Cellular and Molecular Mechanisms Controlling the Migration of Telencephalic Interneurons:

A Role for Postmitotic Nkx2-1 in Neuronal Migration

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Abstract

The homeodomain transcription factor Nkx2-1 plays fundamental roles in the development of the ventral subpallium. As for the majority of NK-2 class of transcription factors, Nkx2-1 function mainly in controlling cell-fate decisions in progenitor cells, such as interpreting Sonic Hedgehog (Shh) graded signalling in early patterning events and conferring subtype-specific properties to medial ganglionic eminence (MGE) and preoptic area (POA) precursors. During development, the MGE is simultaneously the source for several telencephalic cell types, such as cortical and striatal interneurons, but interestingly, the expression of Nkx2-1 is only maintained in the striatal population. This evidence suggests a possible function for Nkx2-1 in controlling the migration and/or differentiation of postmitotic striatal interneurons; however the contribution of this transcription factor at this level has not been characterized.

Using experimental manipulations and mouse genetics, we demonstrated a new postmitotic function for the cell-fate determinant Nkx2-1 in controlling neuronal migration in the developing telencephalon. Downregulation of Nkx2-1 expression in MGE-derived postmitotic cells is necessary for the migration of interneurons to the cortex, whereas maintenance of Nkx2-1 expression is required for interneuron migration to the striatum. The sorting of MGE-derived cortical and striatal interneurons is mediated by the differential expression of receptors for the class 3 semaphorins, neuropilins, which are expressed only by cortical migrating interneurons in order to prevent their accumulation in the developing striatum. We showed that Nkx2-1 regulates the segregation of MGE-derived interneurons by controlling the neuropilin/semaphorin interactions; Nkx2-1 overexpression leaves migrating interneurons insensible to a source of Sema3A/3F and reduces the expression of *Neuropilin-2* (*Nrp2*), the binding receptor for Sema3F. Furthermore, Nkx2-1 exerts this role by direct binding to *Nrp2* regulatory elements in MGE cells and interaction with this sequence is sufficient to repress transcription *in vitro* through a mechanism that requires the Nkx2-1 homeodomain (HD) motif. These results demonstrate that Nkx2-1 postmitotic expression controls the migration of MGE-derived interneurons by direct repression of the *Nrp2* guidance receptor, a transcriptional strategy of guidance selectivity operating in many other migrating neurons.

Furthermore, we provide evidence that, apart from the neuropilin/semaphorin interactions, the precise migration of interneurons to the developing striatum is regulated by additional guidance systems. We demonstrated that a population of MGE-derived interneurons co-expresses Nkx2-1 and ErbB4, and relies in an ErbB4-dependent signalling to specifically accumulate in the striatum. Furthermore, *Nkx2-1*-expressing interneurons are actively prevented from invading the developing cortex by a yet unidentified chemorepulsive activity and we believe that the cooperative action of

these signalling systems will ultimately determine the directionality of striatal migrating interneurons. In addition, these results suggest that postmitotic Nkx2-1 is a fundamental factor in conferring guidance specificity to striatal migrating interneurons and open the possibility for additional downstream target effector genes.

Sumário

O factor de transcrição Nkx2-1 desempenha funções fundamentais durante o desenvolvimento do telencéfalo ventral. Como a maior parte dos factores de transcrição pertencentes à classe NK-2, o Nkx2-1 controla processos de decisão e especificação celular em células progenitoras. Em particular, é responsável por interpretar a via de sinalização do morfogénio "Sonic Hedgehog" (Shh) e pela indução de várias propriedades que conferem identidade às células progenitoras da eminência ganglionar medial ("MGE") e da área pré-óptica anterior ("POA"). Durante o desenvolvimento, as células progenitoras da eminência ganglionar medial originam simultaneamente diversos tipos de neurónios, como os interneurónios do córtex e do estriado e, curiosamente, a expressão deste factor de transcrição mantém-se apenas na população diferenciada de interneurónios do estriado. Esta evidência sugere que o Nkx2-1 poderá controlar a migração ou diferenciação dos interneurónios do estriado; contudo, a contribuição deste factor de transcrição para estes processos não estava caracterizada.

Neste estudo identificámos uma nova função para o factor de transcrição Nkx2-1 no controlo da migração de interneurónios durante o desenvolvimento embrionário do telencéfalo. A realização de estudos funcionais e o uso de murganhos transgénicos permitiu-nos demonstrar que os interneurónios corticais necessitam de deixar de expressar Nkx2-1 para migrarem para o córtex e, por outro lado, a migração dos interneurónios do estriado requer a expressão contínua deste factor de transcrição. A segregação destas duas populações de interneurónios originados na "MGE" tinha sido previamente atribuída à expressão diferencial de receptores para as semaforinas da classe 3, moléculas repulsivas existentes no estriado. Estes receptores, as neuropilinas, expressa-se apenas pelos interneurónios corticais em migração e previnem a sua entrada no estriado. Os nossos resultados permitiram-nos inferir que Nkx2-1 regula a segregação destas populações de interneurónios através do controlo das interacções neuropilina/semaforina; a sobre-expressão de Nkx2-1 tornou os interneurónios derivados da MGE insensíveis a uma fonte externa de semaforinas e reduziu a expressão de *Neuropilina-2* (*Nrp2*), o receptor de ligação para a semaforina 3F. Nkx2-1 exerce esta função por união directa a elementos reguladores do gene de *Nrp2* em células da "MGE" e a interacção com esta sequência é suficiente para reprimir a transcrição *in vitro* por um mecanismo que envolve o homeodomínio (HD) de Nkx2-1. Estes resultados demonstram que Nkx2-1 controla a migração de interneurónios provenientes da "MGE" através de repressão directa do receptor *Nrp*2, uma estratégia de controlo de direcção de movimento adoptada por vários tipos de neurónios em migração.

O trabalho apresentado nesta dissertação sugere que, para além das interacções neuropilina/semaforina, a migração de interneurónios do estriado é regulada por outros sistemas

de sinalização. Observámos que uma população de interneurónios originados na “MGE” expressa o factor de transcrição Nkx2-1 e o receptor ErbB4, e que estes interneurónios usam uma via de sinalização dependente de ErbB4 para migrar para o estriado. Estudos funcionais indicaram também que a sobre-expressão de Nkx2-1 incapacita os interneurónios de invadirem o córtex, possivelmente devido à presença de uma actividade repulsiva nesta região cuja identidade é desconhecida. Estes resultados permitem concluir que a direcção de migração dos interneurónios do estriado é regulada pela acção conjunta de várias vias de sinalização e que o factor de transcrição Nkx2-1 desempenha um papel fundamental no movimento dos interneurónios do estriado.

Abbreviations

Ach	**A**cetyl**ch**oline
AEP	**A**nterior **E**ntopeduncular **A**rea
AP	**A**ntero-**P**osterior
Arx	**A**ristaless **R**elated **H**omeobox
bap	**bagp**ipe
BDNF	**B**rain **D**erived **N**erve **F**actor
bHLH	**b**asic **H**elix-**L**oop-**H**elix
BrdU	**Br**omo**d**eoxy**u**ridine
C	**C**arboxy
cDNA	**c**omplementary **D**eoxyribo**n**ucleic **A**cid
ChAT	**Ch**oline **A**cetyl**T**ransferase
ChIP	**Ch**romatin **I**mmuno**P**recipitation
CNS	**C**entral **N**ervous **S**ystem
CR	**C**al**r**etinin
CRD	**C**ysteine-**R**ich **D**omains
Cre	**Cre** Recombinase Enzyme
CXCL/R	**C**ysteine **X** **C**ysteine Chemokine **L**igand/**R**eceptor
DAPI	4',6-**Dia**midino-2-**P**henyl**i**ndole
Dcx	**D**ouble**c**orte**x** (the gene for Doublecortin)
Dlx	**D**istal-**L**ess Homeobo**x**
DNA	**D**eoxyribo**n**ucleic **A**cid
bp	**b**ase **p**air
DV	**D**orso-**V**entral
E	**E**mbryonic
ErbB	**Er**ythro**b**lastic Leukemia Viral Oncogene
EGF	**E**pidermal **G**rowth **F**actor
Eph	**Eph** receptor tyrosine kinase
EYFP	**E**nhanced **Y**ellow **F**luorescence **P**rotein
GABA	**G**amma-**A**mino**b**utyric **A**cid
GAD	**G**lutamic **A**cid **D**ecarboxylase
GAPDH	**G**lycer**a**ldehyde 3-**P**hosphate **D**e**h**ydrogenase
GDNF	**G**lial-**D**erived **N**eurothrophic **F**actor
GDP/GTP	**G**uanosine **D**i**p**hosphate/**T**ri**p**hosphate
GFP	**G**reen **F**luorescence **P**rotein
Gli3R	**Gli3** **R**epressor Form
Gro/TLE	**Gro**ucho/**T**ransducin-**L**ike **E**nhancer of Split
Gsh	**G**enomic **S**creened **H**omeobox
h/hr	**h**ou**r**

HEK	**H**uman **E**mbryonic **K**idney
HD	**H**omeo**d**omain
HGF	**H**epatocyte **G**rowth **F**actor
Hox	**Ho**meotic homeobo**x**
Ig	**I**mmuno**g**lobulin
Kb	**K**ilo **b**ase
Lhx	**L**IM **H**omeobo**x**
LGE	**L**ateral **G**anglionic **E**minence
LMC	**L**ateral **M**otor **C**olumn
Mash	**M**ammalian **A**chaete-**S**chute **H**omolog
MGE	**M**edial **G**anglionic **E**minence
µg	micro**g**ram
µl	micro**l**itre
µm	micro**m**etre
min	**min**ute
mm	**m**ili**m**etre
MN	**M**otor **N**eurons
mRNA	**m**essenger **R**ibo**n**ucleic **A**cid
N	Ami**n**o
n	**n**umber
NK	**N**irenberg **K**in
Nkx	**N**irenberg **K**in Homeobo**x**
Ngn	**N**euro**g**eni**n**
NPY	**N**euro**p**eptide **Y**
NRG	**N**eu**r**e**g**ulin
Nrp	**N**eu**r**o**p**ilin
Olig	**Olig**odendrocyte Lineage
p	**p**-value
P	**P**ostnatal day
PAK	**P**-21 **A**ctivated **K**inase
Pax	**Pa**ired-Bo**x**
PBS	**P**hosphate **B**uffer **S**aline
PCR	**P**olymerase **C**hain **R**eaction
PFA	**P**ara**f**orm**a**ldehyde
PI	**P**ropidium **I**odine
POA	Anterior **P**re**o**ptic **A**rea
PV	**P**ar**v**albumin
Rho	**R**as **Ho**molog
RNA	**R**ibo**n**ucleic **A**cid
Robo	**Ro**unda**bo**ut
RT	**R**everse **T**ranscription
SD	**S**pecific **D**omain
SEM	**S**tandard **E**rror of the **M**ean

Sema	**Sema**phorin
Shh	**S**onic **H**edge**h**og
SST	**S**omato**st**atin
SVZ	**S**ubventricular **Z**one
t	Student’s **t** test
tin	**tin**mamn
TN	**Tin**man
TTF	**T**hyroid **T**ranscription **F**actor
VIP	**V**asoactive **I**ntestinal **P**eptide
vnd	**v**entral **n**ervous system **d**efective
YFP	**Y**ellow **F**luorescence **P**rotein
VZ	**V**entricular**Z**one

Chapter 1. Introduction

The forebrain comprises an intricate set of structures that are required for some of the most complex and evolved functions of the mammalian brain including homeostasis, learning and behaviour. This complexity is in part achieved by an array of migratory movements that take place after initial specification of neuronal progenitors. Thus, several neuronal types are originated at relatively long distances from their final position within the forebrain and undergo extensive migratory programmes to reach their destiny. In the past, a lot of attention was devoted to the study of extrinsic molecules that shape the directionality of migrating neurons whereas nowadays the big challenge is to understand the cell-autonomous mechanisms that ultimately control the responsiveness of migrating cells to the environment. During central nervous system (CNS) development cell-fate decisions are mainly induced by the action of transcription factors, proteins that bind to promoter or enhancer regions[1] of the genome and interact to active or repress the transcription of a particular gene. Furthermore, the involvement of these proteins in controlling several aspects of the differentiation of committed neurons, such as neuronal migration and axon guidance (Guthrie, 2007; Polleux et al., 2007), is starting to emerge.

1.1. The NK-2 homeodomain transcription factors

There are several families of transcription factors grouped together by the structural similarities of their deoxyribonucleic acid (DNA)-binding sites and mechanism of action (Gilbert, 2003). The Homedomain gene superfamily encodes transcription regulatory proteins that act at critical points in development and ontogeny and are characterized by a 180-nucleotide sequence, the homeobox, encoding a structurally conserved DNA-binding motif known as the homeodomain (HD) (McGinnis and Krumlauf, 1992; Shashikant et al., 1991). The homeobox was originally described in *Drosophila Homeotic* (*Hox*) genes, which are critical in the establishment of body axes during embryogenesis. A remarkable number of homeobox genes have mammalian homologues with conserved developmental functions and biochemical properties (Banerjee-Basu and Baxevanis, 2001; Garber et al., 1983; Scott et al., 1983).

By screening a *Drosophila* DNA library with degenerated homeodomain oligonucleotides, Kim and Nirenberg identified in 1989 four new homeobox genes, which they termed *NK1* to *NK4* (Kim and

[1] Promoters are the sites where the Ribonucleic Acid (RNA) Polymerase binds to the Deoxyribonucleic acid (DNA) to initiate transcription and are typically located around 30 base pairs upstream from the transcription initiation site. Enhancers are DNA sequences that control the efficiency and rate of transcription by either activating or repressing (silencers) the utilization of the promoter. These sequences can be located at great distance and either upstream or downstream of the promoter.

Nirenberg, 1989). Burglin subsequently classified the encoded proteins into two homeodomain protein classes, NK-1 (containing the NK1 protein) and NK-2 (containing the NK2, NK3 and NK4 proteins) (Burglin, 1993). In the fly, *NK-2* genes control cell-type specification and morphogenesis of organs from all three germ layers and have become known by descriptors of their mutant phenotypes. For instance, *NK2-vnd* (*ventral nervous system defective*) controls the specification of the ventral neuronal tissue. *NK4/tin* (*tinmamn*) is implicated in the segregation of cardiac and visceral muscle potentiality in the nascent fly mesoderm, whereas *NK3-bap* (*bagpipe*) appears to be downstream of *tin* in the visceral lineage (Azpiazu and Frasch, 1993; Bodmer, 1993; Bodmer et al., 1990; Jiminez, 1995). Additional *NK-1* and *NK-2* genes have been isolated from diverse phyla and evolutionary duplication events have generated a number of *NK* genes belonging to different classes and subfamilies (Harvey, 1996). For instance, the mouse *Nkx5-1*, *Nkx5-2* and *Nkx5-3* genes (also named *H6 homeo box gene* [*Hmx*] *2, 3* and *4*, respectively) have no direct homologous in *Drosophila* and therefore constitute, most likely, a related but separated class (Bober et al., 1994; Yoshiura et al., 1998).

In vertebrates, *NK* genes have also been shown to participate in cell specification and morphogenetic events. *Nkx2-1* (also known as thyroid transcription factor-1 [TTF-1] and thyroid-specific enhancer binding protein [T/EBP]) was the first vertebrate *NK-2* member to be cloned and is expressed in the developing thyroid, lung and forebrain where it controls the transcription of tissue-specific genes (Guazzi et al., 1990; Lazzaro et al., 1991; Mizuno et al., 1991). The murine *Nkx2* genes can be subdivided according to the structural homology and particular amino acid groupings within the homeodomain of *NK-2* related genes. *Nkx2-1*, *Nkx2-2*, *Nkx2-4* and *Nkx2-9* form one subgroup that is similar to *NK2*. In contrast, Nkx2-3, *Nkx2-5* and *Nkx2-6* show high similarity to the Drosophila *NK3* and *NK4* genes (Figure 1), although it is not clear whether these differences reflect functional properties or merely distinctive evolutionary pathways (Harvey, 1996; Lints et al., 1993; Pabst et al., 1998). Significantly, members of the first group are mainly expressed in ectoderm- and endoderm-derived organs, including neural ectoderm, whereas members of the second group are predominantly expressed in mesodermally-derived organs.

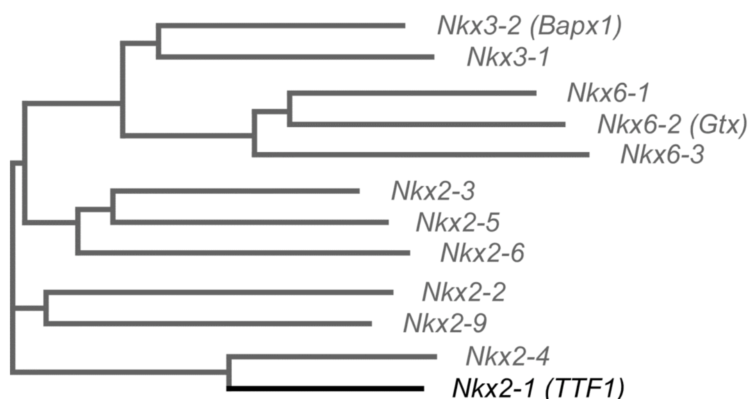

Figure1. Predicted evolutionary relationship between different mouse *Nkx* family members. The mouse *Nkx2-2*, *2-9*, *2-4* and *2-1* present higher homology to the *Drosophila NK-2* homeobox whereas the mouse *Nkx2-3*, *2-5* and *2-6* are homologous to the *NK3* and *NK4* genes. Members of the *Nkx6* subfamily show a closer relationship to the *Drosophila NK3*-related *NK*3 murine group. *NK-1* and *Nkx5* members are not depicted (Dendogram adapted from Alanentalo et al., 2006).

Mouse *NK-2* homologues seem to play similar roles than their *Drosophila* counterparts. For instance, both *vnd* and the murine *Nkx2-1* and *Nkx2-2* homologues control ventral nervous system specification, whereas *Nkx2-5*, the presumably mouse homologue to *Drosophila NK4*, is expressed in heart progenitor cells and controls the development of the mammalian heart (Briscoe et al., 1999; Harvey, 1996; Jiminez, 1995; Kimura et al., 1996; Lints et al., 1993; Pabst et al., 1998; Sussel et al., 1999).

Nkx-related proteins with highly divergent homeodomain sequences [55 to 78% homology as compared to the 68 to 95% homology in NK-like proteins (Lints et al., 1993)] have also been identified. The *Drosophila NK3* homologous *Nkx3-1* is expressed in the adult prostate epithelial cells controlling the differentiation of these cells and the *Nkx3-2/Bapx1* (*bagpipe homeobox homologue 1*) is expressed in the splanchnic mesoderm and embryonic skeleton (Bieberich et al., 1996; Tribioli et al., 1997). The *Nkx6* subfamily members *Nkx6-1*, *Nkx6-2/Gtx* (*Glial and testis specific homeobox gene*) and *Nkx6-3* are expressed during gastro-intestinal tract and central nervous system development and present a closer relationship to the *Nkx3* group than to the *Nkx2* members (Alanentalo et al., 2006; Komuro et al., 1993; Rudnick et al., 1994) (Figure 1).

1.1.1. NK-2 structural and functional domains

Transcription factors can activate gene transcription by remodelling the chromatin or by facilitating the binding of the RNA Polymerase II to the promoter, or alternatively can inhibit transcription through their interaction with histone deacetylase enzymes[2] (Gilbert, 2003). The activity of transcription factors is usually mediated by three distinct functional motifs: (1) a DNA-binding domain that recognizes a particular DNA sequence; (2) a *trans*-activating domain that activates or suppresses transcription; and (3) a protein-protein interaction domain that allows the modulation of its activity by other factors (Gilbert, 2003). NK-2 transcription factors have been described to activate and repress transcription. While proteins like Nkx6-1 are able to simultaneously perform both tasks, the functional analysis of NK-2 proteins is only beginning to reveal its molecular mechanism of action (Chen and Schwartz, 1995; De Felice et al., 1995; Iype et al., 2004; Mirmira et al., 2000; Muhr et al., 2001; Watada et al., 2000).

The homeodomain consists of approximately 60 amino acids that fold into a stable three-α-helices bundle preceded by a flexible amino (N)-terminal arm (Figure 2). Analysis of the three-dimensional structure of individual protein-DNA complexes, directed mutagenesis and biochemical studies have collectively shed light into the mechanisms of sequence-specific DNA binding by

[2] Histone acetyltransferases and histone deacetylases enzymes are responsible for the addition or removal, respectively, of acetyl groups to the histones of the nucleosome. Acetylation destabilizes the nucleosome which facilitates transcription whereas removal of acetyl groups from the histones stabilizes this and prevents the recognition of promoters.

homeodomains (Ades and Sauer, 1995; Gehring et al., 1994; Gruschus et al., 1997; Wolberger, 1996). The homeodomain binds DNA predominantly through interactions between helix 3 (the recognition helix) and the major groove where base-specific contacts made by positions 47, 50, 51 and 54 are believed to be the main determinants in binding specificity. Residues at positions 2, 3, 5-8 on the N-terminal arm have also been shown to influence binding specificity of homeodomains through minor groove interactions (Damante and Di Lauro, 1991; Damante et al., 1996; Ekker et al., 1994; Fraenkel et al., 1998; Laughon, 1991) (Figure 2). Although there is a common DNA-binding structural framework, specific sequence variations between homeodomains lead to different nucleotide recognition preferences among protein classes and members. NK-2 proteins present a conserved tyrosine (Y) in the position 54 of the homeodomain that makes crucial contacts with the 5'-CAAG-3' core of the NK-2 recognition site following its binding to the DNA. A tyrosine to methionine (M) replacement (Y54M) in this position is sufficient to switch the binding specificity towards Antennapedia-type homeodomain proteins that preferentially bind 5'-TAAT-3' core sequences. Moreover, this point mutation inactivates the ability of the NK-2 homeodomain to repress downstream target genes in *Drosophila* (Damante et al., 1996; Gruschus et al., 1997; Koizumi et al., 2003; Tsao et al., 1994; Viglino et al., 1993). Another critical residue of the NK-2 homeodomain is the alanine (A) in position 35; replacement of this residue by a threonine (T) is associated with the lethal *vnd* phenotype in *Drosophila*, presumably due to a pronounce decrease in the binding affinity of NK-2 to its DNA target sequences (Jiminez, 1995; Xiang et al., 1998). Finally, there is variation in the preference for DNA sequences outside the 5'-CAAG-3' recognition core among the different NK-2 members. In the case of Nkx2-1, it was found that it preferentially binds 5'-CCACTG/CAAGTG-3' sequences in the regulatory elements of thyroid and hypothalamus target genes (Bohinski et al., 1994; Damante et al., 1994; Francis-Lang et al., 1992; Lee et al., 2001; Mastronardi et al., 2006).

The homeodomain not only binds DNA, but also mediates protein-protein interactions through which it may influence its activity (Svingen and Tonissen, 2006; Wolberger, 1996). Nevertheless, for the majority of homeodomin proteins such as Nkx2-1, the combinatorial molecular code present in the contacting residues of the homeodomain appears to be the primary force behind its binding specificity (Damante et al., 1994; Damante et al., 1996; Svingen and Tonissen, 2006). Consistently, a recent high-throughput analysis suggests that a large proportion of the *in vivo* binding events involve the monomeric homeodomain preferences that can be predicted from the primary amino acid sequence (Berger et al., 2008; Noyes et al., 2008). Thus, homeodomain protein-protein interactions are more likely implicated in modulating transcriptional activity, for instance, by recruiting other transcription factors. In lung epithelial cells, the Winged Helix/Forkhead box A1 (FOXA1) transcription factor has been shown to interact with the Nkx2-1 homeodomain, blocking its binding to the surfactant protein C promoter (Minoo et al., 2007). In addition, the homeodomain activity can be regulated by post-translational modifications, such as acetylation (Yang et al., 2004).

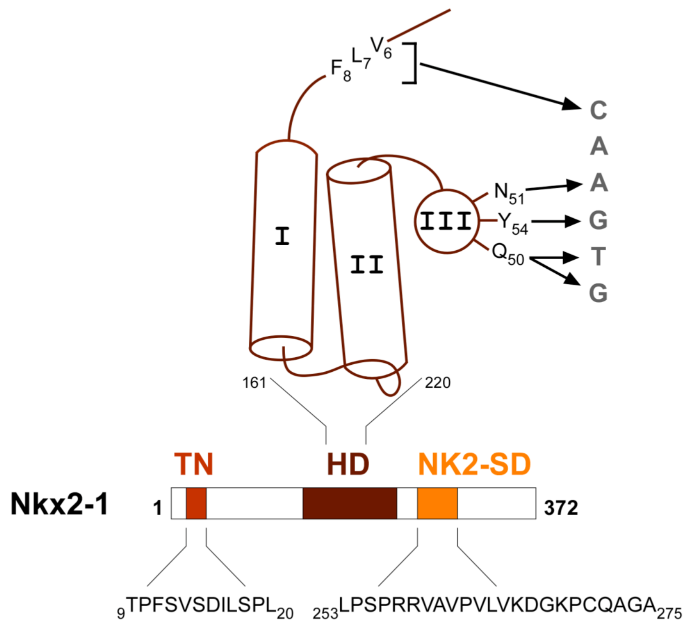

Figure 2. Conserved features of the NK-2 homeodomain proteins. Schematic diagram depicting the position and sequence of the Tinman (TN; light brown), homeodomain (dark brown; HD) and NK2-specific domain (orange; NK2-SD) of the NK-2-related Nkx2-1 protein (372 amino acids). In the HD (residue 161 to 220), helix I is proceeded by an N-terminal arm and separated by a loose loop from helix II which, with helix III, forms a helix-turn-helix motif. Amino acids from the N-terminal arm and helix III that establish critical interactions (arrows) with specific nucleotides of the DNA target sequence (light grey letters) are shown. The NK2-SD (residue 253 to 275) contains a hydrophobic core with valine or isoleucine residues and flanking basic amino acids. The amino acids (black letters) are indicated by the single letter code and the absolute position within the Nkx2-1 protein, except for the HD where the position is within the motif (from residue 1 to 60).

In addition to the homeodomain, two other peptide domains are conserved within most NK-2 proteins (Figure 2). The short TN domain (Tinmam motif) is located in the N-terminus of the protein and presents sequence similarity to the core region of the engrailed homology-1 (eh1) domain present in the transcription repressor Engrailed (En) (Smith and Jaynes, 1996). The TN domain has been proposed to form transcriptional repression complexes by interacting with members of the Groucho/Transducin-like enhancer of split (Gro/TLE) family and this mechanism underlies the repressive activity of Nkx2 and Nkx6 proteins during neural patterning events in the ventral neural tube (Mirmira et al., 2000; Muhr et al., 2001). The NK2-specific domain (NK2-SD), specific for NK-2 proteins, is separated from the carboxy (C)-terminal end of the homeodomain by a short linker and possesses a hydrophobic core sequence that constitutes a putative protein-protein interface (Bodmer, 1993; Lints et al., 1993; Price et al., 1992) (Figure 2). NK2-SD does not seem to be required for binding specificity. Instead, this domain appears to function as an intramolecular inhibitor of a C-terminus transcriptional activator domain (De Felice et al., 1995; Guazzi et al., 1990; Iype et al., 2004; Watada et al., 2000). In addition to the presumably C-terminus activator domain, an amino terminal portion was also reported to mediate the transcriptional activation of some NK-proteins, although the *in vivo* functional relevance of this activity requires further elucidation (Chen and Schwartz, 1995; De Felice et al., 1995).

1.1.2. Nkx transcription factors in mouse CNS development

In the vertebrate CNS, Nkx proteins are expressed within the medial neural plate and ventral neural tube in distinct dorso-ventral (DV) and antero-posterior (AP) domains. Multiple lines of evidence have shown that Sonic hedgehog (Shh) signalling activates the expression of Nkx genes throughout the CNS (Alanentalo et al., 2006; Dale et al., 1997; Ericson et al., 1995; Kohtz et al., 1998; Pabst et al., 2000; Qiu et al., 1998; Shimamura and Rubenstein, 1997). In addition, some

Nkx proteins may contribute to maintaining their expression by directly acting on their own regulatory regions (Chu et al., 1998; Iype et al., 2004; Saunders et al., 1998). Nkx proteins typically function before cell cycle exit by coupling spatial patterning to ventral-fate specification. In the spinal cord, members of the Nkx family respond to graded Shh signalling by instructing progenitor cells to adopt specific identities while repressing the fates of neighbouring cells (Briscoe et al., 2000; Briscoe et al., 1999; Muhr et al., 2001; Sander et al., 2000). For instance, Nkx2-2 establishes the ventral-most progenitor cell domain in the spinal cord (V3 interneuron progenitor domain, p3) by cross-repressing the expression of the homeodomain transcription factor *Paired-box 6* (*Pax6*). In the absence of *Nkx2-2*, neuronal progenitors undergo a ventral-to-dorsal transformation, generating motor neurons (pMN) instead of V3 interneuron progenitors (Briscoe et al., 2000; Briscoe et al., 1999). Within each ventral progenitor domain, Nkx proteins initiate a cascade of transcriptional interactions that will ultimately determine the identity of specific classes of postmitotic neurons. In the motor neuron progenitor domain (pMN), for example, Nkx6-1 and Nkx6-2 instruct the generation of motor neurons by activating the expression of the basic helix-loop-helix (bHLH)[3] transcription factor *Oligodendrocyte lineage 2* (*Olig2*), which then promotes the expression of motor neuron subtype-specific determinants (Briscoe et al., 2000; Novitch et al., 2001; Sander et al., 2000; Shirasaki and Pfaff, 2002; Vallstedt et al., 2001).

