DLX HOMEOBOX GENES AND THEIR ROLE IN INTERNEURONAL DIFFERENTIATION AND MIGRATION IN THE DEVELOPING FOREBRAIN

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Abstract

Understanding the specificity of homeobox genes has been hampered by the lack of verified direct transcriptional targets. The *Dlx* family of homeobox genes is expressed in the ganglionic eminences of the developing forebrain. *Dlx1/Dlx2* double knockout (DKO) mice die at birth. Phenotypic analyses demonstrate abnormal development of the basal telencephalon, including defects in neuronal differentiation in the basal ganglia, reduced expression of GABA in the basal telencephalon, and loss of migration of GABAergic inhibitory interneurons to the neocortex. The mechanisms underlying DLX protein regulation of differentiation and migration of GABAergic interneurons are poorly defined.

We have successfully applied chromatin immunoprecipitation to identify potential direct transcriptional targets of DLX homeoproteins from embryonic tissues *in vivo*. Reporter gene assays demonstrated the transcriptional significance of the binding of DLX proteins to different downstream regulatory elements, which were confirmed *in vitro* by electrophoretic mobility shift assay and site-directed mutagenesis. The functional significance of DLX mediated transcriptional regulation of these targets was further elaborated through several series of loss-of-function assays including gene expression in *Dlx*1/2 knockout embryonic forebrain tissues, as well as siRNA or Lentiviral mediated shRNA knockdown experiments with primary forebrain cultures. Quantitative analysis of the regulatory effect of *Dlx* genes on various forebrain markers of differentiation and migration was performed using *in situ* hybridization, high-performance liquid chromatography coupled with cell counting. Neuronal migration was assessed by forebrain explants and diI labelling of migratory cells from ganglionic eminence to neocortex.

We have demonstrated that DLX1 and DLX2 can transcriptionally activate (*Gad1*, *Gad2*) or repress (*Nrp2*) different downstream targets. In the *Dlx1/2* DKO, reduction of GABA expression and failure of GABAergic interneurons to migrate to the neocortex is partly due to loss or aberrant expression of these DLX downstream targets. In the triple *Dlx1/2; Nrp2*KO, partial restoration of tangential migration of GABAergic interneurons from basal ganglia to the neocortex was successfully established signifying the importance of DLX regulation of Semaphorin-Neuropilin signalling during forebrain development.

List of Abbreviations

AEP-   Anterior Entopeduncular Area

AP-   Anterior-posterior

BG-   Basal Ganglia

bHLH-   Basic Helix-Loop-Helix

BDNF-   Brain Derived Neurotrophic Factor

BrdU-   5-bromo 2'-deoxyuridine

CB-   Calbindin

CDK-   Cyclin dependent kinase

CGE-   Caudal ganglionic eminence

ChAT-   Choline Acetyltransferase

ChIP-   Chromatin Immunoprecipitation

CR-   Calretinin

DARPP32-   Dopamine and cAMP-Regulated Phosphoprotein

DiI-   1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate

Dll-   Drosophila Distal-lessgene

Dlx-   Distal-less family homeobox transcription factor in the vertebrate

DV-   Dorsal-ventral

EMSA-   Electrophoretic Mobility Shift Assay

E-   Glutamate

E #-   Embryonic day #

FITC-   Fluorescein Isothiocyanate

GABA-   Gamma-aminobutyric acid

GAD65-   Glutamic Acid Decarboxylase isoform 65kDa => *Gad*2

GAD67-   Glutamic Acid Decarboxylase isoform 67kDa => *Gad*1

gDNA-   Genomic Deoxyribonucleic Acid

GE-   Ganglionic Eminences

GFP-   Green Fluorescent Protein

HPLC-   High Performance Liquid Chromatography

IF-   Immunofluorescence

IHC-   Immunohistochemistry

ISH-   In Situ Hybridization

kDa-   Kilo Dalton

LGE-   Lateral Ganglionic Eminence

MGE-   Medial Ganglionic Eminence

MI56-   Mouse Dlx5/Dlx6 intergenic enhancer

MZ-   Mantle zone

NCx-   Neocortex

NMDA-   N-methyl-D-aspartate

nNOS-   Neuronal Nitric Oxide Synthase

NPY-   Neuropeptide Y

NRP-   Neuropilin receptor, a high-affinity semaphorin receptor

P #-   Postnatal day #

PV-   Parvalbumin

PCx-   Paleocortex

PFA-   Paraformaldehyde

PKC-   Protein Kinase C

POa-   Preoptic Area

PV-   Parvalbumin

Q-   Glutamine

RMS-   Rostral migratory stream

Sema3-   Semaphorin class 3 protein, a secreted chemorepellent

SHH-   Sonic Hedgehog

shRNA-   small hairpin ribonucleic acid

siRNA-   small interfering ribonucleic acid

SST-   Somatostatin

Str-   Striatum

SVZ-   Subventricular zone

TH-   Tyrosine hydroxylase

TR-   Texas Red

μm-   Micrometer

VZ-   Ventricular zone

WT-   Wild-type – heterozygotes of *Dlx*1/2 genes or *Nrp*2 genes

Chapter 1

General Introduction

1.1 The telencephalon: an overview of its complexity

The forebrain is comprised of a complex set of functional structures that derive from the most anterior region of the neural tube, the prosencephalon (Rubenstein and Beachy, 1998; Rubenstein et al., 1998). The prosencephalon consists of the diencephalon and telencephalic vesicles, which evaginate from the dorsal aspect of the rostral diencephalon. The principle components of the telencephalon are two major regions: the pallium (roof) and the subpallium (base) (**Fig. 1**). The pallium is further subdivided into the medial, dorsal, lateral, and ventral pallium, which will give rise to the hippocampal formation (limbic lobe), the neocortex, the olfactory/piriform cortex, and the claustrum and parts of the amygdale, respectively. The subpallium consists of three primary sub-divisions: the striatal, pallidal, and telencephalic stalk domains, all of which extend medially into the septum. Other telencephalic components, such as septum and amygdala, have both pallial and subpallial origins. Finally, the olfactory bulbs develop as bilateral evaginations from a region of the prosencephalic neural plate intercalated between the septal and the cortical anlagen (Cobos et al., 2001; Rubenstein and Rakic, 1999). Together, these structures are essential for processing sensory information, integration of new sensory information with established memories (both experiential and instinctual), and then formulating and effecting behavioural responses. Thus, in vertebrates, the telencephalon is the seat of consciousness, higher cognition, language, motor control, and emotions. Damage to this structure leads to dementia, specific sensory and motor deficits, language and movement disorders, and changes in personality and emotional state.

Comparative anatomic, genetic, and molecular studies of telencephalic development promise to yield important insights into brain patterning, function, and evolution. Development of the central nervous system (CNS) as a whole begins with neural induction, during which a region of the embryonic ectoderm is specified that will form the neural plate on the dorsal side of the embryo (Barker and Barasi, 1999). Neurulation then occurs during which the neural plate forms the neural tube, which is lined by a pseudostratified columnar epithelium consisting of uncommitted precursor cells. Patterning of the neuroepithelium along the antero-posterior axis leads to the progressive subdivision of the neural tube into regions with distinct fates: the prosencephalon (forebrain), the mesencephalon (midbrain) and the rhombencephalon (hindbrain) (Persaud, 2003). Patterning and regional specification of the forebrain precede cell migration. As in other regions of the CNS, specification of cell types in the forebrain requires the creation of distinct antero-posterior and dorsoventral progenitor domains by the coordinated activity of several morphogenetic centers (Abu-Khalil et al., 2004; Puelles and Rubenstein, 2003). The induction of expression of specific transcription factors in different progenitor domains acts as an intermediary between morphogenetic cues and acquisition of a specific cell phenotype. Through a mechanism that involves mutually repressive interactions, these transcription factors establish boundaries between different progenitor zones, which lead to the consolidation of progenitor domain identity. Once cells are specified, they are set to differentiate and/or migrate to their final position in the mantle zone of the forebrain (Marin et al., 2001).

1.2 Developmental morphology of the telencephalon

Within the forebrain, the two main, interconnected compartments that will be focused solely in this thesis are the neocortex and basal ganglia. We'll start with discussing the general anatomy of the neocortex and basal ganglia.

1.2.1 Neocortex and its progenitors

The neocortex is a complex, highly organized, six-layered structure that contains hundreds of different neuronal cell types and a diverse range of glia (Cobos et al., 2006; Marin and Rubenstein, 2003; Rubenstein and Rakic, 1999; Sur and Rubenstein, 2005). The neocortex is the brain region responsible for cognitive function, sensory perception and consciousness, and as such it has undergone pronounced expansion and development during evolution (Finlay et al., 1998). During early development, there is dramatic expansion of the neuroepithelium in the dorsolateral wall of the rostral neural tube that will give rise to neocortical projection neurons. The layer immediately adjacent to the ventricle is termed the ventricular zone (VZ) (**Fig. 1**). As neurogenesis proceeds, an additional proliferative layer known as the subventricular zone (SVZ) forms adjacent to the VZ (Gotz and Huttner, 2005; Mo et al., 2007). Progenitors residing in the VZ and SVZ produce the projection neurons of the different neocortical layers in a tightly controlled temporal order from embryonic day (E) 11.5 to E17.5 in the mouse (Angevine and Sidman, 1961; Caviness and Takahashi, 1995; Rakic, 1974). Postmitotic neurons position themselves in the developing neocortex through defined modes of radial and tangential migration (Britanova et al., 2006; Noctor et al., 2001; Rakic, 2003).

The early-born cortical neurons (i.e. cells that become post-mitotic and differentiate into neurons early in corticogenesis) are developmentally important for

organizing the cellular and connectional architecture of the cerebral cortex (Rakic, 1972).

The earliest-born neurons appear around E10.5 and form the preplate, a layer of

differentiated neurons superficial to the proliferative cells of the VZ (Allendoerfer and

Shatz, 1994). Subsequent generations of postmitotic neurons migrate into the preplate

and intercalate between the inner and outer cell populations, to form the cortical plate.

Thus, the cortical plate divides the preplate into superficial and deep components,

thereafter termed the marginal zone and subplate, respectively (Allendoerfer and Shatz,

1994). The cortical plate will give rise to the multilayered neocortex such that later-born

neurons arriving at the cortical plate migrate past earlier-born neurons. The cortex is

formed in an "inside-out" order, from layer VI, which contains the earliest-born cortical

plate neurons, to layer II, which contains the latest-born neurons (Angevine and Sidman,

1961; Caviness, 1982; Rakic, 1974).

There are at least three basic types of neurogenic progenitors within the

developing neocortex: neuroepithelial cells, radial glia, and intermediate progenitors

(Gotz and Huttner, 2005). Initially there is a single sheet of pseudostratified

neuroepithelial cells undergoing symmetric cell divisions to expand the pool of

multipotent progenitors as well as a smaller percentage of asymmetric cell divisions to

generate the earliest-born neurons (Chenn and McConnell, 1995; Gotz and Huttner, 2005;

Smart, 1973). As neurogenesis progresses, some progenitors transform into *radial glia*,

which share some but not all antigenicity with the early neuroepithelial cells (Hartfuss et

al., 2001; Malatesta et al., 2003). Possessing long processes that extend from the VZ to

the pial surfaces, radial glia have long been known to have crucial roles in guiding

neurons to their final locations in the cortical plate by serving as a migratory scaffolding

(Rakic, 1972; 2003). In cortical neurogenesis, radial glia generate pyramidal neurons (Anthony et al., 2004; Malatesta et al., 2003). Intermediate progenitors (or basal progenitors) are the other major type of neural progenitor and are located in the SVZ, and in the basal VZ early in neurogenesis before the formation of the SVZ. The SVZ starts to form at E13.5 in the mouse then expands significantly during late corticogenesis, and is a site of gliogenesis and neurogenesis for upper-layer neurons (Smart and McSherry, 1982; Takahashi et al., 1995). Thus, in addition to the output from the VZ, the expansion of the SVZ might represent an evolutionary mechanism to increase the number of neurons within the neocortex especially during the generation of neurons of upper layers (Kriegstein et al., 2006; Smart et al., 2002).

1.2.2 Basal ganglia and its progenitors

The basal ganglia (BG) constitutes key brain structures that play a prominent role in motor functions, in particular in the planning, initiation and execution of movement (Albin et al., 1995). From a developmental point of view, the term refers to the striatal and pallidal components of the basal telencephalon that evolve from the lateral (LGE) and medial (MGE) ganglionic eminences (**Fig. 1**). In mammals, these structures consist of a collection of interconnected subcortical nuclei, namely the caudate nucleus, putamen, nucleus accumbens, olfactory tubercle, globus pallidus and ventral pallidum (Persaud, 2003; Hiemer et al., 1995). Together, these nuclei form multiple loops linking the basal ganglia to the cortex, thalamus and brainstem. Frequently other forebrain and midbrain structures, such as the subthalamic nucleus, ventral tegmental area, and substantia nigra, are also included, merely because they are closely related to the striatopallidal circuitry (Barker and Barasi, 1999). Most of our current knowledge about

the involvement of the BG in motor function has been gained from studies of human disorders, in particular Parkinson's and Huntington's diseases, and from the study of animal (mammalian) models mimicking these diseases (Marsden and Obeso, 1994; Parent and Hazrati, 1995a; b). Moreover, it is now generally accepted that the BG are also involved in a variety of non-motor functions, including those related to incentive and motivated behaviours (McDonald and White, 1993; Redgrave et al., 1999).

The striatum (also known as the dorsal striatum or neostriatum) consists of the caudate nucleus and putamen, two nuclei that develop from the same telencephalic primordium and together make up part of the basal ganglia (**Fig. 1**) (Voorn et al., 2004). The striatum is the largest and major integrative component of the basal ganglia: it receives inputs virtually from all cortical areas and these corticostriatal projections impose upon the striatum a functional subdivision that is maintained throughout the basal ganglia (Parent and Hazrati, 1995b). Association cortices project principally to the head of the caudate nucleus and pre-commissural putamen (associative territory), sensorimotor cortices target preferentially the post-commissural putamen (sensorimotor territory), and the limbic and paralimbic cortices project chiefly to the ventral portion of the striatum (limbic territory), including nucleus accumbens (Parent and Hazrati, 1995a). Each of these three major striatal territories can be further subdivided into two distinct compartments – the striosomes and the extrastriosomal matrix – that differ from one another by their connections and neurochemical makeup (Kawaguchi et al., 1995). The *extrastriosomal matrix*, which occupies about 85% of the total striatal volume (Johnston et al., 1990), is enriched in calbindin (CB) and other neurochemical compounds, including the enzyme tyrosine hydroxylase (TH), and it receives input mainly from the

sensorimotor and associative cortices (Graybiel, 1990). The *striosomes* form a complex tri-dimensional labyrinth embedded within the extrastriosomal matrix; they are poorly stained for both CB and TH and have their principal afferents from both the limbic and paralimbic cortices (Graybiel and Ragsdale, 1978; Prensa et al., 1999).

The striatum begins to appear during the 6th week of development in humans (O'Rahilly and Muller, 1999). Sex differences in the timing of striatal development have been reported in mice, with peak neurogenesis occurring earlier in females (E13 as compared to E15 in males) and extending over a shorter period of time (Frick et al., 2002), suggesting that striatal neurogenesis may be under the control of sex hormones such as oestrogen; how far this finding applies to humans is currently unknown. More specifically, the origin of the striatum is the ganglionic (currently known as 'ventricular') eminences located in the floor of the telencephalic vesicle (Smart and Sturrock, 2000), which protrude into the lateral ventricle and can be delineated into medial and lateral ganglionic eminences by a rostro-caudal furrow. The MGE, which appears first during development, is responsible for generating the cholinergic interneurons of the striatum and the pallidum and basal forebrain (Marin et al., 2000; Song and Harlan, 1994). The LGE has been shown to be the principal source of striatal neurons generating both GABAergic projection neurons as well as a population of striatal interneurons co-localizing GABA and somatostatin (Campbell et al., 1995; Deacon et al., 1994). The neuroepithelium of both ganglionic eminences also contains two distinct proliferative zones that contribute to the generation of neuronal precursors; the VZ located adjacent to the lateral ventricles, where mitotic activity takes place, and the SVZ where dividing cells are scattered and do not form an epithelial structure (**Fig. 1**)

(Halliday and Cepko, 1992). Neuroepithelial cells undergo asymmetric divisions, producing another neuroepithelial cell that is capable of division and a neuroblast that no longer undergoes division. After exit from the cell cycle in the VZ or the SVZ, neuroblasts migrate laterally into the mantle zone where they undergo terminal differentiation. Thus the ganglionic eminences will eventually consist of an accumulation of neurons in the mantle layer of the neuroepithelium. These neurons ultimately must find their final destination by migration along guiding radial glia, which according to other data may themselves be neuronal progenitors (Magrassi et al., 1998). By the time the lateral and medial eminences can be delineated in the developing rodent brain, newborn cells in the VZ and SVZ of the ganglionic eminences appear to be committed to striatal phenotypes according to their site of origin (i.e., have specified fates), but full maturation requires further contact-mediated and/or intrinsic/extrinsic signals that act locally on these cells to complete the process of terminal differentiation.

