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Loss of *Nkx2.1* homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum

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SUMMARY

The telencephalon is organized into distinct longitudinal domains: the cerebral cortex and the basal ganglia. The basal ganglia primarily consists of a dorsal region (striatum) and a ventral region (pallidum). Within the telencephalon, the anlage of the pallidum expresses the *Nkx2.1* homeobox gene. A mouse deficient in *Nkx2.1* function does not form pallidal structures, lacks basal forebrain TrkA-positive neurons (probable cholinergic neurons) and has reduced numbers of cortical cells expressing GABA, DLX2 and calbindin that migrate from the pallidum through the striatum and into the cortex. We present evidence that these phenotypes result from a ventral-to-dorsal transformation of the pallidal primordium into a striatal-like anlage.

Key words: *Nkx2.1*, Telencephalon, Regionalization, mouse

INTRODUCTION

The telencephalic basal ganglia are major components of the extrapyramidal motor system, whose two principal subdivisions are the striatum and the pallidum (Gerfen,1992). The analysis of regulatory gene expression in the telencephalon suggests that the striatum and pallidum are separate longitudinal domains that are under distinct genetic control (Puelles and Rubenstein, 1993; Rubenstein et al., 1994). Whereas the *Dlx* homeobox genes are expressed in the primordia of both the striatum and the pallidum (Bulfone et al. 1993; Liu et al., 1997), other homeobox genes are only expressed in precursor cells of the pallidum. These include *Nkx2.1* (Guazzi et al., 1990; Price et al., 1992; Shimamura et al., 1995).

Nkx2.1 is a member of the vertebrate Nkx family (Price et al., 1992; Qiu et al., 1998; Pera and Kessel, 1998). *Nkx2.1* is also known as *TTF1* and *T/ebp* because of its ability to regulate the expression of thyroid-specific gene products (Guazzi et al., 1990; Mizuno et al., 1991; Kimura et al., 1996). The initial expression of *Nkx* genes in the mouse central nervous system (CNS) is in the medial neural plate which overlies the SHH-secreting axial mesendoderm (Shimamura et al., 1995; Shimamura and Rubenstein, 1997). This observation is consistent with the fact that SHH can induce *Nkx2.1*, *Nkx2.2* and *Nkx6.1* (Barth and Wilson, 1995; Ericson et al., 1995; Shimamura and Rubenstein, 1997; Pera and Kessel, 1997; Dale et al., 1997; Qiu et al., 1998; Kohutz et al., 1998). Within the medial neural plate and ventral neural tube, expression of the *Nkx* genes have distinct anteroposterior (A/P) and dorsoventral (D/V) boundaries (Qiu et al., 1998). For instance, while *Nkx2.2* and *Nkx2.9* are expressed along the entire CNS in a narrow column of cells adjacent to the floor plate (Shimamura et al., 1995; Pabst et al., 1998), *Nkx2.1* expression is restricted to the forebrain (Price et al., 1992; Shimamura et al., 1995; Qiu et al., 1998).

The vertebrate *Nkx* genes are related to the invertebrate *NK* genes. The *Nkx2* subfamily has homology with *Drosophila NK2* or *ventral nervous system defective* (*vnd*). Like the *Nkx2* genes, *NK2/vnd* is expressed in neuroblast precursors along the ventral part of the embryonic CNS (Kim and Nirenberg, 1989; Jiminez et al., 1995). Recent studies have demonstrated that *NK2/vnd* is required to specify ventral CNS cell fates, and in its absence, the ventral CNS appears to be respecified into more dorsal identity (McDonald, et al., 1998; Weiss et al., 1998; Chu et al., 1998). Like the *NK2/vnd* mutants, mice lacking *Nkx2.2* also appear to have a ventral-to-dorsal respecification of the *Nkx2.2*-deficient neurons in the spinal cord (Briscoe et al., 1999).

In this study, we analyzed whether *Nkx2.1* specifies ventral regional fate within the telencephalon. *Nkx2.1* is one of the earliest known genes to be expressed in the forebrain; its transcripts can be observed in the hypothalamic primordium at the one somite stage (Shimamura et al., 1995; Ericson et al.,

1995; Dale et al. 1997; Pera and Kessel, 1997; Shimamura and Rubenstein, 1997; Qiu et al., 1998). By the 11 somite stage, *Nkx2.1* expression is evident in the rostrobasal telencephalon (Shimamura et al., 1995), a region that develops into the preoptic area, anterior entopeduncular area, medial ganglionic eminence (MGE), septum, and parts of the amygdala (Fig. 1; L. Puelles, E. Kuwana, A. Bulfone, K. Shimamura, J. Keleher, S. Smiga, E. Puelles and J. L. R. R., unpublished data). Like the other *Nkx* genes, *Nkx2.1* is expressed in both progenitor and postmitotic cells, suggesting that it may have roles in specification of precursors and maintenance of phenotype in differentiating and mature ventral forebrain cells.

A mouse containing a targeted disruption of the *Nkx2.1* gene was generated; homozygous mutants die at birth and have severe lung, thyroid and pituitary defects (Kimura et al., 1996; Takuma et al., 1998) In addition, morphological abnormalities were observed in the ventral hypothalamus and telencephalon. To determine the mechanism underlying the homozygous *Nkx2.1* mutant telencephalic defects, we studied early molecular properties of the mutant basal telencephalon. We present evidence that although an MGE-like structure forms, it appears to be respecified to the fate of a more dorsal structure: the lateral ganglionic eminence (LGE). Thus, the mutant MGE does not produce its normal derivatives (e.g. the globus pallidus); instead we suggest that it produces an LGE derivative, the striatum. We have also observed that the lack of the MGE results in the loss of cells that migrate from the pallidum into the striatum (cholinergic neurons) and into the cortex (GABAergic neurons and calbindin-positive cells). Thus, *Nkx2.1* is required both for regional specification of the ventral telencephalon and for the production of specific cell types that migrate into the striatum and cerebral cortex.

MATERIALS AND METHODS

Genotyping of *Nkx2.1* mutant mice

Southern analysis and/or PCR was used to genotype offspring resulting from *Nkx2.1* heterozygous matings. Southern analysis was performed as described by Kimura et al. (1996). For genotyping by PCR, two sets of PCR primers were used: one for detection of the *neo* gene present in the *Nkx2.1* mutant allele and the other for detection of the *Nkx2.1* gene. The sequences of the *neo* primers are: 5′-CAAGATGGATTGCACGCAG-3′ and 5′-CATCCTGATCGACAAGAC-3′. These primers produce a 400 bp fragment from the *neo* gene. The primers used for the *Nkx2.1* wild-type allele amplify an approx. 300 bp region across the unique *Xho*I site in the *Nkx2.1* gene. In the mutant allele, a 1.1 kb *neo* cassette was inserted into the unique *Xho*I site of *Nkx2.1* (Kimura, et al., 1996). Since the *Nkx2.1* primers flank the *Xho*I site they amplify an approx.1.4 kb fragment from the mutant *Nkx2.1* allele. The sequences of the *Nkx2.1* primers are: 5′-CGCCGGAAGCGCCGGGTG-3′ and 5′-GCCTGGCAGGGTTTGCCG-3′. We used the same PCR conditions for both primers: 94°C for 5 minutes; 35 cycles of 94°C for 1 minute, 62°C for 1 minute, and 72°C for 1 minute and 72°C for 6 minutes.

Tissue preparation and histology

Embryos were obtained by dissection of pregnant mice at various stages of development. Noon on the day when the vaginal plug was detected was considered as stage E0.5. Embryos were subsequently staged according to morphological criteria (Kaufman, 1992). In embryos older than E15.5, brains were removed and fixed overnight in 4% paraformaldehyde in PBS. Younger embryos were left intact and fixed overnight in 4% paraformaldehyde in PBS. Embryos and brains were then either dehydrated and embedded in paraffin wax or cryoprotected in sucrose, embedded in OCT and frozen. All tissue was sectioned serially at 10 μm. Those sections not used for RNA in situ analysis and immunohistochemistry were stained with cresyl violet.

In situ hybridization

In situ hyridization were performed using ^{35}S-riboprobes on 10 μm frozen and paraffin wax embedded sections as described previously (Bulfone, et al., 1993). We used a full length *Nkx2.1* cDNA probe to detect expression of *Nkx2.1* transcript (Shimamura et al., 1995). This probe also allowed the detection of a truncated *Nkx2.1* transcript made from the intact 5′ *Nkx2.1* genomic DNA that is still present in the homozygous mutant animals. Additional cDNA probes include: *Dlx2* and *Dlx5* (Liu et al., 1997); other probes were kindly provided by A. McMahon (*Shh*), P. Gruss (*Pax6*), V. Pachnis (*Lhx6*, *Lhx7/L3*), H. Ingraham (*SCIP/Oct6/Tst1*), A. Wanaka (*Lhx7/L3*), C. Gerfen (*D2R*), T. Jessell (*ER81*), and R. Axel (*Golf*). Whole-mount in situ hybridization was performed as described by Shimamura et al. 1994).

