Nkx2.1 specifies cortical interneuron fate by activating Lhx6

Tonggong Du, Qing Xu, Polloneal J. Ocbina and Stewart A. Anderson*

In the ventral telencephalon, the medial ganglionic eminence (MGE) is a major source of cortical interneurons. Expression of the transcription factor Nkx2.1 in the MGE is required for the specification of two major subgroups of cortical interneurons – those that express parvalbumin (PV) or somatostatin (SST) – but direct targets of Nkx2.1 remain to be established. We find that electroporation of Nkx2.1 cDNA into the ventral telencephalon of slice cultures from Nkx2.1+/− mouse embryos, followed by transplantation into neonatal cortex to permit postnatal analysis of their fate, rescues the loss of PV- and SST-expressing cells. The LIM-homeobox gene Lhx6 is induced by this rescue experiment, and gain- and loss-of-function studies suggest that Lhx6 is necessary and sufficient to rescue these and other interneuron phenotypes in cells transplanted from Nkx2.1+/− slices. Finally, Nkx2.1 protein binds a highly conserved sequence in the Lhx6 promoter, and this sequence appears to mediate the direct activation of Lhx6 by Nkx2.1. The slice transfection and transplantation methods employed here are beginning to uncover embryonic mechanisms for specifying neuronal fates that only become definable postnatally.

KEY WORDS: Cell fate determination, GABA, Medial ganglionic eminence, Nkx2.1, Parvalbumin, Somatostatin, Mouse

INTRODUCTION

Over the past two decades there has been rapid progress in understanding the molecular mechanisms for neuronal subtype fate determination. Many of the initial discoveries were made in Drosophila, with more-recent studies including other model organisms such as C. elegans, zebrafish and chick. In the last decade, these studies have been extended to mammals, for which, particularly in the spinal cord and retina (Dasen et al., 2005; Livesey and Cepko, 2001; Shirasaki and Pfaff, 2002), further advances have occurred. Despite these advances, and the tremendous relevance to human neuropsychiatric illnesses, there has been relatively little progress in cracking the ‘molecular codes’ for specifying neuronal subtypes of the mammalian forebrain (Schuurmans et al., 2004; Zhao et al., 2003).

Beyond the issues of functional pleiotropy and redundancy, a key reason for this lag is the lengthy delay between the final cell cycle, when much of the crucial specification-related signaling is likely to occur (McConnell and Kaznowski, 1991; Xu et al., 2005), and the appearance of the mature neuronal phenotype. This delay dictates that the readout of any manipulation of cell fate in progenitor cells may only be assessable weeks later, in the context of many potential confounding influences of that manipulation on neuronal development. In this paper we present a transfection/transplantation method for studying the genetic control of neuronal fate determination in the developing mammalian forebrain.

Recent evidence indicates that, at least in rodents and ferrets, the large majority of cortical interneurons originate in the subcortical telencephalon (for a review, see Wonders and Anderson, 2006). Despite advances in determining the origins, migratory pathways and the regulation of interneuron migration, little is known about the specification of distinct subgroups of cortical interneurons. Roughly 70% of cortical interneurons can be divided into two practically non-overlapping groups based on their expression of the calcium-binding proteins parvalbumin (PV) or neuropeptide somatostatin (SST) (Gonchar and Burkhalter, 1997; Tamamaki et al., 2003). Both of these subgroups appear to originate mainly within the medial ganglionic eminence (MGE) (Butt et al., 2005; Valcanis and Tan, 2003; Wichterle et al., 2001; Xu et al., 2004), where their fate determination depends on the transcription factors Nkx2.1 and LHX6 (Liodis et al., 2007; Xu et al., 2004). Expression of SST and PV matures postnatally, with PV expression only beginning in rodent cortex during the second postnatal week (Alcantara et al., 1996).

Nkx2.1 is expressed in the MGE and preoptic region of the pallidal telencephalon (Sussel et al., 1999) (see Fig. S1 in the supplementary material). Over 90% of S-phase cells express Nkx2.1 in both the ventricular and subventricular zones of the MGE (Xu et al., 2005). These progenitors produce oligodendrocytes and interneurons of the striatum and cerebral cortex (Kessaris et al., 2006; Marin and Rubenstein, 2001) and projection neurons of the globus pallidus (Xu et al., 2008). As cells migrate out of the MGE, Nkx2.1 expression is downregulated in cortical interneurons but is maintained in subgroups of striatal interneurons postnatally (Marin et al., 2000). As they exit the proliferative zone, most MGE-derived cells begin to express LHX6 (Grigoriu et al., 1998), a homeodomain-containing transcription factor that is not detectable in the telencephalon of Nkx2.1-null embryos (Sussel et al., 1999). Lhx6 continues to be expressed in many interneurons, including most of those that go on to express PV or SST, as they migrate to and then differentiate within the cerebral cortex (see Fig. S1 in the supplementary material) (Cobos et al., 2005; Fogarty et al., 2007; Gong et al., 2003; Lavdas et al., 1999; Liodis et al., 2007).