1.3 Developmental patterning and specification of the telencephalon

With the general anatomy of the neocortex and the basal ganglia in mind, it is important to review what is known about the spatial and temporal development of these two major compartments.

1.3.1 Induction of anterior neural plate

The telencephalon derives from cells at the margin of the neural plate (Inoue et al., 2000; Rubenstein et al., 1998). While minor differences are apparent in fate maps between species, in general, telencephalic precursors are situated rostral to the diencephalic territory. This places the telencephalic anlagen at the margin of the anterior neural plate, where it is under the influence of signalling pathways that both regulate anterior-posterior (AP) and dorsal-ventral (DV) patterning of neural tissue. Candidate posteriorizing signals include Fgfs, Wnts, retinoic acid, and the Nodal family of TGFβ proteins (Lumsden and Krumlauf, 1996). While evidence exists for the involvement of all such signals, it has remained uncertain how these diverse molecules each contribute to this process. However, recent genetic studies have shown that mutations affecting the function of the embryonic organizer directly or indirectly disturb the AP patterning of the neural plate and can lead to increased or decreased specification of forebrain fates. For example, mutations in the *bozozok* (*boz*) gene (Fekany et al., 1999) or *headless* (*hdl*) gene (Kim et al., 2000), both transcriptional repressors of *Wnt* target genes in zebrafish, exhibit a phenotype in which anterior neural plate fates are variably reduced or lost. Forebrain fates can be rescued in *boz* embryos by expression of a *Wnt* antagonist suggesting that *Boz* normally negatively regulates the *Wnt* pathway and that *Wnt* signalling inhibits forebrain fates (Fekany et al., 1999; Hashimoto et al., 2000). On the other hand, DV

regional patterning of the developing telencephalon gives rise to ventral (subpallial) and dorsal (pallial) territories which consist of GEs and cerebral cortex, respectively. While the morphology of adult pallial and subpallial structures varies enormously between species, highly similar regional subdivisions are observed in the telencephalon of all classes of vertebrates during early development (Fernandez et al., 1998; Puelles et al., 2000), and the same set of signalling pathways regulate early regional patterning of the telencephalon throughout evolution from fish through to mammals.

1.3.2 Hedgehog pathway and ventral telencephalic development

Sonic hedgehog (Shh) is a member of the Hedgehog family of secreted signalling proteins that has been implicated in a wide variety of developmental processes. Mice, fish, and humans that have defects in *Shh* signalling lack ventral telencephalic structures (Chiang et al., 1996; Muenke and Beachy, 2000), but it is currently uncertain how, when, or where *Shh* is required for telencephalic patterning. Although genetic studies have yet to resolve the precise role of *Shh* in telencephalic patterning, gain-of-function and explant studies show that *Shh* can promote ventral telencephalic identity (Corbin et al., 2000; Shimamura and Rubenstein, 1997). Further elucidation of the role of *Shh* in telencephalic patterning will come from analysis of the roles of other components of the Hedgehog signalling pathway. *Patched* is a component of the *Shh* receptor complex and functions as a negative regulator of *Shh* signalling (Kalderon, 2000; Murone et al., 1999). In *Patched*1 mutant mice, expression of the homeobox gene *Nkx*2.1 is expanded, consistent with the notion that localized activity of *Shh* in the ventral telencephalon normally induces this gene (Goodrich et al., 1997). *Shh* may also be involved in the subdivision of the ventral telencephalon into the MGE and LGE. The *Fgf*

signalling pathway also appears to regulate patterning of ventral telencephalic structures (Hebert and Fishell, 2008). Mice with mutation in the *Fgf*8 gene have ventral telencephalic and olfactory bulb deficits (Meyer et al., 1998). Several of the transcription factors downstream of *Shh* and *Fgf* that are key to the subdivision of the ventral telencephalon are homeodomain proteins including *Nkx*2.1 (Kimura et al., 1996; Sussel et al., 1999), *Gsh*2 (Corbin et al., 2000; Yun et al., 2001), and *Pax*6 (Stoykova et al., 2000; Toresson et al., 2000). Within the ventral telencephalon, *Nkx*2.1 mutants have a ventral-to-dorsal transformation such that cells which should normally form MGE are transformed to an LGE identity. A related phenotype is observed in mice with mutations in *Gsh*2. Ectopic expression of pallial markers in the dorsal LGE, indicating dorsalization of this region of the subpallium are found in these mice (Corbin et al., 2000; Yun et al., 2001). Mice lacking functional *Pax*6 aberrantly express subpallial genes within the ventral pallium and MGE-specific genes in the LGE. Together, these results suggest that homeobox gene activity mediates the DV patterning of the telencephalon and establishes boundaries between regional subdivisions.

1.3.3 Pathways involved in dorsal telencephalic development

Loss of function of several different homeodomain transcription factors results in a dorsal-to-ventral transformation within the telencephalon. *Gli*3 is a transcription factor in the *Shh* signalling pathway. Mouse *Gli*3 mutants lose many dorsal telencephalic markers and structures (such as hippocampus) and in one genetic background, markers of the LGE spread into the cerebral cortex (Theil et al., 1999; Tole et al., 2000). Among the genes that exhibit reduced dorsal expression in *Gli*3 mutant mice is the homeobox gene *Emx*2. In the dorsal telencephalon, *Emx*2 is expressed at higher

levels in caudal than in rostral areas, and it has been proposed that graded *Emx*2 activity

may confer regional identity within the cortex (Bishop et al., 2000; Mallamaci et al.,

2000). In support of this, loss of *Emx*2 function leads to expansion of rostral/lateral

cortical domains, while caudal/medial domains are reduced or lost (Pellegrini et al., 1996;

Yoshida et al., 1997). Conversely, within the cortex, *Pax*6 expression is higher rostrally

and ventrally than dorsally and caudally. In addition to the disruptions to ventral pallial

development described above (Stoykova et al., 1997; Toresson et al., 2000), loss of *Pax*6

function also leads to expansion of caudal cortical domains at the expense of rostral

domains (Bishop et al., 2000). Opposing activities of the homeodomain proteins PAX6

and EMX2 may therefore generate graded positional identity within the dorsal

telencephalon and contribute more generally to the regulation of overall regional

subdivision of the telencephalon (Muzio et al., 2002).

Although homeobox gene activity is crucial to telencephalic patterning,

other families of transcription factors also profoundly influence the development of this

brain region. For instance, a striking dorsal-to-ventral fate change occurs in the

telencephalon of mice with altered expression of members of the basic helix loop helix

(bHLH) family of transcription factors, which have been known to regulate neurogenesis

and influence neuronal identity (Fode et al., 2000; Jan and Jan, 1994). In *Ngn*2 or *Ngn*1/2

mutants, there is ectopic induction of ventral molecular markers such as *Mash*1 in the

cerebral cortex, indicating that *Ngn* activity promotes dorsal telencephalic development

by suppressing ventrally expressed genes. Although the loss of subsets of ventral

neurons in *Mash*1 null mutant mice confirms a role for this protein in ventral

telencephalic development (Casarosa et al., 1999), it remains to be resolved how *Mash*1 function relates to other genes involved in DV patterning.

In addition to their early functions during neural plate formation, the bone morphogenetic protein (BMP) and Wnt signalling pathways have later roles in patterning the pallium. Several BMPs are expressed in and around the dorsal telencephalon, and studies using explant cultures and gene mis-expression *in vivo* suggest involvement in the regulation of patterning, cell survival, and proliferation (Furuta et al., 1997). Ectopic *Bmp* activity represses ventral telencephalic markers (such as *Nkx*2.1 and *Dlx*2), while maintaining or increasing expression of dorsal markers, and leads to decreased proliferation, increased apoptosis in the basal telencephalon, and holoprosencephaly (Golden et al., 1999; Ohkubo et al., 2002). Loss-of-function studies have yet to demonstrate a clear role for BMPs in DV patterning of the telencephalon but dorsal development is clearly disrupted in mice lacking both *Bmp*5 and *Bmp*7 (Solloway and Robertson, 1999). Like BMPs, several members of the Wnt family of secreted signalling proteins are likely to be involved in dorsal telencephalic development. Loss-of-function mutations in *Wnt*3a and in a downstream effector of Wnt activity, *Lef*1, lead to the loss or reduction of the hippocampus (Galceran et al., 2000; Lee et al., 2000). Mice lacking *Lhx*5 also lack a hippocampus, but it has yet to be determined whether this *Lim* homeobox gene is regulated by *Bmp* and/or *Wnt* signals (Zhao et al., 1999).

Lastly, while distinct patterning mechanisms regulate the specification of neocortex and basal ganglia, recent studies have revealed an unexpected interdependence between these domains. This has been demonstrated through analysis of the various tangential migrations of neurons between the different telencephalic structures, which

will be discussed later. Hence, in general, DV regionalization of the telencephalon results in the specification of domains that produce neurons that synthesize different subsets of neurotransmitters.

1.4   Cellular and genetic makeup of the telencephalon

From general anatomy and development of the forebrain, the next step is to discuss the overall cellular diversification of individual compartments based on morphology, location, physiology and the neurochemicals expressed.

1.4.1 Neocortex

The cortical preplate is thought to function primarily as a framework for further development of the cortex, organizing its laminar structure and some of its connections. In mice, preplate cells differentiate into at least two distinct types of neurons: Cajal-Retzius cells and subplate cells (Meyer et al., 1998). Cajal-Retzius cells express Reelin and calretinin and have been implicated in controlling cell migrations and radial glia morphology (Rice et al., 2001; Super and Uylings, 2001). In layer I to layer VI, there are two broad classes of cortical neurons: interneurons, which make local connections; and projection neurons, which extend axons to distant intracortical, subcortical and subcerebral targets (Marin and Rubenstein, 2003). Projection neurons are glutamatergic neurons characterized by a typical pyramidal morphology that transmit information between different regions of the neocortex and to other regions of the brain. During development, they are generated from progenitors of the neocortical germinal zone located in the dorsolateral wall of the telencephalon (Gorski et al., 2002; Rakic, 1972; Tan et al., 1998). By contrast, GABA-containing interneurons and Cajal–Retzius cells are generated primarily from progenitors in the ventral telencephalon and cortical hem, respectively, and migrate long distances to their final locations within the neocortex (Marin and Rubenstein, 2001; 2003; Super and Uylings, 2001; Yamamori and Rockland, 2006). In this manner, multiple progenitor zones contribute to the rich variety of neuronal

types and distinct populations of neurons that are located in different cortical layers, have unique morphological features, express different complements of transcription factors, and ultimately serve different functions (Wonders and Anderson, 2006).

1.4.2 Striatum

Cytologically, the striatum contains a multitude of medium-sized projection neurons with spiny dendrites and a smooth nuclear envelope, and a smaller number of medium- to large-sized interneurons with smooth dendrites and a markedly indented nuclear envelope (Parent and Hazrati, 1995b). Except for the large cholinergic interneurons, virtually all striatal neurons, whether projection or local circuit neurons, use GABA as their major neurotransmitter; they can nevertheless be grouped into various classes according to the combination of neuropeptides, transmitter-related enzymes and calcium-binding proteins they express (Cicchetti et al., 2000). Hence, virtually all medium-sized spiny projection neurons of the striatum express calbindin, but about half of them also contain enkephalin and neurotensin, whereas the other half expresses substance P and dynorphin (Cicchetti et al., 2000). In contrast, the striatum also contains a variety of aspiny striatal interneurons which are implicated in regulating striatal projection function in both striosome and matrix compartment (Kawaguchi et al., 1995; Kawaguchi and Kubota, 1997). There are four main classes of striatal interneurons based on their content of various other neurochemicals: (1) large cholinergic neurons with 80% of them co-expressing calretinin; (2) medium GABA-containing (GABAergic) neurons expressing neuronal nitric oxide synthase, neuropeptide Y, and somatostatin; (3) medium GABAergic neurons containing parvalbumin; and (4) medium GABAergic neurons co-expressing calretinin (Cicchetti et al., 2000; Kawaguchi et al., 1995).

1.5 The origin and specification of cortical interneurons

Now it's time to dwell into further details and specificity of the cellular makeup of the forebrain. We will focus on the main neuronal subtype of GABA-containing interneurons.

1.5.1 Overview of cortical interneurons

Since the time of Cajal, who described the "cells with short axons" first detected in cortical sections stained by the Golgi method (DeFelipe and Jones, 1998), scientists investigating the cerebral cortex have studied these neurons that are generally characterized by a locally projecting axon, aspiny or sparsely spiny dendrites, a cell soma that is smaller than most cortical pyramidal neurons of the same species, and that contain the neurotransmitter GABA. As new techniques have been developed, these cells, known as interneurons, have been characterized by their morphology, connectivity, neurochemistry and the expression of ion physiology/channels (Markram et al., 2004). Cortical interneurons accomplish specific functions in both the development and function of the cerebral cortex through a remarkable diversity of subtypes, which are variably defined by their morphological, physiological and molecular characteristics (Monyer and Markram, 2004). This diversity, along with the attainment of subtype-defining characteristics only after weeks of postnatal maturation, had previously hindered efforts to connect the embryonic development of cortical interneurons to their differentiated fate. Consequently, little was known about the generation of cortical interneuron diversity until improved fate-mapping approaches and transgenic mice became available.

1.5.2 GABAergic cortical interneurons

GABAergic interneurons represent ~20% of the neurons in the mouse

cerebral cortex (Tamamaki et al., 2003b). GABA acts predominantly via postsynaptic,

chloride-permeable, ionotropic GABA receptors to shunt or hyperpolarize target cells. As

such, GABAergic interneurons are crucial to regulating the firing activity and entraining

the principal neuronal component in the cerebral cortex: the glutamatergic projection

neurons or pyramidal cells (Cobb et al., 1995; Tamas et al., 2000). Subgroups of

GABAergic interneurons are mainly distinguished by the expression of markers that are

not necessarily relevant to their physiological function. Although this classification

scheme has proved useful for initial efforts to connect interneuron origins and the

effectors of their fate with a mature phenotype, the ultimate goal is to develop a

transcriptional code for the generation of interneuron subgroups defined not by arbitrary

marker proteins, but by their effects on postsynaptic neurons and neural networks.

       Studies from many laboratories have shown that cortical interneurons in

rodents are born in subcortical regions and migrate tangentially over long distances to

populate both of the cortex and the hippocampus (Marin and Rubenstein, 2003; Metin et

al., 2006). Fluorescent dye labelling of cultured telencephali found a robust migration of

cells from the subpallium into the overlying cortex (de Carlos et al., 1996), a result that is

consistent with immunolabelling for the transcription factor distal-less homeobox 2

(DLX2) (Porteus et al., 1994)). Subsequent analyses of *Dlx*1/*Dlx*2 mouse mutants,

together with *in vivo* ablation experiments and co-labelling of migrating cells in slice

cultures, suggested that this migration includes cortical interneurons (Anderson et al.,

1997a; Marin and Rubenstein, 2001). In rodents and ferrets the subpallium appears to be

the primary source of cortical interneurons, whereas one study reported that in human

embryos most cortical interneurons undergo their terminal mitosis in the cortical

subventricular zone (Letinic et al., 2002). As such, it is tempting to speculate that vertically oriented bipolar or bitufted calretinin-expressing interneurons, which are far more numerous in primate cortex than in rodents, have evolved a cortical origin in primates (Tamamaki et al., 2003b).