Immunohistochemistry

Immunohistochemistry was performed as described by Anderson et al. (1997a,b). The following antibodies were used: mouse monoclonal anti-MAP2 (Sigma, 1:2000), mouse monoclonal anti-βIII tubulin (TuJ1) (Promega, 1:2000), rabbit polyclonals anti-calbindin (Swiss antibodies, 1:5,000), anti-calretinin (Chemicon, 1:1000), anti-tyrosine hydroxylase (Pel-Freez, 1:1000), anti-Gad67 (Chemicon, 1:2000), anti-GABA (Sigma, 1:5000), anti-TrkA (kindly provided by L. Reichardt, 1:10,000), anti-DLX2 (1:200; Porteus et al., 1994).

Organotypic cultures

Organotypic cultures were performed exactly as described by Anderson et al. (1997a,b).

RESULTS

Nkx2.1 expression in the basal telencephalon

Nkx2.1 expression is first detectable in the basal telencephalon of the mouse at approximately the 11 somite stage (Shimamura et al, 1995). As the basal telencephalon develops, *Nkx2.1* is maintained in regions that form morphologically distinct structures such as the medial ganglionic eminence (MGE), as well as parts of the septum, anterior entopeduncular area and preoptic area (POA) (Fig. 1a-d; L. Puelles, L. S. and J. R., unpublished data). For this study, we have focused on the role of *Nkx2.1* in the development of the MGE, a proliferative zone that gives rise to pallidal components of the basal ganglia (Rubenstein et al., 1998; Salvador Martinez, personal communication).

At E10.5 in the mouse, the MGE is a neuroepithelial protrusion out of the wall of the anterobasal telencephalon into the lateral ventricle (LV). As shown in Fig. 1a and b, *Nkx2.1* RNA is expressed uniformly throughout the MGE neuroepithelium. Between E11.5 and E12.5, a second morphologically distinct basal ganglia anlage, the lateral ganglionic eminence (LGE), emerges between the MGE and the cortex (Figs 1, 3, 7). At these stages the LGE lacks *Nkx2.1* expression (Fig. 1c). By E12.5, the MGE is composed of three molecularly distinct cell layers. The ventricular zone (VZ), which is adjacent to the telencephalic ventricle, is composed undifferentiated, rapidly dividing cells. VZ cells contribute to the subventricular zone (SVZ), a secondary proliferating population of cells (Halliday and Cepko, 1992; Bhide, 1996). Postmitotic cells migrate from the proliferative zones to

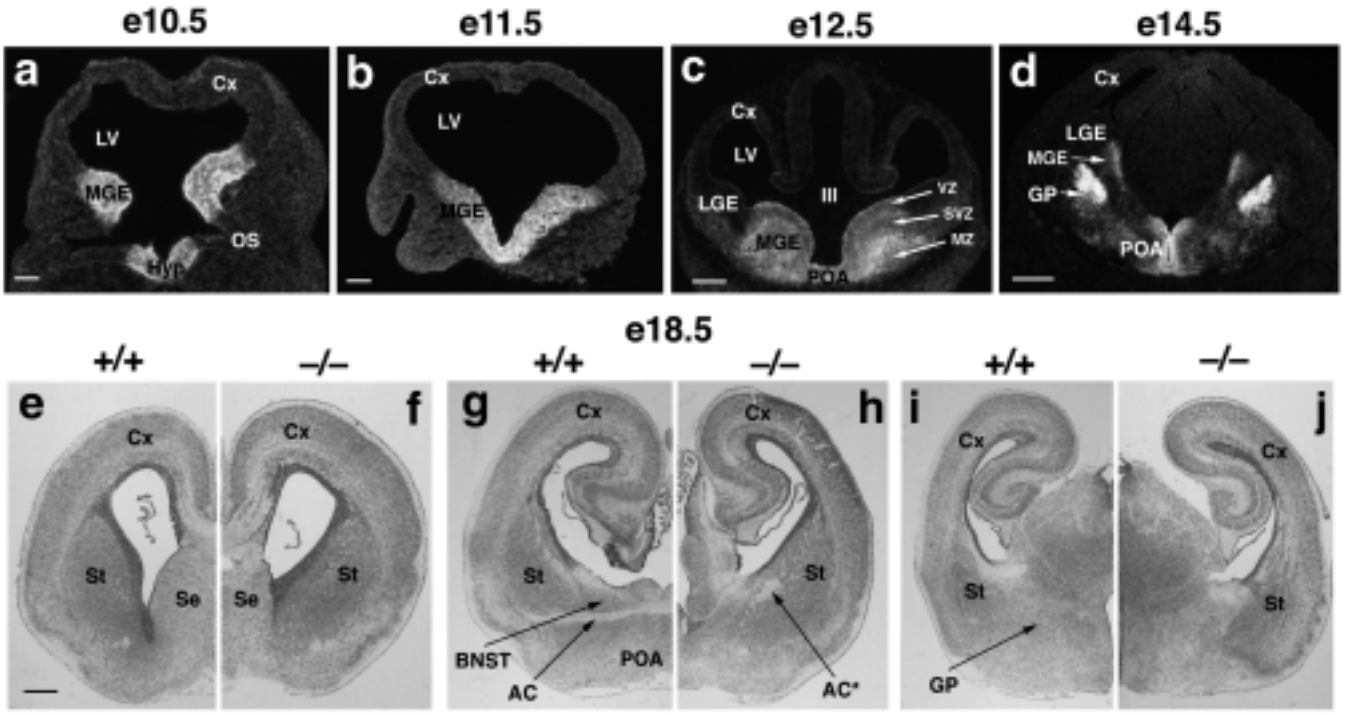

Fig. 1. Developmental expression of *Nkx2.1* in the basal telencephalon and histological analysis of E18.5 wild-type and *Nkx2.1* mutant embryos. (a-d) RNA in situ analysis of *Nkx2.1* expression in coronal sections of the basal telencephalon at four embryonic stages. (e-j) Cresyl violet-stained coronal hemisections through the telencephalon of wild-type (e,g,i) and *Nkx2.1* mutant (f,h,j) E18.5 mice. The sections show different rostral-caudal levels from the same brain, with e and f being the most rostral and i and j being the most caudal. Cx, cortex; LV, lateral ventricle; MGE and LGE; medial and lateral ganglionic eminences; Hyp:hypothalamus; OS:optic stalk; POA,preoptic area; III, third ventricle; GP, globus pallidus; St, striatum; Se, septum, BNST, Bed nucleus of the stria terminalis; AC, anterior commissure; AC*, tract of the mutant anterior commissure does not cross the midline. Scale bar, (a,b) 100 μm; (c) 250 μm; (d) 400 μm; (e) 300 μm.

generate the mantle zone. *Nkx2.1* is expressed in all three layers of the MGE (Figs 1c,d, 7). At E14.5 and later stages, *Nkx2.1* expression is prominent in the developing globus pallidus (GP) (Fig. 1). As development continues, *Nkx2.1* expression can be detected in several other ventral telencephalic structures including the bed nucleus of the stria terminalis (BNST), parts of the septum, the ventral pallidum and parts of the amygdala (Fig. 1d; L. Puelles, E. Kuwana, A. Bulfone, K. Shimamura, J. Keleher, S. Smiga, E. Puelles and J. L. R. R., unpublished data; L. Puelles, L. S. and J. L. R. R., unpublished data).

Nkx2.1 mutants are defective in basal ganglia development

To assess the extent of basal telencephalic defects in the *Nkx2.1* mutant, we compared Nissl-stained sections from E18.5 *Nkx2.1* mutant embryos (Fig. 1f,h,j) to wild-type control littermates (Fig. 1e,g,i). E18.5 was the only age that was previously shown to have morphological defects in the telencephalon (Kimura et al., 1996). At E18.5, we found that the mantle zones of the MGE and LGE are not cytoarchitecturally distinct and that pallidal structures, such as the globus pallidus, are either missing or unrecognizable (n=4). Likewise, the axon tract that traverses this region (anterior commisure, AC), does not cross the midline. Conversely, the striatum, an LGE derivative, appears to be enlarged and extends into the brain region normally occupied by the pallidal structures. These results were confirmed by molecular analyses (see below, Fig. 4i-p).

Because several components of the basal ganglia are affected in the *Nkx2.1* mutant, we studied whether there were corresponding molecular and histological defects within the nascent basal telencephalon. We began our analysis at E10.5, when a morphologically distinct MGE is forming. The *Nkx2.1* mutant allele was created in such a way that we are able to use mutant *Nkx2.1* transcripts as a marker of the MGE (see Materials and Methods). In the *Nkx2.1* mutants, a structure that resembles a small MGE and expresses high levels of *Nkx2.1* RNA is evident at E10.5 and E11.5 (Fig. 2a,d,g,j). We refer to this structure as the MGE*. The MGE* produces postmitotic neurons as determined by the expression of two neuronal antigens in its mantle zone (TuJ1 and MAP2) (Fig. 2q and r; data not shown).