Here we use genetic gain- and loss-of-function manipulations in mouse embryonic slice cultures, followed by transplantation into cortical environments in vitro and in vivo, to further examine the role of Nkx2.1 in the specification of MGE-derived (PV- or SST-expressing) cortical interneuron subgroups. We demonstrate that the LIM-homeodomain transcription factor gene Lhx6 is activated by and appears to be a direct target of Nkx2.1. Like Nkx2.1 itself, Lhx6 is sufficient to rescue both neurochemical and morphological aspects of Nkx2.1−/− MGE-derived interneurons. In addition, at least for the specification of the SST-expressing phenotype, Lhx6 is required around the time of cell cycle exit and not postnatally in mature...
interneurons. These results and the system presented lay important groundwork for further studies on the transcriptional regulation of interneuron fate in the mammalian forebrain.

MATERIALS AND METHODS

Animals
Nkx2.1−/− null mice (Kimura et al., 1996) on a CD1 background (Xu et al., 2004) and non-transgenic CD1 strain mice were used. All animal procedures were undertaken according to the guidelines of the Institutional Animal Care and Use Committee at the Weill Cornell Medical College.

Gene constructs, slice electroporation and transplantation
Slice electroporation (EP) was conducted as described (Stuhmer et al., 2002; Xu et al., 2005) using vectors concentrated with Endotoxin-free DNA Maxiprep Kits (Qiagen). Full-length cDNAs for Nkx2.1 (from John Rubenstein (UCSF, San Francisco, CA) and Oscar Marín (Universidad Miguel Hernández, Alicante, Spain)) and Lhx6 (from Vassilis Pachnis, MRC, London, UK) were cloned into pCAG-IREs-GFP (from Connie Cepko, Harvard Medical College, Boston, MA) and their expression confirmed by immunofluorescence (see Fig. 1 for Nkx2.1). VP16-Nkx2.1 is a gift from Parvis Minoo (Li et al., 2002). The Nkx2.1 homeodomain point mutant (Val45Phe) (Krude et al., 2002) was generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and subcloned into the pCAGGS vector to produce pNkx2.1HD.

To generate the Lhx6 RNAi vector, a sequence encoding a small hairpin (sh) targeting the distal exon of 2s (sense strand, 5′-GTCAGAGCAGGAAGCTGGCTGGGAAATCATG ATTGCTGAGGGA-3′; antisense strand, 5′-gattAAAAATGACGCGAGGGCC-3′) was inserted into the Apal and HindIII sites in pSilencer 1.0 (a gift from Yang Shi) (Sui et al., 2002). This shRNAi species has previously been demonstrated to greatly reduce LHX6 abundance in MGE-derived cells and to reduce interneuron migration to the cortex (Alifragis et al., 2004). To enable visualization of the RNAi-transfected cells with a single colour (blue), the pL6-shLhx6 construct was then inserted into the SpeI site in pCAG-IREs-GFP (shLhx6-GFP). The Scramble RNAi sequence (Ambion) was also subcloned into the same site in the pCAG-IREs-GFP vector. To ensure that nearly all Nkx2.1-transfected cells also received the RNAi vector, 2 mg/ml of this vector and 1 mg/ml of pNkx2.1 were microinjected into the slice for EP. This procedure results in over 90% co-labeling of Nkx2.1 and GFP in transfected cells (data not shown) (see also Stuhmer et al., 2002).

For transplantation studies, 12–16 hours after EP GFP epifluorescence was imaged, then the regions of the MGE with the highest densities of GFP+ cells were dissected out, mechanically dissociated, and counted on a hemocytometer under epifluorescence to calculate the percentage of all cells that were GFP+ (usually 10–15%). In the case of slices from Nkx2.1−/− embryos, in which a morphologically identifiable MGE does not exist (Sussel et al., 1999), tissue was targeted for EP and dissection from the same approximate dorsal-ventral level as the MGE of control slices, as described (Xu et al., 2004).

For in vitro transplants, the feeder cells were prepared from neonatal cortex as described (Xu et al., 2004; Xu et al., 2005) and the transfected cells were plated at a density of 1000 GFP+ cells per well of a 16-well glass chamber slide (36 mm2; Lab-Tek). For in vivo transplants, cells were injected into S1 cortex 1 mm below the pial surface of cold-anesthetized neonatal pups (day of birth or P1), using a microinjector (Nanoinject II, Drummond). Per injection site, 10,000–20,000 cells were placed per hemisphere to obtain at least 1000 GFP+ cells per transplant. At P30 the brains were removed, fixed with 4% paraformaldehyde, and sectioned on a vibratome at 50 μm.