The expression of Nkx proteins is maintained in certain populations of postmitotic neurons, which suggests that these transcription factors also play a role in regulating neuronal subtype-specific and differentiation programs. In the spinal cord, the postmitotic expression of Nkx6-1 defines motor neuron pools within the lateral motor column (LMC) and regulates muscle nerve trajectories and target specificity towards individual limb muscles (De Marco Garcia and Jessell, 2008). In the forebrain, Nkx2-1 determines the specification of hypothalamic progenitor cells and the persistence of Nkx2-1 expression in hypothalamic differentiated neurons controls the precise expression of puberty and sexual development regulatory genes (Kimura et al., 1996; Lee et al., 2001; Mastronardi et al., 2006)

1.2. Nkx2-1 function in the ventral telencephalon

Early in development (around Embryonic day [E] 8.5 in mouse), the anterior neural plate gives rise to the prosencephalon (or embryonic forebrain) that consists of the diencephalon and telencephalic vesicles (Marín et al., 2000; Rubenstein et al., 1998) (Figure 3). The telencephalon comprises two major regions: the pallium (roof) and the subpallium (base). The pallium is organized into four main radial subdivisions (medial, dorsal, lateral and ventral pallium), originating cortical structures, such as the cerebral cortex and hippocampus from the dorsal and medial pallium, respectively, and nuclear structures, such as the cortical amygdala and claustrum from

[3] A class of transcription factors characterized by the presence of two α-helices separated by a loop; the helices mediate dimerization and the adjacent basic region is required for DNA binding.

the lateral and ventral pallium, respectively. The subpallium consists of three primary subdivisions that extent medially into the septum: the striatal domain, derived from lateral ganglionic eminence (LGE) progenitor cells, the pallidal domain, derived from medial ganglionic eminence (MGE) progenitor cells, and the telencephalic stalk domain, containing the anterior entopeduncular and preoptic areas (AEP and POA) (Marín and Rubenstein, 2002; Puelles et al., 2000; Puelles et al., 1999). Recent work has included the AEP within the ventral aspect of the MGE (Flames et al., 2007) (Figure 3).

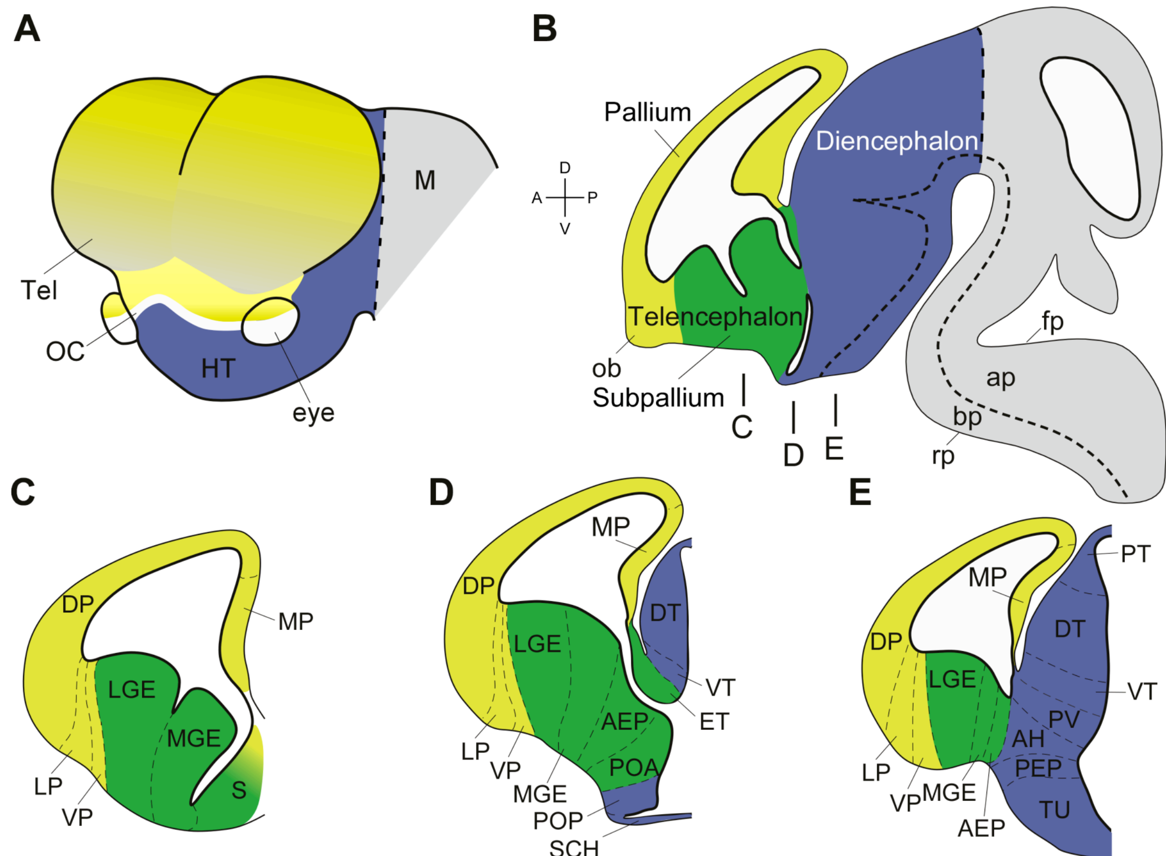

Figure 3. Organization of the prosencephalon. (A) Representation of a mouse brain at embryonic day E13.5 where the most anterior (A) part of the brain, the prosencephalon, is divided in two regions: the telencephalon (yellow) and the diencephalon (blue). (B) Schema of a saggital section through the brain of an E13.5 mouse showing the division of the telencephalon into a dorsal (D) part, pallium (yellow) and a ventral (V) part, subpallium (green). (C-E) Transversal sections through the telencephalon and diencephalon; the planes of section are indicated in (B). The caudal aspect of the LGE (E) can also be designated caudal ganglionic eminence (CGE). Abbreviations: AEP, anterior entopeduncular area; AH, anterior hypothalamus; ap, alar plate; bp, basal pale; DP, dorsal pallium; DT, dorsal thalamus; ET, eminentia thalami; fp, floor plate; HT, hypothalamus; LGE, lateral ganglionic eminence; LP, lateral pallium; M, mesencephalon; MGE, medial ganglionic eminence; MP, medial pallium; ob, olfactory bulb; OC, optic chiasm; P, posterior; PEP, posterior entopeduncular area; POA, anterior preoptic area; POP; posterior preoptic area; PT, pretectum; PV, paraventricular nucleus; SCH, suprachiasmatic nucleus; rp; roof plate; S, septum; Tel, telencephalon; TU, tuberal region; VP, ventral pallium; VT, ventral thalamus.

Expression of Nkx2-1 is restricted to the ventral-most domains of the forebrain, including the ventral telencephalon and the hypothalamus (Guazzi et al., 1990; Price et al., 1992; Shimamura et al., 1995). In the telencephalon, Nkx2-1 is expressed from the eleven somites stage in progenitor

cells of the MGE, POA and septal anlage where it plays a fundamental role in interpreting patterning signals and imposing a specification and differentiation code. The contribution of Nkx2-1 for these developmental processes will be further detailed in the following sections.

1.2.1. Patterning the dorsoventral axis of the subpallium

As in other regions of the CNS, the generation of distinct progenitor domains along the dorsoventral axis of the subpallium is induced by the activity of morphogenes[4] that impose the acquisition of specific cell types through the activation of transcription factors (Marín and Rubenstein, 2002). Shh is initially secreted from the prechordal mesoderm and induces its own expression in the overlying ventral-most region of the telencephalon, including the proliferative region of the POA and the mantle of the MGE (Ericson et al., 1995; Shimamura et al., 1995). Shh expression in the telencephalon forms a reciprocal gradient with the repressor form of the Gli3[5] zinc finger transcription factor (Gli3R) and promotes ventral identity in part by inhibiting the dorsalizing activity of Gli3R (Rallu et al., 2002). In addition, several lines of evidence suggest that activation of fibroblast growth factor 8 (FGF8) by Shh and repression of bone morphogenetic protein (BMP) signalling are also required for the establishment of ventral identity in the telencephalon (Anderson et al., 2002; Hebert and Fishell, 2008; Lupo et al., 2006; Storm et al., 2006). The combinatorial activity of these molecules induces the expression of subpallial-specific genes, such as the transcription factors *Distal-less homeobox 1* and *2* (*Dlx1*, *Dlx2*), *Genomic screened homeobox 2* (*Gsh2*) and the basic helix-loop-helix *Mammalian archaete-schute homologue 1* (*Mash1*). Some other genes, such as *Nkx2-1*, are only induced in the ventral-most domains of the subpallium (Dale et al., 1997; Ericson et al., 1995; Gunhaga et al., 2000; Kohtz et al., 1998; Pera and Kessel, 1997; Rallu et al., 2002; Shimamura and Rubenstein, 1997) (Figure 4). Interestingly, Shh and Nkx2-1 are expressed in overlapping domains in the ventral telencephalon and these genes appear to cross-activate each other (Jeong et al., 2006; Sussel et al., 1999; Xu et al., 2005).

Cross-regulatory interactions between transcription factors sharpen the boundaries between progenitor domains and contribute for the establishment of regional identity along the subpallium (Figure 4). Gsh2 controls the specification of the LGE in part by repressing the expression of cortical specification genes in the subpallium (Toresson et al., 2000; Yun et al., 2001). For instance, analysis of mouse mutants for *Gsh2* and *Pax6* demonstrate that their mutual antagonism is required for the positioning of the pallial-subpallial boundary at the interface between the LGE and the ventral pallium (Toresson et al., 2000; Yun et al., 2001). Gsh2 also cooperates with Nkx2-

[4] Secreted factors that can induce different cell fates in a concentration-dependent manner by forming a gradient.

[5] Transducers of the Shh signalling pathway displaying both activator and repressor activities located respectively in the C-terminus and N-terminus of the protein. Gli transcription factors contain zinc fingers, a protein motif in which a cysteine or cysteine-histidines residues coordinate a zinc ion. This module can operate both in DNA-recognition and protein-protein interactions.

1 in the acquisition of ventral identities in the subpallium, as evidenced by the severe patterning defects observed in *Nkx2-1*$^{-/-}$;*Gsh2*$^{-/-}$ double mutants (Corbin et al., 2003). Furthermore, genetic evidence suggests that Pax6 and Nkx2-1 antagonize each other to establish the boundary between the LGE and MGE; in *Pax6* mutants the expression of *Nkx2-1* expands dorsally into the LGE whereas in the absence of *Nkx2-1* the expression of *Pax6* and other LGE-specific genes extends ventrally (Chapouton et al., 1999; Stoykova et al., 2000; Sussel et al., 1999). Although Pax6 was shown to repress *Nkx* genes in the hindbrain and spinal cord (Briscoe et al., 2000; Briscoe et al., 1999), it is not clear whether these transcription factors cross-repress each other directly or through Shh/Gli3R signalling in the telencephalon (Corbin et al., 2003; Chiang et al., 1996; Sussel et al., 1999; Xu et al., 2005).

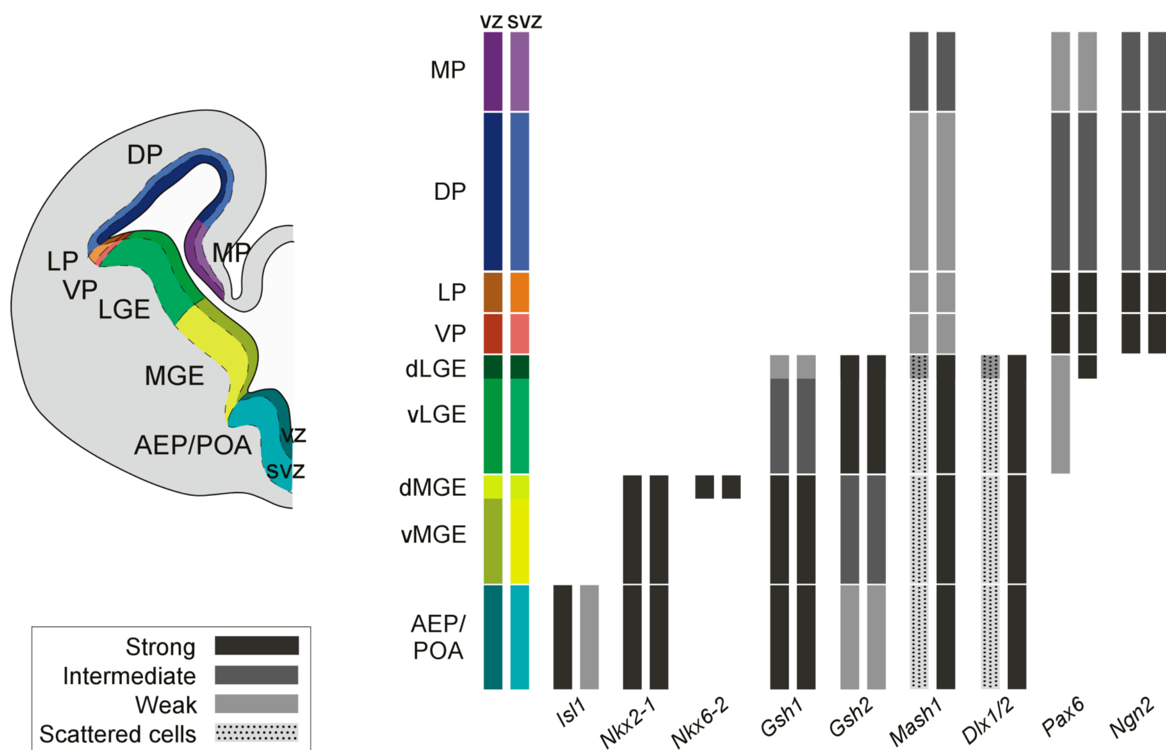

Figure 4. The expression patterns of transcription factors shape progenitor domains boundaries along the dorsoventral axis of the developing telencephalon. Coronal hemisection of an E13.5 telencephalon showing the progenitor cell domains in different colors. Some genes are expressed predominantly in the subpallium (*Isl1*, *Nkx2-1*, *Nkx6-2*, *Gsh1/2*, *Mash1*, *Dlx1/2*), whereas others in the pallium (*Pax6*, *Ngn2*). Repressive interactions between regulatory genes contribute for the generation of boundaries in the subpallium. Abbreviations: AEP, anterior entopeduncular area; DP, dorsal pallium; dLGE/vLGE, dorsal and ventral lateral ganglionic eminence; LP, lateral pallium; dMGE/vMGE, dorsal and ventral medial ganglionic eminence; MP, medial pallium; POA, anterior preoptic area; svz, subventricular zone; VP, ventral pallium; vz, ventricular zone.

Shh signalling regulates several processes during telencephalic development. At early stages (E8.5-E11.5), Shh is fundamental for ventral telencephalic patterning (Chiang et al., 1996; Fuccillo et al., 2004), while loss of *Shh* neuronal expression at later time points (E12.5) does not affect the expression of ventrally restricted genes (Xu et al., 2005). At these stages, Shh function is required to preserve the identity of MGE precursors by sustaining the expression of key transcription

factors, such as *Nkx2-1* and *Nkx6-2*, contributing thereby to the expansion of these progenitor pools (Machold et al., 2003; Xu et al., 2005). In this context, Shh acts through a Gli3R-independent mechanism (Gulacsi and Anderson, 2006).

1.2.2. Specification and differentiation of ventral subpallial cells

Nkx2-1 is required for the specification of MGE and POA precursors and determines the acquisition of cell-specific properties of their derivatives (Corbin et al., 2003; Kimura et al., 1996; Sussel et al., 1999). In the absence of *Nkx2-1* function, MGE and POA progenitors (except the most ventral aspect of the POA) are re-specified to a dorsal fate similar to that of LGE progenitors, a phenotype resembling the ventral-to-dorsal transformation previously described for the *Drosophila NK2/vnd* mutant or *Nkx2-2*-deficient neurons in the spinal cord (Briscoe et al., 1999; Chu et al., 1998; McDonald et al., 1998; Weiss et al., 1998). In *Nkx2-1* mutant mice, genes normally expressed in the MGE and POA progenitor domains, such as the LIM-homeobox[6] transcription factors *Lhx6* and *Lhx7* (Grigoriou et al., 1998; Wanaka et al., 1997), are absent. Concurrently, LGE- (e.g. *Pax6* and the POU-homeodomain transcription factor *Octamer binding protein 6* [*Oct6*]) and CGE-specific genes (e.g. *COUP transcription factor 2* [*Coup-TFII*]) are induced in the mutant MGE/POA region, suggesting that Nkx2-1 both specifies a ventral telencephalic fate and inhibits a LGE/CGE phenotype (Butt et al., 2008; Corbin et al., 2003; Sussel et al., 1999). Analysis of the ventral telencephalon of E18.5 *Nkx2-1*$^{-/-}$ mice revealed a pallidal to striatal transformation. In these mice, neuronal populations derived from MGE/POA precursors (such as globus pallidus or basal forebrain cholinergic projection neurons[7]) are missing (Figure 5A), and LGE-derived striatal projection neurons occupy a much larger region of the subpallium than in wild type mice (Butt et al., 2008; Kimura et al., 1996; Sussel et al., 1999).

Whereas cortical projection neurons derive from the pallium, there is compelling evidence that the majority of cortical gamma-aminobutyric acid (GABA)-producing (GABAergic) interneurons[8] are originated in the subpallium and reach the cortex in several migratory streams (Anderson et al., 1997; De Carlos et al., 1996; Fogarty et al., 2007; Lavdas et al., 1999; Sussel et al., 1999; Tamamaki et al., 1997; Wichterle et al., 1999; Wichterle et al., 2001). In *Dlx1/2* double mutant embryos, which show abnormal differentiation of late born subpallial cells, there is a dramatic reduction (around 75%) in the number of neocortical GABAergic interneurons (Anderson et al., 1997). In agreement with a subpallial origin for cortical interneurons, *Nkx2-1* mutants also display

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[6] Homeodomain transcription factors that contain two tandem zinc finger protein motifs (LIM domain) implicated in protein-protein interactions.
[7] Neurons that send axons outside their local environment. In the subpallium, pallidal and striatal projection neurons use gamma-aminobutyric acid (GABA) as the main neurotransmitter whereas the basal forebrain cholinergic neurons use acetylcholine (Ach). In the pallium, projection neurons use glutamate as the main neurotransmitter.
[8] Neurons that establish synaptic contacts with nearby neurons. The most common type of telencephalic interneurons uses GABA as neurotransmitter and is located in the cortex and striatum. In the striatum, there is an additional interneuronal population that contains Ach.

a severe reduction (around 50%) in the number of cortical GABAergic interneurons, suggesting that many of these indeed derived from MGE and/or POA progenitors that require Nkx2-1 for its specification (Butt et al., 2008; Kimura et al., 1996; Sussel et al., 1999). In addition, GABAergic and cholinergic striatal interneurons are missing in *Nkx2-1* mutant mice (Butt et al., 2008; Kimura et al., 1996; Marín et al., 2000; Sussel et al., 1999), demonstrating that striatal interneurons do not derive from LGE precursors (that give rise to striatal projection neurons) but instead originate from Nkx2-1-expressing progenitor cells. Altogether, these results demonstrate that subpallial Nkx2-1-expressing precursors in the MGE/POA generate many cortical and most striatal interneurons that subsequently migrate to their corresponding target territories (Wichterle et al., 2001) (Figure 5A).

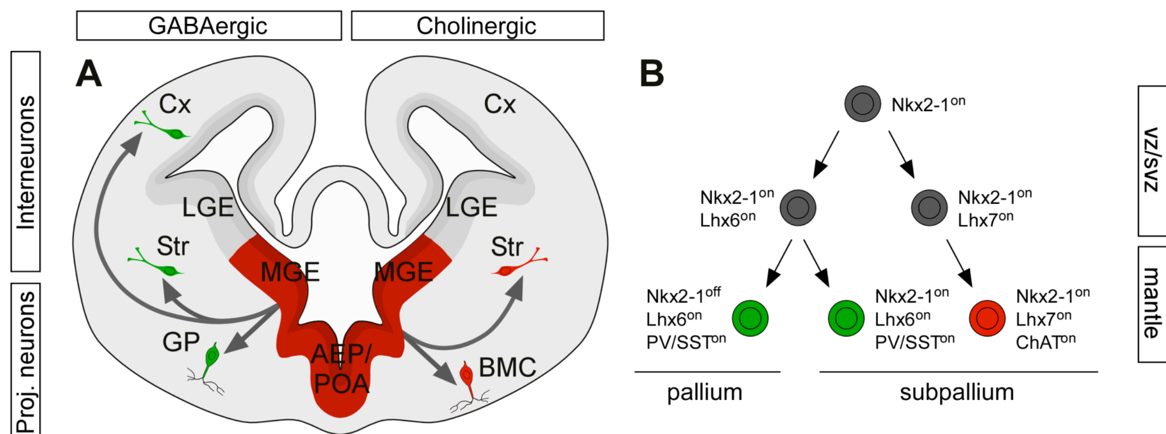

Figure 5. Nkx2-1 controls the specification of several telencephalic neuronal populations. (A) Schema depicting the origin and migration of neurons derived from the Nkx2-1 proliferative domain (red), including both GABAergic (green) and cholinergic (orange) projection neurons and interneurons for pallial and subpallial structures. (B) Nkx2-1 activates the expression of *Lhx6* and *Lhx7* in subventricular zone (svz) progenitor cells. Lhx6 is required for the specification of GABAergic Parvalbumin (PV) and Somatostatin (SST) interneurons for the cortex (Cx) and striatum (Str) whereas Lhx7 induces the expression of cholinergic markers (e.g. the acetylcholine-synthesizing enzyme Choline AcetylTransferase, ChAT) in subpallial neurons. Interestingly, only neurons remaining in the subpallium maintain Nkx2-1 expression postmitotically. Abbreviations: AEP, anterior entopeduncular area; BMC, basal magnocellular complex; GP, globus pallidus; LGE, ventral lateral ganglionic eminence; MGE, medial ganglionic eminence; POA, anterior preoptic area; vz, ventricular zone.

What types of cortical and striatal interneurons require Nkx2-1 function? Telencephalic GABAergic interneurons constitute a heterogeneous group of cells with different morphological, electrophysiological and molecular (i.e. expression of neuropeptides, calcium binding proteins, ionic channels, transcription factors, etc.) characteristics [for a recent update on interneuron terminology, see (Ascoli et al., 2008)]. However, the majority of cortical and striatal GABAergic interneurons can be classified in three large groups based on the non-overlapping expression of the calcium binding proteins Parvalbumin (PV), Calretinin (CR) and the neuropeptide Somatostatin (SST) (Gonchar and Burkhalter, 1997; Kubota and Kawaguchi, 1994) (Figure 6). Interneurons expressing STT and neuropeptide Y (NPY, an interneuronal population that largely overlaps with SST) were almost absent in the cortex and striatum of *Nkx2-1* mutant mice (Anderson et al., 2001), suggesting that at least this large group of interneurons requires Nkx2-1 function. Although the requirement of Nkx2-1 for the specification of PV positive interneurons was not determined in

the initial analysis of *Nkx2-1* mutants due to the late onset of the expression of this protein (Alcántara et al., 1996), both *in vitro* and *in vivo* evidence have shown that Nkx2-1 is also required for the specification of this group of interneurons (Butt et al., 2008; Wonders et al., 2008; Xu et al., 2004). By contrast, several lines of evidence demonstrate that CR positive interneurons do not required Nkx2-1 for their development (Butt et al., 2008; Marín et al., 2000; Sussel et al., 1999; Xu et al., 2004). Remarkably, a recent analysis of *Nkx2-1* conditional mutants has shown that loss of *Nkx2-1* leads to a large increase in the number of CR positive cortical interneurons at the expense of PV and SST populations (Butt et al., 2008), reinforcing the view that a ventral-to-dorsal transformation of progenitor fates occurs in these mutants. In summary, these genetic experiments, along with fate-mapping (Fogarty et al., 2007; Miyoshi et al., 2007; Xu et al., 2008) and transplantation experiments (Anderson et al., 2002; Butt et al., 2005; Flames et al., 2007; Nery et al., 2002; Valcanis and Tan, 2003; Wichterle et al., 2001; Xu et al., 2004), have shown that Nkx2-1-expressing precursors from the MGE are the main source of PV and SST positive interneurons whereas CR-expressing bipolar cortical interneurons are mainly derived from the *Gsh2*-positive and *Nkx2-1*-negative dorsal aspect of the caudal ganglionic eminence (CGE) (Figure 6).

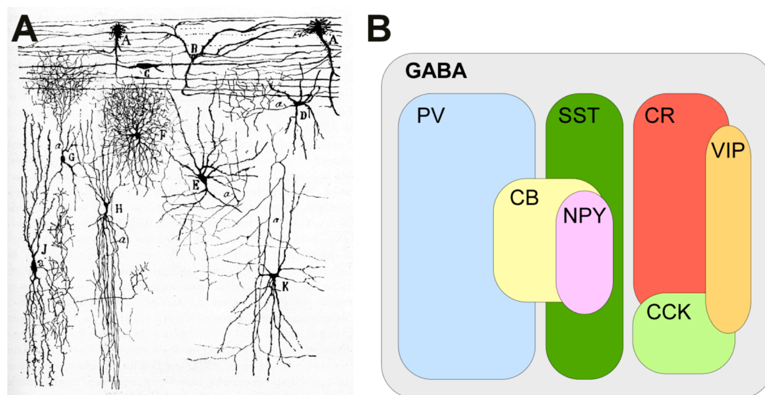

Figure 6. Telencephalic GABAergic interneurons. (A) Morphological diversity of interneurons in the cerebral cortex. Adapted from "Texture of the human and vertebrates central nervous system" Ramón y Cajal, 1899. (B) Based on the expression of calcium-binding proteins and neuropeptides, GABAergic interneurons can be classified in three non-overlapping groups expressing Parvalbumin (PV), Somatostatin (SST) and Calretinin (CR). Abbreviations: CB, calbindin; CCK, colecistoquinin; GABA, gamma-aminobutyric acid; NPY, neuropeptide Y; VIP, vasoactive intestinal peptide.

Nkx2-1 is also required for the specification of distinct populations of striatal interneurons. Analysis of *Nkx2-1-Cre;ROSA-YFP* transgenic mice revealed that the majority of GABAergic (all PV- and most SST/NPY- expressing interneurons) and all cholinergic striatal interneurons derive from *Nkx2-1*-expressing progenitors (Figure 5A). In contrast to MGE-derived cortical interneurons, however, striatal interneurons maintain Nkx2-1 expression until postnatal stages (Marín et al., 2000; Xu et al., 2008). Analysis of E18.5 $Nkx2\text{-}1^{-/-}$ and postnatal $Olig2\text{-}Cre;Nkx2\text{-}1^{Fl/Fl}$ mutant mice revealed that interneurons are either absent or severely reduced in the striatum of these animals (Butt et al., 2008; Marín et al., 2000), which suggests that Nkx2-1 is required for the specification of virtually all striatal interneurons. The only exception to this rule appears to be some SST- and CR- expressing populations. The fact that *Nkx2-1* removal causes a more dramatic phenotype in the striatum than in the cortex, along with the observation that Nkx2-1 expression is maintained in

the majority of postmitotic striatal but not cortical interneurons (Figure 5B), suggests that Nkx2-1 could be additionally required for the migration or differentiation of striatal interneurons.

Experimental evidence therefore suggests that Nkx2-1 acts as a master molecular switch that simultaneously induces MGE/POA and represses LGE/CGE genetic programmes at different developmental points. Which mechanisms are orchestrated by Nkx2-1 to achieve these goals? In the spinal cord, the combinatorial expression of members of the LIM-homeodomain family of transcription factors controls neuronal-subtype specification (Shirasaki and Pfaff, 2002). Similarly, the LIM homeodomain transcription factors *Lhx6* and *Lhx7* are expressed in MGE/POA-derived postmitotic cells and appear to be the main effectors of Nkx2-1 in the specification and differentiation of subpallial cells (Grigoriou et al., 1998; Sussel et al., 1999; Zhao et al., 2003) (Figure 5B). Nkx2-1 protein directly activates the transcription of *Lhx6* in MGE-derived cells and this transcription factor acts downstream of Nkx2-1 in the acquisition of PV and SST subtype-specific properties (Du et al., 2008; Fogarty et al., 2007; Lavdas et al., 1999; Liodis et al., 2007; Zhao et al., 2008). *Lhx6* mutant mice show a dramatic reduction in the number of both PV- and SST- expressing striatal and cortical interneurons. Moreover, ectopic expression of *Lhx6* in *Nkx2-1* mutant mice can rescue the generation of PV and SST positive interneurons (Du et al., 2008; Liodis et al., 2007). However, in contrast to *Nkx2-1* loss-of-function, there is no obvious increase in the number of CR- or VIP- expressing interneurons in *Lhx6* mutant mice, suggesting that the repression of CGE-like properties is not mediated by Lhx6 (Butt et al., 2008; Liodis et al., 2007; Zhao et al., 2008). Lhx6 regulate additional aspects in the differentiation of MGE-derived GABAergic cells, although it does not seem to be required for the acquisition of the GABAergic phenotype (Alifragis et al., 2004; Liodis et al., 2007; Marín et al., 2000). For example, *Lhx6* appears to be required for the migration and final allocation of interneurons into the appropriate cortical layers (Lavdas et al., 1999). Lhx6 may mediate these functions by regulating the expression of receptors that have been previously implicated in controlling the migration of cortical interneurons, such as the tyrosine kinase[9] receptor for Neuregulin-1 (NRG1), *erythroblastic leukemia viral oncogene homolog 4* (*ErbB4*), and the chemokine receptor *CXC chemokine receptor 4* (*CXCR4*) (Flames et al., 2004; Li et al., 2008; Lopez-Bendito et al., 2008; Stumm et al., 2003; Zhao et al., 2008).