Telencephalic *Sonic hedgehog* expression is severely reduced in *Nkx2.1* mutant embryos

Sonic hedgehog (SHH) secretion from the axial mesendoderm is required for patterning of the anteromedial neural plate, including the hypothalamus and basal telencephalon (Chiang et al., 1996). In addition, SHH can induce markers of the basal telencephalon, such as *Nkx2.1* (Ericson et al., 1995; Shimamura and Rubenstein, 1997; Dale et al., 1997; Pera and Kessel, 1997) and Dlx2 (Khotz et al., 1998). *Shh* begins to be expressed in the VZ of the ventral-most regions of the basal telencephalon (preoptic and anterior entopeduncular areas; POA, AEP) between the 10-12 somite stage, at approximately the same time as *Nkx2.1* begins to be expressed in the basal telencephalon

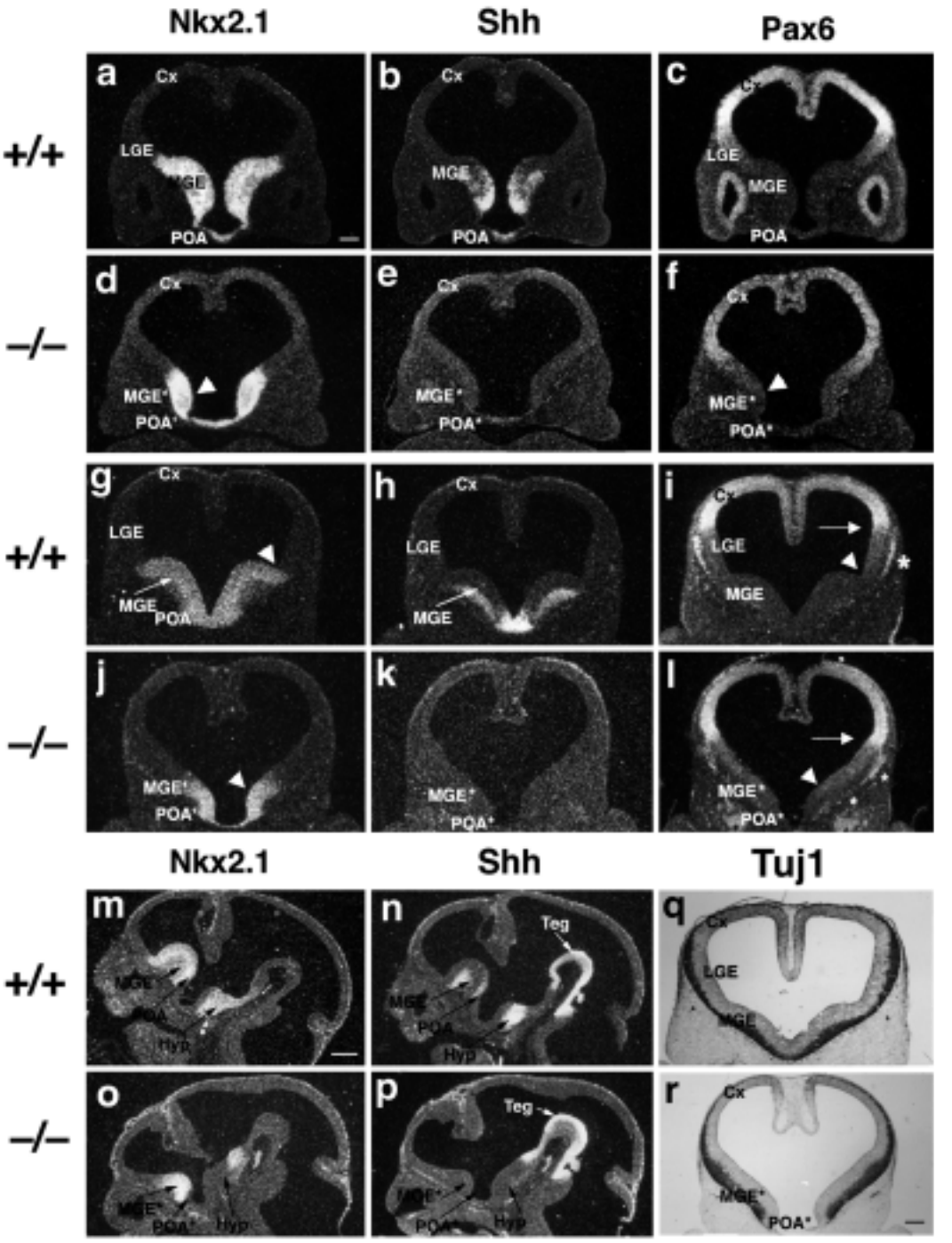

Fig. 2. Dorsalization of the *Nkx2.1* expression domain in E10.5 and E11.5 *Nkx2.1* mutants. (a-f) Serial coronal sections from wild-type (a-c) and mutant (d-f) E10.5 embryos. (a,d) In wild-type and *Nkx2.1* mutant embryos *Nkx2.1* RNA is restricted to the MGE and POA. (e) In the mutant, only a trace of *Shh* expression can be detected in the POA*. (c) *Pax6* is expressed strongly in the cortical (Cx) anlage and weakly in the LGE anlage. The ventral border of *Pax6* marks the beginning of the MGE. (f) In the mutant, LGE levels of *Pax6* expression are found more ventrally (arrowhead in f), and overlap with *Nkx2.1* expression in the MGE* (arrowhead in d). (g-l) Serial coronal sections from wild-type (g-i) and *Nkx2.1* mutant (j-l) E11.5 embryos. (g,j) At E11.5, the MGE in the mutant is less pronounced, but *Nkx2.1* expression remains strong. The arrowhead indicates the ventral border of *Pax6* expression (see i and l). (h,k) *Shh* is expressed in the VZ and mantle of the wild-type MGE, but is undetectable in the mutant. (i) *Pax6* expression is strong in the cortex and weaker in the LGE. The arrowhead marks the ventral border of *Pax6* expression which in wild-type coincides with the boundary of *Nkx2.1* expression in the MGE. The * marks strong *Pax6* signal in the mantle of the LGE. (l) In the *Nkx2.1* mutant, LGE-levels of *Pax6* expression extends into the region expressing *Nkx2.1*. The arrowhead marks the ventral extent of *Pax6* expression which overlaps the *Nkx2.1*-positive region. *Pax6* does not extend into the POA. *Pax6* expression in the LGE mantle also extends ventrally (*). (m-p) Serial parasagittal sections of wild-type (m-n) and mutant (o-p) E11.5 embryos. (o) *Nkx2.1* transcript can be detected in the MGE* and POA*. (p) *Shh* RNA is not detectable in the basal telencephalon, but can still be clearly detected in the tegmental (Teg) region of the midbrain. (q-r) Coronal sections from the same series of wild-type and mutant sections shown in g-l. Maturing neurons are present in the MGE* based upon the TuJ1 immunoreactivity (r). Scale bars, (a,r) 100 μm; (m) 400 μm.

(Shimamura et al., 1995). Subsequently, *Shh* expression spreads into the SVZ and mantle of the MGE (Fig. 2).

Because *Shh* is expressed early in MGE development and has a potential role in telencephalic patterning, we wanted to assess its expression in the developing MGE of the *Nkx2.1* mutant. Surprisingly, at E10.5 and E11.5, *Shh* expression was undetectable in the mutant basal telencephalon and hypothalamus (Fig. 2e,k,p), aside from trace levels of *Shh* in the rostral midline at E10.5 (Fig. 2e; *n*=11). *Shh* expression in the midbrain and more posterior regions of the central nervous system appeared normal at all ages examined (Fig. 2p; data not shown). To determine whether *Nkx2.1* expression is required for induction or maintenance of *Shh* expression in the forebrain, we analyzed E8.75-E9.5 *Nkx2.1* mutant embryos by

whole-mount in situ hybridization. Even at these stages which ordinarily express *Shh* in the forebrain (Shimamura et al., 1995), *Shh* transcripts in the hypothalamus and basal telencephalon were not detectable, while *Shh* expression in the anterior mesendoderm was normal (data not shown).