RT-PCR
Slices from Nkx2.1 nulls were electroporated with pNkx2.1/IREs-GFP or pGFP control (n=3). After 12 hours the MGE-like regions were dissociated and sorted by FACS (Vantage, Becton-Dickinson) producing a yield of 3000–5000 cells. Total RNA was purified (RNeasy Kit, Qiagen) and subjected to reverse transcription (Omniscript reverse transcriptase, Qiagen) and PCR (HotStar Kit, Qiagen). Primers included Lhx6 (5′-TGATTGCCAGGCCCAGCAG and 5′-GTCCACTCTTGACAGTACAT; 422 bp product), Nkx2.1 (5′-AACAGCGGCATGACGAGCAG and 5′-CAATTTCTTGTCACGCTCC; 315 bp) and β-actin (5′-GAGCTGCGCTAGCGCCAGG and 5′-TACTCTGCTTACGATC; 364 bp).

Immunodetection
Immunofluorescence labeling of cells in dissociated cultures was conducted as described (Xu et al., 2004), and labeling of antigens in postnatal brain sections was conducted floating. Primary antibodies used included GFP (rabbit or chick; Molecular Probes), GABA (rabbit), LHX6 (rabbit, a gift from Vassilis Pachnis (Lavdas et al., 1990)), neuropeptide Y (rabbit; rabbit; Sigma), Immunostar, NKX2.1 (mouse; Lab Vision), somatostatin (rat; Chemicon), parvalbumin (mouse; Chemicon), calretinin (rabbit; Chemicon), Kv3.1 (rabbit, a gift from Bernardo Rudy, New York University, NY) and PCNA (mouse IgG; Novocastra). Alexa line secondary antibodies (Molecular Probes) were used.

Lhx6 promoter analyses
Phylogenetic sequence comparisons of the Lhx6 locus were performed using the ECR browser (http://www.dcode.org) sequence alignment and visualization tool (Ovcharenko et al., 2004b).

To generate Lhx6 reporter constructs, a 2.1 kb fragment of Lhx6 genomic region (Fig. 5) was cloned from BAC RP23-D116 by PCR (5′-ACTAGT(SpeI)CACGCTTCTTGAAGCTTGTG and 5′-TCTAGA(XbaI)-CCCTGCTGGCCCAT). This fragment was inserted in place of the CAG promoter in pCAG-IREs-GFP to produce pS-Lhx6-IREs-GFP. Site directed mutagenesis (using the oligo sequence 5′-CCCTTCTCCCGACCTTACACC-3′) was then used to remove the putative NKX2.1 binding domain (GCTCTTGAAGCTTGTG) from 239–250 nt.

RESULTS

Expression of Nkx2.1 cDNA in Nkx2.1−/− slices rescues interneuron fate

Our previous work has shown that progenitors from the MGE-like region of Nkx2.1−/− slices (MGE*, Fig. 1B,G; see Fig. S2 in the supplementary material; Materials and methods), cultured on dissociated cells from neonatal cortex, fail to differentiate into the SST- or PV-expressing interneuron phenotype (Xu et al., 2004). To determine whether rescued expression of Nkx2.1−/− in the ventral telencephalon of slices from Nkx2.1−/− mutants can rescue the PV or SST fate of these cells, telencephalic slices were prepared from Nkx2.1−/− mouse embryos at embryonic day (E) 12.5, and an expression vector, pNkx2.1-GFP, was introduced into the MGE* by electroporation (Fig. 1 and see Fig. S2 in the supplementary material). After 1 day in vitro (DIV), subregions of the MGE* with the highest proportion of transfected cells were dissected, dissociated, plated over a feeder culture made from neonatal cortex and maintained for 14-28DIV (see Fig. S2 in the supplementary material). In other experiments, cells were transplanted directly into the cortical plate of the somatosensory cortex of neonatal pups and then examined in tissue sections after 30 days (Fig. 1). After fixation, the fate of cells with neuronal morphology (the vast
Fig. 1. Transfection of Nkx2.1+/– slices with Nkx2.1 cDNA results in rescue of interneuron phenotypes. (A–D) Schematic showing slice electroporation and transplantation paradigm. (A) The Nkx2.1 domain is shown in the MGE of an E12.5 wild-type (wt) mouse embryo slice. The MGE*, a region that expresses a truncated Nkx2.1 transcript (Sussel et al., 1999), is shown in the slice from an Nkx2.1 mutant embryo. (B) This MGE* region is targeted for electroporation, and after 1 day in vitro (DIV) the region is dissected out, dissociated and transplanted (C,D) directly into the neocortex of neonatal pups (as in H–N), or plated onto a high-density culture of neonatal cortical cells (as in Xu et al., 2004); see Fig. S2 in the supplementary material). (E,F) Coronal sections of a slice from an E12.5 wt embryo that was electroporated with pNkx2.1-GFP, maintained 1DIV, then fixed and examined for GFP fluorescence (E) and NKX2.1 immunolabeling (F). The right-hand, electroporated side of the slice has extensive ectopic NKKX2.1 expression, whereas only native NKX2.1 expression is seen on the left-hand side of the slice (arrow in F). (G) A slice from an Nkx2.1+/– embryo was electroporated with pNkx2.1-GFP. After 1DIV, cells from the MGE* (outlined in white) were transplanted into the cortical plate of a neonatal pup and then examined at postnatal day 30 (P30) in 40 μm coronal sections. (H) Transplanted GFP-expressing cells scattered through the medial cortex. (I–N) Examples of co-labeling for GFP and parvalbumin (PV; I,J), somatostatin (SST; K,L), and neuropeptide Y (NPY; M,N). In control experiments with pGFP vector, almost no cells expressing any of these markers are detected after transplantation of Nkx2.1+/– MGE* progenitors (Table 1). MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence; Ctx, cerebral cortex. Scale bars: 100 μm in G,H.