Lhx7 is expressed by ventral MGE/POA subventricular zone cells and in many derivatives restricted to the subpallium such as striatal cholinergic interneurons (Fragkouli et al., 2005; Zhao et al., 2003). Thus, in contrast to *Lhx6*, *Lhx7* is not expressed in migrating cortical interneurons. *Lhx7* presumably lies downstream of Nkx2-1 in inducing cholinergic fate, since *Lhx7* mutant mice lack most cholinergic neurons throughout the ventral telencephalon, including striatal interneurons and projection neurons of the basal forebrain (Fragkouli et al., 2005; Mori et al., 2004; Zhao et al.,

[9] A family of membrane receptors containing an intracellular domain that catalyses the phosphorylation by adenosine triphosphate (ATP) of specific tyrosine residues on target proteins.

2003) (Figure 5B). The presence of some cholinergic neurons in *Lhx7* mutant mice and the expression of this transcription factor in some non-cholinergic neurons in the basal telencephalon suggest that Lhx7 is not sufficient to induce the cholinergic phenotype in all neurons and, similar to the spinal cord, might cooperate with other LIM transcription factors (e.g. *Isl LIM homeobox 1* [*isl1*]) to induce this phenotype in the subpallium (Pfaff et al., 1996; Wang and Liu, 2001).

1.3. Migration of MGE-derived interneurons

In contrast to projection neurons, which use radial migration to reach their final destination, MGE-derived cortical and striatal interneurons migrate tangentially to their corresponding target territories by following specific guidance cues. Tangentially migrating neurons appears to respond to some of the same molecules that control guidance of growing axons (Tessier-Lavigne and Goodman, 1996) and display a typical morphology that includes a branched leading process (Marín et al., 2006). MGE-derived interneurons adopt preferentially two migratory routes in the developing telencephalon; at early developmental stages (E12.5), interneurons migrate superficial to the striatal mantle whereas and at later time points (E13.5-E15.5) these cells, together with CGE-derived cortical interneurons, adopt a deep route of migration (Butt et al., 2005; Marín and Rubenstein, 2001; Miyoshi et al., 2007). In general, the migration of MGE-derived interneurons is regulated by the action of two types of factors: those that stimulate their movement (motogenic factors; molecules that promote cell motility without giving directionality) and those that direct cells through appropriate pathways towards the corresponding targets (guidance factors) (Figure 7).

1.3.1. Motogenic factors

MGE-derived cells have an outstanding cell-autonomous migratory capability that is maintained even when cultured *in vitro* in an artificial matrix (collagen or matrigel[10]) or after transplantation into an adult mouse brain (Nery et al., 2002; Wichterle et al., 1999). In addition, several molecules expressed in the route of MGE-derived cells have been suggested to further enhance their migration (Figure 7A). For example, several lines of evidence suggest that hepatocyte growth factor (HGF) promote the migration of MGE-derived cells (Powell et al., 2001). Tangential migration of MGE-derived cells is also strongly stimulated by brain derived nerve factor (BDNF), neurothrophin 4 (NT4) and glial-derived neurothropic factor (GDNF) (Polleux et al., 2002; Pozas and Ibañez, 2005). The activity of GDNF in MGE cells is partially mediated by the mitogen-activated protein (MAP) kinase signalling, whereas BDNF seem to act trough phosphoinositide-3-kinase (PI3K), a signalling cascade that regulates the activity of the cytoskeleton by modulating

[10] The trade name for a gelatinous protein mixture obtained from Engelbreth-Holm-Swarm mice sarcoma, which is mainly composed by extracellular matrix proteins such as laminin, collagem type IV and entactin.

the balance of activation of proteins from the Ras homolog gene family (Rho) of GTPases[11] (Marín et al., 2006; Polleux et al., 2002; Pozas and Ibañez, 2005). CXCL12, a member of the CXC subfamily of chemokines (also known as stromal cell-derived factor-1, SDF-1) was also reported to exert a strong motogenic activity over CXCR4-expressing MGE-derived cells. This activity, however, seems to be required primarily for the tangential dispersion of interneurons after they have arrived to the cortex (Li et al., 2008; Lopez-Bendito et al., 2008; Stumm et al., 2003).

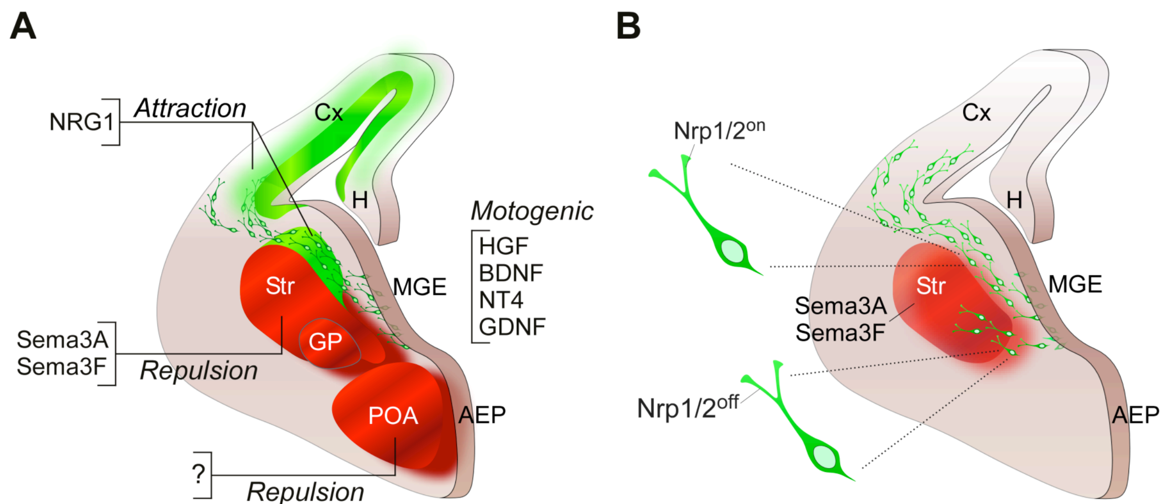

Figure 7. Mechanisms regulating the migration of MGE-derived interneurons. (A) Schematic representation of a transversal hemisection through the telencephalon of an E13.5 embryo depicting MGE-derived interneurons migrating deep to the striatal mantle. This process is regulated by both motogenic factors and attractive (NRG1, green) or repulsive (red) molecules expressed in the migratory path. (B) Sorting of MGE-derived cortical and striatal interneurons is mediated by neuropilin/semaphorin interactions. Interneurons migrating to the cortex express neuropilin (Nrp) receptors whereas interneurons migrating to the striatum do not. Expression of Sema3A and Sema3F in the mantle of the developing striatum (Str) create an exclusion zone for migrating cortical interneurons and channel them into adjacent paths whereas striatal interneurons are able to invade this territory. Abbreviations: AEP, anterior entopeduncular area; Cx, cortex; GP, globus pallidus; H, hippocampus; MGE, medial ganglionic eminence; POA, anterior preoptic area.

1.3.2. Guidance factors

Guidance requires the patterned expression of attractive or repellent molecules within the substrate and the graded expression of diffusible molecules secreted by distance sources (Butler and Tear, 2007). The response of a migrating neuron to the external guidance cues depends on the complement of receptors it expresses. Guidance receptors act either through the activation of second messenger systems that direct local rearrangement of the cytoskeleton and promote growth towards or away from the target or by mediating differential adhesion (Butler and Tear, 2007). Most of the described guidance molecules can be either attractive or repulsive depending on the status of the receiving neuron; different receptor complexes at the membrane and the recent history of second-messenger activation in the cytosol (e.g. levels of cyclic adenosine monophosphate [cAMP]) can provide alternative responses to the same cue (Dickson, 2002; Song

[11] A family of small signalling proteins with hydrolase activity (bind and hydrolyse guanosine triphosphate [GTP]), which regulate downstream proteins involved in numerous processes, such as actin dynamics.

et al., 1998).

Several signalling systems that confer directionality to the migration of MGE-derived interneurons have also been described (Figure 7A). The general direction of migration for MGE-derived interneurons –ventral to dorsal– appears to be established by the simultaneous activity of chemorepulsive and chemoattractive factors produced by the POA and cortex, respectively (Marín et al., 2003; Wichterle et al., 2003). Some studies have suggested that repulsion of interneurons from the subpallium is mediated by Slits (Zhu et al., 1999), a family of large extracellular matrix proteins that possess chemorepulsive activity for growing axons and migrating cells in a variety of systems (Brose and Tessier-Lavigne, 2000). However, the chemorepulsive activity found in the POA is still present in mice deficient for both *Slit1* and *Slit2*, the Slit members expressed in the subpallium (Marín et al., 2003). Netrin1 has also been implicated in repelling striatal projection neurons from the LGE (Hamasaki et al., 2001), but mice simultaneously lacking *Slit1*, *Slit2* and *Netrin1* have normal numbers of cortical interneurons at birth (Marín et al., 2003). Thus, the nature of the interneuron chemorepellent located in the POA remains to be identified (Figure 7A).

Cortical chemoattraction of MGE-derived migrating interneurons appears to be mediated, at least in part, by NRG1 (Flames et al., 2004) (Figure 7A). Neuregulins (NRG1-4) are a family of proteins containing an epidermal growth factor (EGF)-like motif that activates membrane-associated tyrosine kinases related to the EGF receptor (also known as ErbB1). The EGF-like domain of NRG elicits ErbB receptor dimerization, tyrosine phosphorylation and the activation of downstream signalling pathways. NRG1 directly binds to the EGF motif of ErbB3 and ErbB4 receptors, which alone or in combination with ErbB2 mediate the large range of functions attributed to this factor during the development of the nervous system (Buonanno and Fischbach, 2001; Falls, 2003). Unlike ErbB2 and ErbB3, which appear to require heterodimerization, ligand-induced homodimerizarion of ErbB4 has been reported to be functionally competent in some systems (Plowman et al., 1993). The *Nrg1* gene is subjected to differential promoter usage and alternative splicing giving rise to three major classes of proteins; types I and II comprise secreted isoforms that contain an extracellular immunoglobulin (Ig)-like domain (NRG1-Ig) whereas type III contain an extracellular cysteine-rich domains (CRD) and are membrane-bounded (Buonanno and Fischbach, 2001; Falls, 2003). In the developing telencephalon, *Nrg1-CRD* is strongly expressed throughout the LGE, from the subventricular zone to the striatal mantle, whereas *Nrg1-Ig* is expressed in a lateral to medial gradient in the cortex (Flames et al., 2004) (Figure 7A). Nrg1-CRD and Nrg-Ig isoforms constitute, respectively, a subpallial permissive corridor and a strong cortical chemoattractive signal for a population of MGE-derived migrating interneurons expressing the ErbB4 receptor (Flames et al., 2004; Yau et al., 2003). Perturbing *ErbB4* function abolishes the cortical attraction and produces a reduction in the number of MGE-derived interneurons tangentially migrating to the cortex at developmental stages and a deficit in GABAergic interneurons in the postnatal cortex (Flames et al., 2004). The requirement of attractive signals for

the guidance of MGE-derived striatal interneurons has not been reported.

Guidance molecules are also required for the segregation of MGE-derived cells to different telencephalic structures. Thus, sorting of interneurons destined for the cerebral cortex or striatum appears to be mediated by Neuropilin/classIII-Semaphorin interactions (Marín et al., 2001; Tamamaki et al., 2003) (Figure 7A). Semaphorins (Sema) are members of a large, highly conserved family of molecular signals that were initially identified through their role in axon guidance (Kolodkin et al., 1993; Luo et al., 1993), and later implicated in regulating cell motility and attachment in vascular growth, immune system and tumour progression (Kruger et al., 2005; Tamagnone and Comoglio, 2004). All semaphorins contain a conserved sema domain and have been grouped on the basis of their structural elements and amino-acid similarity; the invertebrate classes 1 and 2; the vertebrate classes 3 to 7 and the final group is encoded by viruses. Except for the class 2, 3 and the viral semaphorins that are secreted, proteins from all other classes are membrane-bound. Plexins (type A to D) are the predominant family of semaphorin receptors and can function as both ligand-binding and as signalling receptor, mediating many biological functions of semaphorins through regulating integrins and controlling the activation of Rho-family GTPases (Kruger et al., 2005). Most plexin-semaphorins interactions are mediated through the sema domain of both proteins, except for class 3 semaphorins, where most members require neuropilins as essential semaphoring-binding co-receptors to signal through class-A plexins. Neuropilins contain a short intracellular domain that lack intrinsic enzymatic activity and mediate the repulsive actions of class 3 semaphorins in axons (Gu et al., 2005; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). However, class 3 semaphorins can also be interpreted as attractive signals depending on the receptor complexes expressed by neurons (Castellani et al., 2002; Chauvet et al., 2007). In the developing telencephalon, Neuropilin1 (Nrp1) and Neuropilin2 (Nrp2) are expressed by interneurons that migrate to the cortex but not by interneurons that invade the developing striatum. Expression of neuropilins allows migrating cortical interneurons to respond to a chemorepulsive activity in the striatal mantle, of which the class 3 semaphorins (Sema3A and Sema3F) are components (Figure 7B). In contrast, lack of neuropilin expression by striatal interneurons is fundamental for the invasion of their target territory (Marín et al., 2001). Thus, loss of *Nrp1* or *Nrp2* function increases the number of interneurons migrating to the striatum and decrease the number reaching the cerebral cortex, which leads to a final increase in the number of NPY-expressing interneurons in the adult striatum (Marín et al., 2001). So, the final destination of MGE-derived tangentially migrating interneurons (striatum or cortex) is determined by the expression of the class 3 semaphorin receptors Nrp1 and Nrp2. The mechanisms that control the selective expression of these receptors by MGE-derived cortical but not striatal interneurons and which ultimately determine the sorting of these populations are currently unknown.

1.3.3. What mechanisms control the selective expression of guidance receptors?

During development, axons are instructed to navigate to their corresponding target areas by simultaneously integrating multiple extracellular signals along the pathway (Tessier-Lavigne and Goodman, 1996). The precise guidance of each neuronal population is achieved by the selective perception of environmental cues; migrating axons continuously adapt their response by modulating the expression of guidance receptors and their intracellular signaling cascades. A key mechanism regulating selectivity in axon guidance decisions is transcriptional regulation in postmitotic neurons (Butler and Tear, 2007; Polleux et al., 2007; Shirasaki and Pfaff, 2002). In the vertebrate spinal cord, for example, the combinatorial activities of different LIM-homeodomain transcription factors confer motor neuron subtypes with the ability to select distinct axonal pathways and final targets (Kania et al., 2000; Sharma et al., 2000; Sharma et al., 1998; Shirasaki et al., 2006; Thaler et al., 2004). Studies over the past few years have begun to identify possible downstream guidance effectors regulated by these transcription factors. In spinal motor neurons, Lim1 determines the trajectory of motor axons emerging from the lateral aspect of the lateral motor column (LMCl) by promoting the expression of the Eph receptor tyrosine kinase A4 (EphA4) in these cells. This allows LMCl neurons to select a dorsal trajectory avoiding the ventral limb, which expresses the chemorepellent factor EphrinA5 (Kania and Jessell, 2003). Similar mechanisms appear to control the expression of guidance receptors in other axonal tracts, such as the thalamocortical and retinal projections (Butler and Tear, 2007; Polleux et al., 2007; Shirasaki and Pfaff, 2002). In the retina, for instance, the zinc finger protein of the cerebellum 2 (Zic2) transcription factor determines the ipsilateral projection of ventro-temporal retinal ganglion cells by regulating the expression of EphB1, the receptor for the chemorepulsive cue EphrinB2 expressed in the optic chiasm (Garcia-Frigola et al., 2008; Lee et al., 2008; Williams et al., 2004). Although it remains to be elucidated whether transcription factors directly or indirectly regulate the expression of guidance receptors in all of these processes, recent evidence in spinal commissural neurons strongly suggests that direct transcriptional regulation operates during axon guidance (Wilson et al., 2008).

The migration of MGE-migrating cortical and striatal neurons to their corresponding target territories is subjected to several levels of regulation, ranging from the common activity of motogenic factors to the selective action of environmental guidance cues (Marín and Rubenstein, 2003). The molecular mechanisms controlling neuronal migration have multiple similarities with those described for axon guidance. For instance, migrating neurons and growing axons are instructed towards their final destination by the same guidance molecules (Bagri et al., 2002; Bagri and Tessier-Lavigne, 2002; Brose and Tessier-Lavigne, 2000) and are able to respond to distinct cues by the differential expression of specific guidance receptors (Dickson, 2002). In axon guidance, transcriptional regulation is one of the key mechanisms controlling the repertoire of

receptors expressed by each neuron. Is transcriptional regulation also relevant for neuronal migration? There is increasing evidence that transcription factors also modulate neuronal migration and guidance decisions in the developing mouse brain. However, the precise contribution of such regulators for the migration and sorting of MGE-derived interneurons is currently unknown.

1.4. Aims

The homeodomain transcription factor Nkx2-1 plays fundamental roles in the development of the ventral subpallium. For example, Nkx2-1 interprets Shh signalling and instructs specific cell-fates in MGE-derived cells. During development, the MGE is simultaneously the source for several telencephalic cell types, such as cortical and striatal interneurons. Interestingly, the expression of Nkx2-1 is only maintained in the striatal population, suggesting a possible function for this transcription factor in controlling the migration and differentiation of these cells. Strikingly, the precise sorting of these two MGE-derived populations relies in the differential expression of receptors for the class 3 semaphorins and transcription regulation has been proposed to be the main mechanism controlling target selectivity in axon guidance. Preliminary experiments of *Nkx2-1* gain-of-function in organotypic embryonic slices suggested a potential role for Nkx2-1 in controlling target decisions of MGE-derived interneurons (O. Marín, unpublished observations). With this scenario in mind, the major aim of this study was to investigate the functional relevance of Nkx2-1 postmitotic expression for the migration of MGE-derived striatal interneurons. To achieve this goal, we proposed to investigate:

1. The implication of Nkx2-1 postmitotic expression in controlling the sorting of MGE-derived cortical and striatal interneurons by modulating the Sema/Nrp signalling.

2. The involvement of Nkx2-1 in mediating the responsiveness of MGE-derived striatal interneurons to additional environmental cues required for their migration.

Chapter 2. Postmitotic Nkx2-1 Controls the Migration of Telencephalic Interneurons by Direct Repression of Guidance Receptors

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Postmitotic Nkx2-1 Controls the Migration of Telencephalic Interneurons by Direct Repression of Guidance Receptors

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SUMMARY

The homeodomain transcription factor Nkx2-1 plays key roles in the developing telencephalon, where it regulates the identity of progenitor cells in the medial ganglionic eminence (MGE) and mediates the specification of several classes of GABAergic and cholinergic neurons. Here, we have investigated the postmitotic function of Nkx2-1 in the migration of interneurons originating in the MGE. Experimental manipulations and mouse genetics show that downregulation of Nkx2-1 expression in postmitotic cells is necessary for the migration of interneurons to the cortex, whereas maintenance of Nkx2-1 expression is required for interneuron migration to the striatum. Nkx2-1 exerts this role in the migration of MGE-derived interneurons by directly regulating the expression of a guidance receptor, *Neuropilin-2*, which enables interneurons to invade the developing striatum. Our results demonstrate a role for the cell-fate determinant Nkx2-1 in regulating neuronal migration by direct transcriptional regulation of guidance receptors in postmitotic cells.

INTRODUCTION

During development of the nervous system, migrating neurons and axons are guided to their final destination by the coordinated activity of multiple extracellular cues that act on specific membrane receptors to steer their movement in the right direction (Dickson, 2002; Tessier-Lavigne and Goodman, 1996). Because neuronal guidance typically involves a complex set of instructions even in the simplest organisms, adoption of a specific program of migration requires that neurons respond to guidance cues in a highly regulated pattern, both in time and space. Transcriptional regulation is a key determinant in this process, as it ultimately represents one of the primary mechanisms controlling the repertoire of receptors expressed by neurons (Butler and Tear, 2007; Polleux et al., 2007).

Transcription factors regulating neuronal fate specification typically function before the last division of progenitor cells, while guidance decisions are made at later stages of development (Jessell, 2000). Thus, it is likely that transcription factors expressed in postmitotic neurons are responsible for activating specific migration and axon guidance programs. In the vertebrate spinal cord, for example, the expression of a specific combination of transcription factors in postmitotic motoneurons appears to encode their axon trajectory and final targeting (Kania et al., 2000; Sharma et al., 1998, 2000; Thaler et al., 2004). Interestingly, some of the same transcription factors that regulate axon guidance also play a major role in the early specification of different neuronal pools, suggesting that the same transcription factors may carry on different functions depending on the cellular context (De Marco Garcia and Jessell, 2008; Müller et al., 2003; Shirasaki and Pfaff, 2002). In addition to demonstrating the function of transcription factors in the regulation of axon guidance, studies over the past few years have begun to identify possible candidate genes that would function as downstream effectors of these factors. For example, it has been suggested that Lim1 expression in LMC neurons may regulate the expression of the receptor tyrosine kinase EphA4, which is essential for the final targeting of their axonal projections to the limb mesenchyma (Kania and Jessell, 2003). Similarly, the role of the transcription factor Zic2 in regulating midline crossing by retinal axons appears to involve the regulation of another member of the Eph family, EphB1 (García-Frigola et al., 2008; Lee et al., 2008; Williams et al., 2004).

Since migrating neurons and growing axons are instructed toward their final destination by similar guidance molecules (Bagri and Tessier-Lavigne, 2002; Brose and Tessier-Lavigne, 2000), it seems conceivable that equivalent mechanisms regulate the expression of guidance receptors in both migrating neurons and axons. However, the function of specific transcription factors in the migration and positioning of neurons is still very limited (McEvilly et al., 2002; Sugitani et al., 2002; Ge et al., 2006; Hand et al., 2005; Le et al., 2007). In the developing telencephalon, the

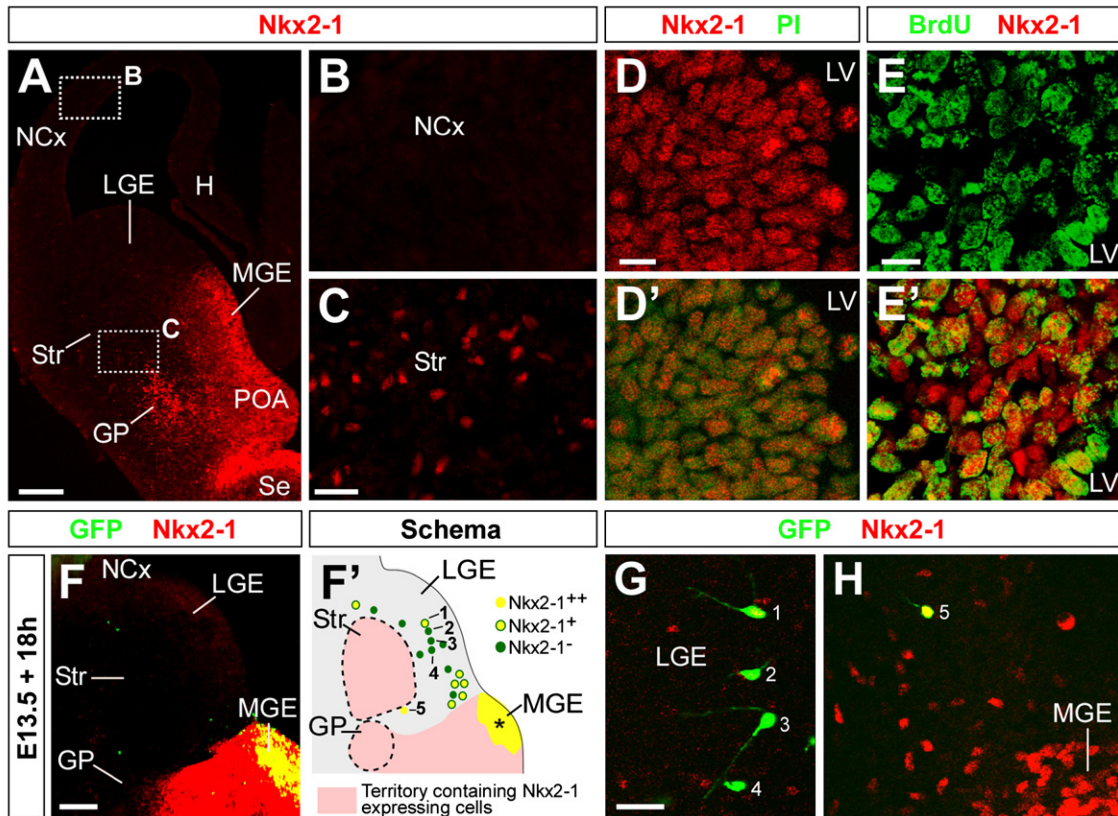

Figure 1. Cortical Interneurons Downregulate Nkx2-1 Protein Expression after Leaving the MGE Progenitor Zone

(A) Coronal section through the brain of an E13.5 mouse embryo showing Nkx2-1 protein expression in the medial ganglionic eminence (MGE), striatum (Str), globus pallidus (GP), preoptic area (POA), and septum (Se).

(B and C) Higher-magnification images of the areas boxed in (A).

(D–E′) MGE ventricular zone (VZ) from an E13.5 embryo depicting MGE cells stained with Nkx2-1 and propidium iodine (PI) (D and D′) and MGE progenitors stained S-phase marker BrdU and Nkx2-1 (E and E′).

(F–H) Expression of Nkx2-1 protein in GFP-expressing cells after transplantation of GFP-expressing progenitors in the MGE. As illustrated in the schematic diagram (F′) and the high-magnification images (G and H), the majority of migrating cells had undetectable levels of Nkx2-1 (green dots), many cells expressed low levels of Nkx2-1 (yellow dots with green circle), and a small number of cells expressed high levels of Nkx2-1 protein (yellow dots). The numbers depicted in the schematic (F′) describe the location of cells shown in (G) and (H).

H, hippocampus; LGE, lateral ganglionic eminence; LV, lateral ventricle; NCx, neocortex. Asterisk, transplant. Scale bars = 200 μm (A and F), 50 μm (B, C, G, and H), and 10 μm (D–E′).

medial ganglionic eminence (MGE) is the origin of interneurons that migrate tangentially to the striatum and cerebral cortex (Lavdas et al., 1999; Marín et al., 2000; Sussel et al., 1999; Wichterle et al., 1999, 2001). We have previously shown that sorting of striatal and cortical interneurons to their respective target territories depends on neuropilin/semaphorin interactions (Marín et al., 2001). Here, we have investigated the transcriptional mechanisms regulating this process. We found that downregulation of postmitotic Nkx2-1 expression is a necessary event for the migration of interneurons to the cortex, whereas Nkx2-1 expression is required for interneuron migration to the striatum. Forced Nkx2-1 expression in MGE-derived cells prevents interneuron migration to the cortex, whereas loss of Nkx2-1 function reduces the number of interneurons that accumulate in the striatum. Nkx2-1 exerts this role in the migration of MGE-derived interneurons by directly regulating the expression of *Neuropilin-2* (*Nrp2*), the receptor of *Semaphorin-3F* (*Sema3F*). Our results demonstrate that direct transcriptional regulation of guidance receptors in postmitotic neurons is an essential mechanism in neuronal migration.

RESULTS

Interneurons Migrating to the Cortex Rapidly Downregulate Nkx2-1 Expression

Nkx2-1 is one of the earliest genes expressed in the mouse forebrain (Sussel et al., 1999). At embryonic day (E) 13.5, the peak of interneuron generation in the mouse, Nkx2-1 is strongly expressed by cells in several progenitor domains of the subpallium, including the MGE, the preoptic area, and part of the septum, whereas it is absent from the lateral ganglionic eminence (Figure 1A). At this stage, Nkx2-1 expression was also observed in many postmitotic neurons derived from the MGE, such as striatal interneurons (Figures 1A and 1C; Marín et al., 2000). In contrast,

Nkx2-1 expression was not detected in cortical interneurons at this or any other embryonic stages (Figures 1A and 1B and data not shown), even though many of them originate in the MGE (Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 1999, 2001). There are two possible hypotheses that would explain the differential expression of Nkx2-1 in postmitotic striatal and cortical interneurons. One possibility is that MGE progenitors producing cortical interneurons never express Nkx2-1. Alternatively, Nkx2-1 might be expressed by MGE progenitors giving rise to both striatal and cortical interneurons, but this latter population would rapidly downregulate Nkx2-1 expression soon after leaving the MGE. To test these alternative hypotheses, we first quantified the percentage of cells expressing Nkx2-1 located within 100 μm of the ventricle in the MGE of E13.5 embryos. Virtually all cells present in the ventricular zone (VZ) of the MGE expressed Nkx2-1 at this stage (Figures 1D and 1D′; 99.2% ± 0.4% of propidium iodine cells, average ± SEM). We next quantified the number of progenitor cells expressing Nkx2-1 in the VZ of the MGE in E13.5 embryos. In agreement with previous reports (Xu et al., 2005), we found that almost every cell in the MGE that incorporated the S-phase marker BrdU at this stage also expressed Nkx2-1 (Figures 1E and 1E′; 96.9% ± 1.1% of BrdU cells, average ± SEM), reinforcing the view that all MGE progenitors express this transcription factor.