Subcortical origins of cortical interneurons have been traced to neuroepithelial cells of mainly the medial, lateral, and caudal (CGE) ganglionic eminences; and to a much lesser extent of the septum and of rostral migratory stream (RMS). Genetic studies using tools that allow indelible labelling of the subcortical telencephalon and its cellular progeny demonstrate that molecularly distinct regions of the neuroepithelium generate distinct cortical interneuron subgroups (Fogarty et al., 2007). The MGE appears to give rise primarily to parvalbumin (PV) - or somatostatin (SST) - expressing subgroups, whereas the CGE gives rise primarily to vertically oriented, bipolar or bitufted cells that express calretinin (CR). Indeed, *in vivo* transplantation studies of fluorescently tagged MGE or CGE precursors followed by electrophysiological analysis of interneurons at postnatal stages demonstrated that interneuron subclasses with different electrophysiological, morphological, and immunohistochemical profiles are generated from different subcortical regions (Butt et al., 2005). These results indicate that the origins of interneurons are predictive of multiple aspects of their mature phenotype. It also suggests that underlying differences in gene expression between different regions are responsible for the different potential of progenitors from these regions to achieve a given interneuron phenotype. Beyond the evidence for distinct spatial origins of interneuron subgroups or subtypes, there are also distinct temporal origins between interneuron subgroups. Within a given layer, the

mainly CGE-derived bipolar vasoactive intestinal peptide- and CR-expressing

interneurons are generally born later than those that express SST (Cavanagh and

Parnavelas, 1988; 1989). Using careful titration of inducible fate mapping with an *Olig*2-

Cre (oligodendrocyte transcription factor 2–enterobacteria phage P1, cyclization

recombinase) transgenic mouse, this analysis has recently been extended to the

physiological level of sub-classification, in which distinct temporal origins of

physiologically defined subclasses of interneurons have been identified (Miyoshi et al.,

2007).

1.5.2.1 Medial ganglionic eminence

The homeobox transcription factors *Dlx*1 and *Dlx*2 are closely

linked genes that are expressed throughout the subcortical telencephalon and are required

for most subcortical to cortical interneuron migrations (Anderson et al., 1997a; Anderson

et al., 2001). Cortical cultures show that the number of GABA-expressing cells in the

neocortical slices is reduced by separating the neocortex from the subcortical

telencephalon. Mice lacking both *Dlx*1 and *Dlx*2 function show no detectable cell

migration from the subcortical telencephalon to the neocortex and also have far fewer

GABA-expressing cells in the neocortex. *Dlx*2, when overexpressed in embryonic mouse

forebrain slices, can induce expression of both *Gad*1 and *Gad*2, which encode the two

glutamic acid decarboxylase enzymes that are important for the synthesis of GABA

(Stuhmer et al., 2002a). The postnatal expression of *Dlx*1 is crucial as *Dlx*1 mutants show

a selective loss of CR-, Neuropeptide Y (NPY) - and SST-containing interneurons,

beginning around the fourth postnatal week. The transplantation of GFP-expressing MGE

progenitors from *Dlx*1 mutants into wild-type neonatal cortex further showed that this

cell loss is due to a cell-autonomous requirement for *Dlx*1 and is preceded by decreased dendritic length and branching (Cobos et al., 2005b; Cobos et al., 2007).

Expression of the transcription factor *Nkx*2.1 in the MGE is required for the specification of the MGE-derived interneuron subgroups that express PV or SST (Sussel et al., 1999; Xu et al., 2004). In *Nkx*2.1-null mice, normal MGE tissue fails to form and there is a ventral expansion of LGE-like tissue, with the absence of cortical SST- and NPY-expressing interneurons (Anderson et al., 2001). Upstream of *Nkx*2.1, the morphogen *Shh* appears to play a critical role in both the establishment of *Nkx*2.1 expression in the MGE (Chiang et al., 1996; Fuccillo et al., 2004) and in the maintenance of this expression during neurogenesis (Xu et al., 2005). Reductions in *Shh* signalling in MGE progenitors result in both a large reduction in *Nkx*2.1 expression and a reduction in the ability of these progenitors to generate PV- or SST-containing interneurons (Xu et al., 2005). Downstream of *Nkx*2.1, the lim-homeodomain transcription factor *Lhx*6 is expressed as cells from the MGE exit the cell cycle and migrate tangentially toward the striatum and cortex (Du et al., 2008; Gong et al., 2003; Grigoriou et al., 1998; Lavdas et al., 1999). More specifically, *Lhx*6 is detected in virtually all of the MGE-derived, *Nkx*2.1-dependent subpopulations (mainly PV- or SST-expressing), whereas the bipolar CR-expressing subpopulation from the CGE are generally *Lhx*6 negative (Fogarty et al., 2007). Analysis of animals homozygous for an *Lhx*6 mutation showed that they maintain a normal number of GABA-positive interneurons in the neocortex and the hippocampus but lack the PV- and SOM-subpopulations (Liodis et al., 2007). It thus appears that *Lhx*6 activity is not required for the migration and specification of the GABAergic neurotransmitter identity of cortical

interneurons but it is necessary for their specification into the *Nkx*2.1 lineage, MGE-derived subgroups. Downregulation of *Nkx*2.1 expression in postmitotic cells is also necessary for the migration of interneurons to the cortex, whereas maintenance of *Nkx*2.1 expression is required for interneuron migration to the striatum. *Nkx*2.1 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 (Nobrega-Pereira et al., 2008).

1.5.2.2 Caudal ganglionic eminence

In addition to the MGE, the caudal ganglionic eminence (CGE) is the other subcortical structure that is most strongly implicated in the generation of cortical interneurons (Anderson et al., 2001; Nery et al., 2002). Morphologically, the CGE exists as a fusion of the more rostral MGE and LGE, beginning at the coronal level of the mid- to caudal thalamus. There are two distinct molecular domains within the CGE that closely resemble caudal extensions of the MGE and LGE. The ventral-most CGE, like the MGE, expresses *Nkx*2.1 (Nery et al., 2003). By contrast, the more dorsal domain that protrudes into the lateral ventricle expresses high levels of the transcription factor *Gsh*2, which is required for the proper patterning of the LGE (Corbin et al., 2003), and *ER*81, a transcription factor that is expressed in olfactory bulb progenitors both in the LGE and in the adult SVZ (Stenman et al., 2003a). Fate-mapping experiments with *in utero* isochronic, homotopic transplants of dissections that included both dorsal and ventral CGE at E13.5 showed that the CGE gives rise to deep-layer cortical interneurons, many of which express PV or SST (Nery et al., 2002). Of note, selective dissection of the dorsal CGE at E14.5 gives rise to many CR-positive, bipolar cells after plating on a

cortical feeder layer as nearly all CR-positive interneurons undergo their final S-phase of cell cycle after E14.5 (Xu et al., 2004). Finally, a recent analysis of a *Gad*65-GFP (green fluorescent protein) transgenic mouse line, together with explant cultures, also suggested that many cells migrating from the CGE become vertically orientated CR-containing interneurons (Lopez-Bendito et al., 2004). Taken together, these data suggest that CR-positive interneurons are primarily generated in the NKX2.1-negative region of the dorsal CGE. In contrast, the ventral CGE might generate PV- or SST-containing interneurons, although the caudal migration of MGE-born progenitors through the CGE en route to the cortex complicates this scenario (Butt et al., 2005; Yozu et al., 2005).

1.5.2.3 Lateral ganglionic eminence

Although several studies have indicated that any LGE contribution to cortical interneurons is far smaller than that of the MGE (Anderson et al., 2001; Wichterle et al., 2001), evidence in support of some LGE contribution deserves mention. First, *Nkx*2.1 mutants lack normal MGE tissue, yet they show only a ~50% reduction of cortical GABA-containing cells at E18.5 (Sussel et al., 1999). Although this could be the result of an enhanced generation of CR-positive cells from dorsal CGE-like neuroepithelium, in these mutants the LGE-like region also shows robust migration to the cortex at E15.5 (Anderson et al., 2001; Nery et al., 2003). Second, slice culture experiments in which progenitors were labelled with the S-phase marker bromodeoxyuridine (BrdU) suggested that a small number of LGE-derived cells, some of which co-label for GABA, do migrate from the LGE to the cortex (Anderson et al., 2001). Finally, explants taken from rat embryos in which the MGE has been removed continue to show robust migration from the LGE to the cortex, implying that the

observed migration is not simply due to MGE cells which are migrating through the LGE

(Jimenez et al., 2002). One possible explanation for these mixed results is the relatively

pleiotropic nature of the morphologically defined LGE, which consists of distinct

progenitor domains along the dorsoventral axis that give rise to the lateral cortex,

olfactory bulb interneurons and medium spiny striatal projection neurons (Stenman et al.,

2003a). In addition, migration from the LGE to the cortex includes oligodendrocytes after

E14.5 (Kessaris et al., 2006). To summarise, the current data support at best a minor

contribution from the LGE to the cortical interneuron population. The identities of these

cells are not known, but do not seem to include many that contain SST, PV or CR (Xu et

al., 2004).

1.5.2.4 Rostral migratory stream

In contrast to cells from the LGE, cells taken from the rostral

migratory stream (RMS) at postnatal day (P) 0 express CR when cultured on cortical

feeder cells (Xu et al., 2004). However, given that nearly all CR-positive cortical

interneurons in the somatosensory cortex at P25 are born before E16.5 (Xu et al., 2004),

it is difficult to assess the relevance of this finding. It might simply be a case of CR-

positive interneurons of the olfactory bulb showing the capacity to survive and

differentiate in a cortical environment. However, it is also possible that cells could exit

the RMS prior to reaching the olfactory bulb and instead migrate to the cortex. In support

of this model, immunohistochemical labelling for DLX1, which labels migrating

interneuron precursors in the RMS, appears to label cells migrating from the RMS to the

cortex (Anderson et al., 1999). Earlier migration from the rostral neuroepithelium of the

lateral ventricle into layer I of the cortex has also been described for cells containing CR,

calbindin and GABA (Ang et al., 2003; Zecevic and Rakic, 2001). Furthermore, immunohistochemical labelling for polysialic acid–neural cell adhesion molecule (PSA–NCAM) in adult rabbits reveals cells migrating from the RMS into the frontal cortical parenchyma (Luzzati et al., 2003). Taken together, these results suggest the involvement of the RMS in the generation of cortical interneurons, with a particular emphasis on the subgroup that contains CR.

1.5.2.5 Septal region

Another subpallial region that might contribute interneurons to the cerebral cortex is the septal area. Initial speculation about the occurrence of migration from the septal region to the cortex was based on immunohistochemical labelling for DLX1 (Anderson et al., 1999). More convincing evidence comes from the recent analysis of mouse mutants lacking the homeodomain-containing transcription factor *Vax*1, which is expressed in a pattern similar to that of *Dlx*1 and *Dlx*2 within the subcortical telencephalon (Taglialatela et al., 2004). At birth, *Vax*1 mutants have a 30–44% reduction in GABA-containing cortical neurons compared with wild-type mice, with the greatest loss occurring in the rostral-most cortex. Whereas the MGE is reduced in size, the septal region is almost completely absent in these mutants. Experiments conducted using slice cultures show cells migrating from the ventrolateral septum into layer I of the rostral cortex in the wild-type mice; this migration is lost in the *Vax*1 mutants. These data therefore provide evidence for a septal contribution to the cortical interneuron population, although further work is needed to definitively show this migration (Ang et al., 2003; Hevner et al., 2004). Whether migration from the septum gives rise to distinct subtypes of interneurons remains to be explored.

1.5.2.6 Cortex

Although several reports have shown that cultures of dorsal telencephalic progenitors have the capacity to generate GABA-containing cells (Gotz et al., 1995; Gulacsi and Lillien, 2003), little evidence supports a cortical origin of cortical interneurons in rodents (Anderson et al., 2002; Xu et al., 2004). However, retroviral labelling of slice cultures of human embryonic forebrain suggested that most GABA-positive cells in the human cortex originate from mitoses in the cortical SVZ (Letinic et al., 2002). This intriguing finding awaits replication in humans and non-human primates, but is supported by the observation that *Nkx*2.1 is strongly expressed in the cortical proliferative zone in humans but not in rodents (Rakic and Zecevic, 2003a). Finally, it is noteworthy that an additional population of cortical interneurons could be born within the postnatal cerebral cortex. A recent study (Dayer et al., 2005) found that a small percentage of postnatally born cells (measured using BrdU incorporation) in the adult rat cortex showed immunoreactivity for the neuron-specific marker NeuN, and that a subset of those cells contained GABA and either CR or calbindin (CB). Although the small number of cells makes the contribution of postnatal interneuronogenesis to the overall interneuron population difficult to assess, this finding might represent an additional source of cortical interneurons.

1.6 Two modes of neuronal migration

The neurons of the adult CNS are generated in specialized germinative centers whose locations are distinct from those where the neurons will reside in the mature organism. This physical separation means that during normal CNS development, neurons (or their immediate precursors) must move from their site of origin to their site of function. As the developing nervous system is topologically a tube, the problem can be simplified: cells must move centrifugally from germinative regions located at the inner surface of the tube wall (the VZ) to their adult location nearer the outer surface (the pia). While the simplest and most direct route is a radial trajectory direct from the VZ to the pia, there are contributions of other more topologically complex pathways. Specifically, I wish to focus on the process of tangential migration that contributes a significant source of neurons for many CNS structures. Consideration of this topic typically focuses on the basal ganglia and the neocortex, but non-radial migrations are found in many sites.

Initial categorizations of radial migration and tangential migration were based on the relative directions taken by migrating neuronal precursor cells. Radial migration is defined by neuronal migration in a direction perpendicular to the surface of the brain, whereas tangential migration describes neurons migrating in a direction parallel to the surface of the brain. During radial migration, the precursors of pyramidal neurons, the major projection neurons of the cerebral cortex, are thought to move from the VZ to the pia along the fibers of radial glial cells (Hatten, 1993; Rakic, 1990). The outwardly migrating neurons form the cortical plate, which separates the preplate. This primitive lamination of the neocortex proceeds in an "inside-out" pattern in that new cells take

more superficial positions whereas old cells are positioned in deeper layers of the cortical plates (Rakic, 1988).

Although tangential migration was first observed in the 1960s (Rakic and Sidman, 1969), its importance was better appreciated in the late 1980s and 1990s (Corbin et al., 2001; Marin and Rubenstein, 2001). Tangential migration does not require glial fibers and is a major mode of migration for cells originating in the basal telencephalon (or the subpallium), particularly from the GEs. Significant proportions of interneurons, such as the GABAergic neurons in the telencephalon, are derived from these tangentially migrating neuronal precursor cells. Neuronal precursor cells from different GEs migrate into different regions of the brain (Anderson et al., 2001; Corbin et al., 2001; Marin and Rubenstein, 2001). Interestingly, tangential migration persists even after birth especially in the SVZ of the forebrain, giving rise to more olfactory interneurons during postnatal life (Garcia-Verdugo et al., 1998; Luskin, 1993).

1.6.1   Radial migration

In the developing mammalian telencephalon, the proliferative zone is mainly found in two distinct areas. The first forms the pseudo-stratified neuroepithelium located in the VZ. The second is found predominantly in the SVZ, which extends from the basal portion of the VZ to a deeper region within the basal ganglia (Takahashi et al., 1995). After their final postmitotic division within these two periventricular germinal zones, neuronal precursors migrate outward away from their place of origin and differentiate in a given structure of the adult brain. Hence, radial migration is the prevalent mode of neuronal cell movement in the developing mammalian brain. It is particularly prominent during formation of the laminar structures such as the neocortex

(Rakic, 1972) and hippocampus (Eckenhoff and Rakic, 1984). It also occurs, to a lesser degree, in the developing diencephalon, brain stem, and spinal cord (Rakic, 1977). The alignment of postmitotic neurons with a system of radial glial fibers during periods of cortical formation led to the general hypothesis that radial glia provide a scaffold for directed migrations in brain (Rakic, 1971; 1978). Support for this model has been widespread, with *in vitro* (Edmondson and Hatten, 1987; Hatten, 1993) and *in vivo* studies (Anton et al., 1996; Gao and Hatten, 1993) demonstrating that 80–90% of the billions of neuronal precursors in mammalian cortex migrate along glial fibers.