Nkx2.1 controls interneuron fate via Lhx6

The ability of Nkx2.1 expression to rescue neurochemical aspects of the Nkx2.1+/– interneuron phenotype provides an opportunity to identify downstream effectors of cortical interneuron fate determination. A lead candidate for such an effector is LHX6, a LIM-homeodomain-containing transcription factor that is expressed in most MGE-derived interneurons from around the time of their final cell cycle through their maturity in the postnatal cerebral cortex (Cobos et al., 2005; Fogarty et al., 2007; Gong et al., 2003; Lavdas et al., 1999; Liodis et al., 2007) (see Fig. S1 in the supplementary material); LHX6 is not detectable in the MGE* of Nkx2.1–null embryos (Sussel et al., 1999). Indeed, RT-PCR detected the induction of Lhx6 expression in the MGE* of Nkx2.1–null embryos transfected with Nkx2.1 (Fig. 2A).

To further confirm that Lhx6 can be induced in Nkx2.1+/– MGE* progenitors after transfection with pNkx2.1-GFP, rescue- and control-transfected neurons were assessed after in vivo

majority of surviving cells from donors at this age of E12.5+1DIV) were determined by immunofluorescence for GFP and the given interneuron subgroup marker. Similar transplantations of cells directly from the MGE of transgenic mice into neonatal cortex have been shown to give rise to neurons with neurochemical and physiological characteristics of inhibitory interneurons (Alvarez-Dolado et al., 2006; Cobos et al., 2005).

Consistent with previous results using in vitro transplants from Nkx2.1 nulls (Xu et al., 2004), Nkx2.1+/– cells transfected with control vector almost never gave rise to PV+, SST+ or NPY+ interneurons (Table 1). In marked contrast, transfection with Nkx2.1 cDNA resulted in substantial rescue of PV or SST expression (Table 1, Fig. 1I–L). In addition, neurons expressing NPY, a neuropeptide that shares about 50% co-labeling with SST in mouse cortex, were also rescued (Fig. 1M,N). Similar results were obtained whether cells were plated onto a cortical feeder layer or transplanted into the neonatal cortical plate in vivo (Table 1, Fig. 1 and see Fig. S2 in the supplementary material).

Lhx6 is induced when Nkx2.1 is expressed in the Nkx2.1+/– MGE*

Table 1. Transfection and transplantation of the MGE-like region in Nkx2.1+/– slices with Nkx2.1 or Lhx6 cDNAs rescues the expression of MGE-derived interneuron markers

<table>
<thead>
<tr>
<th>cDNA, transplanation</th>
<th>PV (%)</th>
<th>SST (%)</th>
<th>NPY (%)</th>
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<tbody>
<tr>
<td>Control (n=4)</td>
<td>0.4±0.2% (272)</td>
<td>17.0±3.1% (177)</td>
<td>0.6±0.5% (895)</td>
</tr>
<tr>
<td>Control (n=3)</td>
<td>0.0±0.0% (134)</td>
<td>12.7±4.0% (157)</td>
<td>0.0±0.0% (161)</td>
</tr>
<tr>
<td>Control (n=3)</td>
<td>0.0±0.0% (167)</td>
<td>17.5±3.5% (464)</td>
<td>0.0±0.0% (159)</td>
</tr>
<tr>
<td>Control (n=3)</td>
<td>0.0±0.0% (282)</td>
<td>17.0±3.1% (177)</td>
<td>0.6±0.5% (895)</td>
</tr>
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</table>