To specifically test whether MGE-derived interneurons downregulate Nkx2-1 expression while migrating toward the cortex, we performed experiments in which a piece of E13.5 MGE VZ from a green fluorescence protein (GFP)-expressing transgenic mouse brain was transplanted into the same region of wild-type host slices. After 18 hr in culture, we analyzed the expression of Nkx2-1 in GFP migrating cells (Figures 1F and 1F′; n = 3 experiments, 132 GFP cells analyzed). The majority of GFP-expressing cells derived from the transplant were found in a corridor deep to the striatal mantle through which most interneurons reach the cortex (Flames et al., 2004), and they did not contain detectable levels of Nkx2-1 protein (Figures 1F′ and 1G; 67.0% ± 14.3% of GFP cells, average ± SEM). About 22% of the GFP-expressing cells were also located in the corridor and contained traces of Nkx2-1 (Figures 1F′ and 1G; 22.2% ± 14.8% of GFP cells, average ± SEM), suggesting that these cells might be undergoing downregulation of this protein. Finally, a small proportion of GFP-expressing cells was located in the ventrolateral aspect of the prospective striatum and expressed high levels of Nkx2-1 protein (Figures 1F′ and 1H; 10.9% ± 4.3% of GFP cells, average ± SEM). These experiments suggest that MGE-derived interneurons migrating toward the cortex rapidly downregulate the expression of Nkx2-1 after leaving the MGE, whereas those interneurons migrating to the striatum maintain the expression of this transcription factor.

Nkx2-1 Expression Prevents the Migration of MGE-Derived Cells toward the Cortex

Our previous observations raised the intriguing possibility that downregulation of Nkx2-1 in MGE-derived interneurons might be necessary to acquire a cortical migratory fate. To test this hypothesis, we forced the expression of *Nkx2-1* in MGE progenitor cells through focal electroporation in E13.5 slices (Figure 2A). In control experiments, *Gfp*-expressing cells migrated to the cortex following their normal route (>90 cells per cortex in 22/22 slices; Figures 2C and 2H), and only a minority of cells was found in the striatum. In contrast, in slices coelectroporated with *Gfp* and *Nkx2-1*, the majority of MGE-derived cells accumulated in the basal ganglia, and only occasional cells were found in the cortex (<20 cells per cortex in 22/22 slices; Figures 2D and 2H). To exclude the possibility that overexpression of *Nkx2-1* prevents interneuron migration rather than changing their direction, we electroporated small MGE explants with either *Gfp* alone or *Gfp* and *Nkx2-1* and cultured them in three-dimensional Matrigel matrices. After 36 hr in culture, cells were found to migrate a similar distance in the two experimental conditions (data not shown; see also Figures 6B and 6D), suggesting that expression of *Nkx2-1* in postmitotic interneurons does not impair cell migration but appears to specifically disrupt their target selection.

Nkx2-1 belongs to the NK-2 class of homeodomain (HD) transcription factors (Harvey, 1996). In addition to the HD, two other peptide domains are conserved within the NK-2 class of transcription factors. The short TN domain (Tinmam motif), located at the N-terminal region, has been suggested to underlie the function of the NK-2 proteins as repressors during neural patterning events in the ventral neural tube (Muhr et al., 2001). The function of the NK-2 specific domain (NK-2-SD) is not fully understood. In Nkx2-2, NK-2-SD has been shown to act as an intra-molecular inhibitor of a transcriptional activator domain located at the C terminus of the protein (Watada et al., 2000). To investigate the mechanism through which Nkx2-1 may regulate the migration of MGE-derived cells, we performed a structure-function analysis in organotypic slices. We first evaluated the role of the TN motif by electroporating a truncated form of *Nkx2-1* missing the first 20 amino acids (*Nkx2-1*$^{\Delta TN}$; Figure 2B). Analysis of slices electroporated with *Gfp* and *Nkx2-1*$^{\Delta TN}$ revealed that MGE-derived cells also failed to reach the cortex in the absence of the TN motif (<20 cells per cortex in 17/17 slices; Figures 2E and 2H). To investigate if a putative C terminus activator domain plays a role in the migration of MGE-derived cells, we used a truncated form of *Nkx2-1* missing four amino acids of the NK-2-SD domain and the remaining C-terminal of the protein (*Nkx2-1*$^{\Delta Ct}$; Figure 2B). Analysis of slices electroporated with *Gfp* and *Nkx2-1*$^{\Delta Ct}$ showed that MGE-derived cells also failed to reach the cortex in the absence of the C terminus activator domain and an intact NK-SD motif (<20 cells per cortex in 11/11 slices; Figures 2F and 2H). Finally, we assessed the role of the HD in this process by performing a single amino acid substitution in the position 35 of the *Nkx2-1* HD (*Nkx2-1*^{A35T}; Figure 2B). This mutated form of Nkx2-1 binds to DNA target sequences with 50-fold less affinity than wild-type Nkx2-1 (Xiang et al., 1998). Analysis of slices electroporated with *Gfp* and *Nkx2-1*^{A35T} revealed that MGE-derived cells expressing this construct were able to migrate toward the cortex like in control slices (>90 cells per cortex in 14/15 slices; Figures 2G and 2H). Similar results were obtained when a different point mutation in the HD (*Nkx2-1*^{Y54M}) was used (>90 cells per cortex in 18/19 slices; data not shown). Of note, the A35T replacement does not appear to decrease the stability of the protein (which would have explained the absence of a phenotype), since *Gfp/Nkx2-1*^{A35T}-expressing cells migrating toward the cortex have detectable

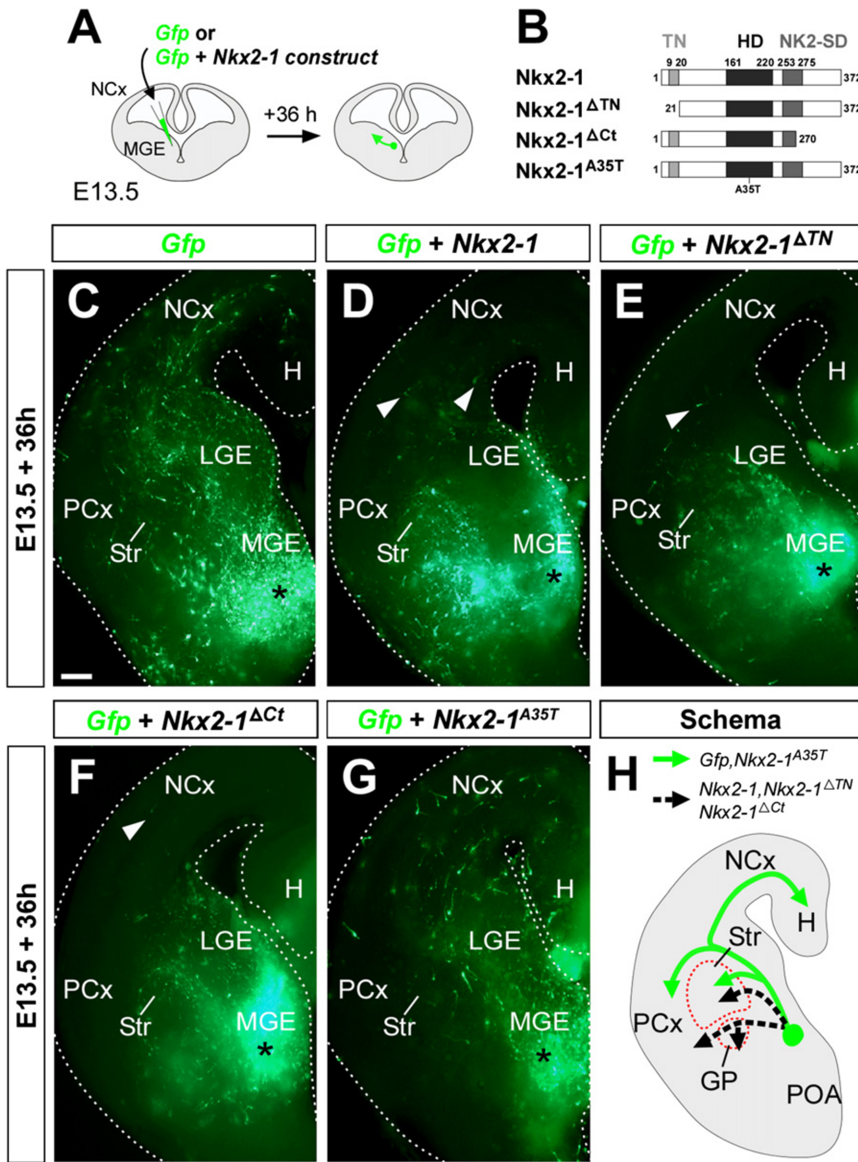

Figure 2. *Nkx2-1* Overexpression in MGE-Derived Interneurons Prevents Their Migration to the Cortex

(A and B) Schematic diagrams of the focal electroporation experiment and the Nkx2-1 (372 amino acids) constructs used in these experiments.

(C–G) Migration of MGE-derived cells electroporated with *Gfp* (C) or with *Gfp* and *Nkx2-1* (D), *Nkx2-1$^{\Delta TN}$* (E), *Nkx2-1$^{\Delta Ct}$* (F), or *Nkx2-1^{A35T}* (G). Arrowheads point to cells that have reached the cortex. Dotted lines indicate the limits of the organotypic slices.

(H) Schematic representation of the migratory routes adopted by MGE-derived cells electroporated with *Gfp* and *Gfp* + *Nkx2-1^{A35T}* (green arrow) or with *Gfp Nkx2-1*, *Nkx2-1$^{\Delta TN}$*, or *Nkx2-1$^{\Delta Ct}$* (black dotted arrow).

GP, globus pallidus; H, hippocampus; HD, homeodomain; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; NK-2-SD, NK-2 specific domain; PCx, piriform cortex; POA, preoptic area; Str, striatum; TN, Tinman motif. Scale bar = 200 μm.

levels of Nkx2-1 (see Figure S1 available online). Altogether, these experiments revealed that the mechanism through which *Nkx2-1* prevents MGE-derived cells from migrating toward the cortex requires an intact HD and does not rely on interactions involving the TN and the C terminus activator/SD domains.

Loss of Postmitotic Nkx2-1 Function Decreases the Number of Striatal Interneurons

If Nkx2-1 regulates the sorting of cortical and striatal interneurons, loss of Nkx2-1 function in postmitotic cells should lead to a reduction in the number of MGE-derived cells that accumulate in the striatum. To test this hypothesis, we bred mice carrying floxed alleles of the *Nkx2-1* locus (Kusakabe et al., 2006) with transgenic mice in which Cre recombinase is expressed under the control of *Lhx6* (Fogarty et al., 2007), a LIM-HD transcription factor expressed by MGE-derived neurons that drives recombination almost exclusively in postmitotic cells (Figure S2). To verify the efficiency and specificity of recombination, we analyzed the distribution of cells expressing Nkx2-1 protein in the subpallium of E15.5 control and *Lhx6-Cre;Nkx2-1$^{Fl/Fl}$* mutant embryos. As expected, we found that expression of Nkx2-1 in the MGE VZ was not affected whereas the number of Nkx2-1 expressing cells throughout the subpallial mantle, including the developing striatum, was dramatically reduced in *Lhx6-Cre; Nkx2-1$^{Fl/Fl}$* mutant embryos (Figure S3). The presence of *Nkx2-1* mRNA in some striatal cells suggests that either they have yet to recombine the two *Nkx2-1* alleles or that a fraction of striatal interneurons may not express *Lhx6* or, at least, the *Lhx6-Cre* transgene (Figure S3).

To assess the impact of postmitotic loss of Nkx2-1 function in the development of striatal interneurons, we analyzed the expression of several markers for striatal interneurons, such as *Lhx6*, *Lhx7* (also known as *Lhx8*), *Er81* and *Somatostatin* (*Sst*) (Kawaguchi et al., 1995; Marín et al., 2000; Stenman et al., 2003). We found that the striatum of E15.5 *Lhx6-Cre;Nkx2-1$^{Fl/Fl}$* mutant embryos contained significantly fewer *Lhx6*-, *Lhx7*-, *Er81*- and *Sst*-expressing neurons than controls (n = 3; Figure 3). We next analyzed the distribution of interneurons in the striatum of postnatal day 25 control and *Lhx6-Cre;Nkx2-1$^{Fl/Fl}$* mutant mice. Quantification of the number of neurons expressing choline acetyltranferase (ChAT), parvalbumin (PV) and SST, markers of the three main classes of mature striatal interneurons (Kawaguchi et al., 1995), revealed that the striatum of P25 *Lhx6-Cre;Nkx2-1$^{Fl/Fl}$* mutant mice contained significantly fewer ChAT- and PV-expressing interneurons than controls (n = 3; Figures 4A–4F), while the number of SST-expressing cells did not differ (n = 3; Figures 4G–4I).

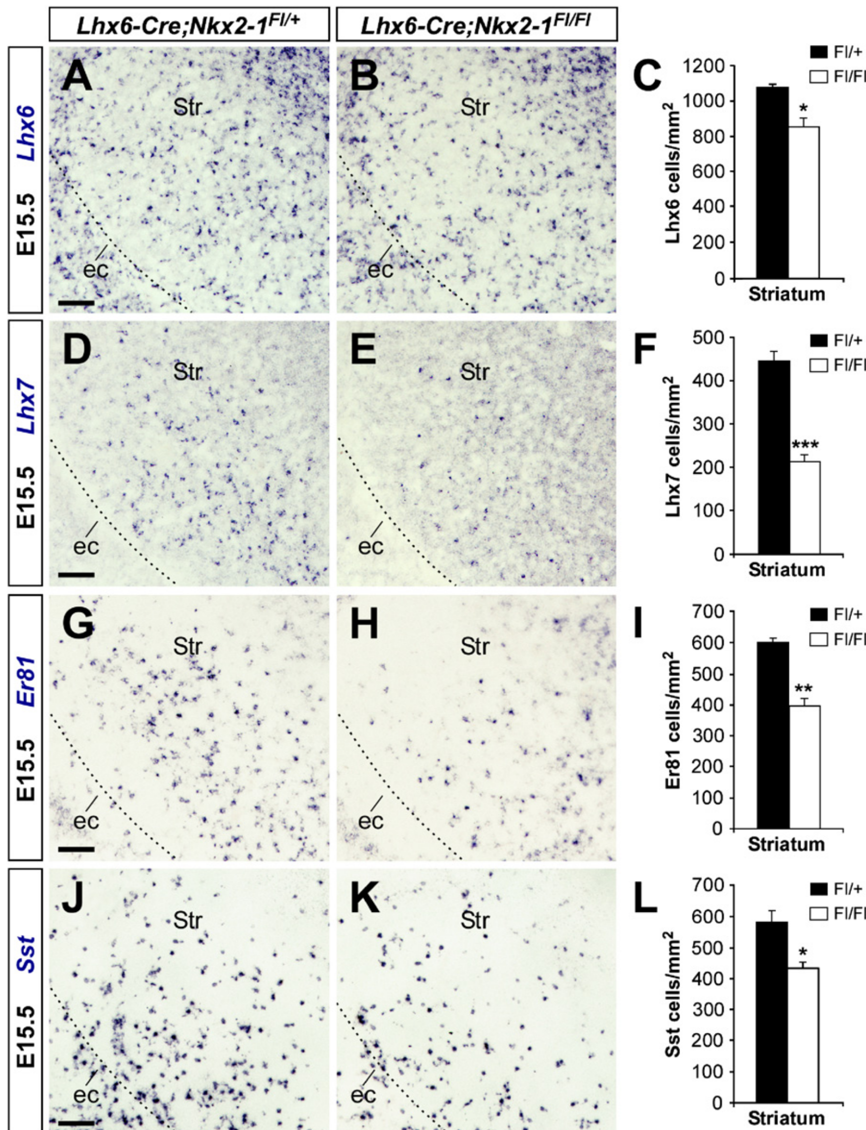

Figure 3. Reduced Numbers of Striatal Interneurons after Postmitotic Loss of *Nkx2-1* Function

(A, B, D, E, G, H, J, and K) Coronal sections through the telencephalon of E15.5 control (A, D, G, and J) and *Lhx6-Cre;Nkx2-1$^{Fl/Fl}$* mutant (B, E, H, and K) embryos showing *Lhx6* (A and B), *Lhx7* (D and E), *Er81* (G and H), and *Sst* (J and K) mRNA expression.

(C, F, I, and L) Quantification of the number of *Lhx6*, *Lhx7*, *Er81*, and *Sst*-expressing cells in the striatum of E15.5 control and *Lhx6-Cre;Nkx2-1$^{Fl/Fl}$* mutant embryos. Histograms show average ± SEM 1083.96 ± 23.47 (*Lhx6* control); 862.07 ± 49.01 (*Lhx6* mutant); 452.32 ± 21.78 (*Lhx7* control); 212.02 ± 18.68 (*Lhx7* mutant); 601.60 ± 12.74 (*Er81* control); 397.05 ± 23.84 (*Er81* mutant); 579.03 ± 39.17 (*Sst* control); 432.18 ± 20.03 (*Sst* mutant). ***p < 0.001, **p < 0.01, and *p < 0.05, t test.

ec, external capsule; Str, striatum. Scale bar = 100 μm.

The previous results were consistent with the hypothesis that loss of Nkx2-1 function prevents the migration of MGE-derived interneurons to the striatum. There were, however, alternative possibilities to explain these results. For example, loss of Nkx2-1 could lead to increased cell death in striatal interneurons. Quantification of the density of apoptotic cells (marked by cleaved Caspase3 expression) revealed no differences in the MGE and striatum of E13.5 control and *Lhx6-Cre;Nkx2-1$^{Fl/Fl}$* mutant embryos (n = 3; MGE, 77.2% ± 3.3% [control] and 70.2% ± 4.4% [mutant]; striatum, 49.0% ± 0.7% [control] and 45.5 ± 5.7% [mutant]; average ± SEM). Similar results were observed at E12.5 and E14.5 (data not shown), suggesting that this was not the case.

Nkx2-1 could also be required to induce or maintain the expression of striatal interneuron markers, and so the observed decrease in the number of striatal interneurons could merely reflect a failure in the expression of those genes. This seems unlikely, because similar numbers of *Lhx6* and *Lhx7*-expressing cells were found throughout the telencephalon outside the striatum in control and mutant mice (Figure S4). However, to completely rule out this possibility, we performed two additional series of experiments. First, we bred a Cre-reporter line (Rosa-LoxP-STOP-LoxP-YFP, also known as Rosa-EYFP mice; Srinivas et al., 2001) into the mutant background to obtain *Lhx6-Cre;Nkx2-1$^{Fl/+}$;Rosa-YFP* (control) and *Lhx6-Cre;Nkx2-1$^{Fl/Fl}$;Rosa-YFP* (mutant) embryos. In these mice, Cre-mediated recombination in *Lhx6*-expressing cells leads to their permanent labeling with EYFP through the ubiquitous Rosa promoter. Consistent with the hypothesis that MGE-derived interneurons fail to migrate to the striatum in the absence of Nkx2-1, the striatum of *Lhx6-Cre;Nkx2-1$^{Fl/Fl}$;Rosa-YFP* mutants at P0 contained fewer YFP cells than controls (n = 3; Figures 5A–5C). This deficit was readily detectable as early as E13.5 (n = 3; Figures S5).

In a second series of experiments, we directly assessed the migration of MGE-derived interneurons by focally electroporating a plasmid encoding *Gfp* (Figure 5D). In control experiments, the ratio between the number of *Gfp*-expressing cells in an equal volume of cortex and striatum was approximately 3:1 (n = 6 slices; Figures 5E and 5G). In contrast, in slices obtained from *Lhx6-Cre;Nkx2-1$^{Fl/Fl}$* mutant embryos, this proportion increased to approximately 7:1 due to a reduction in the number of *Gfp*-cells that migrated to the striatum, while the number of cortical *Gfp*-cells remained similar to controls (n = 6 slices; Figures 5F and 5G). In conclusion, our experiments demonstrate that postmitotic *Nkx2-1* function is required for the migration of MGE-derived interneurons to the developing striatum.

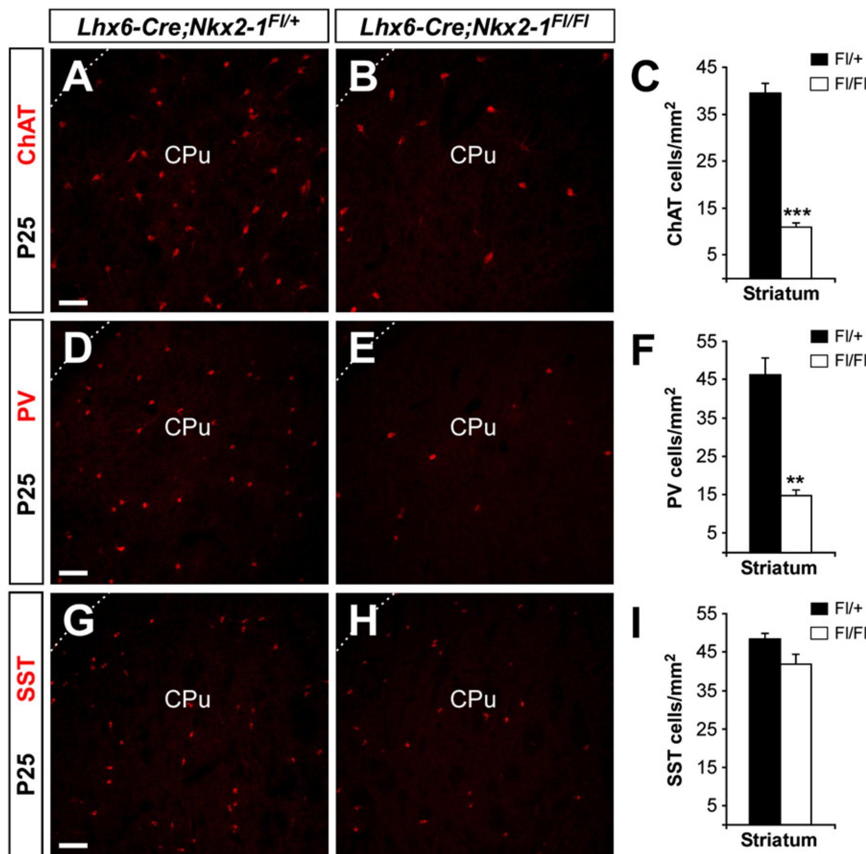

Figure 4. Loss of *Nkx2-1* Function Decreases the Number of Interneurons in the Postnatal Striatum

(A, B, D, E, G, and H) Coronal sections through the striatum of P25 control (A, D, and G) and *Lhx6-Cre;Nkx2-1$^{Fl/Fl}$* mutant (B, E, and H) mice showing ChAT (A and B), PV (D and E), and SST (G and H) expression.

(C, F, and I) Quantification of the number of ChAT, PV, and SST-expressing cells in the striatum of P25 control and *Lhx6-Cre;Nkx2-1$^{Fl/Fl}$* mutant mice. Histograms show average ± SEM 39.42 ± 2.26 (ChAT control); 10.94 ± 1.04 (ChAT mutant); 46.32 ± 4.52 (PV control); 14.71 ± 1.77 (PV mutant); 48.23 ± 1.65 (SST control); 41.73 ± 2.81 (SST mutant). ***$p < 0.001$ and **$p < 0.01$, t test.

CPu, caudate putamen. Scale bar = 100 μm.

Nkx2-1 Expression in Postmitotic Cells Suppresses *Sema3A/3F*-Mediated Repulsion of MGE-Derived Interneurons

Nkx2-1 might control the sorting of MGE-derived cortical and striatal interneurons through regulating the expression of specific receptors for guidance factors in those cells. Our previous work has shown that Neuropilin-1 (Nrp1) and Neuropilin-2 (Nrp2), the binding receptors for the repulsive molecules Semaphorin 3A (Sema3A) and Semaphorin 3F (Sema3F), respectively, are expressed by MGE-derived cortical interneurons, but not by striatal interneurons (Marín et al., 2001). Because the developing striatum expresses both Sema3A and Sema3F, expression of Nrp1 and Nrp2 in cortical interneurons prevents their entry into the striatum, channeling them toward the cortex (Marín et al., 2001). We thus hypothesized that postmitotic Nkx2-1 may participate in the sorting of cortical and striatal interneurons by regulating the expression of the receptors for Sema3A and/or Sema3F. To test this idea, we electroporated E13.5 MGE explants with plasmids encoding *Gfp* or *Nkx2-1-IRES-Gfp* and cultured them along with aggregates of COS cells expressing either *DsRed* or *DsRed* and *Sema3A/3F* in Matrigel matrices (Figure 6A). As expected from our previous work (Marín et al., 2001), we found that Sema3A/3F exerted a potent chemorepulsive effect over *Gfp*-expressing MGE-derived cells (n = 12; Figures 6B, 6C, and 6H). In contrast, *Sema3A/3F*-expressing COS cells did not repel MGE-derived cells expressing *Nkx2-1* (n = 12; Figures 6D, 6E, and 6H). To determine if the Nkx2-1 HD mediates the suppression of Sema3A/3F-mediated chemorepulsion, we confronted *Nkx2-1^{A35T}*-electroporated MGE explants with COS cells aggregates. As in controls, MGE-derived cells expressing the HD mutation *Nkx2-1^{A35T}* were repelled by COS cells expressing *Sema3A/3F* (n = 11; Figures 6F, 6G, and 6H). In sum, these results demonstrate that expression of Nkx2-1 in migrating MGE-derived cells renders them insensitive to Sema3A/3F chemorepulsion through an Nkx2-1 HD-dependent mechanism.

Nkx2-1 Represses *Nrp2* Expression in Migrating MGE-Derived Interneurons

The previous results are consistent with the hypothesis that Nkx2-1 represses the expression of receptors for Sema3A and/or Sema3F in MGE-derived migrating neurons. To directly test this, we developed an in vitro assay in which we could specifically isolate RNA from migrating MGE-derived neurons (Figure 7A). MGE explants were electroporated with *Gfp* or *Nkx2-1-IRES-Gfp* plasmids and migrating cells were collected after 48 hr in culture. A limitation of this experimental approach is that although the population of cells is highly enriched in migratory neurons, the proportion of electroporated cells was relatively low (~30%; Figures 7B–7C′). Despite this caveat, gene expression analysis using semiquantitative RT-PCR revealed a dramatic increase in the expression of *Nkx2-1* in migrating MGE-derived cells expressing *Nkx2-1-IRES-Gfp* compared to those expressing the control plasmid (n = 3, Figure 7D). Compared to controls, we also detected a mild reduction in the expression of *Nrp1* and a prominent decrease in the expression of *Nrp2* transcripts in migrating neurons expressing *Nkx2-1-IRES-Gfp* (Figure 7D). In contrast, we did not detect significant differences in the expression of *PlexinA3* and *PlexinA4* (Figure 7D), signaling components of the receptor complexes for Sema3A and Sema3F (Yaron et al., 2005). Similarly, the expression of

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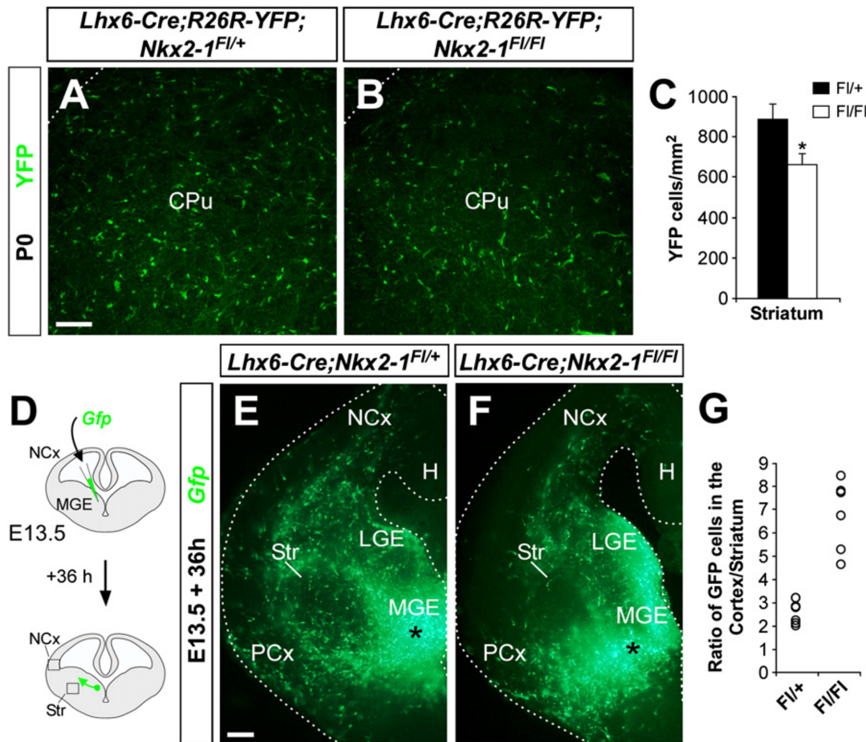

Figure 5. Tracing Experiments Reveal Less Interneurons Invading the Striatum after Postmitotic Loss of *Nkx2-1* Function

(A and B) Coronal sections through the striatum of P0 *Lhx6-Cre;Nkx2-1$^{Fl/+}$;Rosa-YFP* control (A) and *Lhx6-Cre;Nkx2-1$^{Fl/Fl}$;Rosa-YFP* mutant (B) mice showing YFP expression. YFP is also detected in scattered blood vessels, as previously reported (Fogarty et al., 2007).