Dorsalization of the ventral telencephalon in the *Nkx2.1* mutants

To determine whether the defects in *Nkx2.1* and *Shh* expression affect patterning of the ventral telencephalon, we studied the expression of *Pax6*, a homeobox gene that is repressed by SHH and which represses *Nkx* gene expression (Chiang et al., 1996; Ericson et al, 1997). In the telencephalon of wild-type embryos, *Pax6* expression is strong in the cortex, weak in the LGE and not detectable in the *Nkx2.1*-expressing areas (MGE and POA) (Fig. 2c,i; also see Stoykova et al., 1996). In the *Nkx2.1* mutants, at E10.5, prior to the formation of a LGE, low levels of *Pax6* transcripts are detected in the basal telencephalon tissue that expresses the mutant *Nkx2.1* gene (Fig. 2f). The ventral expansion of *Pax6* expression becomes more obvious by E11.5 and E12.5, when it clearly overlaps with *Nkx2.1* expression in the VZ of the MGE*, although it does not expand into the mutant preoptic area (POA*) (Figs 2i,l, 3k-n). This suggests that the mutant MGE* has dorsal characteristics that are not present in the wild-type MGE. In addition to the dorsalization of the VZ, there is a ventral expansion of *Pax6* expression in the subpial mantle of the basal telencephalon (Fig. 3n).

Nkx2.1 mutants lack morphological and molecular maturation of the MGE

By E12.5, the dorsal/ventral molecular abnormalities are reflected by clear histological defects. At this stage in wild-type mice, the MGE and LGE are distinct structures (Fig. 3a). However, in E12.5 *Nkx2.1* mutant embryos, the sulcus that is normally found between the MGE and LGE (see arrowhead in Fig. 3a) is almost completely absent, and there appears to be a single ganglionic eminence (Fig. 3b). Despite, the morphological homogeneity, the medial (ventral) part of the mutant ganglionic eminence still expresses *Nkx2.1* (Fig. 3d), demonstrating that the MGE* still exists.

To further characterize the MGE*, we looked at expression of the LIM-homeobox genes *Lhx6* and *Lhx7* (*L3*), whose onset of expression in the telencephalon is restricted to the *Nkx2.1* territory (Wanaka et al., 1997; Grigoriou et al., 1998). Telencephalic expression of both genes is not detectable in the *Nkx2.1* mutant at E12.5 (Fig. 3f and h). The lack of *Shh*, *Lhx6* and *Lhx7* in the SVZ and mantle suggests that the MGE* does not follow the normal developmental program of the MGE. Furthermore, the morphological appearance of the MGE*, the presence of *Pax6* in the VZ of MGE*, combined with the enlarged striatum that is evident at E18.5, suggest the hypothesis that the MGE* may have become respecified to produce striatal tissue. To test this model, we analyzed the expression of genes that are expressed in the LGE and developing striatum.

Evidence that the MGE* is respecified to form an LGE which differentiates into the striatum

The *Dlx* genes encode homeobox transcription factors that are essential for differentiation of the LGE and MGE

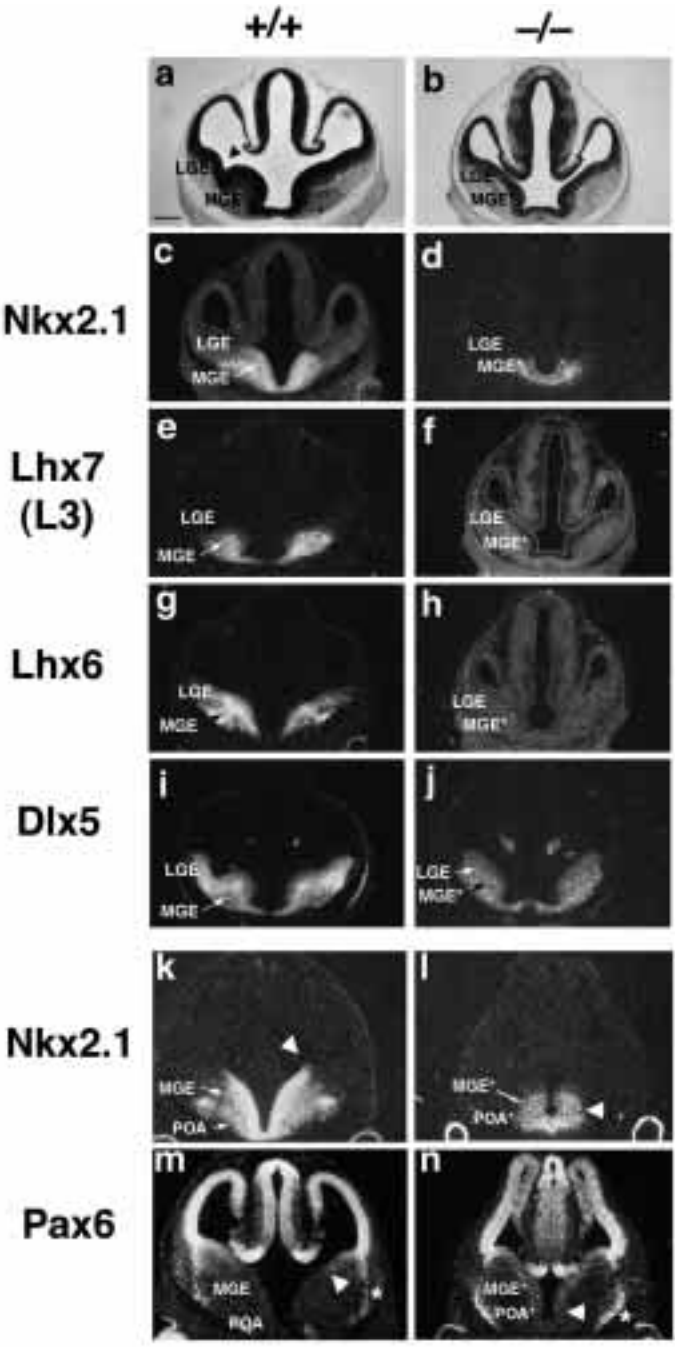

Fig. 3. *Nkx2.1* mutants lack differentiation markers of the MGE at E12.5. (a,c,e,g,i) Serial coronal sections from an E12.5 wild-type embryo showing cresyl violet staining (a), *Nkx2.1* expression in the MGE (c), *Lhx7/L3* expresson in the mantle of the MGE (e), *Lhx6* expression in the SVZ and mantle of the MGE (g), and *Dlx5* expression in the SVZ and mantle LGE and MGE (i). (b,d,f,h,j) Serial coronal sections from an E12.5 *Nkx2.1* mutant embryo showing cresyl violet staining (b), *Nkx2.1* expression (d), loss of *Lhx7/L3* and *Lhx6* expression in the MGE* (f and h), and normal levels of *Dlx5* expression in the MGE* and LGE (j). (k-n) Adjacent coronal E12.5 sections from a different set of animals than shown above; wild-type (k,m); mutant (l,n). The ventral limit of *Pax6* expression, which is indicated by an arrowhead, overlaps with *Nkx2.1* in the MGE* only in the mutants. *Pax6* expression in the mantle of the LGE (marked with an *) also extends more ventrally and overlies the MGE*. *Pax6* is excluded from the POA*. Bar, 300 μm.