The numbers refer to the percentage of transplanted cells with neuronal morphologies that co-label with the given marker (with total counted neurons shown in parentheses).
transplantation by immunofluorescence labeling with an LHX6-specific polyclonal antibody (Lavadas et al., 1999; Liodis et al., 2007). LHX6 protein was detectable in more than half (54/93=58%, n=3) of the Nkx2.1–/– cells transfected with the Nkx2.1 cDNA. Nearly all of those rescued for expression of PV or SST co-labeled with LHX6 (56/60=93%), whereas LHX6 was detectable in very few of those that received the control vector (3/90).

**Lhx6 induction is required for the rescue of the PV or SST phenotype by Nkx2.1**

The expression pattern of Lhx6 and its induction in the Nkx2.1 rescue paradigm raise the possibility that this gene functions in interneuron fate determination. A previous loss-of-function study of Lhx6 by small hairpin RNA interference (shRNAi) in slice cultures found that Lhx6 expression is required for normal interneuron migration to the cortex, but not for the expression of GABA (Alifragis et al., 2004). This result has recently been confirmed and extended to the fate determination of PV- and SST-expressing interneuron subgroups in cortex by loss-of-function analysis (Liodis et al., 2007). To determine whether the induction of Lhx6 is required downstream of Nkx2.1 in the specification of cortical interneurons, we conducted loss-of-function studies using the Nkx2.1 rescue/transplantation paradigm. The same pU6-shRNAi sequence used by Alifragis and colleagues was cloned into a vector that co-labels in the mouse cortex co-label for LHX6. (D) Quantification of the effect of shLhx6 RNAi on the Nkx2.1 rescue of PV+ and SST+ interneuron fate (n=3 donor samples for each condition transplanted into separate pups, Student’s t-test, **p<0.01, *p<0.03).**

**Lhx6 expression also rescues the interneuron fate defect of the Nkx2.1–/– MGE**

The ability of Lhx6 shRNAi to block the Nkx2.1 rescue experiment raises the possibility that Lhx6 functions downstream of Nkx2.1 in the specification of cortical interneurons. To determine whether Lhx6 can rescue the Nkx2.1–/– interneuron defect, full-length Lhx6 CDNA was cloned into the pCAG-IRESCFP vector. When expressed within the MGE* of Nkx2.1–/– slices, pLhx6-GFP also lead to a substantial rescue of PV-, SST- and NPY-expressing cells (Fig. 3, Table 1). In those cells expressing PV, roughly 95% also express the potassium channel Kv3.1 (also known as KCNC1 – Mouse Genome Informatics) (Fig. 3A-D) that is closely associated with PV expression in cortical interneurons (Weiser et al., 1995). Like PV, Kv3.1 was very rarely expressed by control-transfected Nkx2.1+/– cells from the MGE* (1/57=1.8%, from three transplantsations).

To determine whether, in addition to these neurochemical markers, morphological aspects of interneuron differentiation were rescued in this paradigm, Nkx2.1–/– progenitors from the MGE* that were transfected with Lhx6 or control vector were examined for the presence of dendritic spines. Since cortical interneurons are generally smooth or sparsely spiny, the frequency of heavily spiny versus aspiny or sparsely spiny neurons can provide a measure of whether morphological as well as neurochemical characteristics of Nkx2.1–/– MGE*-derived interneurons are rescued by Lhx6. Indeed, Nkx2.1–/– progenitors from the MGE* that were transfected with Lhx6 had a significantly lower likelihood than control-transfected cells of being heavily spiny (42% of GFP+ cells in control transfections, 25% in Lhx6 transfections, P<0.03; Fig. 3L-K). Of Lhx6-transfected Nkx2.1–/– cells that expressed PV or SST, nearly all (70/72 examined) had a non- or sparsely spiny morphology.