(C) Quantification of the number of YFP-expressing cells in the striatum of P0 *Lhx6-Cre;Nkx2-1$^{Fl/+}$;Rosa-YFP* control and *Lhx6-Cre;Nkx2-1$^{Fl/Fl}$;Rosa-YFP* mutant mice. Histograms show average ± SEM 889.31 ± 79.03 (YFP control); 664.12 ± 58.40 (YFP mutant). * $p < 0.05$, t test.

(D) Schematic diagram of the focal electroporation experiment. The number of *Gfp*-expressing cells was counted in a fixed volume of the cortex and striatum (black boxes) and the ratio between these populations was determined for each slice.

(E and F) Migration of MGE-derived cells in E13.5 *Lhx6-Cre;Nkx2-1$^{Fl/+}$* control (E) and *Lhx6-Cre;Nkx2-1$^{Fl/Fl}$* mutant (F) slices. Occasionally, *Gfp*-expressing cells accumulated in the piriform cortex of mutant slices. Dotted lines indicate the limits of the organotypic slices.

(G) Ratio of *Gfp*-expressing cells in the quantified region of the cortex and striatum for each individual E13.5 *Lhx6-Cre;Nkx2-1$^{Fl/+}$* control and *Lhx6-Cre;Nkx2-1$^{Fl/Fl}$* mutant slices. Average ± SEM 2.55 ± 0.41 (control); 6.80 ± 1.22 (mutant). ***$p < 0.001$, t test. Number of *Gfp*-expressing cells in the striatum (30.38 ± 4.03, control; 12.20 ± 3.80, mutant. ***$p < 0.001$, t test) and cortex (76.80 ± 12.76, control; 84.20 ± 21.20, mutant) of electroporated slices.

CPu, caudate putamen; H, hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; PCx, piriform cortex; Str, striatum. Scale bar = 100 μm (A and B) and 200 μm (E and F).

the GABA synthesizing enzyme *Gad67* or the transcription factor *Lhx6,* both present in cortical and striatal interneurons, did not differ between *Gfp*- and *Nkx2-1-IRES-Gfp*-expressing cells. We next used quantitative RT-PCR to precisely determine the influence of Nkx2-1 in the transcription of *Nrp1* and *Nrp2* in migrating MGE-derived cells. In these experiments, we could confirm that *Nrp2* expression was reduced in *Nkx2-1-IRES-Gfp*-expressing MGE-derived cells (n = 6; Figure 7E). In contrast, although we consistently found reduced levels of *Nrp1* expression in *Nkx2-1-IRES-Gfp*-expressing MGE-derived cells compared to controls, these differences were not statistically significant (n = 6; Figure 7E). Together with our previous findings of reduced sensitivity to semaphorin signaling, these results strongly suggest a role for Nkx2-1 in the transcriptional repression of, at least, the *Nrp2* receptor.

Nkx2-1 Directly Represses the Transcription of *Nrp2*

Nkx2-1 could inhibit the expression of *Nrp2* in MGE-derived migrating interneurons by directly interacting with the *Nrp2* promoter. To test this, we first examined whether Nkx2-1 protein directly binds to *Nrp2* regulatory sequences in vivo. A phylogenetic footprinting analysis of the 20 kb sequence upstream from the *Nrp2* transcription initiation site reveal two putative *Nrp2* regulatory regions containing two adjacent Nkx2-1 binding sequences (Francis-Lang et al., 1992), which we designated as *Nrp2*-region2 (from −21375 bp 5′-CTTGC-3′ to −21086 bp 5′-GTGCT-3′) and *Nrp2*-region1 (from −327 bp 5′-CCGGA-3′ to −68 bp 5′-GGGGA-3′; Figures 8A and S6). Chromatin immunoprecipitation (ChIP) analyses demonstrated that Nkx2-1 binds to the *Nrp2*-region1 in E13.5 MGE-derived cells, while it does not seem to complex with the *Nrp2*-region2 (n = 3; Figures 8B and 8C). To demonstrate that Nkx2-1 represses the expression of *Nrp2* through regulatory sequences located in the *Nrp2*-region1, we cloned this 260 bp DNA fragment upstream of a luciferase reporter plasmid containing a *c-fos* minimal promoter (Figure 8D). Cotransfection of HEK293 cells with the reporter plasmid and full-length *Nkx2-1* produced a significant transcriptional repression of luciferase activity (n = 5; Figure 8D). In contrast, the mutated *Nkx2-1^{A35T}* did not repress luciferase gene expression (Figure 8D). These results indicate that Nkx2-1 transcriptional repression of *Nrp2* requires the direct binding of Nkx2-1 to, at least, the *Nrp2*-region1 regulatory sequence, and that the integrity of the Nkx2-1 HD motif is essential for this activity.

DISCUSSION

During development, neurons are instructed to migrate and project to specific regions of the brain in a process that is tightly regulated by a variety of guidance cues. A fundamental question that remains to be clarified is how distinct neuronal populations respond selectively to guidance. Transcription factors are thought to play a major role in regulating the differential expression of

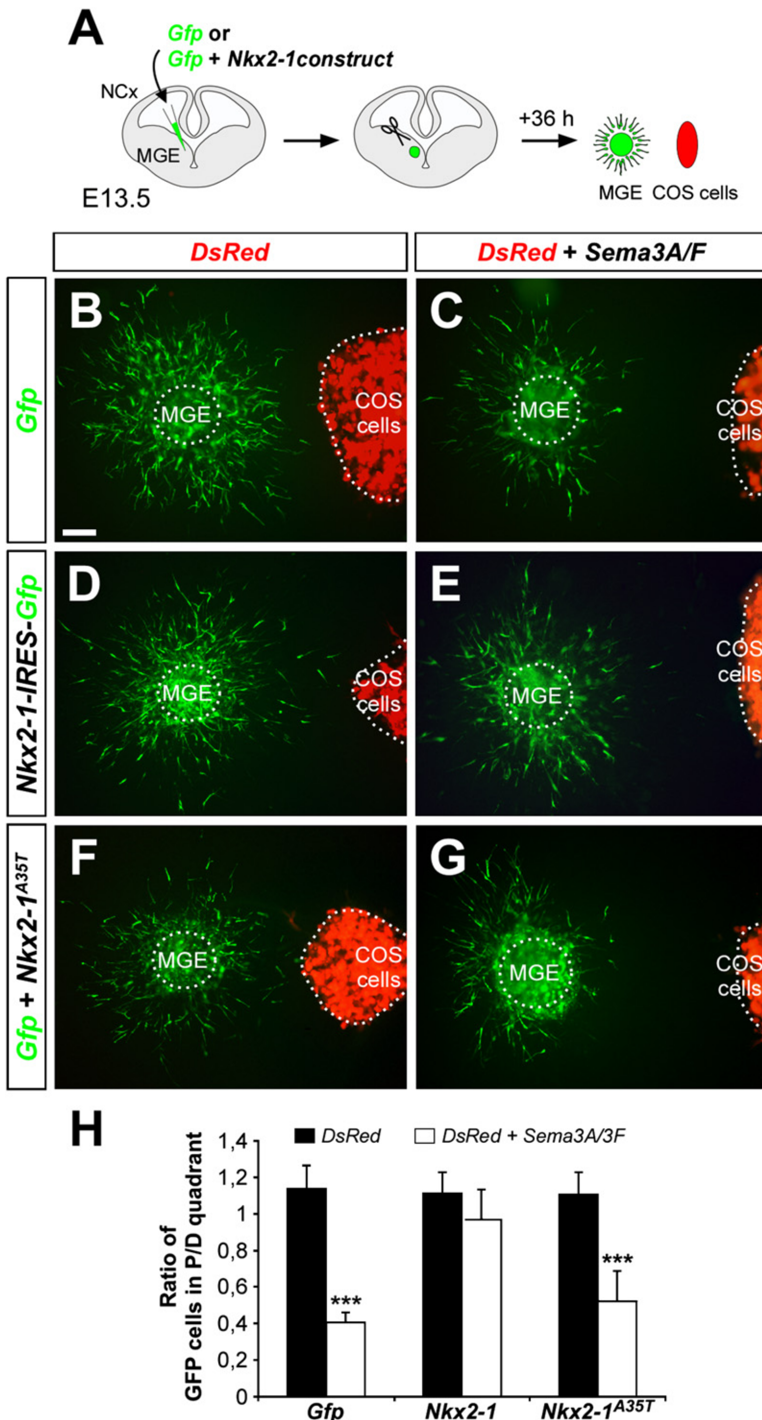

Figure 6. Postmitotic *Nkx2-1* Expression Suppresses Sema3A/3F-Mediated Repulsion in MGE-Derived Interneurons

(A) Schematic diagram of the experimental paradigm used in MGE/COS coculture confrontation assays. E13.5 slices were focally electroporated with *Gfp*, *Nkx2-1-IRES-Gfp* or *Gfp* + *Nkx2-1*^{A35T}. MGE explants were dissected from the electroporated region, confronted to *DsRed* or *DsRed* + *Sema3A/3F*-transfected COS cell aggregates and cultured in Matrigel matrices.

(B–G) Migration of *Gfp* (B and C), *Nkx2-1-IRES-Gfp* (D and E), and *Gfp* + *Nkx2-1*^{A35T} (F and G) electroporated MGE-derived cells in response to mock-transfected (B, D, and F) or *Sema3A/3F*-transfected (C, E, and G) COS cells aggregates.

(H) Quantification of co-culture confrontation assays. P and D, proximal and distal quadrants, respectively. Histograms show average ± SEM 1.13 ± 0.12 (*Gfp* MGE cells, mock-COS cells); 0.40 ± 0.05 (*Gfp* MGE cells, *Sema3A/3F*- COS cells); 1.11 ± 0.12 (*Nkx2-1IRES-Gfp* MGE cells, mock-COS cells); 0.97 ± 0.16 (*Nkx2-1IRES-Gfp* MGE cells, *Sema3A/3F* -COS cells); 1.10 ± 0.12 (*Gfp* + *Nkx2-1A35T* MGE cells, mock-COS cells); 0.52 ± 0.17 (*Gfp* + *Nkx2-1A35T* MGE cells, *Sema3A/3F*-COS cells). ***$p < 0.001$, t test.

MGE, medial ganglionic eminence; NCx, neocortex. Scale bar = 50 μm.

guidance receptors during cell migration and axon guidance (Butler and Tear, 2007; Guthrie, 2007; Polleux et al., 2007), but it is presently unclear how they exert their influence. Using gain and loss-of-function approaches, our results demonstrate that the Nkx2-1 transcription factor is uniquely required for the differential migration of cortical and striatal GABAergic interneurons. Postmitotic interneurons expressing Nkx2-1 become insensitive to semaphorin signaling and migrate toward the striatum, whereas those that lack Nkx2-1 expression migrate to the cortex. ChIP and luciferase assays revealed that Nkx2-1 fulfills this function, at least in part, by directly repressing the expression of *Nrp2*, a receptor for class III semaphorins. Our results therefore demonstrate that postmitotic transcriptional mechanisms play an important role in neuronal migration by directly regulating the repertoire of guidance receptors expressed by migrating neurons.

Transcriptional Control of Telencephalic Interneuron Migration

The MGE gives rise to many cortical GABAergic interneurons while concurrently generating most striatal interneurons (Lavdas et al., 1999; Marín et al., 2000; Sussel et al., 1999; Wichterle et al., 1999), but the mechanisms controlling the segregation of these two neuronal populations remain poorly understood. We have previously shown that Nrp1 and Nrp2, the binding receptors for the striatal repulsive molecules Sema3A and Sema3F (Bagri and Tessier-Lavigne, 2002; Kruger et al., 2005), respectively, are expressed by MGE-derived cortical interneurons and absent from MGE-derived striatal interneurons (Marín et al., 2001). Loss of neuropilin function increases the number of interneurons migrating to the striatum and simultaneously decreases the number of cells reaching the cortex. Since the final destination of tangentially migrating interneurons (striatum or cortex) is determined by the expression of semaphorin receptors, we have investigated the nature of the factors controlling this process.

Our experiments demonstrate that postmitotic Nkx2-1 controls the segregation of MGE-derived cells by regulating

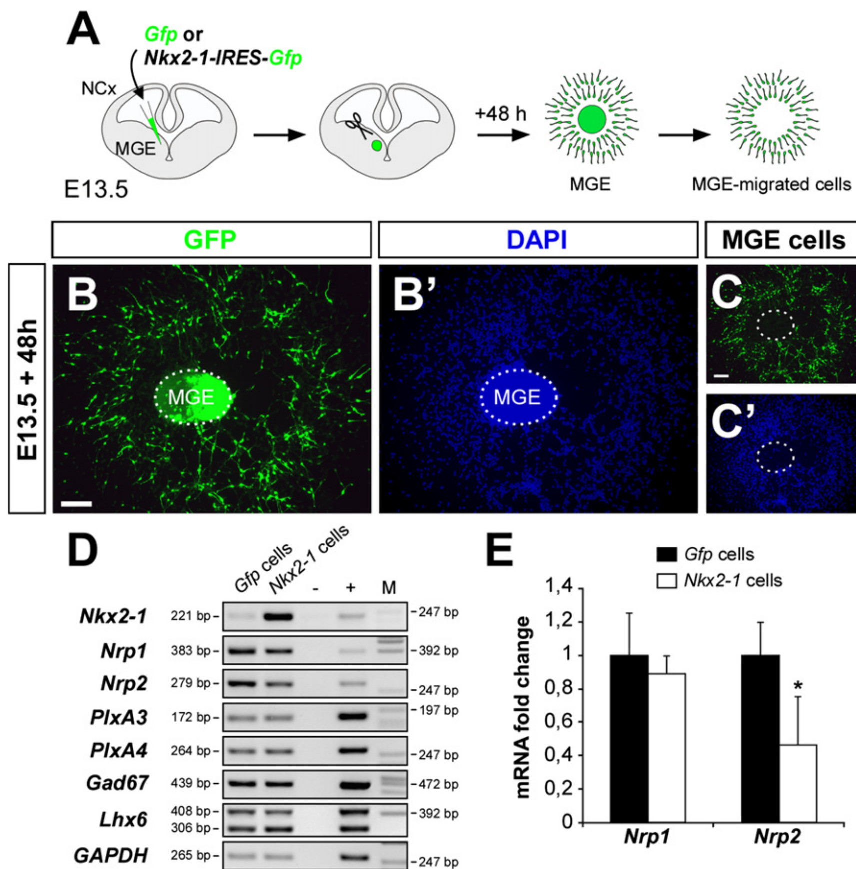

Figure 7. *Nkx2-1* Represses *Neuropilin-2* Expression in MGE-Derived Cells

(A) Schematic diagram of the experimental paradigm used to isolate RNA from migrating MGE-derived cells.

(B–C′) A *Gfp*-electroporated MGE explant stained with DAPI after 48 hr in culture, before (B and B′) and after (C and C′) removing the explant core, which contains progenitor cells.

(D) Semiquantitative RT-PCR analysis comparing gene expression in *Gfp*- and *Nkx2-1*-electroporated MGE-derived cells. Negative (−, all reagents except cDNA) and positive (+, E14.5 MGE cDNA) controls were included in each run. Amplicon and molecular marker (M) base pairs (bp) are shown at the left and right sides of the panels, respectively. The *Lhx6* gene has two transcripts: *Lhx6* or *Lhx6.1a* (408 bp) and *Lhx6.1b* (306 bp). GAPDH was used as loading control.

(E) Quantitative RT-PCR analysis for *Neuropilin-1* and *Neuropilin-2* expression in *Gfp*- and *Nkx2-1*-electroporated MGE-derived cells. Histograms show average ± SEM 1.00 ± 0.25 (*Gfp* cells, *Nrp1*); 0.89 ± 0.11 (*Nkx2-1* cells, *Nrp1*); 1.00 ± 0.19 (*Gfp* cells, *Nrp2*); 0.46 ± 0.29 (*Nkx2-1* cells, *Nrp2*). $^{*}p < 0.05$, t test.

MGE, medial ganglionic eminence; NCx, neocortex. Scale bar = 50 μm.

neuropilin/semaphorin interactions. The maintenance or downregulation of Nkx2-1 expression in migrating cells is linked to their final destination: striatal interneurons maintain Nkx2-1 expression, whereas cortical cells rapidly downregulate Nkx2-1 mRNA, and protein. Moreover, when MGE-derived cells were forced to express Nkx2-1, they failed to reach the cortex and accumulated in the basal telencephalon. In contrast, loss of postmitotic Nkx2-1 function resulted in a reduction in the number of MGE-derived interneurons that populate the striatum. There are two possible caveats in the latter experiment. First, the timing of Cre recombination leaves a very short period between the loss of Nkx2-1 expression (which happens almost exclusively in cells negative for Ki67; Figure S2) and the selection of a target territory. Thus, by the time interneurons lose Nkx2-1 in *Lhx6-Cre;Nkx2-1$^{Fl/Fl}$* mutant embryos, many may have already arrived to the striatum. Second, *Lhx6* or the *Lhx6-Cre* transgene are not expressed by all striatal interneurons, and therefore some of them maintain Nkx2-1 expression (Figure S3). Despite these limitations, many MGE-derived interneurons failed to reach the striatum in the absence of Nkx2-1 postmitotic function, suggesting that Nkx2-1 is an important regulator of this process. One question that remains unresolved is the destiny of *Nkx2-1*-deficient striatal interneurons. Quantification of the number of *Lhx6*-expressing interneurons in the cortex of E15.5 control and *Nkx2-1* conditional mutant embryos revealed no differences (n = 3; Figure S7). However, since cortical interneurons greatly outnumber striatal interneurons, it is very unlikely that rerouting of some striatal cells to the cortex could be easily perceived. Alternatively, loss of Nkx2-1 function may not be enough to redirect all interneurons to the cortex and many cells could have disperse through other subpallial regions, avoiding the striatum. Consistent with view, *Lhx7*-expressing cells were never observed in the cortex of *Lhx6;Nkx2.1$^{Fl/Fl}$* mutant embryos (data not shown).

Expression of Nkx2-1 rendered MGE-migrating cells insensitive to the Sema3A/3F chemorepulsion. This result directly implicates Nkx2-1 function in suppressing the responsiveness of MGE-derived cells to semaphorin signaling. Nkx2-1 suppresses the response of MGE-derived cells to class III semaphorins by directly controlling the expression of, at least, *Nrp2*. Nkx2-1 directly binds in vivo to a region of the *Nrp2* promoter containing two Nkx2-1-specific binding sites located in close proximity (∼151 base pairs). The interaction of Nkx2-1 with this short sequence was sufficient to repress transcription in vitro, reinforcing the notion that Nkx2-1 directly suppresses the expression of *Nrp2* in migrating MGE-derived interneurons. Nkx2-1 regulates the transcription of other genes by interacting with clustered binding sites (Bohinski et al., 1994), and since this transcription factor binds DNA as a monomer, these repeated consensus sequences might represent a unique arrangement for Nkx2-1 binding-site recognition. Our experiments failed to show a conclusive relationship between Nkx2-1 and Nrp1. It should be noted, however, that the relatively low efficiency of Nkx2-1 overexpression in postmitotic migrating interneurons (∼30%) might have precluded the identification of additional targets genes, such as *Nrp1*.

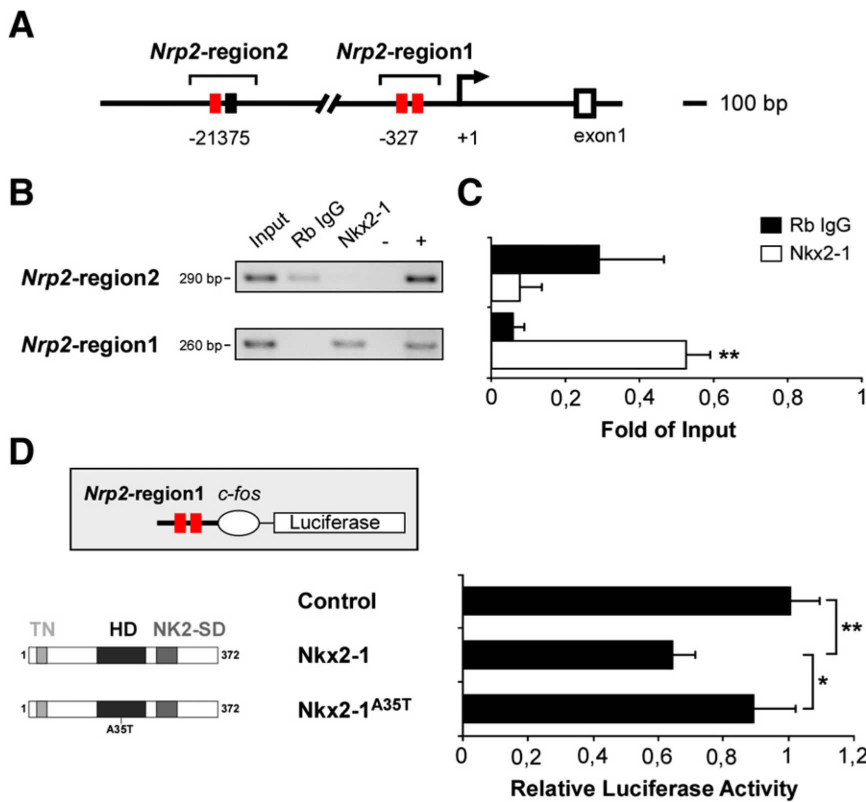

Figure 8. Nkx2-1 Binds the *Neuropilin-2* Promotor In Vivo and Regulates Its Expression

(A) Putative Nkx2-1 DNA binding sites (red and black boxes indicate 8/9 and 6-base pairs [bp] consensus sequences, respectively) in *Nrp2*-region2 (from −21375 bp 5′-CTTGC-3′ to −21086 bp 5′-GTGCT-3′) and *Nrp2*-region1 (from −327 bp 5′-CCGGA-3′ to −68 bp 5′-GGGGA-3′) of the *Neuropilin-2* locus.

(B) ChIP assays were performed using E13.5 MGE cells and a non-specific rabbit anti-IgG (Rb IgG) or a polyclonal antibody against Nkx2-1. Input chromatin represents 1% of the total chromatin. Negative (−, all reagents except DNA) and positive (+, E13.5 mouse genomic DNA) controls were included in each run. *Nrp2*-region 2 and *Nrp2*-region 1 amplicon size (bp) are indicated.

(C) The intensity of each PCR band was quantified and normalized against the input band. Histograms show average ± SEM For *Nrp2*-region2: 0.29 ± 0.17 (Rb IgG) and 0.07 ± 0.06 (Nkx2-1). For *Nrp2*-region1: 0.06 ± 0.02 (Rb IgG) and 0.52 ± 0.06 (Nkx2-1). **p < 0.01, t test.

(D) A luciferase reporter plasmid containing the *Nrp2*-region1 sequence upstream of the *c-fos* minimal promoter driving luciferase (*pGL3-Nrp2-cfos-Luc*) was cotransfected with either mock, *Nkx2-1*, or *Nkx2-1^{A35T}* expression vectors. For each condition, the relative luciferase activity corresponds to the ratio of normalized activities from the promoter-luciferase (*pGL3-Nrp2-cfos-Luc*) and empty-luciferase (*pGL3-cfos-Luc*) reporter vectors. Histograms show average ± SEM 1.00 ± 0.09 (control), 0.64 ± 0.07 (*Nkx2-1*), and 0.89 ± 0.13 (*Nkx2-1^{A35T}*). **p < 0.01, *p < 0.05, one-way ANOVA followed by Tukey's post test.

Nkx2-1 transcriptional activity depends on the interactions of its HD motif with specific DNA target sequences (Harvey, 1996; Damante et al., 1994). Other highly conserved domains of the protein, such as the TN motif and the NK-2-specific domain (SD), could further modulate Nkx2-1 transcriptional activity (Muhr et al., 2001; Watada et al., 2000). Our experiments demonstrate that Nkx2-1 regulates the segregation of MGE-derived cells through transcriptional interactions involving specific residues of the HD (Ala35 and Tyr45). These amino acid residues are needed to suppress the responsiveness to semaphorins and are involved in the interactions between Nkx2-1 and the *Nrp2* regulatory sequence. In contrast, neither the TN nor the C terminus/SD domains appear to regulate the migration of MGE-derived cells to the cortex. Apart from binding to specific DNA sequences, the Nkx2-1 HD has been shown to regulate transcriptional activity by interacting with other transcription factors or through posttranslational modifications (Minoo et al., 2007; Yang et al., 2004). Thus, in addition to conferring binding specificity to the *Nrp2-region1* promoter, it is possible that specific amino acid residues of the Nkx2-1 HD establish additional interactions that mediate the repression of *Nrp2* expression in MGE-derived migrating cells.

The transcriptional regulation of telencephalic interneuron migration is likely to involve additional factors. For example, the Dlx1/2 transcription factors appear also to repress *Nrp2* in the developing forebrain (Le et al., 2007), although the functional consequences of this regulation for the migration of MGE-derived cells have not been examined. In addition to neuropilin/semaphorin interactions, it is likely that Nkx2-1 may control other guidance systems in MGE-derived neurons. Of note, migrating neurons expressing Nkx2-1 do not simply migrate through the striatum as they fail to sense semaphorins, but indeed they seem to be attracted to that location. This result suggests that the striatum may also contain an attractive factor for MGE-derived interneurons, although the molecular nature of this activity remains unknown. Alternatively, Nkx2-1 may confer striatal interneurons with sensitivity for a cortical chemorepulsive cue, which will prevent migrating MGE-derived interneurons expressing Nkx2-1 from entering the cortex.

A question that remains to be elucidated is the mechanism regulating the postmitotic expression of Nkx2-1 in migrating MGE-derived neurons. Sonic hedgehog (Shh) signaling induces and maintains Nkx2-1 expression in MGE progenitors during development (Ericson et al., 1995; Shimamura et al., 1995; Xu et al., 2005) and supports the expansion of progenitors through neurogenesis (Machold et al., 2003). However, the analysis of *Dlx5/6Cre;Smo$^{Fl/Fl}$* conditional mutant mice suggests that Shh does not control the expression of *Nkx2-1* in striatal postmitotic interneurons (Xu et al., 2005). Future work should address this issue, as it seems critical for understanding how appropriate

numbers of different inhibitory populations are generated during development.

Multiple Roles for Nkx2-1 in the Development of Telencephalic Interneurons

Previous studies have revealed that Nkx2-1 is critical for the development of the mammalian subpallium. The emerging idea, however, is that Nkx2-1 plays diverse roles in closely related cells depending on their relative stage of differentiation. During early patterning of the telencephalon, Nkx2-1 regulates the specification of the MGE and preoptic area (POA) progenitor cells (Corbin et al., 2003; Sussel et al., 1999). In the absence of Nkx2-1 function, the MGE and POA progenitors are respecified to a more dorsal fate, similar to that of LGE progenitors. This transformation leads to a dramatic reduction in the number of cells derived from those structures, such as GABAergic cortical and striatal interneurons, as well as projection neurons of the globus pallidus and other basal forebrain structures (Sussel et al., 1999). Moreover, conditional deletion of Nkx2-1 in the telencephalon demonstrates that this transcription factor is required for the specification of parvalbumin and somatostatin cortical interneuron subtypes (Butt et al., 2008 [this issue of *Neuron*]).

In addition to its master role in ventral subpallial identity, Nkx2-1 also instructs the selection of specific fates by controlling the expression of differentiation genes in subpallial-derived neurons, such as the LIM-HD transcription factors *Lhx6* and *Lhx7* (Sussel et al., 1999). As shown for *Lhx6*, Nkx2-1 appears to control the expression of these genes through direct transcriptional regulation (Du et al., 2008). *Lhx6* and *Lhx7* are essential regulators of the fate of several types of GABAergic and cholinergic neurons derived from Nkx2-1-expressing progenitors (Fragkouli et al., 2005; Mori et al., 2004; Wonders and Anderson, 2006; Zhao et al., 1999), reinforcing the idea that Nkx2-1 expression prior to cell cycle exit influences the fate of MGE-derived cells.

Our results demonstrate that Nkx2-1 plays an additional role in postmitotic MGE-derived cells by controlling the repertoire of guidance receptors expressed by migrating interneurons. As for the induction of the cell-fate determinant *Lhx6* (Du et al., 2008), the postmitotic function of Nkx2-1 in neuronal migration is achieved through the direct transcriptional repression of a guidance receptor, *Nrp2*. Thus, the cellular context in which Nkx2-1 operates at different stages of differentiation greatly influences the functional outcome of its transcriptional activity. The mechanisms conferring Nkx2-1 with time and context-dependent transcriptional specificity remain to be elucidated.

EXPERIMENTAL PROCEDURES

Mouse Lines

Wild-type mice and GFP-expressing transgenic mice (Hadjantonakis et al., 2002) maintained in a CD1 background were used for expression analysis and tissue culture experiments. *Lhx6-Cre* (Fogarty et al., 2007), *Rosa-EYFP* (Srinivas et al., 2001) and *Nkx2-1*$^{Fl/Fl}$ (Kusakabe et al., 2006) mice were maintained in a mixed C57Bl/6 × 129/SvJ x CBA background. Animals were kept under Spanish, UK, and EU regulation.