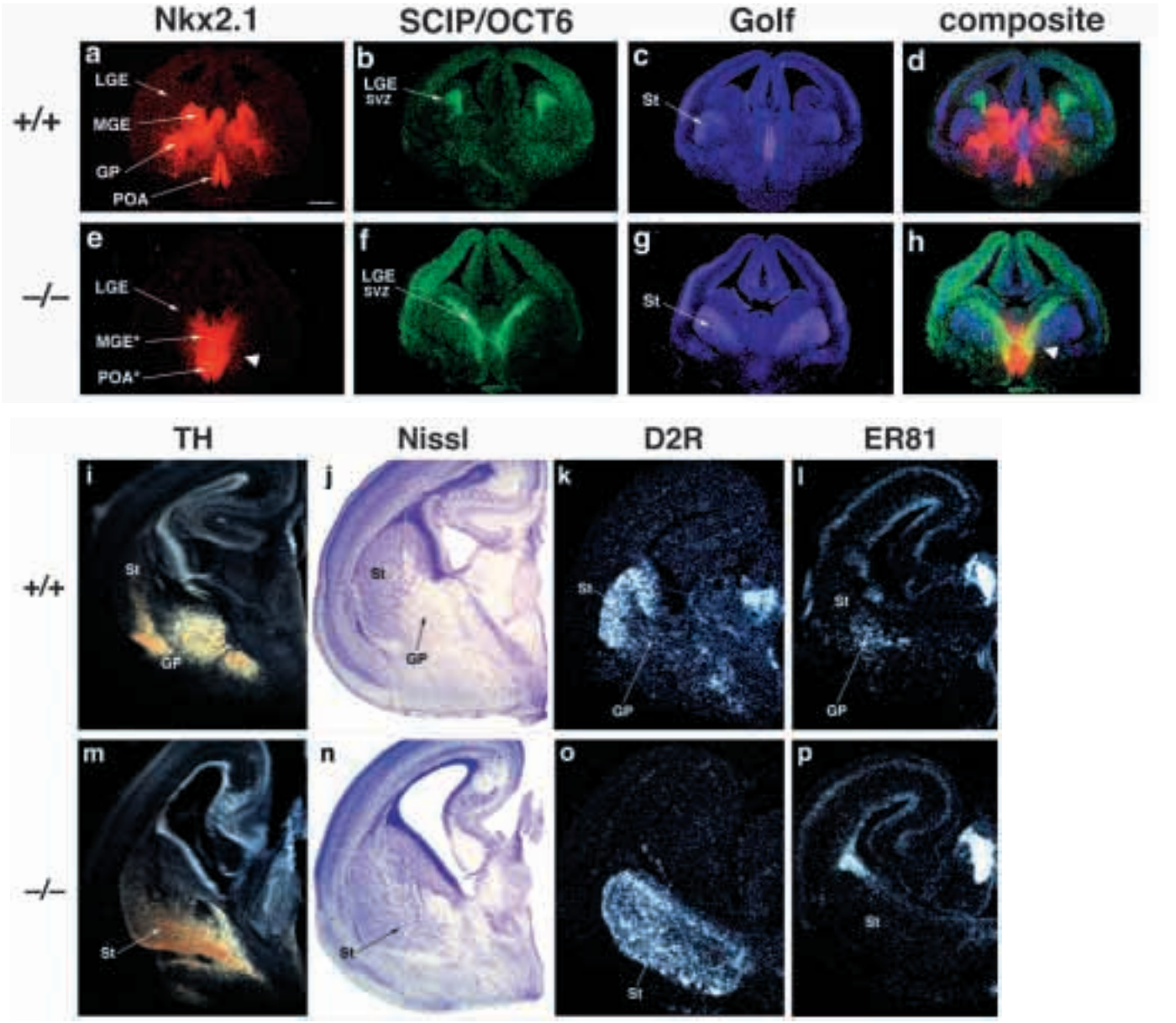

Fig. 4. Molecular respecification of the MGE*: expression of LGE-specific markers at E14.5 and E18.5. Analysis at E14.5 (a-h) and E18.5 (i-p). (a-h) Serial coronal sections from E14.5 wild-type (a-c) and *Nkx2.1* mutant (e-g) telencephalons assayed for RNA expression of *Nkx2.1* and two LGE markers, *SCIP* and *GOLF*. The in situ signal for each has been colored using Adobe Photoshop: *Nkx2.1*, red; *SCIP/Oct6*, green; *GOLF*, purple. (a,e) In the wild-type, *Nkx2.1* is expressed in the MGE and POA proliferative zones, and in a nucleus that is probably the globus pallidus (GP). In the mutant, *Nkx2.1* expression remains strong in the VZ and SVZ, but there are only scattered cells expressed in the mantle and no evidence for a globus pallidus (arrowhead in e). (b,f) In the wild-type basal telencephalon, *SCIP/OCT6* expression is restricted to the SVZ of the LGE. In the mutant, *SCIP/OCT6* has extended ventrally into the SVZ of MGE*, where it overlaps with *Nkx2.1*-expressing cells. *SCIP/OCT6* expression is excluded from the POA*. (c,g) *GOLF* expression in the basal telencephalon marks the mantle of the LGE and is excluded from the MGE mantle. In *Nkx2.1* mutants, *GOLF* expression has expanded ventrally into the region normally occupied by the globus pallidus. (d and h) The three wild-type serial sections and three mutant serial sections have each been overlayed in Adobe photoshop to more clearly represent the data. In the wild-type composite (d), *Nkx2.1* expression does not overlap with *SCIP/OCT* in the SVZ or *GOLF* in the mantle. In the mutant composite (h), *SCIP* extends ventrally into the MGE*, where it overlaps with the *Nkx2.1*-expressing domain (yellow color). The arrowhead points to a region of the MGE mantle where a few *Nkx2.1*-expressing cells are present in the enlarged *GOLF*-positive domain. (i,m) Innervation of the basal telencephalon by tyrosine hydroxylase fibers is intact in the *Nkx2.1* mutant at E18.5. Nissl staining (j,n) and expression of *dopamine receptor 2* (*D2R*) (k,o) shows expansion of the striatal tissue and loss of the globus pallidus. (l,p) Expression of *ER81* shows loss of the globus pallidus. Scale bar, 400 μm.

(Anderson et al., 1997a,b; K. Yun, T. Stuehmer and J. L. R. R., unpublished results). Four Dlx genes are expressed in progressively more advanced stages of differentiation in the MGE and LGE (Liu et al., 1997; Anderson et al., 1997b). There is also evidence that SHH may be required for their expression (Kohut et al., 1998). We assayed for the expression of *Dlx2* and

Dlx5, and found that their expression appears normal in both the LGE and MGE* (Fig. 3i,j, and data not shown). Thus, unlike the MGE-specific markers, *Dlx* expression is not affected by the loss of *Nkx2.1* function. There are at least two explanations for this result: either *Dlx* expression in the MGE* represents an *Nkx2.1*-independent population of MGE cells, or the MGE* has taken on LGE-like properties.

To distinguish between these possibilities, we analyzed at E14.5 the expression of two LGE-restricted markers: *SCIP/Tst1/Oct6* encodes a POU-homeodomain transcription factor that is expressed in the SVZ of the LGE (Fig. 4b; Alvarez-Bolado et al., 1995; Anderson et al., 1997a) and *GOLF* encodes a guanine nucleotide binding protein that is expressed in the mantle of the LGE (Fig. 4c; Belluscio, 1998). Neither gene is expressed at high levels in the MGE or its derivatives at E14.5 (Fig. 4d). However, in the *Nkx2.1* mutant, *SCIP* expression has expanded into the SVZ of the MGE* and now overlaps with the *Nkx2.1*-expressing region (Fig. 4f,h). In addition, *GOLF* expression is ventrally expanded into the region normally occupied by the MGE mantle (Fig. 4g). Although there are only a small number of *Nkx2.1*-positive cells present in the MGE* mantle, these cells appear to be in the region containing the ventral-most *GOLF*-expressing cells (arrowhead in Fig. 4h). Therefore, this evidence suggests that in *Nkx2.1* mutants, the MGE* becomes respecified to produce striatal-like tissue, instead of the globus pallidus.

Histological analysis of the basal telencephalon at E18.5 also revealed expansion of striatal tissues and loss of pallidal structures in the *Nkx2.1* mutants. For instance, expression of *dopamine receptor 2* (*D2R*), which marks striatal projection neurons is greatly expanded (Fig. 4k,o), whereas expression of the *ER81* ETS transcription factor, which labels neurons in the globus pallidus, is lost (Fig. 4l,p). Striatal histology and connectivity in the *Nkx2.1* mutants is indistinguishable from that in normal littermates based on Nissl staining (Fig. 4j,n), expression of *D2R* and its innervation by tyrosine hydoxylase (TH) fibers (Fig. 4i,m).

Not all of the *Nkx2.1*-expressing region is equally dorsalized. As noted above, *Pax6* expression is not detectable in the VZ of the POA*. Likewise, expression of *SCIP* and *Golf* also do not expand into this region. Consistent with these molecular results, striatal tissue does not expand into this ventral-most region of the telencephalon.

Nkx2.1 cells migrate from the MGE into the LGE

During our analysis of *Nkx2.1* expression in the wild-type basal telencephalon, we noticed the appearance of scattered *Nkx2.1*-expressing cells in the striatum by E14.5 (Fig. 5a). As development proceeded, the expression of *Nkx2.1* was maintained in a subset of striatal cells (Fig. 5b). To determine whether the *Nkx2.1*-positive striatal cells arose in situ or whether they migrated from the MGE, we tracked the migration of cells out of the MGE in slice cultures (Anderson, et al., 1997). Crystals of DiI were placed into the VZ and SVZ of the MGE in brain slices from E12.5 mice. After 24 to 36 hours in culture, many labeled cells were present throughout the striatum, suggesting that a population of striatal cells arrive via a tangential migration from the MGE (n=13; Fig. 5c).