The large majority of Lhx6-transfected Nkx2.1–/– cells also expressed detectable levels of GABA (data not shown), but the interpretation of this result is complicated by the fact that GABA is also expressed by most subcortical projection neurons and is not reduced in the pallidum of Nkx2.1 mutants (Sussel et al., 1999), in pallidal cells transfected by RNAi for Lhx6 (Alifragis et al., 2004), or in the cortex of Lhx6-null mutants (Liodis et al., 2007).
Fig. 3. Lhx6 expression can rescue interneuron phenotypes in transplanted cells from Nkx2.1−/− MGE*. pLhx6-GFP was electroporated into the MGE-like region of E12.5 Nkx2.1−/− slices, then after 1DIV the transfected regions were dissociated and transplanted into the cortex of neonatal pups. Shown are coronal sections through a P30 mouse that had received the transplantation into the cortical plate at P1. (A–H) Examples of co-labeling for GFP together with Kv3.1 and parvalbumin (PV; A–D), somatostatin (SST; E,F), and neuropeptide Y (NPY; G,H). In control experiments with pGFP vector, few cells expressing these markers are detected after transplantation of Nkx2.1−/− MGE-like progenitors (see text and Table 1). (I,J) Transfected neurons (I, pGFP control; j, pLhx6-GFP) photographed at higher magnification to reveal dendritic spines. Insets show the boxed regions at higher magnification. (K) The frequency of heavily spiny neurons is significantly lower in the Nkx2.1−/− MGE* cells transplanted with Lhx6 than in controls (41.9% versus 24.7%, n=3, *P<0.03). In addition, those Nkx2.1−/− cells ‘rescued’ for expression of PV or SST by Lhx6 are nearly all non- or sparsely spiny. These results suggest that Lhx6 can act downstream of Nkx2.1 to direct some aspects of both the neurochemical and morphological fates of MGE-derived cortical interneurons.

summary, these results suggest that Lhx6 directs both neurochemical and morphological aspects of MGE-derived interneuron fate, independently of the expression of GABA.

Nkx2.1 appears to directly activate Lhx6 expression in the MGE

The requirement for Nkx2.1 for expression of Lhx6 (Sussel et al., 1999), and the induction of Lhx6 in the Nkx2.1 rescue experiment (Fig. 2), raise the question of whether Nkx2.1 directly activates Lhx6 expression. Whereas Nkx2.2 regulates neuronal fate in the ventral spinal cord by transcriptional repression (Muhr et al., 2001), Nkx2.1 is known to directly activate the transcription of target genes in the lung and thyroid (Li et al., 2000; Mizuno et al., 1991; Tell et al., 1998). To examine this issue we first compared the mouse, human, chicken, fugu and frog sequences over 1991; Tell et al., 1998). To examine this issue we first compared the mouse, human, chicken, fugu and frog sequences over.

To determine whether Nkx2.1 binds this region, chromatin immunoprecipitation was conducted on lysates of MGE from E12.5 embryos. PCR on the DNA pulled down using an anti-NKX2.1 monoclonal antibody (see Materials and methods) indicated that a 119 bp fragment including the above sequence appears to bind NKX2.1 in vivo (Fig. 4C).

To determine whether this sequence promotes the transcription of Lhx6 within the Nkx2.1 expression domain, an IRES-GFP construct was cloned into the 3′ end of a 2.1 kb fragment of the Lhx6 promoter (p5′-Lhx6-GFP). Electroporation (EP) of this construct into the MGE of E13.5 slices resulted in robust expression of GFP (Fig. 5A–C; n=5). By contrast, little expression was apparent upon EP into either the dorsal midline of wild-type embryos (Fig. 5A–C; n=5), or into the lateral ganglionic eminence (LGE) or cortex (Fig. 6A–C). Consistent with the requirement for Nkx2.1 to drive the expression of Lhx6, no GFP expression was seen after EP into the MGE-like region of Nkx2.1−/− slices (Fig. 5D–F; n=5). However, Co-EP of p5′-Lhx6-GFP together with an Nkx2.1 expression vector restored GFP expression in the MGE-like region of Nkx2.1−/− slices (Fig. 5G–I; n=5). Similarly, Co-EP of p5′-Lhx6-GFP together with an Nkx2.1 expression vector was able to drive p5′-Lhx6-GFP expression in the LGE and cortex of wild-type slices (Fig. 6D–F; n=5).

The above results suggest that expression of this Lhx6 promoter fragment in the telencephalon requires the presence of Nkx2.1. To determine whether the Nkx2.1 binding sequence in the promoter fragment of p5′-Lhx6-GFP is necessary for Lhx6 expression within the MGE, this sequence was removed from the reporter construct, generating pΔ5′-Lhx6-GFP. EP of pΔ5′-Lhx6-GFP into the MGE of wild-type embryos resulted in very limited expression of GFP (Fig. 5J–L; n=5). Since ectopic expression of Nkx2.1 was able to drive expression of pLhx6-GFP in the LGE and cortex (Fig. 6D–F), and this effect was nearly eliminated when the Nkx2.1 consensus binding sequence is removed from the reporter construct (Fig. 6J–L), we next tested whether the DNA-binding region of Nkx2.1 is required for this effect. A point mutation that is associated with a hereditary movement disorder in humans (Krude et al., 2002), resulting in a Val45Phe alteration in the homeodomain, was introduced into the Nkx2.1 expression vector (pNkx2.1△HD). This mutation greatly reduced the ability of Nkx2.1 to bind to its consensus target sequence. Co-EP of pNkx2.1△HD together with pLhx6-GFP into the LGE resulted in minimal activation of the reporter (Fig. 6, compare G–I with D–F). Finally, a vector containing the VP16 transcriptional activation sequence fused to Nkx2.1 was tested (VP16Nkx2.1) (Li et al., 2002). VP16Nkx2.1 strongly
activated the pLhx6-GFP reporter, suggesting that NKX2.1 does not indirectly activate Lhx6 transcription by repressing the expression of an intermediate gene.