DNA Constructs

A cDNA encoding *Nkx2-1* (accession number NM_009385) was used. The truncated constructs *Nkx2-1*$^{\Delta TN}$*-IRES-Gfp* (deletion [Δ] of amino acids 1–20) and *Nkx2-1*$^{\Delta Ct}$ (Δ amino acids 271–372) were generated by PCR. The single-amino acid substitutions *Nkx2-1*^{A35T} and *Nkx2-1*^{Y54M} were prepared using the QuickChange II XL Kit (Stratagene). All constructs (*Gfp*, *Nkx2-1-IRES-Gfp*, *Nkx2-1*$^{\Delta TN}$*-IRES-Gfp*, *Nkx2-1*$^{\Delta Ct}$, *Nkx2-1*^{A35T}, and *Nkx2-1*^{Y54M}) were inserted into the *pCAGGS* chicken β-actin promoter expression vector.

In Vitro Focal Electroporation

E13.5 organotypic coronal slice cultures from wild-type or *Gfp* transgenic embryos were obtained as described previously (Anderson et al., 1997). Expression vectors were electroporated at a concentration of 1 μg/μl and mixed in a 0.9/1.5 ratio when coelectroporated. Expression vectors were focally injected into the MGE, and embryonic slice cultures were electroporated as previously described (Flames et al., 2004).

In Situ Hybridization and Immunohistochemistry

For in situ hybridization, brains were fixed overnight in 4% paraformaldehyde in PBS (PFA). Twenty micrometer frozen sections were hybridized with digoxigenin-labeled probes as described before (Flames et al., 2007). Immunohistochemistry was performed on culture slices, MGE explants in Matrigel pads, or 20 μm cryostat sections. Slices, explants, and embryos were fixed in 4% PFA at 4°C from 2–6 hr. The following primary antibodies were used: rat anti-BrdU (1/100, Accurate), chicken anti-GFP (1/1000, Aves Labs), rabbit anti-Nkx2-1 (1/2000, Biopat), rabbit anti-PV (1/3000, Swant), goat anti-ChAT (1/100, Chemicon), rat anti-SST (1/200, Chemicon), rabbit anti-cleaved Caspase3 (1/150, Cell Signaling), and rabbit anti- Ki67 (1/500, Novocastra). The following secondary antibodies were used: donkey anti-rat 488, goat anti-chicken 488, donkey anti-rabbit 555, donkey anti-goat 555 (Molecular Probes), and donkey anti-rat Cy3 (Jackson Laboratories). The immunofluorescence detection of EYFP was performed using an anti-GFP antibody. DAPI (Sigma) and propidium iodine (Molecular Probes) were used for fluorescent nuclear counterstaining.

Quantification

For the quantification of interneurons in E13.5, E15.5, P0, and P25 control and *Lhx6-Cre;Nkx2-1*$^{Fl/Fl}$ mutant brains, the outline of the striatum or cortex at rostral, intermediate, and caudal levels was delineated in 20 or 60 μm sections, different interneuron markers (*Lhx6*, *Lhx7*, *Er81*, *Sst*, ChAT, PV, and SST) or YFP-expressing cells were counted for three different brains from each genotype, and the cell density (number of cells per mm^2) was calculated. In the transplantation experiments, the intensity of Nkx2-1 fluorescence in *Gfp*-expressing cells was quantified and classified into high or low levels when presenting >90% or <30%, respectively, of the Nkx2-1 fluorescence intensity found in MGE progenitors (considered as 100%).

MGE Explants Cultures

MGE explants were dissected out from organotypic slices after electroporation. For gene-expression analysis, MGE explants were cultured on glass coverslips coated with poly-L-Lysine and laminin in Neurobasal medium containing 0.3% methylcellulose (Sigma). In coculture experiments, MGE explants were confronted with COS7 cells aggregates expressing *DsRed* alone or *DsRed* and *Sema3A/3F* in Matrigel matrix (Beckton-Dickinson) as described previously (Flames et al., 2004). Sema3A and Sema3F cDNA sequences used have been described elsewhere (Marín et al., 2001). For quantification, each explant was divided into four quadrants, the number of *Gfp*-expressing cells was quantified in the proximal and distal quadrant (in relation to the COS7 cells) and the proximal/distal ratio was calculated.

Semiquantitative and Quantitative RT-PCR

Total RNA from MGE-derived cells was extracted with TRIzol according to the manufacturer's instructions (Invitrogen). RNA (150 ng) was treated with DNaseI RNase-free (Fermentas) for 30 min at 37°C prior to reverse transcription into single-stranded cDNA using SuperScriptII Reverse Transcriptase and Oligo(dT)$_{12-18}$ primers (Invitrogen) for 1 hr at 42°C. For semiquantitative PCR, 2 μl of cDNA, the appropriate primers (Figure S8), and recombinant *Taq* DNA polymerase (Invitrogen) were used. For *Nrp1* and *Nrp2*, a preamplification PCR step (multiplex) was performed using 10 μl of cDNA and a primer mix containing *GAPDH*, *Nrp1*, and *Nrp2* multiplex primers. PCR products were analyzed by electrophoresis on a 2% agarose gel. Quantitative (q) PCR was

carried out in an Applied Biosystems 7300 real-time PCR unit using the Platinum SYBR Green qPCR Supermix UDG with ROX (Invitrogen), 5 μl of cDNA, and the appropriate primers (Figure S8). Each independent sample was assayed in duplicate. Gene expression levels were normalized using GAPDH.

Phylogenetic Footprinting Analysis

In silico analysis of the predicted 5′ flanking region of the *Mus musculus Nrp2* locus (accession number NT_039170) was performed using Vista and University of California Santa Cruz (UCSC) Genome Browsers. Putative Nkx2-1 binding sites match between six and nine nucleotides the described Nkx2-1 consensus sequence 5′-CCACTC/GAAGTG-3′.

Chromatin Immunoprecipitation Assay

ChIP assay was performed using the EZ ChIP Kit (Upstate) according to the manufacturer's instructions. Briefly, mouse E13.5 MGE cells (1–2 × 10^7) were crosslinked with 1% PFA for 25 min at room temperature. Sonication of cells in SDS lysis buffer on ice (Bioruptor, Diagenode; 200W potency; 40 s on, 20 s off; 5 × 5 min) generated soluble chromatin fragments between 150 and 400 bp. Chromatin was immunoprecipitated with 5–6 μg of rabbit anti-Nkx2-1 (Biopat) and rabbit anti-immunoglobulin G (IgG) antibodies. Immunoprecipitated DNA sequences were analyzed by PCR using primer pairs spanning the *Nrp2*-region2 and *Nrp2*-region1 (Figure S6). PCR products were analyzed by electrophoresis on a 2% agarose gel.

Promoter Luciferase Assay

HEK293 cells were cotransfected with 200 ng of a luciferase reporter plasmid (*pGL3-cfos-Luc* or *pGL3-Nrp2-cfos-Luc*), 8 ng of *CMVβgal*, and 200 ng of an empty (mock), full-length *Nkx2-1*, or *Nkx2-1^{A35T}* expression vector using Fugene reagent (Roche). After 1 day in culture, cells were collected and assayed for luciferase and β-galactosidase activity using the Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Each independent sample was assayed in duplicate and luciferase activities were normalized using β-galactosidase activity.

SUPPLEMENTAL DATA

The Supplemental Data include eight figures and can be found with this article online at http://www.neuron.org/cgi/content/full/59/5/733/DC1/.

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Neuron, Volume *59*

Supplemental Data

Postmitotic Nkx2-1 Controls the Migration of Telencephalic Interneurons by Direct Repression of Guidance Receptors

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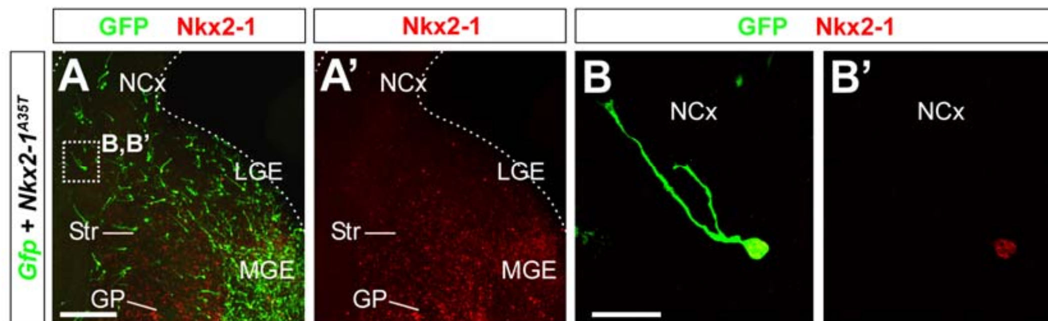

Figure S1. MGE-Derived Cells Electroporated with *Gfp* and *Nkx2-1*^{A35T} Migrate to the Cortex and Express Nkx2-1 Protein

(A and A') Expression of GFP and Nkx2-1, respectively, in MGE-derived cells electroporated with *Gfp* + *Nkx2-1*^{A35T} in organotypic slices. Dotted lines indicate the limits of the section.
(B and B') Higher magnification of the area boxed in (A) showing a cell derived from the MGE and electroporated with *Gfp* and *Nkx2-1*^{A35T}. This cell is entering the cortex and expresses a form of Nkx2-1 that is recognized by the polyclonal antibody raised against this protein.
GP, globus pallidus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str, striatum.
Scale bars equal 200 µm (A and A') and 20 µm (B' and B').

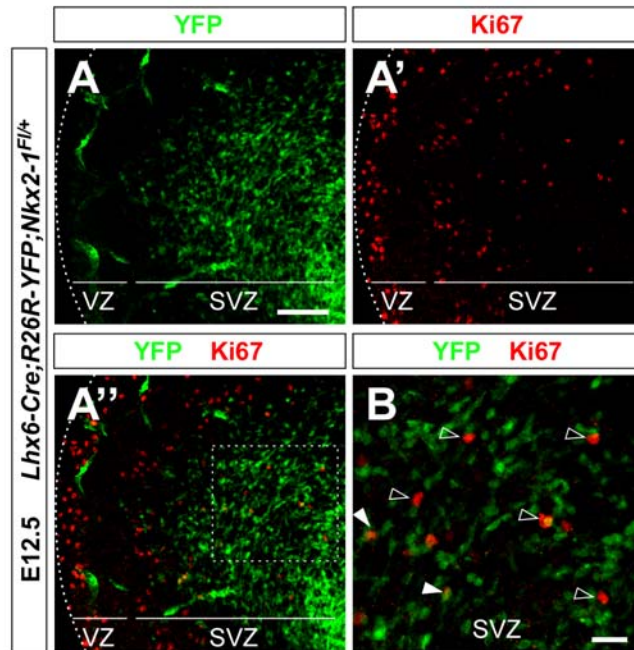

Figure S2. Recombination Driven by the *Lhx6-Cre* Transgenic Line Is Restricted Almost Exclusively to Postmitotic Cells

(A–A'') Coronal sections through the MGE of an E12.5 *Lhx6-Cre;Nkx2-1*$^{Fl/+}$*;Rosa-YFP* embryo showing that almost all YFP-expressing cells are located in the subventricular zone and do not co-label for the progenitor marker Ki67. YFP is also detected in scattered blood vessels, as previously reported (Fogarty et al., 2007).

(B) Higher magnification of the area boxed in (A'') depicting YFP-expressing cells that are negative (open arrowhead) or positive (white arrowhead) for Ki67 expression.
VZ, ventricular zone; SVZ, subventricular zone.
Scale bars equal 50 µm (A, A' and A'') and 20 µm (B).

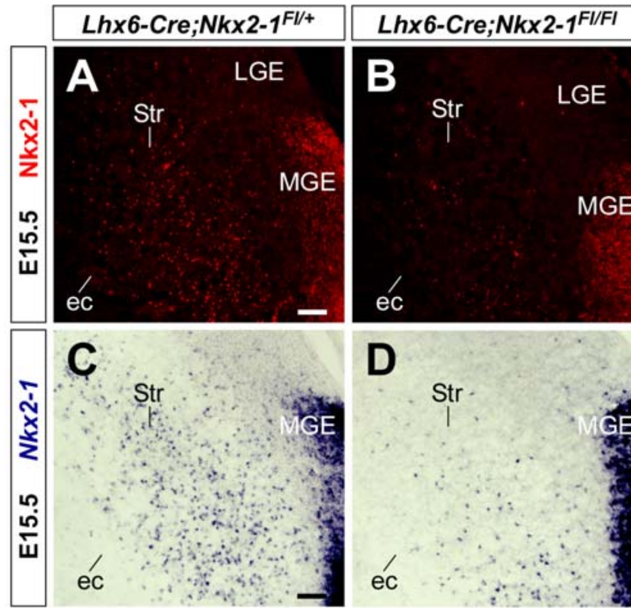

Figure S3. Nkx2-1 Expression in Control and *Lhx6-Cre;Nkx2-1*$^{Fl/Fl}$ Mutant Embryos

(A–D) Coronal sections through the telencephalon of E15.5 control (A and C) and *Lhx6-Cre;Nkx2-1*$^{Fl/Fl}$ mutant (B and D) embryos showing Nkx2-1 protein (A and B) and mRNA (C and D) expression.

ec, external capsule; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Str, striatum.
Scale bar equals 100 µm.

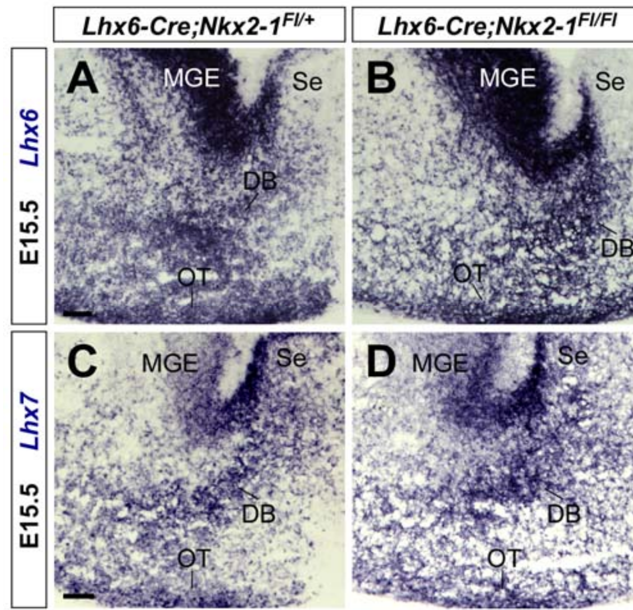

Figure S4. *Lhx6* and *Lhx7* mRNA Expression Is Unchanged in the Ventral Telencephalon of *Lhx6-Cre;Nkx2-1*$^{Fl/Fl}$ Mutant Embryos

(A–D) Coronal sections through the telencephalon of E15.5 control (A and C) and *Lhx6-Cre;Nkx2-1*$^{Fl/Fl}$ mutant (B and D) embryos showing *Lhx6* (A and B) and *Lhx7* (C and D) mRNA expression.

DB, diagonal band; MGE, medial ganglionic eminence; Se, septum; OT, olfactory tubercle. Scale bar equals 100 µm.

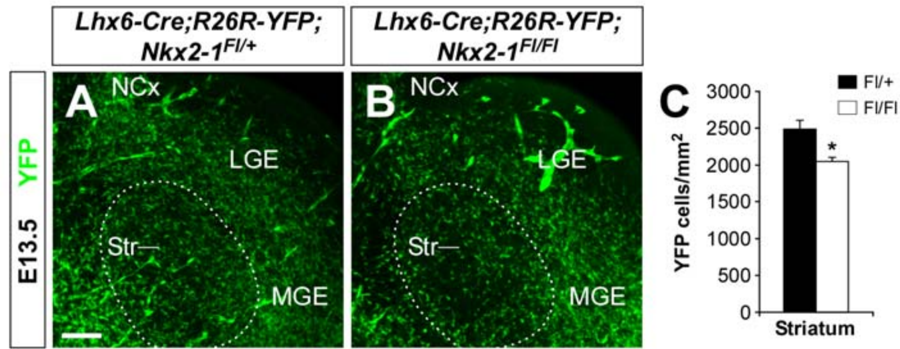

Figure S5. Tracing Experiments Reveal a Reduction in the Number of Interneurons that Invade the Embryonic Striatum upon Loss of *Nkx2-1* Function

(A and B) Coronal sections through the telencephalon of E13.5 *Lhx6-Cre;Nkx2-1*$^{Fl/+}$*;Rosa-YFP* control (A) and *Lhx6-Cre;Nkx2-1*$^{Fl/Fl}$*;Rosa-YFP* mutant (B) embryos showing YFP expression. Dotted lines indicate the limits of the developing striatum. YFP is also detected in scattered blood vessels, as previously reported (Fogarty et al., 2007).

(C) Quantification of the number of YFP-expressing cells in the striatum of E13.5 *Lhx6-Cre;Nkx2-1*$^{Fl/+}$*;Rosa-YFP* control and *Lhx6-Cre;Nkx2-1*$^{Fl/Fl}$*;Rosa-YFP* mutant embryos. Histograms show average ± s.e.m. 2488.03 ± 134.24 (YFP control); 2043.78 ± 55.54 (YFP mutant). * $p < 0.05$, *t*-test.

LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str, striatum.

Scale bar equals 100 µm.

Mus musculus Neuropilin2 putative promoter regions

***Nrp2*-region2**

(chr1: 62.617.785) 5' - **cttgccagggtgttatgaggatta**attaatgtttgtaaagtgcttggaat
t***ccactgaagaa***atgtaccttgtcgatgcaaattattatcattatatagtgcctttcatcccagaatctc
aaagtgcttccccaacaattaattaagcctcactacacccctgtgagagagttaagcatcactctgccct
cactaatacaggcagcatctgcacctgagtcacagagtctaatgttcaaatc***actctccagga***gggagtg
agactc**cattagcaaccatccctaagtgct** - 3' (chr1: 62.618.074)

***Nrp2*-region1**

(chr1: 62.638.833) 5' - **ccggaggggaggcagag**gaggcgagca
aggcaccagcctgcagcccgcccccggcacat***cctctgaagca***cagacactcggccgggcgctgggcgag
gtggaggtgagggcgggcgccagcgaactcggagagccgctcgcacactcgcgggcgatcccagccgccg
cacccgcagcaacaccagcagcaccggccgcagcagcttcctgcctcgcact***cccctccagag***actggcc
aagcgg**gtgtaaccgccggggga** - 3' (chr1: 62.639.092)

Figure S6. Putative Promoter Regions for *Mus musculus Neuropilin-2*

Nrp2 regulatory sequences (Genbank AF022855), designated *Nrp2*-region2 (chr1: 62.617.785-62.618.074) and *Nrp2*-region1 (chr1: 62.638.833- 62.639.09), containing Nkx2-1 consensus sequences (red letters in bold and italic for 8/9 base pairs sequences and black letters in bold and italic for a 6 base sequence). The oligonucleotide primers used for PCR detection of the ChIP assays are shown in bold and underlined.

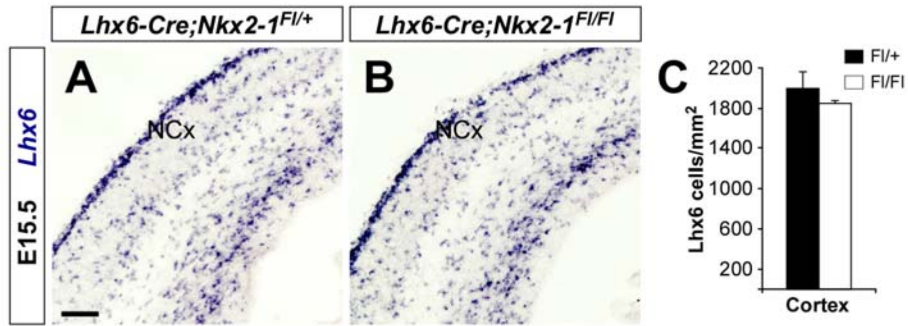

Figure S7. *Lhx6* mRNA Expression Is Unchanged in the Cortex of *Lhx6-Cre;Nkx2-1*$^{Fl/Fl}$ Mutant Embryos

(A and B) Coronal sections through the telencephalon of E15.5 control (A) and *Lhx6-Cre;Nkx2-1*$^{Fl/Fl}$ mutant (B) embryos showing *Lhx6* mRNA expression.

(C) Quantification of the number of *Lhx6*-expressing cells in the cortex of E15.5 control and *Lhx6-Cre;Nkx2-1*$^{Fl/Fl}$ mutant embryos. Histograms show average ± s.e.m. 2004.43 ± 170.50 (*Lhx6* control); 1855.71 ± 35.33 (*Lhx6* mutant).
NCx, neocortex.
Scale bar equals 100 µm.

Sequence (5'-3'), amplicon length, and accession number of primers

Primer	Sequence	Amplicon	Acession number
Nkx2-1 F	CGAGCGGCATGAATATGAG	221	NM_009385
Nkx2-1 R	GACCTGCGTGGGTGTCAG		
Nrp1 Fm	GTGGGCTTGGGCTGAG	410	NM_008737
Nrp1 Rm	CAGGCGGGCTACTTTG		
Nrp1 F	TGGGCTGTGAAGTGGAA	383	
Nrp1 R	CAGGCGGGCTACTTTG		
Nrp1 Fq	GGGCTGAGGATGGAGCTACTGG	106	
Nrp1 Rq	AGTTGGCCTGGTCGTCGTCACACT		
Nrp2 Fm	CTCCGCACGTTACTATTTGAT	725	NM_010939
Nrp2 Rm	TGACCCCTTTCACTGTCTTG		
Nrp2 F	CCGAGGTGGTGCTAAACAAG	279	
Nrp2 R	CTGGCTGGGCTTGAGGGTTC		
Nrp2 Fq	CCACTGCTGACTCGGTTCATC	109	
Nrp2 Rq	TGTTGGAGCAGGGTGCATCT		
PlxA3 F	TGGAGGCACTCGGCTTA	172	NM_008883
PlxA3 R	GATGGCAAGGGTGATAGGG		
PlxA4 F	GAAGCCCAACCGAGGAC	264	NM_175750
PlxA4 R	GGTTCAATCCGCACAATG		
Gad67 F	CCGCCTCCCAGTCTGACATC	439	Z49976
Gad67 R	CCATCCGCCCTGTAGTTGCT		
Lhx6 F	CACGGCTACATTGAGAGTCA	408/306	AJ000337
Lhx6 R	GACAGGCTGCTTGTTTTCAT		
GAPDH Fm	CAGCCTCGTCCCGTAGA	382	NM_008084
GAPDH Rm	GGAGATGATGACCCTTTTC		
GAPDH F	AAAATGGTGAAGGTCGGTGT	265	
GAPDH R	CTCACCCCATTTGATGTTAG		
GAPDH Fq	CGGTGCTGAGTATGTCGTGGAGT	143	
GAPDH Rq	CGTGGTTCACACCCATCACAAA		

F, Forward; R, Reverse; m, multiplex; q, quantitative RT-PCR

Figure S8. List of Primers Used in Semi-quantitative and Quantitative RT-PCR Experiments

Supplemental References

Fogarty, M., Grist, M., Gelman, D., Marín, O., Pachnis, V., and Kessaris, N. (2007). Spatial genetic patterning of the embryonic neuroepithelium generates GABAergic interneuron diversity in the adult cortex. J. Neurosci. *27*, 10935–10946.

Chapter 3. Directional Guidance of MGE-Derived Striatal Interneurons Relies on ErbB4-Dependent Signalling and Cortical Repulsion

Sandrina Nóbrega-Pereira and Oscar Marín

Manuscript in preparation

3.1. Summary

In the developing telencephalon, the medial ganglionic eminence (MGE) gives rise to many cortical and virtually all striatal interneurons. While the molecular mechanisms controlling the migration of interneurons to the cortex have been extensively studied, very little is known about the nature of the signals that guide interneurons to the striatum. Here we report that the coordinated action of ErbB4 signalling and a cortical repulsive activity control the migration of MGE-derived striatal cells. Our results also suggest that the responsiveness of MGE-derived striatal interneurons to those cues is at least in part controlled by the postmitotic action of the Nkx2-1 transcription factor.

3.2. Introduction

During the development of the telencephalon, the MGE is the source of several neuronal populations, including striatal and cortical interneurons (Lavdas et al., 1999; Marín et al., 2001; Sussel et al., 1999; Wichterle et al., 1999; Wichterle et al., 2001). The mechanisms controlling the migration of cortical interneurons have been extensively studied over the past years, and they seem to involve the simultaneous activity of several chemorepulsive and chemoattractive factors (Flames et al., 2004; Marín et al., 2003; Marín et al., 2001; Wichterle et al., 2003). Initially, MGE-derived interneurons avoid migrating in ventral direction due to the existence of a currently uncharacterized chemorepulsive activity in the preoptic area (Marín et al., 2003). Subsequently, expression of neuropilin receptors in migrating cortical interneurons instructs them to avoid the developing striatum, which is enriched in two chemorepulsive molecules, Sema3A and Sema3F (Marín et al., 2001). Different isoforms of the Neuregulin-1 gene (*Nrg1*) contribute to the migration of MGE-derived cortical interneurons via the ErbB4 receptor (Flames et al., 2004). MGE-derived interneurons migrate to the cortex through a narrow corridor deep to the striatal mantle that expresses a membrane-bound isoform of Neuregulin-1 (CRD-Nrg1), and this molecule constitutes a very permissive substrate for these cells (Flames et al., 2004). In addition, the secreted isoform of Nrg1 (Ig-Nrg1) is expressed in the developing cortex and exerts a long-range chemoattractive effect over MGE-derived cortical interneurons (Flames et al., 2004). Other factors, such as glial-derived neurotrophic factor (GDNF) and hepatocyte growth factor (HGF) also seem to influence the migration of cortical interneurons by promoting their migration (Powell et al., 2001; Pozas and Ibañez, 2005).

In contrast to our extensive knowledge on the mechanisms directing the migration of cortical interneurons, our understanding of the events controlling the targeting of interneuron to the developing striatum is very limited. We have recently shown that Nkx2-1, a transcription factor required for the specification of MGE progenitor cells (Butt et al., 2008; Sussel et al., 1999), is also

involved in regulating the migration of striatal interneurons (Nobrega-Pereira et al., 2008). Virtually all MGE progenitor cells express Nkx2-1, but its expression is differentially regulated in striatal and cortical interneurons. While most MGE-derived striatal interneurons maintain Nkx2-1 expression postmitotically, expression of this gene is downregulated in migrating cortical interneurons (Marín et al., 2000; Nobrega-Pereira et al., 2008). Loss of Nkx2-1 in postmitotic MGE-derived cells decreases the number of interneurons migrating to the striatum (Nobrega-Pereira et al., 2008), which suggest that Nkx2-1 controls at least some of the genetic programmes required for the correct guidance of these neurons. For example, postmitotic Nkx2-1 directly represses the expression of semaphorin receptors, which renders striatal interneurons insensitive to semaphorins expressed in the developing striatum (Nobrega-Pereira et al., 2008).

In this study, we analyzed further the function of Nkx2-1 in the migration of striatal interneurons. We found that MGE-derived striatal cells express the ErbB4 receptor and partially rely in ErbB4-mediated interactions to precisely migrate to the developing striatum. In addition, *Nkx2-1* leaves MGE-derived cells susceptible to a yet unidentified chemorepulsive activity present in the developing cortex. These results reveal the implication of several guidance signals in controlling the migration of MGE-derived striatal cells and the involvement of the Nkx2-1 transcription factor in modulating the responsiveness of striatal interneurons to those cues.

3.3. Results

We have previously shown that forced expression of Nkx2-1 in all MGE-derived neurons prevents migration of interneurons to the cortex, while migration to the striatum is greatly enhanced (Nobrega-Pereira et al., 2008) (Figure S1). Nkx2-1 seems to facilitate interneuron migration to the striatum by repressing the expression of receptors for semaphorins (Nobrega-Pereira et al., 2008), which during normal development prevent cortical interneurons from invading this territory (Marín et al., 2001). However, the observation that Nkx2-1-expressing interneurons accumulate in the striatum rather than passing through in their way to the cortex also suggests that: (i) striatal projection neurons produce a chemoattractant for Nkx2-1-expressing interneurons, and/or (ii) the cortex releases a chemorepellent activity for Nkx2-1-expressing interneurons.

3.3.1. ErbB4-dependent signalling is required for the migration of MGE-derived interneurons to the striatum

CRD-Nrg1, the membrane bound isoform of Nrg1, is strongly expressed in the developing striatum at the time of cortical and striatal interneuron migration (Flames et al., 2004; Figure 1A and 1B). This suggests that, in addition to regulating the migration of cortical interneurons through the LGE corridor, Nrg1 may also guide MGE-derived striatal interneurons to their target. To test this hypothesis, we first analyzed the expression of the Nrg1 receptor ErbB4 in migrating striatal

interneurons. At embryonic stage (E) 13.5, *ErbB4* transcripts were found in cells migrating to the cortex and in the developing striatum (Figure 1C). Because *ErbB4* expression in the striatum may correspond to cells passing through this structure rather than immature striatal interneurons, we next examined the expression of ErbB4 protein in MGE-derived interneurons migrating *in vitro*. To unequivocally identified striatal interneurons emanating from the MGE, we used antibodies against Nkx2-1, a transcription factor that is expressed in most migrating striatal interneurons, but not in cortical interneurons (Marín et al., 2000; Nobrega-Pereira et al., 2008). Double labelling staining demonstrated that migrating striatal interneurons express the ErbB4 receptor (Figure 1D and D').