During development, glial cells assume forms and functions that subserve those of the developing neurons. These radial glial cells provide the primary pathway for directed migrations (Rakic, 1972). After the epoch of cell migration, glial cells transform into stellate astrocytes (Schmechel and Rakic, 1979a; b). Once migration across the scaffold is completed, the radial glial cells disappear and cells are locked in position by the formation of specific axon-target interactions (Baird et al., 1992).

A number of neuronal and glial receptor systems have been implicated in the directed migration of CNS neurons along radial glial fibers. Antibody perturbation studies on granule neuron migration *in vitro* demonstrate that the neural glycoprotein astrotactin (*Astn*) provides a neural receptor system for migration along glial processes (Edmondson et al., 1988; Fishell and Hatten, 1991). Analysis of mice with a targeted null mutation of *Astn* indicates that the size of the neuronal layers in cortex and cerebellum are smaller, which suggests that *Astn* null mice may mimic the disorder of microencephaly (Hatten, 1993). Several components of the extracellular matrix (ECM) have been proposed to influence granule cell migration. Antibody perturbation

experiments of thrombospondin in explant cultures of cerebellar cortex demonstrated

reduced granule cell migration (O'Shea et al., 1990). Tenascin appears to have the

opposite role, namely of stimulating neurite production and thereby stimulating migration

(Husmann et al., 1992). It is important to note that both thrombospondin and tenascin

influence migration indirectly by altering the rate of parallel fiber production, not directly

by affecting the migration of cell soma. Other studies demonstrate a role for neuregulin, a

growth factor expressed in granule cells as they migrate on Bergmann glial fibers in the

cerebellum (Anton et al., 1997; Rio et al., 1997).

In generation of embryonic layers of cerebral cortex, as proliferation in

the neuroepithelium thickens the cortical wall, a system of radial glial fibers appears

across the radial plane. Postmitotic neurons migrate away from the inner surface of the

neural tube along the trajectory set forth by the radial glial fiber system (Rakic, 1972). As

the first neuronal populations migrate away from the VZ, a zone of axons appears

between the germinative zone and the mantle of postmitotic cells. This intermediate zone

(IZ) consists of pioneer axons laid down by the emigration of the first wave of neurons to

become postmitotic away from the VZ. As one discusses migration, the issue of what

makes the neuron stop migration always emerges. First, cell-cell (neuron-glial) adhesion

systems were assumed to stop the neuron along the glial pathway. Experiments on

cerebellar neurons provide evidence that the stop signal for neuronal migration is not a

simple de-adhesion but rather a cue provided by target axons projecting toward the

neuron (Baird et al., 1992). The second emerging class of stop signals is the ECM

component, Reelin. A zone of Reelin appears to stop the earliest-generated neurons in the

cortex, and the earliest-generated neurons in the cerebellum (Miyata et al., 1997). The

similarity in the mouse phenotype of *reeler* and *scrambler* mice has led to the hypothesis that their gene products are part of a signalling pathway that regulates neuronal lamination (Sheldon et al., 1997; Ware et al., 1997). As well, early steps in neuronal development, in particular programs of gene expression during neurogenesis, can set forth and/or alter the program of cell migration. An example of a class of gene that disrupts the cell cycle, thereby influencing cell patterning, is *Cdk*5. Targeted disruption of this cyclin-dependent kinase gene leads to abnormal cell positioning within the neocortex; humans with the mutations in this gene have abnormal corticogenesis and perinatal death (Ohshima et al., 1996). The more complete analysis of mice lacking p35 shows aberrant cell migration (Chae et al., 1997). The *reeler*-like preplate does not form. Instead, the cells move tangentially in the IZ and never move onto the glial fiber system. Hence, these findings lend further support to the general idea that radial migration is essential for the proper lamination of the cortex.

1.6.2   Tangential migration

In contrast to the cortical projection neurons, which are generated in the dorsal telencephalon (pallium) and migrate radially toward the marginal zone (MZ), GABAergic neurons have been found to migrate tangentially in the lower IZ beyond area boundaries in the embryonic rat cortex (DeDiego et al., 1994). Cells in the GE of the ventral telencephalon cross the corticostriatal boundary and migrate to the developing cortex (de Carlos et al., 1996). The cells that migrate tangentially from the GE to the cortex in mice include GABAergic interneurons (Anderson et al., 1997a; Tamamaki et al., 1997), and their migration was found to depend on the transcription factors *Dlx*1 and *Dlx*2 (Anderson et al., 1997b). As discussed above, although the cortical interneurons of

rodents are primarily produced in the ventral telencephalon (Lavdas et al., 1999; Wichterle et al., 2001), two lineages of interneurons have been reported in the human neocortex (Letinic et al., 2002). One possible explanation for the difference between rodents and humans is that some of the *Mash*1-positive progenitors in the human neocortical VZ/SVZ may have arrived from the GE at earlier embryonic stages and then continued to divide locally. However, retroviral labelling of the proliferative VZ/SVZ cells in slice cultures of human fetal forebrain has shown that these cells divide several times before starting radial migration, supporting their cortical origin (Letinic et al., 2002). Interestingly, expression of *Nkx*2.1, which is required for the specification of the MGE-derived interneurons (Sussel et al., 1999), in the proliferative zone has been found to spread dorsally from the GE to the cortical areas in humans, whereas *Nkx*2.1 is not expressed in the rodent cortex (Rakic and Zecevic, 2003b).

A number of studies have revealed the existence of multiple pathways of tangential migration in the developing mouse cortex (Metin et al., 2006). In the early stages (E12 in mice), the tangentially migrating interneurons arise primarily from the MGE and anterior entopeduncular area (AEP), and follow a superficial route to enter the preplate, where at least some of them differentiate into Cajal-Retzius neurons. In the intermediate stages (E13.5) of corticogenesis, at the peak of tangential migration, the interneurons arise primarily from the MGE and follow a deep route to the developing striatum as well as superficial route to travel through the IZ as well as through the MZ. In the late stages (E15.5), the cells arise from the LGE and follow a deep route to migrate tangentially, mainly in the lower IZ/SVZ, subplate (SP), and MZ (Marin and Rubenstein, 2001). After the GABAergic interneurons reach the cortex through the IZ/SVZ stream,

about 70% dive down to the surface of the ventricle ('ventricle-directed migration''), make contact, and, after a pause in this proliferative zone, they migrate radially to the cortical plate (CP) (Nadarajah et al., 2002). It has been speculated that these neurons may seek the cortical VZ to receive some layer information. Within the cortex, GABAergic neurons exhibit multidirectional tangential migration, at least in the MZ and VZ, and probably also in the CP and SP (Tanaka et al., 2006). These findings indicate that after reaching the cortex by tangential migration from the GE, cortical interneurons undergo a second phase of tangential migration in all directions. Migrating interneurons that have initially travelled through the MZ or the SP/IZ/SVZ then enter the CP radially from either location to reside in their proper layers (Hevner et al., 2004; Polleux et al., 2002).

It seems likely that a variety of guidance systems are needed to direct these diverse migrations. As described above, once a cell has been specified to take a tangential migratory course, there are at least three different types of factors that can regulate this process: first, factors that stimulate the movement of interneurons; second, structural elements that constitute the substrate for their migration; and third, cues that direct interneurons toward their target through the appropriate pathways.

Motogenic factors:. Nucleokinesis in MGE-derived cells comprises two phases. In the first phase, the centrioles and Golgi apparatus migrate forward, and in the second phase, the nucleus translocates toward these organelles. During this phase of nuclear translocation, the leading growth cone either stops migrating or divides, revealing a close correlation between leading edge movements and nuclear movements (Bellion et al., 2005). In mice heterozygous for *Lis*1, which is an important gene responsible for type I lissencephaly, the rate of nuclear movement in migrating interneurons is significantly

reduced, and the leading process becomes longer, and the number of branches decreases (Nasrallah et al., 2006). Tangential migration of cortical interneurons might also be regulated by doublecortin (DCX), which is another major molecule responsible for type I lissencephaly, and doublecortin-like kinase (DCLK), although how these molecules are involved at the cellular level remains to be clarified (Friocourt et al., 2007). Other factors have also been shown to possess motogenic activity via stimulating the undirected movement of neurons from their original position. Neurotrophic factors, brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT4), were shown to stimulate the tangential migration of MGE-derived cells to the cortex by TrkB signalling via PI3-kinase activation (Brunstrom et al., 1997; Polleux et al., 2002). Consistent with the idea that TrkB receptor activation might stimulate neuronal migration in the developing telencephalon, tyrosine kinase inhibitors block BDNF-induced migration in cortical slice culture (Behar et al., 1997). Hepatocyte growth factor/scatter factor (HGF/SF) also acts as a motogen for tangentially migrating interneurons via its receptor, MET (Powell et al., 2001). Mice lacking the urokinase-type plasminogen-activator receptor (uPAR), one of the factors that can cleave the inactive pro-form of HGF/SF to a biologically active protein, have fewer cortical calbindin-positive interneurons at birth (Powell et al., 2001). It remains to be determined whether the distinct migratory behaviour of LGE- and MGE-derived precursors depends on the differential expression of receptors for these motogenic factors.

Extracellular substrates: The results of research on the substrates that support the tangential migration of interneurons have suggested that corticofugal axons in the IZ and MZ act as a scaffold (Metin and Godement, 1996; Parnavelas, 2000). The

neural cell adhesion molecule TAG-1 expressed on corticofugal fibers, including those derived from the pioneer neurons in the MZ (Morante-Oria et al., 2003), has been implicated in this migration *in vitro* (Denaxa et al., 2001; Morante-Oria et al., 2003), although there has been controversy regarding this notion (Tanaka et al., 2003). The absence of TAG-1 *in vivo* does not affect interneuron migration from the GE to the cortex, indicating that TAG-1 is not required for this migration *in vivo* (Denaxa et al., 2005). Interestingly, early-born GABAergic cells colocalized with TAG-1-positive axons *in vivo*, whereas later-born cells colocalized with TAG-1-negative fibers, suggesting interneurons may have a stage-dependent or temporal substrate preference (McManus et al., 2004). In addition to the tangentially oriented fibers, radial glial scaffolds may also be involved either directly or indirectly in the interneuron migration from the cortical IZ to the CP (Bystron et al., 2005; Poluch and Juliano, 2007).

   Guidance factors: On the basis of their expression in the subpallial telencephalon, several families of ligands/receptors are candidates for guiding the trajectories of tangentially migrating interneurons. Neuropilin (*Nrp*) 1 and *Nrp*2 are transmembrane receptors for Semaphorin ligands that are known to be important guidance molecules for interneurons by mediating the sorting of striatal and cortical interneurons during their migration (Marin et al., 2001). In the subpallial telencephalon, neuropilins are expressed by interneurons that migrate to the cortex, but not by interneurons that invade the developing striatum. Migrating interneurons expressing *Nrp*1/2 are repelled away from the striatum, where their repulsive ligands Semaphorin (Sema) 3A and 3F are expressed (Marin et al., 2001). Loss of neuropilin function increases the number of interneurons that migrate into the striatum and decreases the

numbers that reach the embryonic cortex. So, the final destination of tangentially migrating interneurons (striatum or cortex) is determined by the expression of the class 3 semaphorin receptors, Neuropilin1 and Neuropilin2 (Marin and Rubenstein, 2001). It has been proposed that although both the *Dlx* genes have both redundant and distinct roles in induction of the GABAergic phenotype and cross-regulation of the *Dlx* genes functions, they may have specific function in regulating tangential migration (Stuhmer et al., 2002b). DLX1 and DLX2 can repress transcription of *Neuropilin*2 through direct binding via their homeodomains, and regulate tangential interneuron migration via down-regulation of *Neuropilin*2 (Le et al., 2007).

The Slit/Robo signalling system has also been proposed to affect tangential migration. Slit ligands are large ECM molecules that possess chemorepulsive activity for growing axons and migrating cells in a variety of systems (Brose and Tessier-Lavigne, 2000). Indeed, Slits, the ligands for Robo receptors, have been shown to repel GABAergic neurons *in vitro* (Zhu et al., 1999). However, cortical interneuron migration was found to be unaffected and these interneurons avoided the striatum normally in *Slit*1/2 double knockout mice (Marin et al., 2003). The authors stated that the results obtained *in vitro* by (Zhu et al., 1999) did not reflect the effect of this signalling system on the tangentially migrating neurons to the cortex but on subsets of subcortical GABAergic neurons and cholinergic neurons that normally remain in the ventral telencephalon. Thus, it is also important to clarify whether the expression of other guidance molecules such as *Nrp*1/2 in tangentially migrating cells and *Sema*3A/3F in the striatum is altered in *Robo*1-deficient mice (Andrews et al., 2006).

Different isoforms of *Neuregulin*1 (*Nrg*1), i.e., soluble NRG1-Ig and membrane-bound NRG1-CRD, are expressed in the embryonic cortex and along the migratory path of interneurons toward the cortex (Flames et al., 2004). These NRG1 isoforms act as long- and short-range attractants through NRG receptor ErbB4, and NRG1/ErbB4 signalling directly controls tangential interneuron migration (Flames et al., 2004). Signalling by glial cell line-derived neurotrophic factor (GDNF) via the GPI-anchored receptor (GFRα1) has also been found to act as a potent chemoattractant of GABAergic cells, in addition to its roles in promoting GABAergic differentiation of the ventral precursors and stimulating axonal growth in cortical GABAergic neurons (Pozas and Ibanez, 2005). CXC chemokine receptor 4 (CXCR4) is expressed in tangentially migrating interneurons and its ligand stromal cell-derived factor-1 (SDF-1), which is also known as CXCL12 and is secreted by embryonic meninges, is another potent chemoattractant for these migrating interneurons. Interneurons in mice deficient in CXCR4 or SDF-1 are severely underrepresented in the superficial cortical layers and ectopically located in the deep layers (Stumm et al., 2003).

Dopamine D1 receptor activation promotes interneuron migration from the MGE and CGE to the cortex and D2 receptor activation decreases this migration (Crandall et al., 2007). Since the embryonic basal forebrain is rich in dopamine, the balance between D1 and D2 receptors may control interneuron migration to the cortex. Nogo-A, which is abundant in the leading process of tangentially migrating interneurons, is also involved in the regulation of tangential migration of early cohorts of cortical interneurons (Mingorance-Le Meur et al., 2007).

Research on molecules that function in the migrating cells themselves has shown that the cell cycle regulator retinoblastoma (Rb) protein controls tangential migration of cortical interneurons in a cell-autonomous manner (Ferguson et al., 2005). Although Rb deficiency has not been found to impact the specification or generation of the ventrally derived calbindin- and *Lhx*6-expressing interneurons, their tangential migration into the neocortex is dramatically and specifically reduced in the mutant MZ (Ferguson et al., 2005; Liodis et al., 2007). In addition, in mice with a *Foxg*1-*Cre*-mediated deletion of *Rac*1, interneurons halted in the mantle of the ventral telencephalon and were severely reduced in number in the cortex and olfactory bulb, suggestting cell-autonomous defects in the MGE- and LGE-derived migrating cells (Chen et al., 2007). Of interest, alcohol administration to pregnant rodents causes faulty migration of immature neurons, which leads to abnormal neuronal cytoarchitecture and ectopic cells (Miller, 1986; 1993), but little is known about the mechanisms underlying this abnormality. Recently, it has been found that Ca2+ signalling and cyclic nucleotide signalling are the central targets of the action of alcohol in neuronal cell migration (Komuro and Kumada, 2005; Kumada and Komuro, 2004). Most importantly, the aberrant migration of immature neurons caused by alcohol exposure is significantly ameliorated by controlling the activity of these second-messenger pathways (Kumada et al., 2006). However, the molecular mechanisms underlying aberrant forebrain development in children with Fetal Alcohol Syndrome remain to be defined.