**DISCUSSION**

Despite its likely relevance to neuropsychiatric disease, progress in revealing the molecular control of cell fate determination in the mammalian telencephalon has been slow. In this paper we demonstrate that the homeodomain transcription factor NKX2.1 acts to specify neurochemical and morphological aspects of cortical/ striatal interneuron fate by directly activating the LIM-homeodomain gene Lhx6. The combined use of slice EP and transplantation of transfected cells should be applicable to the study of other characteristics of interneuron subgroups, and possibly to other neurons whose defining characteristics are achieved long after crucial fate-determining events have occurred during embryogenesis.

NKx2.1–null mice fail to generate normal MGE tissue (Sussel et al., 1999) and are unable to generate cortical interneurons expressing PV or SST (Xu et al., 2004), distinct subgroups that are known to originate primarily from the MGE (Wonders and Anderson, 2006). By transfecting NKx2.1 back into the MGE-like region of NKx2.1−/− slices, cutting the slice for 24 hours and then transplanting the transfected cells into the cortex of neonatal pups, both the PV and the SST phenotypes can be rescued in vivo (Fig. 1, Table 1). Parallel experiments in which the NKx2.1-transfected cells are cultured on a feeder layer of dissociated neonatal cortex also show substantial rescue of these phenotypes, whereas the expression of these phenotypes in NKx2.1−/− null cells transfected with the control plasmid is almost non-existent (Table 1; see Fig. S2 in the supplementary material).

**Lhx6 specifies interneuron fates downstream of NKx2.1**

The expression patterns of Nkx2.1 and Lhx6 (see Fig. S1 in the supplementary material), the loss of Lhx6 expression in Nkx2.1 nulls (Sussel et al., 1999), and the induction of Lhx6 by Nkx2.1 (Figs 2, 5, 6), raise the possibility that Lhx6 functions directly downstream of Nkx2.1 in the specification of PV and SST interneuron fate. Co-transfection of the Nkx2.1−/− MGE-like region in slices with expression vectors for both Nkx2.1 and pLhx6-shRNAi produces a dramatic reduction in the frequency of PV+ and SST+ phenotypes (Fig. 2). This result suggests that Lhx6 expression is required for the acquisition of these phenotypes, a notion that is strongly supported by the cortical interneuron phenotype observed in Lhx6 nulls in which GABA expression in cortex is grossly normal but the number of PV- or SST-expressing interneurons is very dramatically reduced (Liodis et al., 2007).

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In addition to the neurochemical phenotype, two additional lines of evidence suggest that the rescued cells are interneuron-like. First, they display morphological features of interneurons, including curved terminals characteristic of large PV+ and smaller SST+ basket cells, and aspiny or sparsely spiny dendrites. Second, more than 90% of the Nkx2.1−/− neurons rescued for the PV or SST phenotype are immunopositive for LHX6, a transcription factor expressed in most MGE-derived interneurons of the striatum and cortex from around the time that they exit the cell cycle through maturity (Cobos et al., 2005; Fogarty et al., 2007; Gong et al., 2003; Lavdas et al., 1999; Liodis et al., 2007) (see Fig. S1 in the supplementary material).
NKX2.1 controls interneuron fate via Lhx6

The loss-of-function evidence raises the question of whether Lhx6 is not only required for acquisition of the PV+ and SST+ phenotypes, but is also sufficient to restore this phenotype in the Nkx2.1-null context. Indeed, expression of Lhx6 cDNA within the MGE-like region of Nkx2.1 nulls also results in a substantial rescue beyond PV and SST because most of the ‘rescued’ PV-expressing cells also express the Kv3.1 potassium channel. In addition, in contrast to controls, Lhx6-rescued PV- and SST-expressing cells are nearly all aspiny or sparsely spiny, suggesting that Lhx6 promotes both morphological and neurochemical aspects of interneuron fate (Fig. 3). The control of multiple aspects of MGE-derived interneuron characteristics suggests that Lhx6 might function to drive multiple transcriptional cascades to direct the specification of several subgroups of this telencephalic neuronal subclass.