Expression of ErbB4 in migrating striatal interneurons is consistent with the hypothesis that these neurons rely on Nrg1/ErbB4 signalling to target the striatum. To begin to test this hypothesis, we asked whether ErbB4 signalling is required for interneurons to migrate to the striatum. Because the proportion of MGE-derived cells that migrate to the striatum is very low compared to the number of cortical interneurons under normal circumstances (Nobrega-Pereira et al., 2008), we artificially increased the number of interneurons migrating to the striatum by forcing Nkx2-1 expression in all MGE-derived cells, and asked whether the accumulation of Nkx2-1-expressing interneurons in the striatum requires ErbB4 function. To this aim, we focally electroporated a plasmid encoding *Nkx2-1-IRES-Gfp* in the MGE of embryonic organotypic slices obtained from control or *ErbB4*$^{-/-}$*HER4*^{heart} mutant embryos (Figure 2A). In these mice, expression of human *ErbB4* (*HER4*) under a cardiac-specific myosin promoter avoids the embryonic lethality caused by loss of *ErbB4* function in the myocardium (Tidcombe et al., 2003). Consistent with previous results (Nobrega-Pereira et al., 2008), the majority of *Nkx2-1*-expressing MGE-derived cells accumulate in the developing striatum in wild types slices (n = 4 slices; Figure 2B). In *ErbB4*$^{-/-}$*HER4*^{heart} organotypic slices, however, *Nkx2-1*-expressing cells were still confined to the subpallium, but fewer reached the striatum and instead accumulated throughout the MGE subventricular zone and mantle (n = 4 slices; Figure 2C; number of *Gfp*-expressing cells in the striatum [average ± SEM]: 58.88 ± 2.12 [control] and 21.50 ± 3.00 [mutant], *** $p < 0.001$, t test). To discard the possibility that *ErbB4* function is required for interneuron migration and not just guidance, we cultured wild type and *ErbB4*$^{-/-}$*HER4*^{heart} MGE explants that were previously electroporated with *Gfp*. We found that MGE-derived *Gfp*-expressing cells lacking *ErbB4* migrate similar distances that control cells (Figure S2), suggesting that loss of *ErbB4* function does not impair cell migration. Finally, we analyzed the distribution of interneurons in the striatum of *ErbB4*$^{-/-}$*HER4*^{heart} mutant mice. We found that the striatum of E15.5 *ErbB4*$^{-/-}$*HER4*^{heart} mutant embryos contained fewer Nkx2-1-expressing neurons than controls (n = 3; Figure 2D-F). Altogether, these results suggest that the correct allocation of MGE-derived striatal interneurons partly relies on ErbB4 signalling through a mechanism that it is likely to involve CRD-Nrg1 chemoattraction.

3.3.2. The cortex contains a chemorepulsive activity for striatal interneurons

The cerebral cortex contains high levels of expression of secretable forms of Nrg1, such as Ig-Nrg1 (Flames et al., 2004), which contribute to direct cortical interneurons to their final target. Since striatal interneurons also express ErbB4 receptors, additional mechanisms might exists to prevent striatal interneurons from populating the cortex. To start testing this possibility, we transplanted small E13.5 MGE explants from green fluorescence protein (GFP)-expressing embryos (MGE^{GFP}) into the cortex of telencephalic slices obtained from isochronic wild type mouse embryos (Figure 3A). After 36 h in culture, many MGE-derived GFP-expressing cells were found through the cortex. However, virtually none of the GFP cells that invaded the cortex expressed Nkx2-1 (n = 5; Figure 3B and 3B'; 1.2 ± 0.5% of GFP/Nkx2-1 cells, average ± SEM). These findings suggested two possibilities: (i) the cortex contains an activity that represses the expression of Nkx2-1 in MGE-derived neurons, and/or (ii) striatal interneurons (Nkx2-1-expressing MGE-derived cells) cannot migrate into the cortex. To test this, we repeated the previous experiments after placing a small piece of striatal tissue in the cortex of host slices (Figure 3C). Under those circumstances, many MGE-derived GFP cells expressing Nkx2-1 left the explants and invade the ectopic striatum, where they accumulated (n = 8; Figure 3D and 3D'; 24.0 ± 6.2% of GFP/Nkx2-1 cells, average ± SEM). Thus, striatal interneurons can migrate in close proximity to cortical tissue, but they preferentially accumulate in the striatum rather than invading the cortex.

The previous experiments suggested that the cortex might contain a non-permissive or repulsive activity for striatal interneurons. To directly test this hypothesis, we focally electroporated a plasmid encoding for *Nkx2-1* in the MGE of E13.5 organotypic slices and then placed small MGE-electroporated transplants in the cortex (Figure 3E). In control experiments, many *Gfp*-expressing cells migrated out of the MGE explant and colonized the entire developing cortex (Figure 3F). In contrast, expression of *Nkx2-1* prevented MGE-derived cells to invade the cortex (n = 7; Figure 3G; number of cells in cortex [average ± SEM]: 23.6 ± 1.4 [GFP] and 6.6 ± 0.9 [GFP/Nkx2-1], *** $p < 0.001$, t test). These results demonstrate that MGE-derived Nkx2-1-expressing cells, putative striatal interneurons, are not able to invade the cortex, suggesting that this transcription factor may render MGE-derived cells receptive to a repulsive or non-permissive activity present in the developing cortex.

3.4. Discussion

Our results suggest that the migration of striatal interneurons is regulated through the combination of both chemoattractive and chemorepulsive signals, similar to what it has been described for cortical interneurons (Marín and Rubenstein, 2003). The migration of MGE-derived striatal cells partially relies in ErbB4/Nrg1 interactions. Some MGE-derived Nkx2-1-expressing cells express the Nrg1 receptor ErbB4, suggesting that only a fraction of striatal interneurons use Nrg1/ErbB4

signalling to migrate to the striatum. In the postnatal brain, ErbB4 is expressed primarily in Parvalbumin-expressing interneurons in the striatum (Fox and Kornblum, 2005; Yau et al., 2003), suggesting that only this population may require ErbB4 for their migration. Consistently, loss of *ErbB4* function decreases the number of Nkx2-1-expressing neurons that migrate towards the striatum, but the reduction in the total number of striatal interneurons is not very prominent. This suggests that, in addition to Nrg1, additional chemoattractive cues might be required for the migration and final allocation of striatal interneurons. Alternatively, ErbB4 function might be required for the proliferation of MGE precursors (Ghashghaei et al., 2006), which may directly influence the final pool of migrating neurons. This possibility should be addressed in future experiments.

ErbB4 receptor is expressed and required for the migration of both MGE-derived cortical and striatal interneurons, raising the possibility that its expression in MGE and POA cells is controlled by the cell-fate determinant Nkx2-1. Previous studies have shown that that Nkx2-1 binds and activates *in vitro* the promoter of *ErbB2*, a closely related member of the ErbB family of receptors (Lee et al., 2001). Consistent with this possibility, we found that electroporation of *Nkx2-1* in organotypic slices could lead to the induction of *ErbB4* expression in MGE-derived cells (8/17 slices; Figure S3) but not in LGE-derived cells or in *Nkx2-1*$^{-/-}$ tissue (data not shown). These results suggest that Nkx2-1 can activate the expression of *ErbB4* in MGE cells but its action seems to be conditioned by additional factors. It has been recently shown that expression of *ErbB4* is lost in *Lhx6* mutant mice exclusively in MGE-derived cells migrating to the cortex, but it is maintained throughout the subpallium (Zhao et al., 2008). Since *Lhx6* is a direct target of Nkx2-1 (Du et al., 2008), this LIM transcription factor could be acting downstream of Nkx2-1 to maintain *ErbB4* activation in MGE-derived cells after they down-regulate Nkx2-1 expression postmitotically.

Our experiments demonstrate that MGE-derived striatal interneurons may only invade the developing cortex if they down-regulate the expression of Nkx2-1. When transplanted into the cortex, MGE-derived Nkx2-1-expressing cells fail to leave the explants, even though expression of Nkx2-1 does not perturb the migratory potential of these cells (Nobrega-Pereira et al., 2008). This suggests that Nkx2-1 makes these cells susceptible to a yet unidentified cortical repulsive activity. It is tempting to speculate that the Slit/Robo signalling pathway could mediate this cortical repulsive activity. In the developing telencephalon, the Slit1 and 2 diffusible molecules are expressed across the midline and in restricted laminar patterns in the cortex or in the prospective hippocampus, respectively (Bagri et al., 2002; Marillat et al., 2001; Zhu et al., 1999), whereas the Slit receptors *Robo1/2* are expressed by some MGE-derived cells (data not shown; Andrews et al., 2006; Bagri et al., 2002). Slit proteins have been implicated in controlling the positioning of neuronal populations, such as the cholinergic basal magnocellular complex, across the midline of the ventral telencephalon (Marín et al., 2003). In addition, in *Slit1/2* double mutant mice there is a reduction in the number of NPY-expressing GABAergic interneurons in the dorsolateral striatum

(Marín et al., 2003), suggesting a possible involvement of this signalling in controlling the migration of striatal interneurons. Future experiments will test the possible role of this signalling pathway in controlling the migration of striatal interneurons. Altogether, our results shed light into the molecular mechanisms that regulate the migration of striatal interneurons and ultimately contribute for the correct wiring of the telencephalon.

3.5. Experimental Procedures

Mouse lines. Wild-type mice, GFP-expressing transgenic mice (Hadjantonakis et al., 2002) and *Nkx2-1* mutant mice (Kimura et al., 1996) maintained in a CD1 background were used for expression analysis and tissue culture experiments. $HER4^{heart}$ transgenic mice (Tidcombe et al., 2003), which express a human *ErbB4* (*HER4*) cDNA under the control of the cardiac-specific α-HMC (myosin heavy chain) promoter, were mated to *ErbB4* heterozygous mice (Gassmann et al., 1995) to generate $ErbB4^{+/+}HER4^{heart}$ and $ErbB4^{-/-}HER4^{heart}$ littermate mice and these animals were maintained in a mixed C57Bl/6 x 129/SvJ x CBA background. Animals were kept under Spanish and EU regulation.

In vitro focal electroporation. A cDNA encoding *Nkx2-1* (accession number NM_009385) was used. All constructs (*Gfp*, *Nkx2-1* and *Nkx2-1-IRES-Gfp*) were inserted into the *pCAGGS* chicken β-actin promoter expression vector. Expression vectors were electroporated at a concentration of 1 μg/μl and mixed in a 0.9/1.5 ratio when co-electroporated. Expression vectors were focally injected into the MGE and embryonic slice cultures were electroporated as previously described (Flames et al., 2004).

Slice and MGE explants culture. E13.5 organotypic coronal slice cultures from wild-type or GFP transgenic embryos were obtained as described previously (Anderson et al., 1997). Slice transplantation was performed immediately after the preparation of the organotypic slices, and the slices returned to the incubator for the appropriate time. MGE explants were dissected out from organotypic slices after electroporation and placed in the cortex of the host slice in transplantation experiments. For gene-expression analysis, immunofluorescence detection and migratory performance, MGE explants were cultured on glass coverslips coated with poly-L-Lysine and laminin in Neurobasal medium containing 0.4% methylcellulose (Sigma) or in three-dimensional matrigel® matrices.

In situ hybridization and immunohistochemistry. For in situ hybridization, brains were fixed overnight in 4% paraformaldehyde in PBS (PFA). Twenty μm frozen sections and 250 μm de-hydrated organotypic slices were hybridized with digoxigenin-labeled probes as described before (Flames et al., 2007). Immunohistochemistry was performed on: 20 μm cryostat sections, 60 μm vibrotome re-sectioned organotypic slices, MGE explants in matrigel® pads and MGE explants in

Poly-L-Lysine and Laminin substrates. Slices, explants and embryos were fixed in 4% PFA at 4ºC from 2-6 h. The following primary antibodies were used: chicken anti-GFP (1/1000, Aves Labs), rabbit anti-Nkx2-1 (1/2000, Biopat), and rabbit anti-ErbB4 (1/300, a gift from Dr. Cary Lai). The following secondary antibodies were used: goat anti-chicken 488, donkey anti-rabbit 555, donkey anti-rabbit 488 (Molecular Probes) and donkey anti-rabbit Cy3-conjugated Fab fragment (Jackson Laboratories). For the ErbB4/Nkx2-1 double staining, sections were first processed for the rabbit anti-ErbB4 immunofluorescence using anti-rabbit Cy3-conjugated Fab fragment as secondary antibody, fixed in 4%PFA-4%Sucrose for 10 min, and then processed for the rabbit anti-Nkx2-1 staining. DAPI (Sigma) was used for fluorescent nuclear counterstaining.

Quantification. For the quantification of interneurons in E15.5 control and *ErbB4$^{-/-}$HER4^{heart}* mutant brains, the outline of the striatum at rostral and caudal levels was delineated in 20 μm sections, Nkx2-1-expressing cells were counted for three different brains from each genotype and the cell density (number of cells per mm^2) was calculated. In the slice over-expression experiments, 60 μm vibratome re-sectioned organotypic slices were used to count the number of *Gfp*-expressing cells in a fixed volume of the striatum for control and *ErbB4$^{-/-}$ HER4^{heart}* mutant slices. For the MGE^{GFP} transplants into the cortex or cortex and striatum, the percentage of Nkx2-1/*Gfp*-expressing cells was counted for a fixed volume of tissue adjacent to the ectopic MGE explant. For the MGE-electroporated transplants into the cortex, the number of *Gfp*-expressing cells was counted in a fixed volume of cortical tissue adjacent to the ectopic MGE explant.

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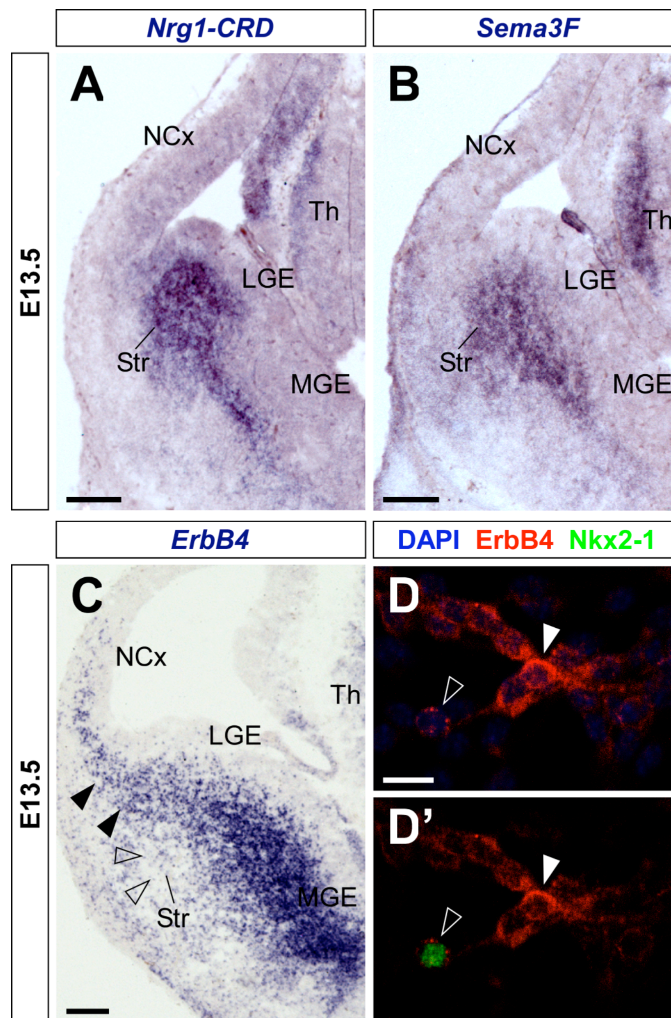

Figure1. The ErbB4 receptor is expressed in MGE-derived cortical and striatal migrating interneurons.

(A-C) Coronal sections through the telencephalon of E13.5 embryos depicting *Nrg1-CRD* (A) and *Sema3F* (B) mRNA expression in the developing striatum. (C) *ErbB4* mRNA expression is detected in cells migrating towards the cortex (NCx; solid arrowheads) and passing through or accumulating in the striatum (Str; open arrowheads).

(D and D') MGE-derived cells stained with DAPI and ErbB4 after 12h in culture (D) where the majority is Nkx2-1-negative (D', solid arrowheads) and a few co-label for Nkx2-1 (D', open arrowheads). Of note, not all Nkx2-1 positive cells stained for ErbB4.

LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Th, thalamus.

Scale bars = 200 µm (A, B and C) and 10 µm (D and D').

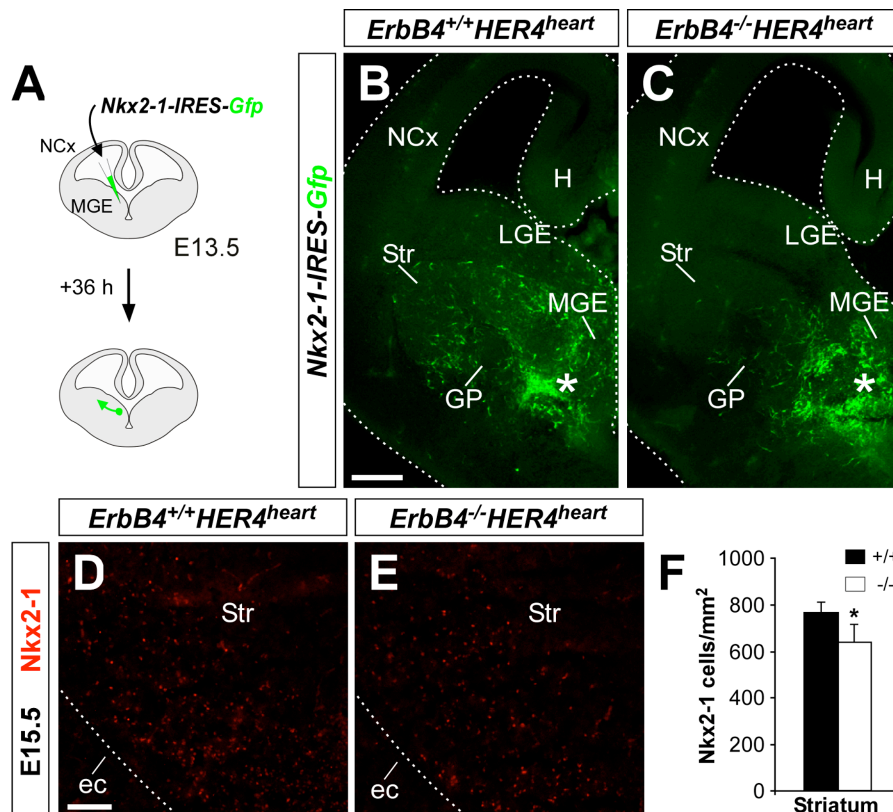

Figure 2. *ErbB4* is required for the migration of MGE-derived interneurons to the striatum.

(A) Schematic diagram of the focal electroporation experiment.

(B and C) Migration of MGE-derived cells over-expressing *Nkx2-1-IRES-Gfp* in E13.5 control (B) and *ErbB4$^{-/-}$HER4^{heart}* mutant (C) re-sectioned slices.

(D and E) Coronal sections through the telencephalon of E15.5 control (D) and *ErbB4$^{-/-}$HER4^{heart}* mutant (E) embryos showing Nkx2-1 protein expression.

(F) Quantification of the number of Nkx2-1-expressing cells in the caudal striatum of E15.5 control and *ErbB4$^{-/-}$HER4^{heart}* mutant embryos. Histograms show average ± SEM 771.46 ± 45.05 (Nkx2-1 control); 643.49 ± 76.77 (Nkx2-1 mutant). * $p < 0.05$, *t* test.

Dotted lines indicate the limits of the slices.

ec, external capsule; GP, globus pallidus; H, hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str, striatum.

Scale bars = 200 µm (B and C) and 100 µm (D and E).

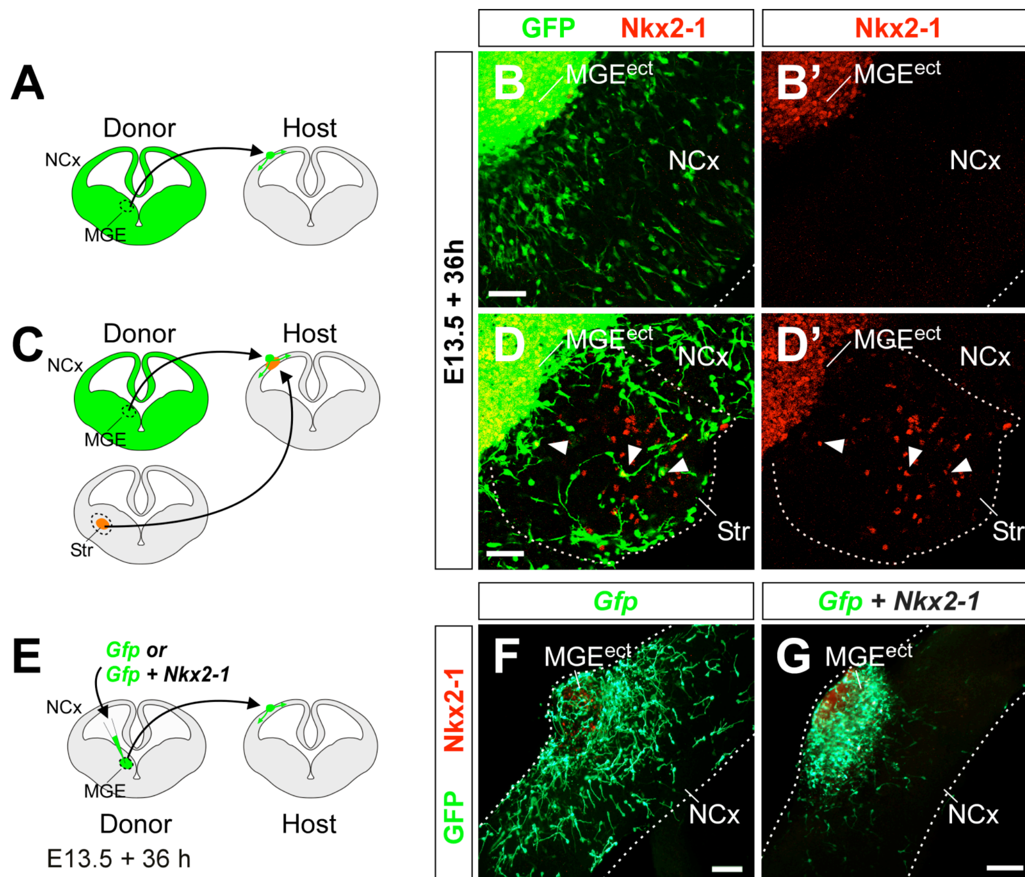

Figure 3. Downregulation of Nkx2-1 in MGE-derived interneurons is a necessary event for cortical invasion.

(A, C, E) Schematic of the slice transplantation paradigms used.

(B and B') MGE^{ect} -derived GFP migrating cells in the cortex of E13.5 wild-type host slices are Nkx2-1 negative.

(D and D') MGE^{ect} -derived GFP migrating cells only co-label for Nkx2-1 (arrowheads) in the striatal explants and not in the cortex of E13.5 wild-type host slices.

(F and F') Migration of MGE^{ect} -derived cells electroporated with *Gfp* (F) or with *Gfp* and *Nkx2-1* (G).

Dotted lines indicate the limits of the organotypic slices.

MGE, medial ganglionic eminence; MGE^{ect}, ectopic medial ganglionic eminence; NCx, neocortex; Str, striatum.

Scale bars = 50 µm (B and D) and 200 µm (F and G).

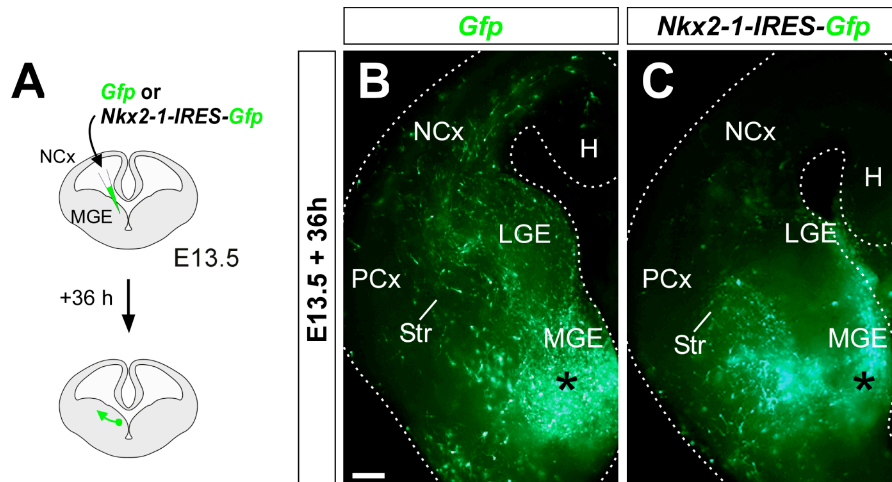

Figure S1. Nkx2-1 overexpression prevents the migration of MGE-derived interneurons to the cortex.

(A) Schematic diagram of the experimental paradigm used.

(B and C) Migration of MGE-derived cells over-expressing *Gfp* (B) or *Nkx2-1-IRES-Gfp* (C) in E13.5 organotypic slices.

Dotted lines indicate the limits of the organotypic slices.

H, hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; PCx, piriform cortex; Str, striatum.

Scale bar = 200 µm.

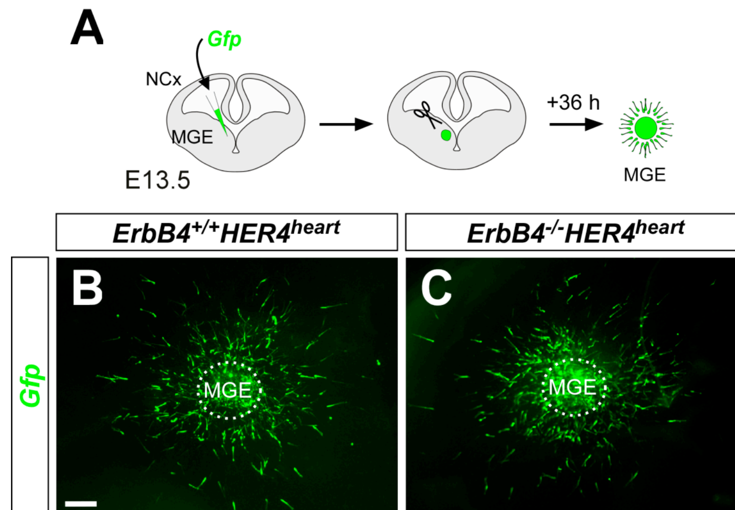

Figure S2. Loss of *ErbB4* function does not impair the migration of MGE-derived cells *in vitro*.

(A) Schematic diagram of the experimental paradigm used. E13.5 organotypic slices were focally electroporated with *Gfp*, MGE explants were dissected from the electroporated region and cultured in matrigel matrices.

(B and C) Migration of MGE-derived cells over-expressing *Gfp* in E13.5 control (B) and $ErbB4^{-/-}$ $HER4^{heart}$ mutant (C) explants cultured *in vitro*.

MGE, medial ganglionic eminence; NCx, neocortex.

Scale bar = 50 μm.

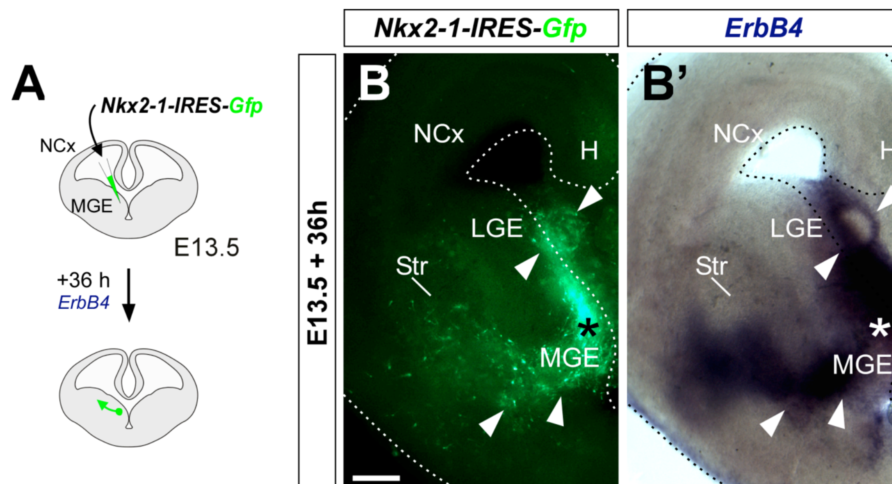

Figure S3. A potential role for Nkx2-1 in activating *ErbB4* expression in MGE cells.

(A) Schematic diagram of the focal electroporation experiment. After 36h, the slices were processed for *ErbB4* transcript detection by *in situ* hybridization.

(B and B') Migration of MGE-derived cells electroporated with *Nkx2-1-IRES-Gfp* (B) and *ErbB4* mRNA expression in the same organotypic slice (B'). Dotted lines indicate the limits of the organotypic slices and arrowheads point to *Nkx2-1-IRES-Gfp*-expressing cells that seem to co-label for *ErbB4.*

H, hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str, striatum.

Scale bar = 200 µm.

Chapter 4. General Discussion

During development of the telencephalon, newborn neurons migrate away from the germinal zones to populate specific regions. To achieve their correct targeting, neurons integrate multiple external signals that will determine their final location. So, guidance selectivity is ultimately achieved by the specific set of receptors and effectors expressed by each neuronal population. Transcriptional regulation has been pointed as the main mechanism regulating this process but, for most cases, is presently unclear how transcription factors exert their influence.