1.7 *Dlx* homeobox genes

Homeobox genes comprise a large class of transcription factors that are crucial regulators of many developmental processes, ranging from organization of the body plan to differentiation of individual tissues. Binding sites for homeodomain proteins contain a core nucleotide motif, TAAT/ATTA, with adjacent bases being responsible for the interactions between specific homeodomain factors and target genes (Gehring et al., 1994; McGinnis and Krumlauf, 1992). The Distal-less (*Dll*) homeobox-containing gene, initially characterized in *Drosophila*, was shown to be essential for the proximodistal patterning of insect limbs (Cohen and Jurgens, 1989). The expression of *Dll* in protostomes and deuterostomes is also found to be a common feature of appendage outgrowth from arthropods to mammals (Panganiban et al., 1997). The vertebrate distal-less (*Dlx*) genes share the highly conserved homeodomain region with the Drosophila *Dll* gene and constitute an evolutionary-conserved group of homeobox-containing factors that play a fundamental role in the early patterning of embryonic structures. In the mouse and human genomes, the *Dlx* family comprises six members, which are grouped into pairs or bigenic clusters (*Dlx*1 and *Dlx*2, *Dlx*5 and *Dlx*6, *Dlx*3 and *Dlx*4) (the latter also reported as *Dlx*7 and *Dlx*8), and organized into three closely linked, convergently transcribed loci (McGuinness et al., 1996). Based on amino acid sequences of the homeodomains of these proteins, the *Dlx* genes fall into two major homology subgroups: Type A (*Dlx2, Dlx3* and *Dlx5*) and Type B (*Dlx1, Dlx6* and *Dlx7*) (Liu et al., 1997; Stock et al., 1996). This subgroup category is supported by analyses of inheritance from an ancestral cluster, homology of nucleotide sequence outside of the homeodomain, and chromosomal location (Ellies et al., 1997b). The proteins encoded by members of the

group of *Dlx*2, *Dlx*3, and *Dlx*5 also contain a short highly conserved amino acid sequence in the amino-terminus (Akimenko et al., 1994). Gene expression analysis of members of the *Dlx* gene family has demonstrated expression in the developing nervous system, neural crest derivatives, branchial arches, and developing appendages (Panganiban and Rubenstein, 2002).

1.7.1   *Dlx* and invertebrate development: *Distal-less*

Distal-less (*Dll*), as its name suggests, is required for distal limb development. The Drosophila *Dll* gene was identified in the early 1980s by means of dominant and recessive mutations that caused both striking antenna-to-leg homeotic transformations and leg truncations. Subsequent studies have revealed that *Dll* encodes a homeodomain transcription factor that is expressed throughout limb development from embryogenesis onwards (Cohen, 1990; Weigmann and Cohen, 1999). *Dll* is required for the elaboration of distal pattern elements in the antenna, the legs, the limb-derived gnathal structures (Cohen et al., 1989), and the anal plate (Gorfinkiel et al., 1999) and can initiate proximodistal axis formation when expressed ectopically (Gorfinkiel et al., 1997). *Dll* homologs are expressed in developing appendages in at least six coelomate phyla, including chordates (Akimenko et al., 1994; Simeone et al., 1994), consistent with requirements for *Dlx* function in normal limb development across the animal kingdom.

In mammalian species, *Distal-less* also has been implicated in various aspects of vertebrate neurogenesis (Beanan and Sargent, 2000; Kraus and Lufkin, 1999). Whether *Dll* is required in the CNS of the fly is unknown, although it is expressed in both the optic lobe of the brain (Kaphingst and Kunes, 1994) and in the glial cells of the ventral nerve cord (Panganiban and Rubenstein, 2002). In addition, the *Drosophila Dll*

gene is required for the formation of parts of the peripheral nervous system. In the *Dll*-null animals, the larval antennal, maxillary and labial sense organs do not form, nor do the mechanosensory vestigial larval legs called 'Keilin's organs' (Cohen and Jurgens, 1989).

The regulation of *Dll* expression in the embryo requires activity of the Wnt family member, *Wingless* (*Wg*) (Cohen, 1990), and is repressed both by a BMP homolog, *Decapentaplegic* (*Dpp*) (Goto and Hayashi, 1997) and by the epidermal growth factor (EGF) signalling pathway (Raz and Shilo, 1993). By contrast, maintenance and refinement of the *Dll* expression pattern through the larval stages requires cooperative positive inputs from both *Dpp* and *Wg,* as well as autoregulatory inputs from *Dll* itself (Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). In adult *Drosophila*, *Dll* expression and limb formation are repressed in the abdomen by products of two *Hox* genes, *Ultrabithorax* (*Ubx*) and *abdominal A* (*abdA*) (Vachon et al., 1992). Several genes lie genetically downstream of *Dll* in the developing leg, and represent candidate targets for direct regulation by *Dll*. These include *bric a brac* (*bab*) (Gorfinkiel et al., 1997), *spineless* (*ss*) (Duncan et al., 1998), *aristaless* (*al*) (Campbell and Tomlinson, 1998), *BarH1/BarH2* (Kojima et al., 2000), *Dwnt5* (Eisenberg et al., 1992), *disconnected* (*disco*) (Cohen et al., 1991), and *Serrate (Ser)* (Rauskolb, 2001). Three other genes have been identified as potential targets of *Dll* activation specifically in the developing antenna. These are *spalt* (*sal*) (Dong et al., 2000), *dachshund* (*dac*) (Dong et al., 2002) and *atonal* (*ato*) (Dong et al., 2002). Thus, it is possible that these genes are evolutionarily conserved *Dll/Dlx* targets.

1.7.2 Sequence, structure, genomic organization of *Dlx* genes in vertebrate

Vertebrate *Dlx* genes share a highly conserved homeodomain with the Drosophila *distal-less* gene. As mentioned above, at least 6 of the known vertebrate *Dlx* genes are organized as convergently transcribed, linked gene pairs, with each gene pair containing one gene from each subgroup (Stock et al., 1996). Whether there are functional differences between the two subgroups is not yet known. However, mouse *Dlx*1 and *Dlx*2, mouse *Dlx*5 and *Dlx*6, and zebrafish *dlx*3 and *dlx*7 are partially redundant (Qiu et al., 1997; Robledo et al., 2002; Solomon and Fritz, 2002), suggesting that some key functions are shared between the two types of Dlx-homeodomain regions, even though the encoded protein sequences outside of the homeodomains are fairly divergent. Each gene pair is linked to a *Hox* cluster. In humans, *Dlx*1 and *Dlx*2 are linked to *Hox*D on chromosome 2 (McGuinness et al., 1996); *Dlx*3 and *Dlx*7 are linked to *Hox*B on chromosome 17q21 (Nakamura et al., 1996); and *Dlx*5 and *Dlx*6 are linked to *Hox*A on chromosome 7 (Simeone et al., 1994). The same linkage of *Dlx* genes to *Hox* clusters also occurs in the mouse genome (Panganiban and Rubenstein, 2002).

Phylogenetic analysis indicates that the adjacent tandem duplication of an ancestral *Dlx* gene, most probably in the primitive chordates, followed by two rounds of genome duplication and a subsequent loss of the *Dlx* pair linked to *Hox*C, accounts for the present arrangement found in the mammalian *Dlx* genes (Stock et al., 1996; Sumiyama et al., 2003). *Drosophila* and amphioxus *Dll* are most closely related to *Dlx*1 (Holland et al., 1996; Stock et al., 1996). *Dlx*1 may thus be the founding member of the vertebrate *Dlx* family.

The conserved homology of the vertebrate *Dlx* genes extends to the genomic structure, which presents a common exon–intron organization (McGuinness et al., 1996) (**Fig. 2**). All *Dlx* genes consist of three exons and two introns; individual genes of each pair are transcribed in the opposite direction from the other. The region encoding the homeobox is split between exons 2 and 3, and this splicing site is also conserved in the Drosophila *Dll* gene. Several of the *Dlx* genes have multiple transcripts due to usage of alternative transcriptional initiation sites or alternative splicing (Liu et al., 1997; Nakamura et al., 1996).

Dlx promoter analyses have been limited thus far to *Dlx*3 and *Dlx*2. A fragment of slightly less than one kilobase located upstream of the *Xenopus Dlx*3 was shown to direct expression to the epidermis when assayed in *Xenopus* embryos and to the hair follicles and mammary epithelium when assayed in transgenic mice (Morasso et al., 1995). A 3.8-kb fragment of the mouse *Dlx*2 5'-flanking sequence was recently shown to confer expression of a reporter transgene to a subset of epithelial cells in both the mandibular and maxillary components of the first branchial arch, with patterns identical to endogenous *Dlx*2 expression (Thomas et al., 2000).

1.7.3 *Dlx* genes as transcription regulators of other *Dlx* gene expression

DLX proteins bind DNA *in vitro* and are expected to act as homeodomain transcription factors (Liu et al., 1997). Transcriptional activation by the DLX3 protein on a reporter construct *in vitro* depends on two subdomains located on either sides of the homeobox (Feledy et al., 1999b). The transcriptional activity of DLX proteins might be modulated by other proteins. In particular, MSX has been shown to

bind to DLX *in vitro*. The binding is mediated by their homeodomain, and results in a

mutual functional antagonism (Zhang et al., 1997).

Gain-of-function experiments demonstrate that *Shh* can induce *Dlx*

expression in the forebrain (Gaiano et al., 1999), while mice lacking *Shh* have greatly

reduced levels of *Dlx*2 expression in the forebrain (Ohkubo et al., 2002). Bone

morphogenetic protein 2 (BMP2) can induce *Dlx*2 expression in chondrocytes (Xu et al.,

2001), whereas BMP4 can induce *Dlx*5 expression in osteoblasts (Miyama et al., 1999),

and *Dlx*1 and *Dlx*2 expression in dental mesenchyme (Bei and Maas, 1998), and *Dlx*3 in

embryonic ectoderm (Feledy et al., 1999a). Fibroblast growth factors (FGFs) can

maintain or induce *Dlx* expression (Ferrari et al., 1999; Mullen et al., 1996). Given the

large number of FGF family members and their overlapping patterns of expression, there

may be compensatory mechanisms for maintaining *Dlx* expression in the absence of any

single *Fgf* gene. Retinoids have also been implicated in *Dlx* repression. For example,

administration of retinoic acid to zebrafish embryos prior to or during cranial neural crest

migration reduces *Dlx* gene expression in ectomesenchymal cells (Ellies et al., 1997a).

It has been observed that the expression of pairs of linked *Dlx* genes is

generally very similar or even identical (Simeone et al., 1994). This finding suggests that

the linked *Dlx* genes may share *cis*-acting sequences that control their pattern of

expression in the embryo and in the adult (Zerucha et al., 2000). Indeed, a transgene

equipped with *lacZ*-reporter and the zebrafish intergenic enhancer was found to be

regulated by *Dlx*1 and *Dlx*2 *in vivo* and in *in vitro* (Stuhmer et al., 2002b; Zerucha et al.,

2000; Zhou et al., 2004). Additional evidence for cross-regulatory interactions between

Dlx genes was obtained in studies of overexpression of truncated forms of *dlx3* mRNA in

zebrafish embryos. These experiments resulted in specific decreases in *dlx4* expression in the inner ear and branchial arches (Zerucha et al., 1997). The intergenic regions of mouse *Dlx*1 & *Dlx*2 and *Dlx*3 & *Dlx*7 also appear to contain shared *cis* elements (Ghanem et al., 2007; Sumiyama et al., 2002). Thus, cross-regulatory interactions between *Dlx* genes may occur in several regions of the embryo (Ghanem et al., 2008).

1.7.4   *Dlx* and vertebrate development: Patterns of expression

During murine mid-gestational stages, all six mouse *Dlx* genes are expressed in discrete domains in both neural and non-neural components of the surface ectoderm, the latter demarcating the regions that will give rise to body appendages, such as the apical ectodermal ridge of the limb buds, genital tubercle, branchial arches, and the ectodermal and mesenchymal components of the developing teeth (Bulfone et al., 1993; Panganiban and Rubenstein, 2002; Qiu et al., 1997). For example, during gastrulation, expression of *Dlx*3 (Akimenko et al., 1994) and *Dlx*5 (Yang et al., 1998) is observed around the lateral parts of the neural plate. Several of the *Dlx* genes (*Dlx*2, *Dlx*3, *Dlx*5 and *Dlx*6) are expressed in the otic placode, and later regionally expressed in the otic vesicle (Depew et al., 1999; Ekker et al., 1992). In the developing retina, *Dlx*1 and *Dlx*2 are expressed in neuronal precursors and in subsets of neurons (de Melo et al., 2003; Eisenstat et al., 1999). At later developmental stages, *Dlx* gene expression is found in differentiating skeletal tissues. For example, *Dlx* genes are found in both ectodermal and mesenchymal compartments of developing teeth (Panganiban and Rubenstein, 2002).

Only four of the genes, *Dlx*1, *Dlx*2, *Dlx*5, and *Dlx*6, are expressed in the developing CNS (Eisenstat et al., 1999; Liu et al., 1997; Price, 1993). Within the neural tube, their expression appears to be highly restricted to the forebrain, where they are

expressed in two domains: one diencephalic and one telencephalic. Where it has been studied, *Dlx* gene expression follows a temporal sequence: *Dlx*2, *Dlx*1 and *Dlx*5, then *Dlx*6 (Eisenstat et al., 1999; Liu et al., 1997). The general trend is for *Dlx*2 to be expressed in subsets of VZ neuroepithelial cells. *Dlx*1, *Dlx*2 and *Dlx*5 are expressed together in most SVZ cells, while *Dlx*5 and *Dlx*6 are expressed in many of the postmitotic differentiating neurons (Eisenstat et al., 1999). This temporal sequence suggested that a regulatory cascade might exist among the *Dlx* genes themselves.

All of the *Dlx* genes are expressed in ectomesenchymal cells derived from the cranial neural crest. The migratory neural crest cells populate the branchial arches, which then give rise to most of the facial connective tissues and bone (Panganiban and Rubenstein, 2002). Within the branchial arches, the *Dlx* genes are expressed in nested patterns along the proximodistal axis, where in the proximal area only *Dlx*1 and *Dlx*2 are expressed; and in the intermediate region, *Dlx*1, *Dlx*2, *Dlx*5, and *Dlx*6 are detected; whereas the distal regions express all six genes. The overlapping pattern suggests that there are both redundant and distinct functions for each *Dlx* gene in craniofacial morphogenesis (Qiu et al., 1997). In addition, they exhibit a temporal sequence of expression that is reminiscent of that observed in the forebrain. Some of the *Dlx* genes also are expressed in and required for development of other neural crest-derived cells, including the peripheral and enteric nervous systems (Depew et al., 1999; Qiu et al., 1995).

1.7.5   *Dlx* and vertebrate development: *Dlx* function in development

The roles of the *Dlx* genes in vertebrate development have primarily been studied through the analysis of loss-of-function mutations in mice, and to a lesser extent,

zebrafish. Gain-of function studies have also led to important insights into *Dlx* gene function particularly in the development of the forebrain. Linked *Dlx* genes share *cis*-regulatory sequences, intergenic regions containing enhancer elements that control their expression pattern through embryogenesis (Ellies et al., 1997b; Ghanem et al., 2003; Sumiyama et al., 2003; Zerucha and Ekker, 2000). This linkage has enabled the simultaneous, targeted deletion of a bigene pair, with the redundant and distinct functions of the *Dlx* genes being confirmed by analysis of the *Dlx*1, *Dlx*2, *Dlx*1/2, *Dlx*5, and *Dlx*5/6 mutants (Acampora et al., 1999; Depew et al., 1999; Qiu et al., 1997; Robledo et al., 2002). Disruption or ablation of *Dlx*1, *Dlx*2, *Dlx*1/2, or *Dlx*5 results in craniofacial, bone, and vestibular defects, but with the limbs lacking any overt abnormalities, probably due to overlapping function of other *Dlx* family member(s). However, the targeted disruption of the *Dlx*5/6 pair led to severe craniofacial, limb, and inner ear defects (Merlo et al., 2002; Robledo et al., 2002), demonstrating the requirement of these *Dlx* genes in limb development. In *Dlx*5/6 null embryos, there is also a homeotic transformation of the proximal structures of the jaw, indicating that these genes play crucial roles in establishing the proximal-distal identities in the pharyngeal arches (Depew et al., 2002). In one study, *Dlx*3 was found to be expressed in the central nervous system (CNS) of a vertebrate (Zhu and Bendall, 2006); *Dlx*3 has an essential role in epidermal and placental development (Beanan and Sargent, 2000). Furthermore, although the linked gene, *Dlx*4, is also expressed in placenta (Quinn et al., 1998), the functional role of each of these genes is specific and cannot be compensated by the other, as *Dlx*3-null mice die at midgestation due to deficiency in placental development. To date, *Dlx*4 expression has not been detected in the CNS.