The dynamic expression pattern of the Lim homeobox gene, *Lhx6*, supports this hypothesis. At E12.5, *Lhx6* expression is restricted to cells of the MGE (SVZ and mantle zone)

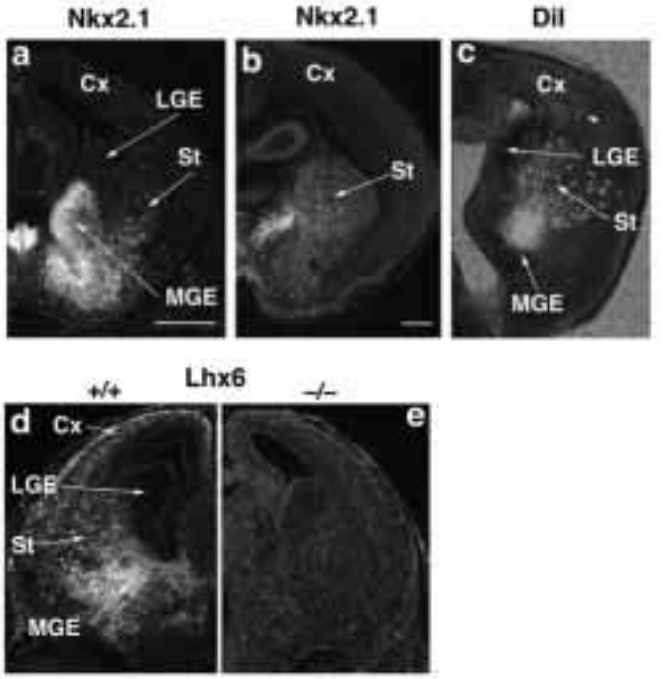

Fig. 5. MGE cells migrate into the LGE and cortex. (a) At E14.5, *Nkx2.1* is expressed in the MGE and in a small population of cells emanating from the MGE into the striatum. (b) By P6, there is a large population of *Nkx2.1*-expressing cells scattered throughout the striatum. (c) DiI was placed in the MGE SVZ in 250 μm thick coronal brain slices from E12.5 embryos. After 36 hours in culture, labeled cells are present in the striatum and a few have migrated into the cortex (arrowhead). (d-e) *Lhx6* RNA expression in coronal hemisections of E14.5 wild-type (d) and mutant (e) brains. By E14.5 in wild-type mice, *Lhx6* expression extends through the striatum and into the cortex. In the *Nkx2.1* mutant, *Lhx6* transcripts are not detectable. Scale bars, 500 μm.

(Grigoriou, et al., 1998; Fig. 3g). By E14.5, expression of *Lhx6* has extended into the striatum similar to the expansion of *Nkx2.1* expression (Fig. 5d). Unlike *Nkx2.1*, *Lhx6* expression also extends into cerebral cortex (Fig. 5d). In the *Nkx2.1* mutant at E14.5, *Lhx6* expression in the MGE*, LGE and cortex is not detectable (Fig. 5e). This finding suggests that *Lhx6*-positive cells originate from the MGE and migrate into the LGE and cortex. Consistent with this premise, we have observed MGE-derived DiI-labeled cells entering the cortex in our slice culture migration studies (arrowhead in Fig. 5c). This result has been substantiated by additional studies on sub-cortical to cortical migrations originating from the MGE (Lavdas et al., 1998; S. Anderson and J. L. R. R., unpublished data).

Cortical interneurons are reduced in *Nkx2.1* mutant mice

Recent studies have identified a migration of cells from the basal ganglia to the cerebral cortex (De Carlos et al, 1996; Anderson et al., 1997b; Tamamaki et al., 1997). There is evidence that these migrating cells are GABAergic and may be the source of many of the neocortical interneurons (Anderson et al., 1997b). Furthermore, neonatal mice lacking *Dlx1* and *Dlx2* expression, which have a block in differentiation of the

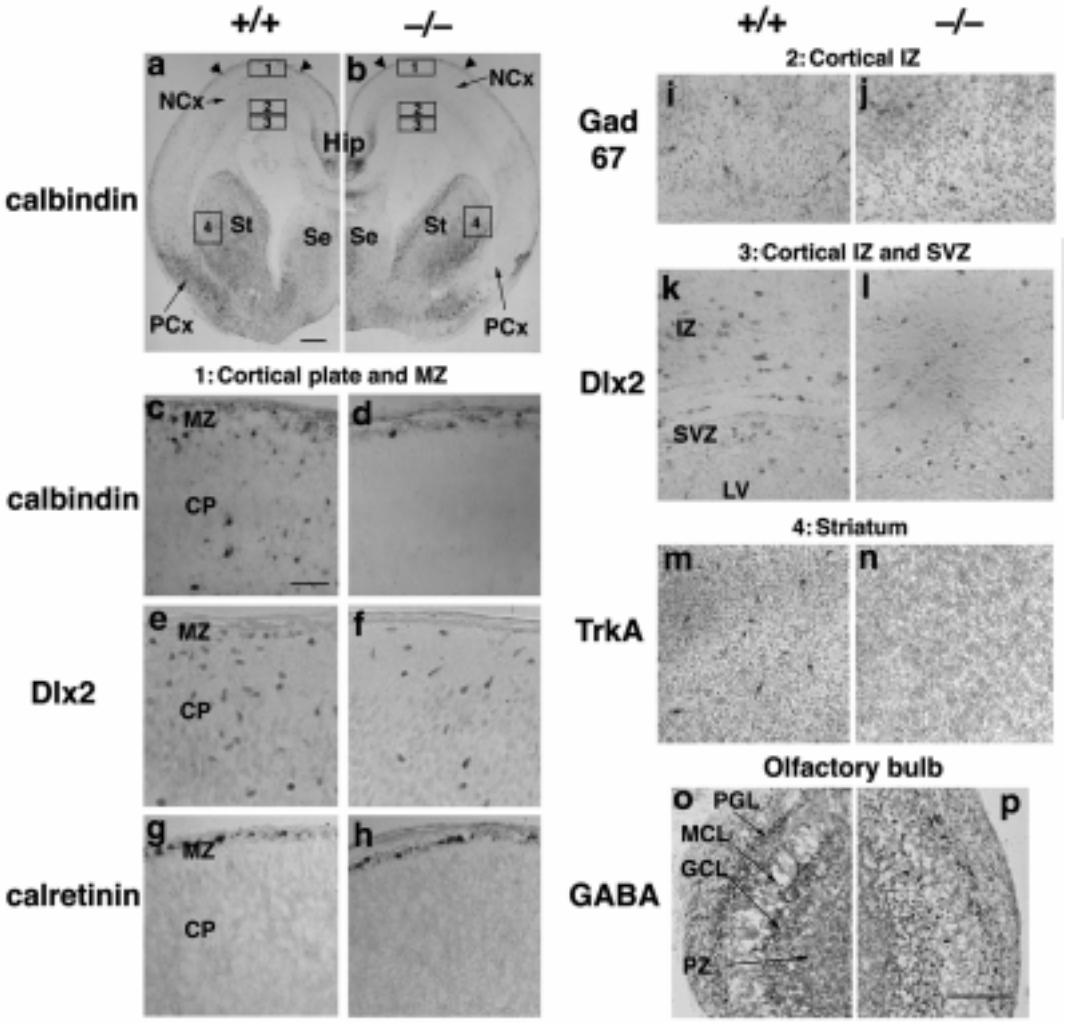

Fig. 6. Subpopulations of neurons are missing from the cortex and striatum of Nkx2.1 mutant animals. (a-p) Immunohistochemical analysis of *Nkx2.1* wild-type and *Nkx2.1* mutant brains at E18.5. (a-b) Calbindin immunoreactivity in coronal hemisections through the rostral telencephalon. The numbered boxes delineate the approximate regions shown at higher magnification in c-n: 1=cortical plate (CP) and marginal zone (MZ) shown in c-h; 2=cortical intermediate zone (IZ) shown in i-j; 3=cortical IZ and subventricular zone (SVZ) shown in k-l; and 4=striatum shown in m-n. Sections shown in c and d are higher magnifications of the hemisections shown in a and b. The data shown in e-n represent immunohistochemical analysis on different sections from the same wild-type or mutant embryos. The region between the arrowheads (a-b) indicates the approximate area from which immunopositive cells were counted in each neocortical layer (see text). (o-p) GABA immunoreactivity in coronal sections of the olfactory bulb at E18.5. GCL:granular cell later; Hip: hippocampus, MCL:mitral cell layer; PGL:periglomerular layer; PZ: proliferative zone. Bars, (a) 250 μm; (c) 50 μm; (p) 150 μm.

LGE, have a four-fold reduction in neocortical GABAergic interneurons and have no detectable GABAergic interneurons in their olfactory bulbs (Anderson et al., 1997b, Bulfone et al., 1998). Our previous studies did not establish whether the migrating cells were coming from the LGE or the MGE. Our finding that MGE cells can migrate to the cortex, and that *Lhx6* expression spreads from the MGE to the cortex prompted us to assess the cortical phenotype in the *Nkx2.1* mutants.