Identifying the extrinsic and intrinsic molecular mechanisms that confer directionality and guidance specificity to subpallial migrating interneurons is fundamental to understand the correct assembly and wiring of the telencephalon. A large class of neuropathological conditions (e.g. epilepsy, autism, Rett syndrome) collectively known as “interneuronopathies” may arise from impairment in the tangential migration of cortical GABAergic interneurons (Kato and Dobyns, 2005). Mutations in subpallial transcription factors have been linked to these pathologies both in humans and animal models (Butt et al., 2008; Cobos et al., 2005; Hamilton et al., 2005; Horike et al., 2005; Kato, 2006; Kato and Dobyns, 2005; Nawara et al., 2006). For instance, removal of both alleles of the *Nkx2-1* gene specifically in subpallial progenitors leads to profound behavioural abnormalities in juvenile animals, in which the occurrence of visible seizures is associated with prolonged abnormal bursting activity in the cortex (Butt et al., 2008). These behavioural abnormalities correlate with a dramatic reduction in the number of MGE-derived GABAergic interneurons in the cortex (Butt et al., 2008), similar to what it has been described in other mutants with reduced number of forebrain GABAergic interneurons (Cobos et al., 2006; Colombo et al., 2007; Kitamura et al., 2002; Powell et al., 2003). These studies highlight the crucial contribution of specific transcription factors in the development of the telencephalon, suggesting that in-depth analysis of their function will contribute to increase our knowledge on the mechanisms that shape a functional brain.

4.1. Transcriptional control of neuronal migration in the developing telencephalon

Neurons can migrate either along the radial axis, interacting with the radial glial scaffold, or disperse tangentially in a plane orthogonal to radial glial cells along the antero-posterior or medio-lateral axis (Hatten, 1999). Thus, neuronal migration is subjected to several levels of regulation, ranging from the selective action of environmental guidance cues to the establishment of interactions with specific substrates (Hatten, 2002; Marín and Rubenstein, 2003). Guidance molecules controlling neuronal migration have multiple similarities with those described for axon

guidance where transcriptional regulation has been shown to control the repertoire of receptors expressed by each neuron (Butler and Tear, 2007; Polleux et al., 2007). Apart from the research reported here, there is increasing evidence that transcriptional regulation plays an important role in tangential and radial migration in the developing telencephalon.

4.1.1. Transcription factors in the migration of telencephalic interneurons

The MGE is a proliferative structure located in the subpallium that gives rise, among other neuronal populations, to interneurons that migrate tangentially to the striatum and cerebral cortex (Lavdas et al., 1999; Marín et al., 2000; Sussel et al., 1999; Wichterle et al., 1999; Wichterle et al., 2001). Since MGE-derived interneurons undergo extensive migration to reach their target territories (Marin and Rubenstein, 2001), they represent a good model to study the contribution of extrinsic and intrinsic determinants in controlling this remarkably complex process. Previously it was shown that Neuropilin-1 (Nrp1) and Neuropilin-2 (Nrp2), the binding receptors for the striatal repulsive molecules Semaphorin-3A (Sema3A) and Semaphorin-3F (Sema3F), respectively, are expressed by MGE-derived cortical and absent from MGE-derived striatal interneurons, mediating thereby the segregation of these populations (Marín et al., 2001). Therefore, one of our aims was to investigate the upstream regulators controlling neuropilin/semaphorin interactions in MGE-derived migrating interneurons.

Using experimental manipulations and mouse genetics, we provide evidence that the homeodomain transcription factor Nkx2-1 modulates the selective responsiveness of MGE-derived interneurons to class 3 Semaphorins (Figure 8A). All MGE progenitor cells express Nkx2-1, which plays a crucial role in their specification (Butt et al., 2008; Sussel et al., 1999). Interestingly, Nkx2-1 expression is maintained in MGE-derived postmitotic striatal interneurons but is downregulated by cortical migrating cells (Marín et al., 2000; Nobrega-Pereira et al., 2008) (Figure 8A). Our data indicate that forced expression of *Nkx2-1* in MGE-derived cells prevented the migration of interneurons to the cortex. Conversely, conditional deletion of *Nkx2-1* in MGE-derived postmitotic cells resulted in a reduction in the number of striatal interneurons at developmental and postnatal stages. We could demonstrate that Nkx2-1 mediates the sorting of these interneuronal populations by negatively regulating their sensitivity to class 3 Semaphorins; MGE-migrating cells expressing *Nkx2-1* are no longer repelled by a source of semaphorins and exhibit a significant reduction in the expression of *Nrp2*. Moreover, Nkx2-1 directly binds to the *Nrp2* locus in MGE cells and interaction with this sequence is sufficient to repress transcription *in vitro*, suggesting a direct and cell-autonomous role for Nkx2-1 in controlling the expression of the *Nrp*2 guidance receptor in MGE-derived cells (Figure 8A). Our data reveal that the segregation of MGE-derived interneurons largely depends on Nkx2-1 postmitotic function, however, and as discussed previously (Nobrega-Pereira et al., 2008), the factors controlling *Nkx2-1* expression at this level are unknown but seem to be Shh-independent. The Nkx6-1 transcription factor is able to maintain the expression of its

own gene in pancreatic β-cells (Iype et al., 2004); similarly Nkx2-1 could mediate a positive feedback loop to sustain its own expression in postmitotic striatal interneurons.

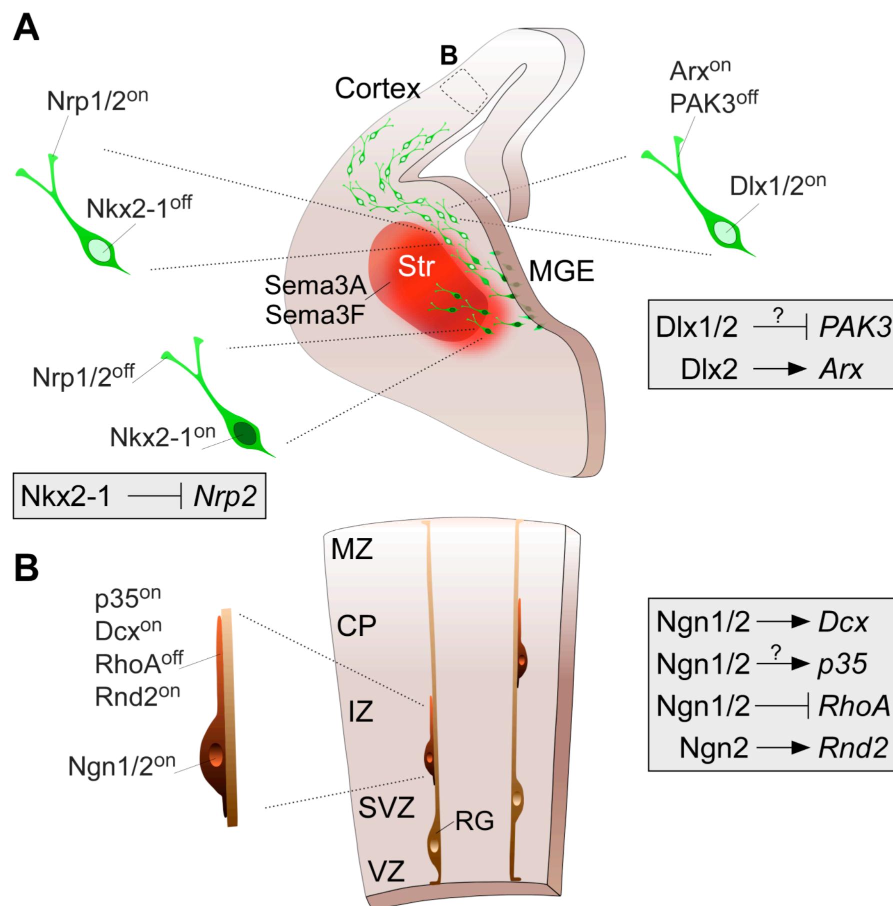

Figure 8. Function of postmitotic transcription factors in controlling neuronal migration and guidance in the telencephalon. (A) Schematic representation of a transversal hemisection through an E13.5 developing telencephalon, depicting tangentially migrating interneurons from the medial ganglionic eminence (MGE) to the cortex and striatum (Str). (i) MGE progenitor cells and migrating MGE-derived striatal interneurons express Nkx2-1 (dark green nuclei), whereas cortical interneurons down-regulate Nkx2-1 after leaving the MGE (light green nuclei). Postmitotic Nkx2-1 directly represses the expression of *Nrp2*, the binding receptor for Sema3F, which enables interneurons to invade the striatum and cortical interneurons to avoid this territory. (ii) The Dlx1/2 genes are expressed by MGE progenitors and migrating interneurons. These transcription factors promote the tangential migration of MGE-derived cells by preventing the premature expression of cytoskeleton regulators that induce neurite outgrowth and by activation the expression of the homeobox *Arx*. (B) Higher magnification of the area boxed in (A) depicting radially migrating projection neurons in the developing cortex, where the ventricular zone (VZ) is to the bottom and the marginal zone (MZ) is to the top. The Ngn1/2 transcription factors induce the neuronal commitment of radial glial (RG) progenitor cells located in the ventricular and subventricular zone (SVZ) and in addition potentiate the radial migration of projection neurons in the SVZ and intermediate zone (IZ) by directly controlling the expression of actin and microtubule cytoskeleton regulators. CP, cortical plate.
Questions marks indicate proposed but not proven transcriptional interactions.

Our experiments showed that Nkx2-1 does not seem to significantly affect the expression of additional components of the Sema3A/3F signalling, such as the *Nrp1* binding receptor and the *PlexinA3/A4* signalling-receptors, components of the Sema3A/3F signalling in perypheric neurons (Yaron et al., 2005). Although *PlexinA3/A4* are expressed in MGE-derived interneurons (Nobrega-Pereira et al., 2008), the association of these receptors with Nrp1/2 co-receptors has not been

demonstrated in these neurons. The factors controlling the activation of *Nrp* receptors in MGE-derived cortical migrating interneurons are currently unknown. Since Nkx2-1 is expressed in virtually all MGE precursors (Nobrega-Pereira et al., 2008) and Nrp1/2 expression is largely confined to postmitotic MGE-derived cells (Marín et al., 2001), it is tempting to speculate that Nkx2-1 could additionally contribute to prevent the premature expression of *Nrp2* in progenitor cells, which otherwise could result in abnormal neuronal migration. The postmitotic maintenance of Nkx2-1 only in striatal neurons will ensure the *Nrp2* repressive state whereas the fast downregulation of Nkx2-1 in cortical interneurons will relieve this repression and enable cortical migrating neurons from avoiding the striatal territory. All our functional experiments tested the contribution of Nkx2-1 postmitotic function and the role of this transcription factor at the progenitor level was not the scope of this study. Nevertheless, our ChIP and luciferase assays reveal that Nkx2-1 is able to bind and repress *Nrp2* transcription in MGE cells and *in vitro*, respectively, leaving this possibility open. In this context, the Dlx1 and Dlx2 homeodomain transcription factors have been described as potential negative regulators of *Nrp2* expression in MGE cells; Dlx1 and Dlx2 bind to a specific region of the *Nrp2* locus *in vivo* and interaction with this sequence promotes repression *in vitro* (Le et al., 2007). Since these factors are expressed by subpallial progenitor cells and in both cortical and striatal migrating interneurons (Eisenstat et al., 1999), the functional relevance of this interaction for the segregation of MGE-derived cells is unlikely and rather suggests a regulation at the level of progenitors.

The Dlx1/2 transcription factors also play a prominent role in the migration of MGE-derived interneurons but appear to control different target effectors. The analysis of *Dlx1/2* double mutants revealed severe defects in the differentiation and tangential migration of subpallial-derived GABAergic interneurons (Anderson et al., 1997; Marín et al., 2000; Pleasure et al., 2000). *Dlx1/2*-deficient MGE cells have increased expression of several cytoskeleton regulators, such as microtubule-associated protein 2 (MAP2), growth associated protein 43 (GAP43) and p-21 activated kinase 3 (PAK3), which may in turn cause their premature differentiation and block their migration (Cobos et al., 2007). PAK3 is a downstream effector of the Rho family of GTPases (Bokoch, 2003) and is primarily expressed in post-migratory MGE-derived cells in which it controls the growth of axons and dendrites. The premature expression of PAK3 in migratory MGE-derived cells causes excessive neurite length, which in turns block their normal movement (Cobos et al., 2007). Thus, Dlx1/2 transcription factors promote tangential migration, in part, through negatively regulating the neurite differentiation program in migratory neurons (Figure 8A). In addition, a recent study reported that Dlx2 is required to activate the GABAergic enhancer element of *Aristaless related homeobox* (*Arx*) (Colasante et al., 2008), a transcription factor previously implicated in the migration of subpallial-derived interneurons (Bonneau et al., 2002; Colombo et al., 2007; Kitamura et al., 2002). This factor is a key mediator of Dlx-dependent migration, since *Arx* over-expression in *Dlx1/2* mutant embryonic slices is able to partially rescue the migration of subpallial interneurons in these mutants (Colasante et al., 2008) (Figure 8A).

4.1.2. The migration of cortical projection neurons is transcriptionally regulated

Cortical projection neurons are born from progenitor cells located in the dorsal telencephalon (i.e. the pallium) and migrate radially towards the pial surface to form the layers of the developing cortex (Kriegstein and Noctor, 2004) (Figure 8B). Regulation of the actomyosin and microtubule cytoskeletons, adhesion molecules and non-receptor kinases is believed to regulate the migration of cortical projection neurons (Ayala et al., 2007; Marín and Rubenstein, 2003).

The bHLH transcription factors Neurogenin1 (Ngn1) and Neurogenin2 (Ngn2) are well known for their proneural activity in several regions of the CNS, including the pallium (Guillemot, 2007). In addition, they have been proposed to potentiate cortical neuronal migration (Figure 8B). For instance, Ngn1/2 may enhance cortical cell migration by increasing the expression of p35 and doublecortin (Dcx) and diminishing the expression of the GTPase Rho member A (RhoA), key modulators of the actin and microtubule cytoskeleton (Ge et al., 2006). Consistent with this notion, the migration defects observed in the cortex of *Ngn2* mutants can be rescued by reducing the function of RhoA (Hand et al., 2005). These genes are likely direct targets of Ngn1 and Ngn2, since both factors bind directly to E-box elements (i.e. consensus binding sites for bHLH factors) located in the *Dcx* promoter in cortical neurons and are able to induce and repress, respectively, the expression of the *Dcx* and *RhoA* promoters *in vitro* (Ge et al., 2006) (Figure 8B). The *Rho-related GTP-binding protein 2* (*Rnd2*), another member of the Rho family of small GTPases, is transiently expressed by cortical migrating neurons and has been identified as a potential *Ngn2* downstream target in a recent genomic screen (Heng et al., 2008) (Figure 8B). Knockdown and overexpression of *Rnd2* cause striking defects in the radial migration of projection neurons. In particular, deregulation of *Rnd2* activity leads to the persistence of an immature multipolar morphology, which may prevent the migration of projection neurons (Heng et al., 2008; Nakamura et al., 2006). As previously shown for a dominant-negative form of *RhoA*, weak and transient expression of *Rnd2* in newborn *Ngn2*-deficient cortical neurons rescued the morphological and migratory abnormalities found in *Ngn2* mutants, reinforcing the view that Rnd2 acts as a downstream mediator of Ngn2 during cortical migration (Heng et al., 2008). Furthermore, ChIP assays suggest that Ngn2 directly regulates *Rnd2* expression in newborn cortical projections neurons (Heng et al., 2008) (Figure 8B).

As for axon guidance, increasing evidence suggest that neuronal migration is controlled by the precise transcriptional regulation of effector genes that regulate a variety of events, from cytoskeleton dynamics to the response to guidance cues. It is also now evident that the same transcription factor can regulate several steps during the development of a given neuronal population. Understanding the mechanisms that modulate transcriptional selectivity will further uncover the action of transcription factors through development.

4.2. Nkx2-1 plays several roles in telencephalic development

Nkx2-1 is expressed in the ventral subpallial progenitor zones and in many postmitotic neurons derived from those structures where it controls several developmental events. At early stages, Nkx2-1 interprets Shh patterning signal to impose regional specification in the subpallial telencephalon, whereas at neurogenic stages Nkx2-1 function determines cell-fate by restricting subtype-specific identity on MGE-derived cells (Anderson et al., 2001; Butt et al., 2008; Du et al., 2008; Marín et al., 2000; Sussel et al., 1999; Xu et al., 2004). Our work reveals a new postmitotic function for Nkx2-1 in neuronal migration by controlling the repertoire of guidance receptors expressed by MGE-derived interneurons which will determine the selective responsiveness to the Sema3A/3F-striatal activity (Nobrega-Pereira et al., 2008). Previous studies reported that deletion of Nkx2-1 in differentiated neurons at postnatal stages perturbed hypothalamic-dependent gene expression and sexual maturation, without affecting basal ganglia-associated behavioural tasks (Mastronardi et al., 2006). This suggests that Nkx2-1 function in the ventral telencephalon is more critical during developmental stages (Butt et al., 2008). In agreement, Nkx2-1 is not expressed in the adult rat and human pallidal and striatal neurons (Krude et al., 2002) and the neurological symptoms (e.g. hypotonia, dyskenesia, and choreoathetosis) associated with *Nkx2-1* heterozygous loss of function mutations in humans and mice are attributed to a failure in basal ganglia development (Breedveld et al., 2002; Kleiner-Fisman et al., 2005; Krude et al., 2002; Pohlenz et al., 2002).

Nkx2-1 is required for the specification of virtually all striatal interneurons (Butt et al., 2008; Marín et al., 2000; Sussel et al., 1999). Our results, however, suggest that the postmitotic function of this transcription factor is restricted to certain subtypes. Removal of *Nkx2-1* postmitotic expression lead to a dramatic reduction in the number of Cholinergic and Parvalbumin-expressing GABAergic interneurons whereas the number of SST/NPY- and CR- positive interneurons was unchanged in the striatum of P25 *Lhx6-Cre;Nkx2-1*$^{Fl/Fl}$ mutant mice (Nobrega-Pereira et al., 2008). These results suggest that the later interneuron subtypes use mainly Nkx2-1-independent mechanisms to migrate to the striatum; whether this requires the involvement of other transcription factors in controlling neuropilin/semaphorin interactions or additional signalling systems remain to be investigated. In addition, we have demonstrated that Nkx2-1 postmitotic function directly controls neuronal migration, as shown by the decreased number of YFP neurons in the striatum of *Lhx6-Cre;Nkx2-1*$^{Fl/Fl}$*;ROSA-YFP* mutant mice, and is not required for the specification of MGE-derived neurons. In agreement, in the striatum of *Lhx6-Cre;Nkx2-1*$^{Fl/Fl}$ mutant mice we did not observed an increase in other interneuronal subtypes (CR, SST or NPY) at the expense of the depleted ChAT and PV populations, and the number of *Lhx6*- and *Lhx7*- expressing neurons was unaffected in other subpallial regions besides the striatum, or in the cortex (data not shown; Nobrega-Pereira et al., 2008). These observations suggest that Nkx2-1 functions in progenitor and postmitotic cells through different genetic programmes, which apart from targeting different genes could implicate

the use of specific transcriptional mechanisms.

Identification of Nkx2-1 downstream molecular mechanisms and target effectors is fundamental to understand the contribution of this factor in telencephalic development. The research reported here provided evidence that Nkx2-1 directly represses the expression of the *Nrp2* guidance receptor in MGE-derived migrating interneurons. Nkx2-1 binds to two tandem consensus regions in the *Nrp2* regulatory region located (300 bp) upstream from the transcription initiation site and interaction with this sequence is sufficient to repress transcription *in vitro*. Furthermore, postmitotic Nkx2-1 ability to leave MGE-derived cells insensitive to a source of Sema3A/3F and to repress *Nrp2* transcription *in vitro* relies in the homeodomain whereas the TN and NK2-SD motifs are not required (Nobrega-Pereira et al., 2008). Apart from necessary, it remains to be determined whether the homeodomain motif is sufficient to mediate Nkx2-1 postmitotic function, and particularly *Nrp2* repression, in MGE-derived neurons. To our knowledge, this is the first Nkx2-1 downstream target gene identified in postmitotic neurons and it will be interesting to investigate if an HD-dependent and TN/NK2-SD-independent mechanism constitutes a signature for Nkx2-1 postmitotic function or if alternatively is gene- and context- specific. For instance, the Ngn2 transcription factor uses partially distinct transcriptional mechanisms in progenitor or postmitotic cells (Ge et al., 2006; Hand et al., 2005) (see also section 4.2.1.). In the developing telencephalon, *Lhx6* is so far the only proven direct transcriptional target of Nkx2-1 in subpallial-derived neurons and transcriptional activation of *Lhx6* also requires an intact Nkx2-1 homeodomain (Du et al., 2008). In this study, we also provide evidence that the *ErbB4* receptor is a potential downstream target for Nkx2-1 in MGE progenitor cells. The expression of *ErbB4* is almost absent in the telencephalon of *Nkx2-1* mutant mice (data not shown) and overexpression of *Nkx2-1* in embryonic organotypic slices is able to induce *ErbB4* expression in MGE-derived cells. It remains to be elucidated whether this interaction occurs though direct or indirect (e.g. *Lhx6*) transcriptional regulation. The ErbB4 receptor has been previously described to mediate Nrg1-chemoattractive signalling in MGE-derived cortical migrating interneurons (Flames et al., 2004) and a possible role for this receptor in the guidance of striatal interneurons will be discussed later (section 4.3.).

Is Nkx2-1 the only transcription factor with such transversal function in the developing telencephalon? As mentioned earlier, there are other examples of transcription factors that play pleiotropic functions in specific neuronal populations (e.g. subpallial Dlx1/2 and pallial Ngn2), and as for Nkx2-1, the mechanisms that regulate their complex function are not totally understood.

4.2.1. Transcription factors as multitasking regulators

It has been typically assumed that different families of transcription factors control distinct events during development, such as the specification of progenitor cells or the differentiation of neuronal populations. This prediction, however, turned out to be largely incorrect (Guillemot, 2007). Thus, while some transcription factors seem to function primarily in the differentiation of neurons (e.g.

LIM-HD transcription factors); many others regulate distinct events at different developmental stages (De Marco Garcia and Jessell, 2008; Muller et al., 2003; Shirasaki and Pfaff, 2002). For instance, the homeodomain transcription factors Nkx2-1 and Hoxa2 are first required for the early specification of specific neuronal populations in the developing telencephalon and hindbrain, respectively, and later regulate the migration of the same neurons (Geisen et al., 2008; Nobrega-Pereira et al., 2008).

An obvious question that emerges is how time and context-dependent transcriptional selectivity is accomplish for a given transcription factor. These proteins are able to recognize and bind to specific DNA target sequences through the interaction of its DNA binding motif with unique nucleotide sequences present in the regulatory regions of selected genes (Damante et al., 1996). In addition, several other mechanisms have been proposed to further modulate transcriptional selectivity, including post-translational modifications (e.g. phosphorylation or acetylation) of specific amino acid residues in protein- or DNA-binding motifs (Hand et al., 2005; Yang et al., 2004). Interactions with specific co-regulators have also been described, such as the cooperative binding of Mash1 with class III POU domain transcription factors Brain (Brn) to the *Delta1* promoter in cortical neurons (Castro et al., 2006) or the interaction of Hox transcription factors with Pbx cofactors (Samad et al., 2004). However, how these mechanisms control the switch in transcriptional selectivity is still unclear. Recently, the Ngn1/2 transcription factors have been proposed to control neurogenesis and migration of cortical projections neurons through partially distinct mechanisms. A Tyrosine (T) to Alanine (A) replacement in the position 241 of the Ngn2 protein (*Y241A-Ngn2)* blocks cortical cell migration without changing Ngn2 ability to regulate the neurogenic *NeuroD* promoter, whereas the mutated *AQ-Ngn1/2*, containing two amino acid substitutions in the C-terminus DNA-binding domain, fails to activate the neurogenic transcriptional programme but still promotes neuronal migration (Ge et al., 2006; Hand et al., 2005). Phosphorylation of the Y241 residue was proposed to mediate the Ngn1/2 migratory promoting activity by displacing the CREB binding protein (CBP) co-activator, which in turn interacts with the *RhoA* and *Dcx* promoters during migration (Figure 8B). In contrast, Ngn1/2 proneural function does not seem to heavily rely on CBP function and instead requires binding to E-box elements. In any case, Ngn1/2 function in neuronal migration also depends on their binding to E-box elements (Ge et al., 2006; Hand et al., 2005; Heng et al., 2008), suggesting that the segregation of the migratory and proneural functions of Ngn1/2 is not exclusively regulated by these mechanisms. In sum, even for the same developmental process, transcription factors use a combination of strategies to recognize and modulate the expression of different effector genes.

4.3. Additional guidance systems in the migration of striatal interneurons

The migration of neurons to the corresponding target territory is achieved by the coordinated action of extrinsic signalling systems that channel migrating cells along the appropriate paths into their final destiny. Several guidance activities have been demonstrated to control the migration of MGE-derived interneurons to the cortex (Flames et al., 2004; Marín et al., 2000; Marín et al., 2003). However, evidence from previous reports and this study suggest that the migration of Nkx2-1- expressing striatal interneurons is mainly controlled by neuropilin/semaphorin interactions (Marín et al., 2001; Nobrega-Pereira et al., 2008). Analysis of *Nrp2* mutant mice and *Nkx2-1* gain-of-function in organotypic slices revealed than desensitization to the Sema3F striatal repulsive activity not only prevented the migration of MGE-derived interneurons to the cortex but also lead to a specific accumulation of these cells in the striatum (Marín et al., 2001; Nobrega-Pereira et al., 2008). This evidence suggests that additional guidance molecules could contribute for the final directionality of striatal interneurons and we decided to investigate the possible activity and identity of these signalling systems.

Our results indicate that a population of Nkx2-1-positive migrating neurons express the ErbB4 receptor and the specific accumulation of Nkx2-1-expressing interneurons in the developing striatum is reduced in the absence of this receptor. As previously discussed (see 3.3.1.), membrane-bound forms of Nrg1 (CRD-Nrg1) are expressed in the striatal mantle and could trigger the ErbB4-signalling in Nkx2-1-migrating interneurons by exerting an attractive/permissive activity or acting as a “stop” signal for differentiation. The direct action of Nrg1-CRD in the migration of striatal interneurons was not tested and the requirement of the ErbB4-signalling for the proliferation and survival of MGE precursors also remain to be clarified. Furthermore, using experimental embryology techniques we could demonstrate that MGE-derived striatal interneurons are able to invade the cortical territory if down-regulating Nkx2-1 and forced expression of this transcription factor prevents cortical invasion. These results suggest that Nkx2-1-expressing migrating interneurons are actively prevented from invading the cortex although the identity of this signalling is currently unknown (see 3.3.2).

Overall, these results propose that striatal migrating interneurons integrate multiple external signals that specifically direct these cells to the striatum and prevent their accumulation in other forebrain structures (e.g. cortex). Thus, previous reports demonstrate that MGE-derived striatal interneurons do not express the binding receptors for the Sema3A/3F-repulsive activity ($Nrp1/2^{off}$) present in the developing striatum, allowing the invasion of this territory (Marín et al., 2001). In this study, we demonstrate that a population of striatal migrating interneurons express the ErbB4 receptor ($Nrp1/2^{off}$; $ErbB4^{on}$) which mediates the targeting to the striatum hypothetically through Nrg1-CRD attractive/permissive interactions. Furthermore, striatal interneurons ($Nrp1/2^{off}$; $ErbB4^{on}$;

receptor X^{on}) are actively prevented from invading the Nrg1- expressing developing cortex (Flames et al., 2004) due to the presence of a yet unidentified cortical repulsive activity. Since the action of these signalling systems has been tested in Nkx2-1-expressing striatal interneurons, it is tempting to speculate that, as for the *Nrp2* receptor (Nobrega-Pereira et al., 2008), Nkx2-1 could also be controlling the responsiveness to the proposed striatal attractive and cortical repulsive activities by regulating the expression of their receptors. Thus, similarly to the neuropilin/semaphorin interactions, cortical and striatal interneurons will have different responsiveness to the presumably cortical repulsive activity, suggesting a role for the postmitotic Nkx2-1 in conferring sensitivity specifically to striatal neurons. On the other hand, the ErbB4-dependent signalling operates in both cortical (Flames et al., 2004) and striatal interneurons, which suggest a role for Nkx2-1 in MGE precursors.

Chapter 5. Conclusions

1- Transcriptional regulation is one of the fundamental mechanisms controlling guidance decisions in navigating neurons. In this study, we found that Nkx2-1 mediates the sorting of MGE-derived cortical and striatal interneurons by direct transcriptional repression of the *Neuropilin-2* guidance receptor, which enables striatal interneurons to invade the semaphorin-expressing striatal mantle and cortical interneurons to avoid this territory.

2- We describe a new postmitotic function for the cell-fate determinant Nkx2-1 in controlling the migration of MGE-derived interneurons. The multitasking potential of Nkx2-1 is not a feature unique to this protein and appears to be shared by many other transcription factors during telencephalic development, although the precise regulation of this complex action is still not understood.

3- The migration of MGE-derived interneurons to the striatum appears to be controlled by the coordinated activity of several environmental activities. Nkx2-1 is a fundamental intrinsic factor in mediating the responsiveness of striatal interneurons to those molecules.

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