1.7.6   *Dlx* genes and development of lateral neural plate and derived tissues

At least one *Dlx* family member, *Dlx*3, has been shown to be regulated by BMP and canonical Wnt/β-catenin signalling that provide ventralizing signals in pre-gastrula stage *Xenopus* embryos (Beanan and Sargent, 2000). These data suggest that *Dlx* genes might reinforce or refine the early ectodermal pattern established by BMP and Wnt signalling. Interestingly, the medial expression borders, adjacent to the neural plate, differ among *Dlx* family members, leading them to propose that distinct cell fates might arise through differential action of *Dlx* family members (Luo et al., 2001). These studies suggest a model in which *Dlx* activity may regulate the position of the neural plate border.

*Dlx*3 is expressed in the ectoplacental cone, chorionic plate, and the labyrinthine layer of the placenta, and functions as a transcriptional activator in placental trophoblasts (Morasso et al., 1999). The *Dlx*3 gene is also broadly expressed in the embryonic ectoderm, as well as in the tooth, hair follicle, and mammary gland, and later has a role in the interfollicular epidermis (Morasso et al., 1995; Morasso et al., 1996). A role for *Dlx*3 in differentiation was shown by its transgenic overexpression in the basal proliferative layer of the stratified epidermis. This led to cessation of proliferation of the basal cells and premature differentiation, with precocious induction of terminal differentiation markers (Morasso et al., 1996). Recently, a transcriptional enhancer that regulates the visceral arch mesenchyme-specific expression of *Dlx*3 was also identified in a highly conserved sequence within the intergenic region between *Dlx*3 and *Dlx*4 (Sumiyama et al., 2003). These results support the hypothesis that *cis*-acting elements responsible for epithelial and mesenchymal expression are independent and located

separately, 5′ for ectodermal and intergenic for mesenchymal expression. Interestingly, both of the *Dlx*1/2 and *Dlx*5/6 bigenic clusters also have intergenic enhancers that control their nested expression in the mesenchyme of the branchial arches (Park et al., 2004).

1.7.7 *Dlx* genes during limb and appendage development

In support of a role for *Dlx* genes in appendicular development, a recent study showed that *Dlx* genes are also crucial players in limb patterning. Analogous to *dll* expression in the appendages of *Drosophila* (Cohen et al., 1989), the mammalian orthologues *Dlx*1, *Dlx*2, *Dlx*5, and *Dlx*6 are found to be expressed in the developing vertebrate limb, with *Dlx*2, *Dlx*5, and *Dlx*6 being co-expressed in the Apical Ectodermal Ridge (AER) of the fore- and hindlimb buds (Panganiban and Rubenstein, 2002). During mouse limb development, the AER is a transient structure of the limb bud and a signalling center directing Proximal–Distal (Pr-Di) limb patterning (Summerbell, 1974). While *dll* had been demonstrated to be required for patterning of the distal leg in *Drosophila*, loss of any single *Dlx* gene has not resulted in an obvious limb phenotype in the mouse, suggesting there is functional redundancy. The simultaneous targeted inactivation of *Dlx*5 and *Dlx*6 in mouse resulted in a phenocopy of the split-hand/split-foot malformation (SHFM1, MIM 183600) in humans (Robledo et al., 2002). Mouse embryos devoid of functional *Dlx*5 and *Dlx*6 showed a disturbance in patterning along the Pr–Di axis of the developing limb indicating that *Dlx*5/*Dlx*6 are crucial for the maintenance of the medial AER during a critical time period of Pr–Di limb patterning. The SHFM1-like limb phenotype in the *Dlx*5/6 mutant embryos displays in the most severe cases a lobster claw-like hand plate, with significant cell death and reduced cell proliferation in the mesenchyme of the medial limb after E11.5, resulting in a lack of

critical cell mass in the mesenchyme to initiate or properly form a cartilage condensation of AER. On the other hand, overexpression of the *Msx2-Dlx5* transgene on a wild-type background does not cause any limb phenotype (Robledo et al., 2002) suggesting that *Dlx* function in this tissue is already at maximal levels. This result supports the theory that there is functional redundancy of members within the *Dlx* homeobox gene family in the limb, and there is a critical threshold concentration of *Dlx* gene products for proper limb patterning.

1.7.8 *Dlx* genes regulate endochondral ossification

*Dlx*5 and *Dlx*6 are the only members of the mammalian *Dlx* homeobox gene family shown to be expressed in the perichondrial region of the embryonic axial and appendicular skeleton, from the beginning of cartilage initiation (Acampora et al., 1999; Simeone et al., 1994). Furthermore, *in vitro* data has indicated a role for *Dlx*5 in regulating osteocalcin expression (Ryoo et al., 1997), suggesting a role for *Dlx*5 during osteoblast differentiation. However, *Dlx*5 or *Dlx*6 single mutants showed only minor, if any, irregularities in the axial or appendicular skeleton (Acampora et al., 1999; Depew et al., 1999). Interestingly, the combined *Dlx*5/6 loss-of-function animals describe a phenotype of a kinked tail by E11.5, indicating a malformation of the axial skeleton (Robledo et al., 2002). By E14.5, the SHFM-like phenotype became apparent in the hindlimbs, where often the central digit and to some variable extent, depending on the severity of the preceding AER defect, adjacent digits were missing. The forelimb was only rarely affected as discussed above. By E16.5, when mineralization of the previously cartilaginous long bones is generally proceeding, the *Dlx*5/6 homozygous mutant embryos showed a delay in ossification, with smaller or absent ossification centers in the

appendicular skeleton as well as the ribs and vertebrae of the axial skeleton. By E18.5, despite some minor mineralization in endochondral ossification centers, it is obvious that the ossification remains severely delayed, rendering *Dlx*5/6 null embryos severely dysmorphic. The studies demonstrated that *Dlx*5 and *Dlx*6 are playing a critical role in cartilage maturation, which subsequently determines the onset of osteogenesis.

1.7.9 *Dlx* genes are required for craniofacial development

All known members of the *Dlx* homeobox gene family are expressed in migratory neural crest cells that will populate the branchial arches (BAs) and are by E9.5 strongly expressed in the BAs (Depew et al., 1999; Depew et al., 2002). The BAs represent another transient developmental structure, that eventually gives rise to craniofacial elements such as the upper (maxillary) and lower (mandibular) jaws with associated soft tissues, structures of the middle ear, the styloid ligament, body of the hyoid as well as thyroid and cricoid cartilages. In the BAs, the *Dlx* genes are expressed in a nested pattern along the Pr–Di axis, with *Dlx*1/*Dlx*2 being expressed broadly, whereas *Dlx*5/*Dlx*6, and *Dlx*3/*Dlx*7 become progressively more distally restricted (Depew et al., 1999; Qiu et al., 1997). It has been hypothesized that this nested expression pattern could function as a code to establish regional identity within the BAs (Depew et al., 2002). Craniofacial phenotypes resulting from mutations in the *Dlx* genes can roughly be divided in malformations affecting (1) skull morphology: *Dlx*1, *Dlx*2, *Dlx*1/2, *Dlx*5, and *Dlx*5/6 (Acampora et al., 1999; Depew et al., 1999; Qiu et al., 1997; Robledo et al., 2002); (2) the inner ear; as seen in embryos lacking *Dlx*1, *Dlx*2, *Dlx*1/2, *Dlx*5, and *Dlx*5/6 (Acampora et al., 1999; Depew et al., 1999; Robledo et al., 2002), (3) the jaws: *Dlx*2, *Dlx*1/2, *Dlx*5, and *Dlx*5/6 (Depew et al., 2002; Qiu et al., 1997), and (4) the teeth: *Dlx*1/2,

*Dlx*3, *Dlx*5 (Depew et al., 1999; Qiu et al., 1997). Skull morphology is affected to a variable extent in the above listed single and double *Dlx* loss-of-function mice, ranging from malformation, misplacement, and lack of alignment of BAs to non-neural crest derived facial bones, cartilage elements, and ligaments.

All *Dlx*1/2 double mutant embryos have a cleft of the secondary palate, seen in 80% of the *Dlx*2 null embryos, while only 10% of *Dlx*1 null embryos had a mild cleft palate (Qiu et al., 1997). In *Dlx*5 and *Dlx*5/6 deficient embryos, nasal bones are foreshortened giving the snout a compressed appearance (Acampora et al., 1999; Depew et al., 2002). *Dlx*5/6 double mutant embryos frequently and some *Dlx*5 null embryos develop a cleft palate (Depew et al., 2002; Robledo et al., 2002). More interestingly, in *Dlx*5/6 double mutant embryos, a striking homeotic transformation from the lower into the upper jaw occurred, supporting the hypothesis that the nested *Dlx* expression pattern in the BAs could be specifying intra-BA identity (Depew et al., 2002).

In dental development a spectrum of defects can be observed, ranging from the complete absence of maxillary molars in mice lacking *Dlx*1/2 (Qiu et al., 1997), to malformation and poor mineralization of molars and incisors in *Dlx*5 mouse mutants (Depew et al., 1999). Patients with tricho-dento-osseus (TDO) syndrome (OMIM 190320) have a frame shift mutation in the human DLX3 gene, with taurodontism and enamel hypoplasia (Price et al., 1998). Human DLX5 is an imprinted gene and is misregulated in individuals carrying mutations in the MECP2 gene (encoding methyl-CpG-binding protein 2), which suggests a molecular mechanism for the pathophysiology of patients suffering from Rett syndrome (RTT, OMIM 312750) an X-linked dominant neurodevelopmental disorder (Bapat and Galande, 2005; Horike et al., 2005).

1.7.10 *Dlx* genes in sensory organ morphogenesis

*Dlx*5 and *Dlx*6 are expressed as early as E8.5 in the otic and olfactory placode, as well as in the ventral cephalic epithelium (Simeone et al., 1994; Yang et al., 1998); followed by expression in the inner ear: the semicircular canals and the endolymphatic duct (Acampora et al., 1999; Robledo et al., 2002). All studied *Dlx* loss-of-function mutants have abnormal inner ear development, possibly indicating less functional redundancy and hence less tolerance for mutations within the *Dlx* gene family during the patterning and forming of the inner ear. The *Dlx*5/6 double mutants appear severely affected, with external ear cartilage absent and fusion of the inner ear capsule and middle ear cartilage to a misshapen temporal bone primordium, rendering inner ear structures unrecognizable (Robledo et al., 2002). The inner ear phenotype in the absence of functional *Dlx*5 was milder; the auditory ossicles were normal in size and formed proper contacts to the tympanic membrane and the vestibular window; however, the vestibulum was malformed and rotated (Depew et al., 1999). In *Dlx*2 and *Dlx*1/2 mutants, the auditory ossicles were affected and with lower penetrance in *Dlx*1 mutants (Qiu et al., 1997).

A peculiarity of the *Dlx*5 and *Dlx*6 genes is their early expression in defined regions of the frontonasal ectoderm: the olfactory placodes, and subsequently in the olfactory and respiratory epithelium that lines the nasal cavities and the vomeronasal organ of the mouse (Yang et al., 1998). At present, defects affecting the olfactory epithelium in *Dlx*5 deficient mice have not been unequivocally demonstrated, but new insights have demonstrated that *Dlx*1&2 and *Mash*1 regulate parallel molecular pathways underlying olfactory bulb development (Long et al., 2007). The olfactory placode,

similarly to the otic placode, invaginates and delaminates in a complex series of morphogenetic events, induced in part by signals from the surrounding mesenchyme (Acampora et al., 1999). Although the *Dlx*5 gene is expressed very early in both the olfactory and otic placodes, its activity does not seem to be required for their initial specification, since invagination and early morphogenesis takes place normally in the *Dlx*5 null mice (Depew et al., 1999).

1.7.11 *Dlx* genes and hematopoiesis

*Dlx*4 is expressed in normal bone marrow cells and at a particularly high level in cell lines of the erythroid phenotype (Shimamoto et al., 1997). Inhibition of *Dlx*4 gene expression by an antisense oligonucleotide directed against DLX4 in erythroleukemia cell lines reduced the plating efficiency and induced apoptosis. This antisense treatment was accompanied by a reduction in *Gata*-1 and *c-myc* mRNA levels. These results suggested that the function of the *Dlx*4 gene may be linked to some aspect of erythropoiesis, possibly in the regulation of apoptosis that occurs during normal erythropoiesis (Chiba et al., 2003). There is also evidence showing a severe dysregulation of several *Dlx* genes in acute lymphoblastic leukemia patients, suggesting a function of these genes in the control of apoptosis (Ferrari et al., 2003a; Ferrari et al., 2003b)(Dell'Orto et al., 2007).

1.7.12 *Dlx* genes in brain development

Several transcription factors are expressed in subpopulations of neurons in the developing forebrain and olfactory bulb. Their restricted domains of expression define distinct regions of the early forebrain. Among these are members of the *Dlx* gene family (Bulfone et al., 1993; Price, 1993). As mentioned above, several *Dlx* genes are

expressed in the primordia of the basal ganglia, in overlapping patterns according to the stage of cell differentiation (Liu et al., 1997). *Dlx*1 and *Dlx*2 are expressed in the least mature cells both in the VZ and in the SVZ. In contrast, *Dlx*5 is expressed in cells of the SVZ and in post-mitotic cells of the mantle, but not in the VZ, while *Dlx*6 expression is higher in the mantle cells (Liu et al., 1997). These data suggest that each *Dlx* gene may play a specific role as well as redundant function in the differentiation of the cell types comprising the basal ganglia.

Indeed, while *Dlx* single mutants only have subtle defects in forebrain development (e.g. *Dlx*2 mutants have reduced numbers of dopaminergic neurons in the olfactory bulb) (Anderson et al., 1997b; Eisenstat et al., 1999), the *Dlx*1/2 double mutants exhibit a major block in neurogenesis within the subcortical telencephalon (Anderson et al., 1997a; Marin et al., 2000). In the *Dlx*1/2 double mutants, the first wave of neurogenesis (from approximately E10-12) appears to be undisturbed, whereas the differentiation of later born neurons (from E13) is largely aborted. This leads to abnormalities in the SVZ, the region that contains the secondary proliferative population (SPP) of neuroblasts, while the primary proliferative population (PPP; which is in the VZ) is apparently unaffected. This block in differentiation not only reduces the production of basal ganglia late-born projection neurons, it also blocks the development of several types of GABAergic, dopaminergic and cholinergic interneurons (Anderson et al., 1997b; Anderson et al., 2001; Pleasure et al., 2000). Furthermore, *Dlx*1 and *Dlx*2 are expressed in cells of the subcortical telencephalon that migrate across the pallial-subpallial boundary and enter the mantle zone and SVZ of the cerebral cortex of E12.5 mouse embryos (Eisenstat et al., 1999). Mice with mutations in both *Dlx*1/2 genes exhibit

a reduction in number and a defective differentiation of both striatal projection neurons and neocortical interneurons due to the lack of tangentially migrating immature interneurons from the subcortical telencephalon into the cerebral cortex (Anderson et al., 1997a; Anderson et al., 1997b). This supports the current hypothesis that cortical projection neurons and interneurons are derived from distinct regions of the telencephalon (Anderson et al., 1999).

Later in development, *Dlx*1 and *Dlx*2 are also expressed in the interneurons of the olfactory bulb cells derived from proliferation and migration from the SVZ (Porteus et al., 1994). The *Dlx*1/2 knockout mice lack mature periglomerular and granule cells of the olfactory bulbs, which are GABAergic interneurons. This defect results from a block in the migration and differentiation of SVZ-derived cells from the basal telencephalon (Qiu et al., 1997). Thus, a model has been suggested in which olfactory bulb projection neurons are generated from progenitors in the VZ of the developing bulb and express transcription factors characteristic of the cerebral cortex, whereas most interneurons in the bulb are generated in the SVZ that express subcortical transcription factors (Anderson et al., 1999; Bulfone et al., 1998).