Overall, the cortex appears histologically normal in E18.5 *Nkx2.1* mutants (Fig. 6a,b). However, immunohistochemical analysis of calbindin, GAD67, and DLX2 (Fig. 6) shows that both the neocortex (NCx) and paleocortex (PCx) have reductions in the numbers of interneurons. Calbindin is almost completely missing (>99%; *n*=3) from the cortical plate (CP), intermediate zone and subventricular zone of the NCx and deep layers of the PCx (Fig. 6b and d). In the subpial region of marginal zone, there are normal numbers of calbindin-expressing cells, which may correspond to the calretinin-positive cajal-retzius cells that are postulated to be derived from a proliferative zone proximal to the olfactory bulb (Meyer et al., 1998). On the other hand, in deeper regions of the marginal zone there is a large reduction of calbindin-positive cells (Fig. 6c,d). These results suggest that the majority of calbindin-expressing neocortical interneurons are derived from the MGE. Since many of the calbindin neurons are also GABAergic, we tested the *Nkx2.1* mutants for GAD67 immunoreactivity, and found an approximately 40% reduction of GAD67-positive cells (*n*=3; Fig. 6j). We also see a reduction (approx. 50%) in DLX2-expressing cells in the cortical plate, intermediate zone and subventricular zone of the NCx, and there is almost a 75% reduction of DLX2-expressing cells in the marginal zone (Fig. 6f and 6l; *n*=3). While there are large

reductions in DLX2-positive cells in the marginal zone, calretinin expression in this region is normal, suggesting that Cajal-Retzius neurons are not derived from the MGE (Fig. 6g,h).

We next studied interneuron development in the *Nkx2.1* mutant olfactory bulb because *Dlx1* and *Dlx2* mutants lack detectable GABAergic cells in this structure (Bulfone et al., 1998). Surprisingly, in the *Nkx2.1* mutant, interneuron development in the olfactory bulb appears normal, based on the expression of GABA, calbindin and DLX2 (Fig. 6o,p and data not shown). This would suggest that the MGE/*Nkx2.1* region does not contribute GABAergic or calbindin-positive interneurons to all regions of the telencephalon.

Cholinergic neurons are missing from the striatum of *Nkx2.1* mutant mice

The cells migrating from MGE to LGE may be the cortical interneurons that are passing through the LGE, or they may represent a distinct population of cells that end their migration in the striatum. Since *Shh* and *Nkx* genes have been implicated in cholinergic neuron development in the spinal cord (Chiang et al., 1996; Ericson et al., 1996; M. Sander, S. Paydar, M. German and J. L. R. R., unpublished data), and cholinergic neurons constitute some striatal, septal and pallidal projection neurons, we tested whether basal telencephalic cholinergic neurons were affected by the *Nkx2.1* mutation. To test for the presence of cholinergic neurons, we assayed for the TrkA neurotrophin receptor, a recognized marker for cholinergic cells (Sobreviela, et al., 1994). As shown in Fig. 6n, TrkA is not detectable in the *Nkx2.1* mutant striatum at E18.5. In fact, no TrkA cells could be identified in the entire basal telencephalon (data not shown).

DISCUSSION

Loss of *Nkx2.1* function causes a ventral-to-dorsal molecular transformation within the anlage of the basal ganglia: evidence that the primordium of the globus pallidus (the MGE) forms striatal tissue

In this study, we demonstrate that *Nkx2.1* is expressed in the proliferative and postmitotic cells of the MGE from its inception, and continues to be expressed in maturing MGE derivatives (Figs 1a-d, 5a,b). Furthermore, *Nkx2.1* is required

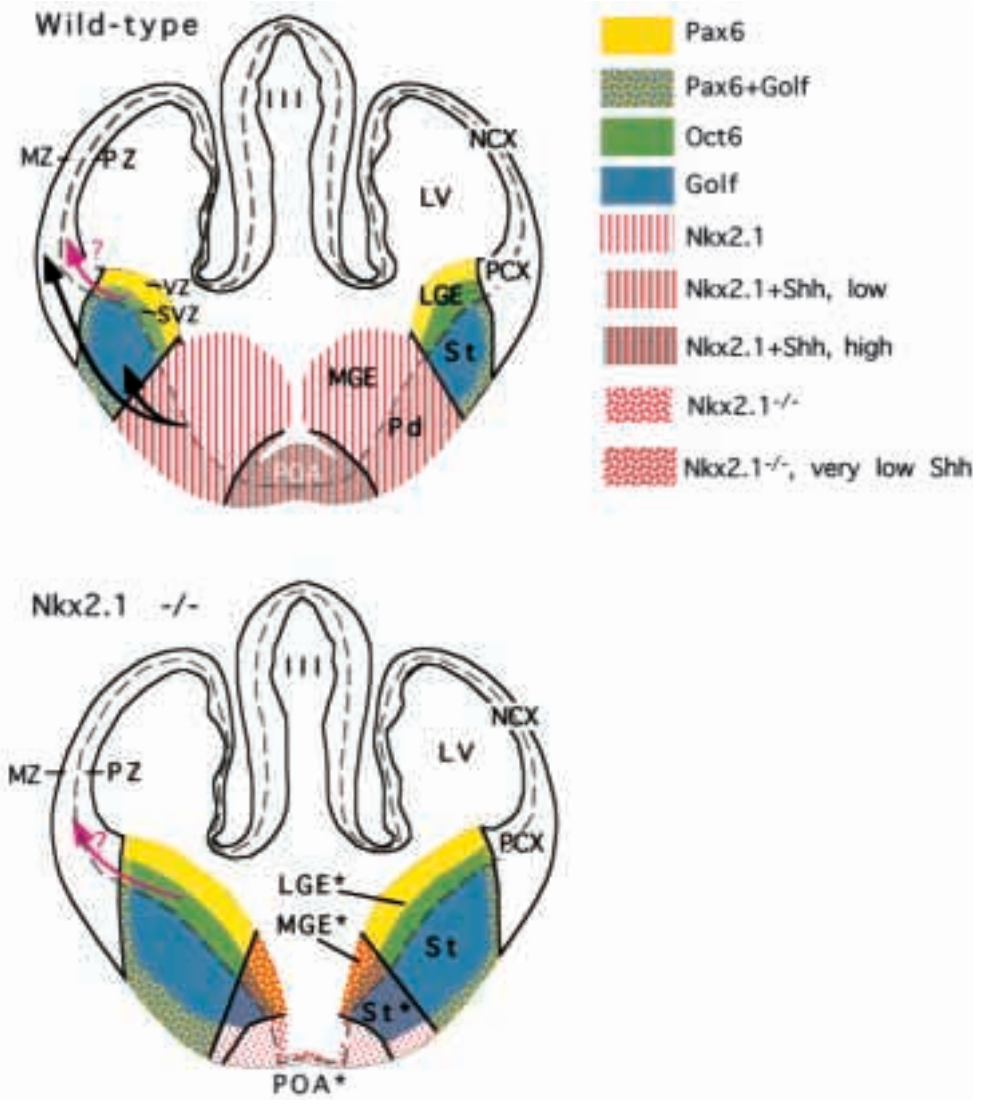

Fig. 7. Schemata illustrating the molecular repatterning of the subcortical telencephalon in *Nkx2.1* mutants. The drawings are based on E12.5 forebrain morphology and integrate data from in situ hybridization experiments done on E12.5-E14.5 embryos. Top, wild-type; bottom, *Nkx2.1*$^{-/-}$. Gene expression patterns in the basal telencephalon are color coded as indicated in the key; expression of these genes is not shown in the cerebral cortex or in the diencephalon. Wild-type *Nkx2.1* expression: vertical red lines; mutant *Nkx2.1* expression: red dots; the size of the dots reflects the strength of expression. Solid black lines indicate the approximate position of the boundaries between the longitudinal subdivisions of the telencephalon. Dashed gray line separates the PZ from the MZ. Yellow dots correspond to *Pax6*-positive cells that are radially migrating through the lateral striatum to the striatal subpial zone. Black arrows indicate tangential cell migrations from the MGE into the LGE and cortex. Red arrow indicates hypothesized migration from the LGE to the cortex. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MGE*, mutant MGE; MZ, mantle zone; LV, lateral ventricle; NCX, neocortex; PCX, paleocortex; Pd, pallidum; POA, preoptic area; POA*, mutant POA; St, striatum; St*, striatal-like tissue that forms in place of the pallidum in the mutant. SVZ, subventricular zone; VZ, ventricular zone; III, third ventricle.

for development of pallidal-related structures of the ventral telencephalon; in its absence, none of the derivatives from *Nkx2.1*-positive telencephalic primordia are identifiable (Figs 1, 4). Our analysis suggests that loss of *Nkx2.1* function results in a molecular repatterning and respecification of the MGE into an LGE-like tissue (Fig. 7). The evidence supporting this hypothesis is based on the following results. First, at E10.5 a MGE-like structure (MGE*), which expresses *Nkx2.1* (Fig. 2d,j,o), is morphologically present (best seen in Fig. 2q,p). Second, the MGE* lacks additional molecular characteristics of the MGE (*Shh*, *Lhx6*, *Lhx7* are absent; Fig. 3) and subsequently acquires molecular characteristics of the LGE (*Pax6* in the VZ; *SCIP* in the SVZ; *GOLF* in the mantle) (Figs 3n and 4f-h). By late in gestation, we observe an enlargement of LGE derivatives (e.g. the striatum) and a lack of MGE derivatives (e.g. the globus pallidus) (Figs 1, 4). It is important to note that we have not proved at single cell resolution that the cells expressing *Pax6*, *SCIP* and *GOLF* also express the *Nkx2.1* truncated transcript. This level of analysis is needed to strengthen the argument supporting a fate respecification of MGE* cells. However, aside from this caveat, we suggest that the evidence is strong enough to hypothesize that *Nkx2.1* has a primary role in regional specification of the basal telencephalon; in its absence, the MGE takes on an LGE fate. We also suggest that the co-expression of the *Nkx2.1* and *Dlx* genes co-define pallidal identity, whereas expression of the *Dlx* genes alone defines striatal identity.

