of some cortical interneuron subtypes, such as those containing PV or VIP, has not been demonstrated, we hypothesize that they also may derive from the basal telencephalon. Migrations of cholinergic (*ChAT*) cells from the NKX2.1$^+$ progenitor zone are indicated on the *right*. A radial migration produces the cholinergic projection neurons of the basal magnocellular complex (*BMC*), whereas tangential migrations produce cholinergic interneurons of the striatum (this study). *E*, Model describing some of the genes that regulate the production of tangentially migrating cells from NKX2.1$^+$ progenitor cells. *Dlx1/2* and *Mash1* regulate the production of secondary progenitor cells that express *Lhx6* and *Lhx7* (we do not know whether a single cell expresses both *Lhx* genes). From these cells we hypothesize that three types of interneurons are formed, two of which migrate to the striatum and a third that contributes interneurons to both the striatum and cortex. *CB,* Calbindin; *CR,* calretinin; *Cx,* cortex; *NOS,* nitric oxide synthase; *NPY,* neuropeptide Y; *Pd*, pallidum; *PV,* parvalbumin; *SOM,* somatostatin; *Str,* striatum; *SVZ,* subventricular zone; *VIP,* vasointestinal peptide; *VZ,* ventricular zone.

they are generated at approximately the same time (Semba et al., 1988; Brady et al., 1989; Phelps et al., 1989). Moreover, the present data demonstrate that cholinergic neurons in the ventral telencephalon derive from a common germinal source, the MGE/POa/AEP region, and that their development requires *Nkx2.1* function.

As in the case of the cholinergic neurons, the neurotransmitter GABA is expressed both in local circuit and projection neuronal populations of the basal telencephalon that may share a common origin. For example, GABA is expressed in subsets of striatal interneurons and globus pallidus projection neurons (Kita and Kitai, 1994; Kawaguchi et al., 1995; Parent and Hazrati, 1995); in both cases their development appears to be dependent on *Nkx2.1* function (Sussel et al., 1999; present study; Marín and Rubenstein, unpublished observations).

It has been proposed that neurons sharing the same chemical phenotype (e.g., cholinergic neurons) from different areas of the telencephalon are neurogenetically homologous and that their final morphology and connectivity depend primarily on extrinsic factors (Gähwiler and Hefti, 1985; Semba et al., 1988; Campbell et al., 1995; Sadikot and Sasseville, 1997). An alternative model is that intrinsic factors distinguish projection and interneurons, even when they share the same neurotransmitter phenotype. This would be necessary to explain their distinct migratory pathways. For instance, basal forebrain cholinergic and pallidal GABAergic projection neurons are superficial to the progenitor zones from which they derive and thus seem to follow a radial migration, whereas cholinergic and GABAergic interneurons migrate tangentially to the striatum.

What are the genes that regulate the choice between becoming a radially migrating projection neuron or a tangentially migrating local circuit neuron? The answer to this question is not known, but clues may be provided by studies of dorsoventral patterning of the spinal cord and hindbrain. In these structures *Nkx* genes (*Nkx2.2* and *Nkx6.1*) specify the identity of the progenitor cells of distinct longitudinal domains in the ventral neural tube (Qiu et al., 1998; Briscoe et al., 1999; M. Sander, S. Paydar, J. Ericson, J. Briscoe, E. Berber, M. German, T. Jessell, and J. Rubenstein, unpublished observations). *Nkx6.1* is upstream of several homeodomain tran-

scription factors that are required for the development of projection neurons (cholinergic motor neurons) and interneurons (V2 cells) (Sander, Paydar, Ericson, Briscoe, Berber, German, Jessell, and Rubenstein, unpublished observations). Among the downstream genes are *Isl1* and *Lhx3*, which encode LIM-homeodomain proteins that have essential roles in motor neuron development (Pfaff et al., 1996; Sharma et al., 1998). The combinatorial expression of LIM-homeodomain proteins defines specific populations of neurons in the ventral CNS (Tsuchida et al., 1994; Appel et al., 1995; Tanabe and Jessell, 1996).

These results drew our attention to the *Lhx6* and *Lhx7* LIM-homeobox genes, which are expressed in the MGE and are dependent on *Nkx2.1* function (Sussel et al., 1999). Thus, these genes are candidates for defining cell type specification within the basal telencephalon. However, they are expressed both in pallidal projection neurons and tangentially migrating interneurons (see Fig. 9), implying that other factors are required to define projection neurons from interneurons. At this point the intrinsic factors that determine whether a cell migrates radially or tangentially are not known. On the other hand, there is evidence that the secreted molecule SLIT1 regulates the radial migration of GABAergic neurons derived from the LGE (Zhu et al., 1999).

Although *Lhx6* and *Lhx7* may not regulate the choice between projection and interneurons, they may have a role in regulating the development of distinct subsets of striatal interneurons (Fig. 10*E*). We base this hypothesis on our observations of their expression in the *Dlx1/2* mutants. In these animals the patterns of *Lhx6* and NPY/SOM/NOS expression are correlated (see Figs. 7*G,I,J,* 9*K*), whereas the expression of *Lhx7* correlates with the distribution of $ChAT^+$ neurons (see Figs. 8*C,* 9*J*).

Telencephalic GABAergic and cholinergic interneurons are derived from the basal ganglia

The results of this and other studies strongly suggest that the basal ganglia primordia are the origin of the majority of interneurons present in the mature striatum, cortex, and olfactory bulb (de Carlos et al., 1996; Anderson et al., 1997b, 1999; Tamamaki et al., 1997; Casarosa et al., 1999; Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 1999; Anderson, Marín, and Rubenstein, unpublished observations). Several lines of evidence suggest that the MGE is a major source of tangentially migrating interneurons to both the striatum and cortex (Anderson et al., 1999; Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 1999; Anderson, Marín, and Rubenstein, unpublished observations). Interestingly, the LGE appears to be the source of a different pool of cortical and olfactory bulb interneurons, as demonstrated by the comparative analysis of *Dlx1/2* and *Nkx2.1* mutants (Anderson et al., 1997a,b, 1999; Sussel et al., 1999; Anderson, Marín, and Rubenstein, unpublished observations).

Theoretical issues

The findings described in this and other studies (de Carlos et al., 1996; Anderson et al., 1997b, 1999; Tamamaki et al., 1997; Casarosa et al., 1999; Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 1999; Anderson, Marín, and Rubenstein, unpublished observations) raise several interesting theoretical issues. First, whereas radially migrating cells could translate the positional information values of their precursors to the overlying mantle zone, tangentially migrating cells would not. Thus, tangentially migrating local circuit neurons may not have a role in the initial formation of topographic connectivity maps, whereas radially migrating cells do.

Second, why are striatal, cortical, and olfactory bulb interneurons generated in the basal telencephalon, instead of locally? It may be that cell type specification is tightly coupled to regional specification. Thus, induction of the transcription factors that regulate development of cholinergic neurons (e.g., *Nkx2.1*) (Sussel et al., 1999; this study) and GABAergic neurons (e.g., *Dlx1* and *Dlx2*) (Anderson et al., 1997a,b, 1999; Bulfone et al., 1998) may take place only in proximity to morphogens that are implicated in specification of the basal telencephalon (e.g., SHH) (Ericson et al., 1995; Chiang et al., 1996; Shimamura and Rubenstein, 1997; Kohtz et al., 1998). This would imply that the LGE is incapable of producing cholinergic cells and that the cortex is incapable of producing GABAergic cells, thus requiring that these cell types tangentially migrate from proliferative zones where they can be produced.

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