The *Dlx*5 and *Dlx*6 genes are expressed in the developing forebrain, with a very similar profile (Simeone et al., 1994). Transcripts are detected early in the primordium of the GEs, and in the ventral diencephalon. At E12.5, these genes are expressed in the ventral thalamus, in both the MGE and LGE, and in the basal telencephalic vesicle anterior to the preoptic area (Eisenstat et al., 1999). At later stages, *Dlx*5 and *Dlx*6 are expressed in the SVZ of the olfactory area and in the developing

olfactory bulbs. Finally, at birth expression is found also in the olfactory tuberculum and in the neocortex (Acampora et al., 1999).

Within the forebrain, the *Dlx* genes are expressed virtually in all neurons that use GABA as their neurotransmitter. This suggests that the *Dlx* genes may have a central role in the development of this neuronal cell type (Anderson et al., 1997a; Anderson et al., 1997b; Stuhmer et al., 2002b). Indeed, ectopic expression of *Dlx2* or *Dlx5* in cortical neurons, using either retroviral vectors or electroporation techniques, induces expression of the GABAergic phenotype (Anderson et al., 1999; Stuhmer et al., 2002a). In our laboratory, consistent with a role for DLX proteins in the differentiation of GABAergic neurons, DLX proteins were shown to activate transcription from glutamic acid decarboxylase (GAD) isoform promoters (Le T., in preparation). GAD isoforms synthesize GABA from glutamic acid. Thus, *Dlx* function is tightly linked to the development of neurons derived from the basal telencephalon that produce GABA, acetylcholine and dopamine (Marin and Rubenstein, 2001; Marin et al., 2001). Reduced numbers or function of cortical GABAergic neurons could lead to hyperactivity states, such as seizures (Cobos et al., 2005b), or could result in defects in the function of local cortical circuits (Cobos et al., 2007). Likewise, GABAergic dysfunction in the basal ganglia could disrupt the learning and/or deployment of complex motor and cognitive behaviours (Cobos et al., 2006; Rubenstein and Merzenich, 2003). *Dlx* dysfunction in the diencephalon could disrupt the operation of the hypothalamic-pituitary circuitry and of the thalamus, through *Dlx* expression in the reticular nucleus (Rubenstein and Rakic, 1999). It is intriguing that two chromosomal regions that are associated with Autism on chromosomes 2q and 7q, contain the *Dlx1/2* and *Dlx5/6* loci, respectively (Hamilton et

al., 2005, Liu et al., 2009). Although there are many putative candidate genes within the implicated regions, these results underscore the potential roles of these genes in human neuropsychiatric disorders (Moldin et al., 2006).

1.7.13 *Dlx* genes and their downstream targets

DLX proteins are implicated as regulators of transcription; however based on the available literature, the downstream targets of DLX proteins are largely unknown. A consensus DNA sequence, (A/C/G)TAATT(G/A)(C/G), recognized by these proteins has been identified (Feledy et al., 1999b). Another study suggests that DLX proteins bind a specific sequence in an enhancer of the *Wnt*1 gene in mouse (Iler et al., 1995). The molecular mechanisms by which DLX proteins exert their influences on transcription are also largely unknown. It has been suggested, however, that specific DLX proteins are able to form homodimers as well as heterodimers with other DLX proteins and members of the *Msx* family of homeodomain proteins (Zhang et al., 1997). These dimerizations are mediated by the homeodomains of both these transcription factor families and are independent of DNA binding.

A variety of genes have been identified as targets of Dlx regulation, including the *Dlx* genes themselves. For example, *Dlx*1, *Dlx*2 and *Dlx*5 all can activate transcription from the mouse *Dlx5/Dlx*6 and zebrafish *dlx5a/dlx6a* (previously *dlx4/dlx6*) intergenic enhancers in tissue culture cells (Yu et al., 2001; Zerucha et al., 2000; Zhou et al., 2004) and in slices obtained from embryonic mouse brain (Stuhmer et al., 2002a). Both gain- and loss-of function experiments indicate that *Dlx*1&2 regulate the expression of the vertebrate ortholog *Aristaless* (*Arx*) homeobox gene in the developing forebrain (Cobos et al., 2005a, Colasante et al., 2008). In our laboratory, *Dlx*1 and *Dlx*2 have been

established to directly activate both *Gad* gene promoters, *Gad*1 and *Gad*2 (Le T., in preparation), as well as the *Neuropilin*2 promoter *in situ* (Le et al., 2007). As described above, this transcriptional regulation is likely to be important in the differentiation and migration of particular GABAergic neurons in the brain. DLX-binding sites that mediate this regulation have been identified (Zerucha et al., 2000). *Dlx*2 also is thought to regulate *Wnt*1 directly in the developing telencephalon (Iler et al., 1995). A single binding site, termed HBS-1 (Iler et al., 1995), mediates this *Dlx*2 regulation. Finally, there is evidence that *Dlx2* is involved in BMP-mediated induction of chondroblast differentiation and *collagen*2a1 expression (Xu et al., 2001).

DLX3 has been implicated directly in the activation of several genes, including those encoding a human chorionic gonadotropin subunit in the placenta (Roberson et al., 2001), and profilaggrin in differentiating keratinocytes (Morasso et al., 1996). The binding sites through which DLX3 regulation occurs have been identified (Morasso et al., 1996; Roberson et al., 2001). These sites share a TAAT core with the recognition sites for other DLX (and other homeodomain) proteins. DLX4 (previously DLX7) activates both *Gata*1 and *Myc* in hematopoeitic cells (Shimamoto et al., 1997). Ectopic DLX4 also can inhibit apoptosis via upregulation of expression of intercellular adhesion molecule 1 (ICAM-1) (Shimamoto et al., 2000). However, it is not known whether the activation of *Gata*1, *Myc* or ICAM1 by DLX proteins is direct or indirect.

Several targets of DLX5 have been identified during bone formation. The first of these, osteocalcin, initially was found to be repressed by DLX5 (Ryoo et al., 1997). However, more recent work suggests that DLX5 actually is a weak activator of osteocalcin transcription but that it potentiates osteocalcin transcription mainly by

interfering with the function of the osteocalcin repressor MSX2 (Newberry et al., 1998).

Two other genes activated by DLX5 during bone differentiation are those encoding

collagen 1A1 (Dodig et al., 1996) and bone sialoprotein (Benson et al., 2000). The

DLX5-binding sites in the regulatory regions for these genes have been characterized

(Benson et al., 2000; Dodig et al., 1996).

Chapter 2

Rationale

Rapid progress is being made in describing the genetic programs that regulate regional specification, morphogenesis, cell type specification, neuronal migration and connectivity in the mammalian forebrain. As the preceding introduction has clearly demonstrated, the development of the forebrain involves complicated processes at the molecular and cellular levels. Yet, few molecular pathways have been elucidated. When looking at the contribution of interneuronal development to that of the telencephalon overall, *Dlx*1 and *Dlx*2 homeobox genes are essential for interneuronal differentiation and migration in the developing forebrain. From a thorough characterization of several *Dlx* downstream targets, we have discovered that *Dlx*1&2 regulate other *Dlx* genes (Zhou et al., 2004), axonal guidance molecules (Le et al., 2007), as well as GABA production (Le et al, in preparation). These studies have helped to elucidate how *Dlx* mutations result in defects in forebrain development, and provide important insights into the mechanism of interneuronal differentiation and migration in the forebrain. Further loss- and gain-of-function studies will likely to continue to explain why hypomorphic or regionally restricted defects in *Dlx* function might alter the function of forebrain GABAergic, cholinergic and dopaminergic neurons. Reduced numbers or function of cortical GABAergic neurons could lead to hyperactivity states such as seizures or autism spectrum disorders (Cobos et al., 2005b). GABAergic dysfunction in the basal ganglia could disrupt the learning and development of complex motor and cognitive behaviours (Rubenstein and Merzenich, 2003). By combining genetic and molecular approaches to

identify *Dlx*-dependent downstream target genes, we hypothesize the role of *Dlx* genes as critical effectors of forebrain development.

Hypotheses

1. *Dlx*1 and *Dlx*2 regulate the expression of the homologous homeobox genes *Dlx*5 and *Dlx*6 through transcriptional activation.

2. *Dlx*1 and *Dlx*2 regulate the differentiation of GABAergic interneurons through transcriptional activation of *Gad* genes.

3. *Dlx*1 and *Dlx*2 regulate the tangential migration of GABAergic interneurons from the basal ganglia to the neocortex through transcriptional repression of Neuropilin2 receptor.

4. Development and characterization of the *Dlx*1/2$^{-/-}$; *Neuropilin*2$^{-/-}$ "triple" mutant mice will demonstrate partial restoration of tangential migration of GABAergic interneurons that was lost in the *Dlx*1/2 double mutant.

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Chapter 3

Materials and Methods

3.1 Animal and tissue preparation

The CD-1 strain [CD-1 (ICR) BR. Swiss strain of albino mice] was used as wild-type animals (Charles River Laboratories, Worcester, MA). *Dlx*1/*Dlx*2 knockout mice were generated as previously described (Anderson et al., 1997b; Qiu et al., 1997) and maintained on the CD-1 background. These mice were a kind gift of Dr. John Rubenstein, University of California at San Francisco. *Neuropilin*2 knockout mice (Chen et al., 2000) on the CD-1 background were a kind gift of Dr. Marc Tessier-Lavigne, Genentech, San Francisco. Mice homozygous for this mutant allele are viable and fertile (although the average litter size for a homozygous female appears small – usually five or fewer embryos) (Chen et al., 2000). *Dlx*1/*Dlx*2/*Nrp*2 triple knockout mice were generated as follows. First, *Dlx*1/*Dlx*2 heterozygous mice (*Dlx*1/2$^{+/-}$) were crossed with *Neuropilin*2 homozygous mice (*Nrp*2$^{-/-}$). Then, F1 generation mice that were heterozygous for all 3 alleles (*Dlx*1/2$^{+/-}$; *Nrp*2$^{+/-}$ were bred to produce the F2 generation in which the triple knockout mice (*Dlx*1/2$^{-/-}$; *Nrp*2$^{-/-}$) can be produced. *Dlx*1/2 null mice, *Neuropilin*2 null mice, and *Dlx*1/2/*Nrp*2 null mice were genotyped as previously described (Chen et al., 2000; Qiu et al., 1995). For comparative studies, all mutants were paired with wild-type littermate controls. Embryonic and newborn tissues were obtained from timed-pregnant mice. Embryonic age was determined by the day of appearance of the vaginal plug (taken as E0.5) and confirmed by morphological criteria. Adult animals were euthanized by cervical dislocation and brains were dissected. Embryonic animals were euthanized by decapitation prior to brain dissection. Brains taken from animals E13.5 or older were

dissected from embryos and newborns, while whole heads of animals younger than E13.5 were collected without brain dissection. Subsequently, dissected brains were left intact for later sectioning or they were further dissected to obtain different anatomical regions (forebrain, ganglionic eminence, neocortex, olfactory bulb, or hindbrain). All animal protocols were conducted in accordance with guidelines set by the Canadian Council on Animal Care and were approved by the University of Manitoba animal care committee.

E13.5, E16.5, E18.5 and P0 tissues were fixed in 4% paraformaldehyde (PFA) (Polysciences Inc. Warrington, PA) in phosphate buffered saline (PBS) (pH 7.4) for 40 minutes. Tissues were transferred to a 20% sucrose (Sigma, St. Louis, MO) solution in PBS overnight and then transferred to a 1:1 mixture of 20% sucrose/O.C.T. cryo-compound (Sakura Finetek, Torrance, CA) for 20 minutes before freezing in O.C.T. Tissues were sectioned (12-25µm) coronally on a Microm HM 510 cryostat (MICROM Laborgeräte GmbH, Walldorf, Germany) or a ThermoShandon Cryotome cryostat (Thermo Electron Corporation, Waltham, MA). Sections were captured onto Superfrost Plus slides (Fisher Scientific, Nepean, ON) and stored at -80°C.

3.2 Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed as described previously (Zhou et al., 2004). The medial and lateral GEs (express *Dlx*1/*Dlx*2) and hindbrain tissues (used as a negative control since this tissue does not express any *Dlx* family members) were dissected from E13.5 CD-1 mice and mechanically triturated to obtain single cells. Then 1-2x10^7 cells were fixed or cross-linked with 1% PFA for 30 minutes at room temperature in the presence of protease inhibitors (Invitrogen, Burlington, ON). Cross-linked cells were resuspended in SDS lysis buffer on ice (1% SDS, 50 mM Tris-HCl pH 8.1, 10 mM

EDTA), and then sonicated. Sonication of cells (Sonifier cell disruptor 350) in SDS lysis buffer and on ice generated soluble chromatin complexes with DNA fragment lengths ranging between 100-500 bp. Protein A-Sepharose beads (Pharmacia Biotech) were added to the supernatant to pre-clear any protein or DNA that bound non-specifically to the beads. DLX1 (Anderson et al., 1997a; Eisenstat et al., 1999) or DLX2 antibodies (Eisenstat et al., 1999; Porteus et al., 1994) were repurified to obtain fractions with high-affinity binding. These purified antibodies were added to the pre-cleared chromatin solution and incubated overnight at 4°C. Then Protein A and Protein G-Sepharose (Sigma) were added and incubated for 3 hours with mixing. Pelleted beads were then washed sequentially with Low Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), High Salt Wash Buffer (500 mM NaCl, LiCl Buffer (0.25 M LiCl, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1, 1% NP-40)) and TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). The enriched homeoprotein DNA complex was eluted twice using freshly prepared Elution Buffer (1% SDS, 0.1 M NaHCO$_3$) and the beads were separated from the supernatant by centrifugation. Then 4 M NaCl was added to the supernatant and incubated at 65°C for 4 hours to reverse cross-linking. Finally, proteinase K was added for 1 h at 45°C. Phenol/chloroform was used to extract residual proteins from the final ChIP DNA fragments and DNA was precipitated with ethanol.

3.3 Thermal cycling/Polymerase Chain Reaction

Oligonucleotide primer pairs were designed to amplify distinct regions of the DLX targets of interest:

- Mouse *Dlx*5/6 intergenic enhancer (MI56)

- Glutamic acid decarboxylase promoters (*Gad*)

- *Neuropilin* promoters (*Nrp*)

  For a complete listing of target gene promoter regions and the oligonucleotide primer

  pairs used, refer to **Table 1**.

Table 1: Primers designed for downstream target regions of DLX proteins

DLX Target	Target GenBank #	Target Region	Primers for specific regions
MI56	AY168010	I56i	sense primer: 5'-GACATTGGGGACAATTTA-3' antisense primer 5'-AATTTGTGTATGAATAAC-3'
		I56ii	sense primer 5'-TTTGCACACCCCAGCACCTCT-3' antisense primer 5'-CAGCCATTATTTAGACCCTA-3'
*Gad*1	z49978	GAD67i	sense primer: 5'-CGCCCTCTGTGGGAAATTTT-3' antisense primer:5'-CCTGGAGAGGGGTAAAAGAA-3'
		GAD67ii	sense primer: 5'-GATACGGGATGGAGGGCTAA-3' antisense primer: 5'-GACTGCCTCTGGAGCTTTGT-3'
*Gad*2	AB032757	GAD65i	sense primer: 5'-TTTCTGGGTGGCTCACAGT-3' antisense primer: 5'-TCCGGGTTGTTGATAACAAA-3'
		GAD65ii	sense primer: 5'-AAAAGGGAAACAGAAAGGA-3' antisense primer: 5'-AGAAAGGCTGCTGATTGAA-3'
*Nrp*1	AF482432	*Nrp*1i	sense primer: 5'-GGAACCGGACTACATGG-3' antisense primer: 5'-AAGACTGGCAACGACCC-3'
		*Nrp*1ii	sense primer: 5'-AACCTCAGGCTGACACC-3' antisense primer: 5'-ACGAGATCTCTGCACCC-3'
*Nrp*2	AF022854	*Nrp*2i	sense primer: 5'-GAGATCACACAGCTGCC-3'

			antisense primer: 5'-CCTACAACATCACGAGG-3'
		*Nrp*2ii	sense primer: 5'-CGTTGATCGTTAGAGACC-3'
			antisense primer: 5'-GACAGAGAGGCTCTCTC-3'

PCR was carried out using the above primer pairs with the isolated ChIP DNA, and with genomic DNA derived from E13.5 mouse embryo used as a positive control. PCR products were separated by gel electrophoresis, purified, then ligated into the pCR2.1 TOPO vector using a TOPO TA cloning kit (Invitrogen, Burlington, ON). Recombinant plasmid DNA was extracted using a Plasmid MiniPrep Kit (Qiagen Inc., Mississauga, ON) and the M13 reverse universal primer was used for sequence confirmation.