Nkx2.1 is related to a *Drosophila* gene named *ventral nervous system defective* (*vnd*). *Vnd* encodes the NK2 homeodomain protein which is expressed in the ventral part of central nervous system (Kim and Nirenberg, 1989; Jiminez et al., 1995). The CNS in fly embryos lacking *vnd* have a ventral-to-dorsal transformation (McDonald et al., 1998; Weiss et al., 1998; Chu et al., 1998), analogous to the phenotype in the *Nkx2.1* mutants. There are several *Nkx* genes expressed in the ventral CNS of vertebrates, including *Nkx2.2* and *Nkx6.1* (Price et al., 1992). Mutations of *Nkx2.2* and *Nkx6.1* also have ventral-to-dorsal transformations (Briscoe et al., 1999; M. Sander, S. Paydar, M. German and J. L. R. R., unpublished data). In both of these cases, *Shh* expression was unaffected, suggesting that *Nkx2.2* and *Nkx6.1*, like *vnd*, have primary roles in ventral specification. We cannot be sure that *Nkx2.1* alone is necessary for fate specification of the MGE, because *Shh* expression, which is essential for ventral specification (Chiang et al., 1996), is also reduced in the *Nkx2.1* knockout mice. However, the described functions of *vnd*, *Nkx2.2* and *Nkx6.1* support the hypothesis that *Nkx2.1* has a primary role in regional specification, like its homologues. Furthermore, in all regions of the CNS, the *Nkx* genes are expressed before *Shh* (Shimamura et al., 1995; P. Crossely and J. L. R. R., unpublished observations), supporting the model that *Nkx2.1* is upstream of *Shh* in the basal telencephalon. Finally, in *Gli2* mutant mice, *Shh* is not expressed in the floor plate, yet *Nkx2.2* is expressed and motor neurons form (Matise et al., 1998). Thus, while *Shh* expression in the axial mesendoderm is essential for ventral specification of the CNS (Chiang et al., 1995), *Shh* expression in neural tissue may not have a major role in regionalization.

The fact that a morphologically distinct MGE* forms in the mutants (Fig. 2p,q) suggests that its early growth is not dependent on *Nkx2.1*. This implies that there are distinct genetic pathways for MGE regional specification and proliferation. We are unaware of other Nkx genes that are expressed in the proper pattern to be candidates for these genes.

Previous studies have demonstrated that SHH can induce *Nkx2.1* expression (Ericson et al., 1995; Shimamura and Rubenstein, 1997; Dale et al., 1997; Pera and Kessel, 1997; Qiu et al., 1998; Kohtz et al., 1998). Here we find that the *Nkx2.1* gene transcription is induced and maintained, despite the massive reduction in *Shh* expression in the forebrain. We suggest that SHH produced in the anterior mesendoderm is sufficient to induce *Nkx2.1* at neural plate stages, and that high levels of SHH expression in the forebrain is not required to maintain *Nkx2.1* expression. In addition, neuroepithelial expression of *Shh* appears to require *Nkx2.1*. It will be important to test whether neuroepithelial expression of *Shh* in other CNS regions also depends upon expression of other *Nkx* genes.

The trace levels of *Shh* in the region of the mutant preoptic area (POA*) (Fig. 2e), may explain why *Pax6* expression does not extend into this region (Figs 2f,l, 3n). This may also explain why the ectopic striatal tissue doesn't enter this territory (compare expression of *Nkx2.1* with *SCIP* and *GOLF* in the POA; Fig. 4). We do not know the identity of the tissue that develops in the mutant POA*.

SHH can induce DLX expression, and anti-SHH immunoglobulins reduce DLX expression in forebrain explants (Kohtz et al., 1998). However, despite the lack of *Shh* expression in the MGE*, there are roughly normal levels of *Dlx2* and *Dlx5* transcripts in *Nkx2.1* mutants (Fig. 3i,j and data not shown). This could be due to the extremely low level of *Shh* expression in the POA* (Fig. 2e), or due to long lasting effects of SHH produced in the anterior mesendoderm at neural plate stages (Shimamura et al., 1997) (note: *Dlx* genes turn on around E9.5, about 24 hours after the medial neural plate begins to be exposed to SHH). In either case, it suggests that normal levels of *Shh* expression in the forebrain are not required for induction or maintenance of *Dlx* expression, and perhaps suggests that other secreted substances have a more direct role for inducing *Dlx* expression in this region.

Despite the loss of *Shh* and *Nkx2.1* expression, and the apparent transformation of ventral telencephalon into a more dorsal fate, *Nkx2.1* mutants form a LGE/striatum, have a normal subcortical/cortical boundary (interface of *Dlx5* and high *Pax6* expression; Fig. 3i,j,m,n) and form the major regions of the cerebral cortex (see Fig. 6). Thus, disruption of ventral patterning does not significantly affect dorsal and anteroposterior patterning. For instance, *Fgf8* and *Otx1*, two genes implicated in anteroposterior patterning of the forebrain (Shimamura et al., 1997; Acampora et al., 1997), continue to be expressed normally (data not shown).

Nkx2.1 mutants have reduced basal forebrain cholinergic neurons and cortical interneurons: evidence for migrations from the ventral telencephalon into the striatum and cerebral cortex

In the absence of *Nkx2.1*, the MGE* does not produce normal ventral telencephalic cell types. Evidence is accumulating that dorsoventral patterning of the vertebrate CNS is regulated by common mechanisms along the entire anteroposterior axis (Hynes et al., 1995; Lumsden and Graham, 1995; Ericson et

al., 1997; Rubenstein and Beachy, 1998). For instance, the *Nkx* genes, which are induced by SHH in all ventral CNS regions (Ericson et al., 1995; Barth and Wilson, 1995; Shimamura et al., 1997; Dale et al., 1997; Pera and Kessel, 1997; Qiu et al., 1998; Kohtz et al., 1998), are required for ventral specification in the forebrain (herein; Kimura et al., 1996) hindbrain and spinal cord (Briscoe et al., 1999; M. Sander, S. Paydar, M. German and J. L. R. R., unpublished results). While there are a variety of ventral CNS cell types, all axial levels produce cholinergic neurons (e.g. basal forebrain cholingeric neurons and motor neurons in the midbrain, hindbrain and spinal cord). *Nkx2.1* mutants lack TrkA-expressing cells in their telencephalons (Fig. 6m,n), and therefore presumably lack cholinergic neurons (the animals die at an age before we can detect other cholinergic cell markers).

In addition to the loss of TrkA/cholinergic cells, *Nkx2.1* mutants lack cells that migrate from the MGE through the LGE and into the entire cerebral cortex. Thus, the *Nkx2.1* mutants, like the *Dlx1/Dlx2* double mutants (Anderson et al., 1997a), have a reduction of GABAergic neurons in the cerebral cortex. However, whereas the *Dlx1/Dlx2* double mutants have abnormal differentiation/migration of late born cells from both the LGE and the MGE, the *Nkx2.1* mutants lack an MGE, but have a LGE. This has allowed us to begin dissecting the relative contributions of the MGE and the LGE to telencephalic tangentially migrating cells. For instance, whereas the *Dlx1/Dlx2* mutants lack GABAergic interneurons in the olfactory bulb (Anderson et al., 1997b; Bulfone et al., 1998), the olfactory bulb in the *Nkx2.1* mutants appears normal (Fig. 6 o,p). This shows that the MGE is not required to make interneuron precursors that follow the rostral migratory stream from the basal ganglia to the olfactory bulb (Goldman and Luskin, 1998). Likewise there are differences between the *Dlx1/Dlx2* and the *Nkx2.1* mutants in the distribution of interneuron loss in the paleocortex and neocortex.

These results suggest that there are several distinct basal telencephalon sources that produce cells which migrate to the cortex (Fig. 7). We hypothesize that the *Nkx2.1*-positive ventral telencephalon (including the MGE, POA and AEP) produces cells expressing acetylcholine, calbindin and GABA, that migrate dorsally into the LGE. At least some of the calbindin-positive and GABAergic cells continue to migrate and populate the paleocortex, neocortex and hippocampus. We suggest that the LGE produces GABAergic cells that migrate rostrally into the olfactory bulb and perhaps the cortex, and there may be a separate dorsal migration of GABAergic cells from the LGE to the cortex.

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