**NKX2.1 appears to directly activate Lhx6 expression**

The results presented above suggest that NKX2.1 functions to specify interneuron subgroups in the MGE largely or entirely by activating Lhx6 transcription. Unlike the prominent repressor functions of other Nkx family members in directing cell fate within the ventral spinal cord (Vallstedt et al., 2001), NKX2.1 directly activates target genes in the thyroid and lung (Liu et al., 2002; Mizuno et al., 1991; Moya et al., 2006), although these targets are not known to include LIM-homeodomain transcription factors. Comparative genomic sequence and transcription factor binding site analyses reveal a highly conserved consensus NKX2.1 binding sequence about 240 bp from the Lhx6 translation initiation site (Fig. 4), and this site is present in a 119 bp fragment identified by chromatin immunoprecipitation. In addition, a 2.1 kb fragment upstream of the translation initiation site drives expression of GFP reporter specifically in Nkx2.1-expressing regions of wild-type slices (Figs 5, 6). This expression is lost in Nkx2.1-null slices, can be rescued by exogenous addition of Nkx2.1 to the Nkx2.1-null slices, and is abolished when the Nkx2.1 binding sequence is removed from the reporter (Fig. 5). Moreover, the ability of ectopic Nkx2.1 to drive the Lhx6 reporter construct in the LGE or cortex (Fig. 6) is abolished by a point mutation in the homeodomain that has previously been shown to greatly diminish the ability of NKX2.1 to bind its target DNA sequence (Krude et al., 2002). Although establishment of the definitive role played by the identified NKX2.1 binding sequence requires in vivo confirmation, taken together these results suggest that NKX2.1 drives specification of the SST+ and PV+ phenotypes via the direct activation of Lhx6.

Role of Lhx6 in cortical interneuron specification

Interestingly, ectopic expression of either Lhx6 or Nkx2.1 in the ventral half of the E12.5 LGE, which normally gives rise primarily to medium spiny neurons of the striatum (Stenman et al., 2003), does not produce PV+ or SST+ neurons (T.D. and S.A.A., unpublished). This result suggests that, consistent with the residual expression of a truncated Nkx2.1 transcript within the MGE-like region of Nkx2.1 nulls (Sussel et al., 1999), the MGE* is molecularly distinct from the LGE proper despite the presence of ventricular zone, subventricular
zone and mantle zone gene expression that is normally restricted to
the LGE (Sussel et al., 1999). The absence or presence of such a
factor would supply competence to attain a PV+ or SST+ phenotype
in response to Lhx6 expression despite the absence of Nkx2.1.

Although these results indicate that Nkx2.1 function, as it pertains
to some crucial aspects of cortical interneuron specification, acts via
the activation of Lhx6, they do not address the extent to which other
aspects of Nkx2.1 function in the MGE depend on Lhx6. For
example, Nkx2.1 is also required for the expression of Lhx7 (also
known as Lhx8 – Mouse Genome Informatics) (Sussel et al., 1999),
and both genes are required for the specification of most cholinergic
neurons of the basal forebrain (Fragkouli et al., 2005; Marin et al.,
2000; Sussel et al., 1999; Zhao et al., 2003). However, although the
MGE or the underlying preoptic/anterior endopeduncular region
gives rise to cholinergic interneurons of the striatum (Marin et al.,
2000), cholinergic phenotypes are not seen in transplants of these
regions into cortex (T.D. and S.A.A., unpublished results), such that
a role for Lhx6 in the specification of these cells was not tested in
this study. Interestingly, although essentially all cholinergic
interneurons of the striatum derive from Nkx2.1-expressing
progenitors, a bipolar-morphology Nkx2.1-lineage-negative
cholinergic interneuron has recently been described in mouse
neocortex (Xu et al., 2008).

Our previous work showed that sonic hedgehog signaling during
the age range of neurogenesis is required to maintain Nkx2.1
expression within, and interneuron generation by, cycling
progenitors of the MGE (Xu et al., 2005). This paper extends that
work in suggesting that Nkx2.1 specifies PV+ or SST+ interneuron
subgroups and other neurochemical, as well as morphological,
aspects of MGE-derived interneuron fates by directly activating
Lhx6. Several lines of evidence suggest that progenitors giving rise
to these subgroups might be partially segregated on the dorsal-
ventral axis of the MGE (Flames et al., 2007; Fogarty et al., 2007;
Ghanem et al., 2007; Wonders et al., 2008). As Lhx6 itself does not
appear to be differentially expressed along the dorsal-ventral axis of
the MGE, a key remaining question is how Lhx6 function is
modified to differentially specify the MGE-derived interneuron
subgroups of the cerebral cortex.

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Supplementary material
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