3.4 Electrophoretic Mobility Shift Assay (EMSA)

For a complete list of the selected regions of putative *Dlx* targets obtained from ChIP and PCR screening as potential targets of DLX proteins, see **Table 2**.

Table 2: Potential DLX target regions obtained from ChIP and PCR

DLX Target	Target GenBank #	Target Region	Nucleotide position
MI56	AY168010	I56i	8688-8917
*Gad*1	z49978	GAD67i	187- 460
		GAD67ii	743-1010
*Gad*2	AB032757	GAD65i	1425-1630
		GAD65ii	2761-3120
*Nrp*2	AF022854	*Nrp*2ii	444-562

The above cloned DNA fragments were excised from the pCR2.1-TOPO vector (Invitrogen, Burlington, ON) with *Eco*RI (NEB, Pickering ON), then the 5' overhang was filled in with the large fragment of DNA polymerase I (Klenow) (NEB, Pickering, ON) in the presence of radiolabeled [α32P] dATP (University of Manitoba). Purified recombinant DLX proteins or nuclear extracts from the E13.5 striatum were incubated with 1X binding buffer, poly(dI-dC), 1 mM PMSF and ^{32}P-labelled DNA probes (2-3x10^4 c.p.m.) for 30 min at room temperature. For 'cold' competition assays, double-stranded unlabelled DNA fragments were added at the same time. For 'supershift' assays, the specific polyclonal DLX1 or DLX2 antibodies were added. A rabbit polyclonal antibody to human Secretory Component (Dako, Mississauga, ON) was used as an 'irrelevant' control antibody. Reactions were incubated for 30 min at room temperature (RT). The DNA-protein complexes were resolved on a 4% non-denaturing polyacrylamide (37.5:1 acrylamide/bisacrylamide) gel in 0.5x Tris-Borate-EDTA solution. Gels were exposed to film overnight at -70°C.

Within each target region, sequences of putative TAAT/ATTA homeodomain binding motifs (Zerucha et al., 2000) were used to generate individual synthetic oligonucleotides (16-32 bp). These double-stranded oligonucleotides were subjected to further EMSA analysis. For the complete list of binding motifs studied, see **Table 3**.

Table 3: Putative TAAT/ATTA homeodomain binding motifs of individual regions of potential DLX targets

DLX Target	Target Region	Motif #	Nucleotide position
MI56	I56i	6	9793-8808
		9	8850-8869
*Gad*1	GAD67i	3	400-420
	GAD67ii	4	971-990
*Gad*2	GAD65i	3	1571-1603
	GAD65ii	3	3086-3107
*Nrp*2	*Nrp*2ii	2	505-524

3.5 Constructs for reporter gene assays

Effector plasmids expressing the mouse *Dlx*1 and *Dlx*2 genes separately under control of a CMV promoter were constructed by inserting a PCR-amplified 790 bp *Dlx*1 cDNA and 1020 bp *Dlx*2 cDNA (gifts from Dr. John Rubenstein, University of California at San Francisco) into the pcDNA3 vector (Invitrogen, Burlington, ON). Reporter plasmids were constructed by inserting potential DLX targets obtained from ChIP and PCR into the pGL3-promoter (for MI56i) or pGL3-Basic vector (for *Gad* or *Nrp*2 promoters) (Promega, Madison, WI), in front of the luciferase gene. For the complete list of the potential DLX targets, see **Table 2**. In addition, the mouse full-length *Gad*1 (GAD67) promoter was cloned using a standard PCR protocol with primers designed according to the mouse *Gad1* promoter sequence (Genbank accession number Z49978): Sense primer: 5'-GCGGTACCAAGCCAGGAGGTAGCGGCGAGA-3';

Antisense primer: 5'-GCCTCGAGTCTGGCAAGTCCGGGTGATCCGGTA-3'.

PCR products were first cloned into the PCR 2.1 vector, then were subcloned into the pGL3-Basic vector (Promega, Madison, WI). The correct orientation of all of the cloned fragments was verified by restriction digestion and sequencing.

Site-directed mutagenesis of putative DLX-binding sites (TAAT/ATTA) was performed using the Quick-Change Mutagenesis kit (Stratagene). For the potential DLX targets, **Table 3** lists only the important candidate binding motifs that were subjected to site-directed mutagenesis, and the nucleotide position in which the mutations were made. Mutations were either a complete deletion of candidate TAAT/ATTA motifs or point mutations in which the nucleotides were changed (A/T=>C/G). All mutations were verified by DNA sequencing.

Generation of Q50E variants (Gehring et al., 1994) of DLX1 and DLX2 proteins to abolish DNA binding was performed using the Quick-Change Mutagenesis kit (Stratagene). DLX-VP16 (activator) and DLX-Engrailed (repressor) fusion constructs were generated as follows: the N-terminal domains of either *Dlx*1 or *Dlx*2 were excised by *Hind*III/*Ppu*MI or *Eco*RI/*Bsm*BI (NEB, Pickering, Ontario) restriction enzyme digestions, respectively, leaving the nuclear localization signal, homeodomain, and C-terminal domains intact. Engrailed (888 bp) and VP16 (255 bp) domains (Kessler, 1997) were ligated to the 5' end of these N-terminal modified Dlx constructs.

3.6 Reporter gene assays

Transient co-transfection experiments were performed in the HEK293 human embryonic kidney cell line (courtesy of Dr. S. Gibson, University of Manitoba) or the P19 murine embryonic carcinoma cell line (a gift from Dr. M. McBurney, University of

Ottawa, Canada) or the C6 rat glioma cell line (American Type Culture Collection, Rockville, MA). The cell lines were grown and maintained in alpha Dulbecco's Modified Eagle's medium (αMEM) supplemented with 7.5% fetal bovine serum, 2.5% calf serum, and 1% penicillin-streptomycin at 37°C with 5% CO_2. Cells were seeded 24 hours before transfection at a density of $1x10^7$ per $36mm^2$ dish. Cells were transiently transfected using Lipofectamine 2000 reagent (Invitrogen) with luciferase gene reporters (1µg), effector plasmids (1µg), and pRSV-βgal (Promega) (0.4µg) as an internal control for transfection efficiency. Subsequently, cells were harvested 48 hrs later, and luciferase activities were measured with the Luciferase Reporter Assay System (Promega) and a standard luminometer, normalizing luciferase activity with β-gal activity which was co-transfected previously.

3.7 Histological staining, Immunohistochemistry (IHC), Immunofluorescence (IF), and Immunoblotting of DLX proteins

For IHC experiments, frozen sections were pre-incubatedfor 2 hours at room temperature in blocking solution: PBS, 5% normal goat serum (GIBCO, Rockville, MD), 0.2% Triton X-100 (Sigma), 0.1% BSA fraction V (Sigma), and 0.02% sodium azide (Sigma). Incubation was performed at 4°C for two hours. Sections were then incubated overnight at 4°C with a primary antibody diluted in blocking solution. For a complete list of the primary antibodies used in IHC and IF experiments, refer to **Table 4**. Sections were washed three times (3x) in PBS/0.05% Triton X-100 (PBS-T) pH 7.4 and then incubated with biotinylated secondary antibodies for 2 hours at room temperature. For a complete list of secondary antibodies and tertiary molecules used, see **Table 5**. Sections were again washed 3 x 5min in PBS with Triton (PBS-T) and then treated with 0.3%

H_2O_2 in PBS-T for 30 minutes. Slides were then washed 3 times in PBS-T and then developed using the Vectastain ABC system with DAB substrate reagent (Vector Laboratories, Burlingame, CA).

For IF experiments, tissue was pre-incubated in blocking solution as described above. For double IF experiments in which the primary antibodies were raised in different host species, incubation with both antibodies was performed concurrently. For experiments in which primary antibodies were raised in the same species, primary antibodies were added separately. Sections were incubated in primary antibody overnight at 4°C. Slides were washed 3 x 5min in PBS-T and secondary antibodies were applied individually thereafter for 2 hour incubations at RT. When secondary antibodies were not directly conjugated to a fluorescent reagent, an appropriate streptavidin-conjugated fluorescent tertiary reagent (**Table 5**) was applied for 2 hours following washing 3x in PBS-T. All labelled sections were mounted using Vectashield fluorescence mounting medium (Vector Laboratories). Secondary antibodies and streptavidin-conjugated fluorescent reagents, if necessary, were then applied prior to treatment with the second primary antibody. Negative controls were performed by omitting the primary antibody.

Table 4: Primary antibodies used for immunohistochemistry and immunofluorescence

Primary Antibody	Dilution	Source	Reference
Mouse anti-β-actin	1:500	Sigma, St. Louis, MO	(Herman, 1993)
Rabbit anti-β-galactosidase	1:500	ICN Pharmaceuticals	(Stuhmer et al., 2002a)
Mouse anti-BrdU	1:200	Chemicon, Temecula, CA	(Yang et al., 2005)
Rabbit anti-Calbindin	1:1000	Chemicon, Temecula, CA	(Di Cunto et al., 2000)
Rabbit anti-Calretinin	1:1000	Swant, Switzerland	(Rogers, 1987)
Rabbit anti-Caspase3	1:100	Cell Signalling Tech., Beverly, CA	(Reis and Edgar, 2004)
Mouse anti-ChAT	1:100	Chemicon, Temecula, CA	(Hagg et al., 1992)
Rabbit anti-DARPP-32	1:300	Chemicon, Temecula, CA	(Kawaguchi and Hirano, 2007)
Rabbit anti-DLX1	1:200	Dr. J. Rubenstein, University of California, San Francisco	(Eisenstat et al., 1999)
Rabbit anti-DLX2	1:400	Dr. J. Rubenstein, University of California, San Francisco	(Eisenstat et al., 1999)
Rabbit anti-GABA	1:1000	Sigma, St. Louis, MO	(Le et al., 2007)
Rabbit anti-GAD65	1:500	Dev. Studies Hybridoma Bank, University of Iowa	(Chang and Gottlieb, 1988)
Rabbit anti-GAD67	1:1000	Chemicon, Temecula, CA	(Bickford et al., 2000)
Rabbit anti-GFAP	1:2000	Dako, Mississauga, ON	(Kuhrt et al., 2004)
Rabbit anti-Glutamate	1:10000	Sigma, St. Louis, MO	(Ottersen and Storm-Mathisen, 1985)
Rabbit anti-	1:500	Affinity Bioreagents, Inc., CO	(Zerucha et al., 2000)

HDAC1			
Mouse anti-MAP2	1:300	Roche Diagnostics, Laval, QC	(Binder et al., 1984)
Mouse anti-MASH1	1:200	BD Biosciences, Mississauga, ON	(Sommer et al., 1995)
Rabbit anti-MSX	1:500	Covance, Richmond, CA	(Iler et al., 1995)
Rabbit anti-nNOS	1:500	Zymed (Invitrogen), ON	(Snyder, 1995)
Rabbit anti-NPY	1:1000	ImmunoStar, Hudson, WI	(Guy et al., 1988)
Rabbit anti-NRP1	1:200	Dr. A. Kolodkin and Dr. D. Ginty, Johns Hopkins University	(Cloutier et al., 2002)
Rabbit anti-NRP2	1:200	Dr. A. Kolodkin & Dr. D. Ginty, Johns Hopkins Univ.	(Cloutier et al., 2002)
Rabbit anti-parvalbumin	1:5000	Swant, Switzerland	(Kagi et al., 1987)
Rabbit anti-PAX6	1:500	Sigma, St. Louis, MO	(Inoue et al., 2002)
Rabbit anti-phosphohistone H3	1:500	Upstate Biochemicals, Charlottesville, VA	(Ajiro et al., 1996)
Mouse anti-Reelin	1:200	Chemicon, Temecula, CA	(Rice et al., 2001)
Goat anti-Somatostatin	1:100	Santa Cruz, Santa Cruz, CA	(Meyerhof et al., 1992)
Rabbit anti-TBR1	1:1000	Chemicon, Temecula, CA	(Wang et al., 2005a)
Rabbit anti-TH	1:1000	Pel-Freeze, Rogers, AR	(Haycock, 1987)
Rabbit anti-TRKB	1:100	Santa Cruz, Santa Cruz, CA	(Douma et al., 2004)

Table 5: Secondary antibodies and tertiary molecules used for IHC and IF

Secondary antibody or Tertiary molecule	Dilution	Source
Biotin-SP conjugated goat anti-rabbit	1:200	Jackson Immunoresearch, West Grove, PA
Biotin-SP conjugated goat anti-mouse	1:200	Jackson Immunoresearch, West Grove, PA
Biotin-SP conjugated goat anti-goat	1:200	Jackson Immunoresearch, West Grove, PA
FITC-conjugated goat anti-rabbit	1:100	Sigma, St. Louis, MO
Streptavidin conjugated Oregon Green-488	1:200	Molecular Probes, Eugene, OR
Streptavidin conjugated Texas Red	1:200	Vector Laboratories, Burlingame, CA
Texas Red-conjugated donkey anti-goat	1:100	Jackson Immunoresearch, West Grove, PA
Texas Red-conjugated donkey anti-mouse	1:100	Jackson Immunoresearch, West Grove, PA
Texas Red-conjugated donkey anti-rabbit	1:200	Jackson Immunoresearch, West Grove, PA

For immunoblotting, we followed established protocols in our laboratory (Zhou et al., 2004).

3.8 Tissue preparations and *in situ* hybridization (ISH)

ISH was done with the help of Dr. Guoyan Du and Tracie Parkinson, Manitoba Institute of Cell Biology, University of Manitoba. Non-radioactive *in situ* hybridization using digoxigenin (DIG)-UTP and non-radioactive *in situ* hybridization combined with immunohistochemistry were performed as previously described (Eisenstat et al., 1999). For a complete list of antisense probes used, see **Table 6**. Single or combined *in situ*

hybridization and immunohistochemistry were performed with sense probes used as

controls.

Table 6: Antisense riboprobes used in *in situ* hybridization studies

Riboprobe cDNA	Source	Reference
*Cux*2	Dr. C. Schuurmans, University of Calgary	(Zimmer et al., 2004)
*Dlx*1	Dr. J. Rubenstein, University of California, San Francisco	(Eisenstat et al., 1999)
*Dlx*2	Dr. J. Rubenstein, University of California, San Francisco	(Eisenstat et al., 1999)
ER81	Dr. C. Schuurmans, University of Calgary	(Arber et al., 2000)
*Gad*65	Dr. J. Rubenstein, University of California, San Francisco	(Erlander et al., 1991)
*Gad*67	Dr. J. Rubenstein, University of California, San Francisco	(Erlander et al., 1991)
*Lhx*2	Dr. C. Schuurmans, University of Calgary	(Bulchand et al., 2001)
*Math*2	Dr. C. Schuurmans, University of Calgary	(Bartholoma and Nave, 1994)
*Neurogenin*2	Dr. C. Schuurmans, University of Calgary	(Gradwohl et al., 1996)
*Nrp*1	Dr. M. Tessier-Lavigne, Genentech, CA	(Kolodkin et al., 1997)
*Nrp*2	Dr. M. Tessier-Lavigne, Genentech, CA	(Kolodkin et al., 1997)
*Robo*1	Dr. C. Schuurmans, University of Calgary	(Anselmo et al., 2003)
*ROR*β	Dr. C. Schuurmans, University of	(Schaeren-Wiemers et al.,

	Calgary	1997)
*Sema*3A	Dr. Kolodkin, Howard Hughes Medical Institute, MD	(Cloutier et al., 2002)
*Sema*3F	Dr. Kolodkin, Howard Hughes Medical Institute, MD	(Cloutier et al., 2002)
*Slit*1	Dr. C. Schuurmans, University of Calgary	(Anselmo et al., 2003)
*Tbr*1	Dr. C. Schuurmans, University of Calgary	(Hevner et al., 2001)
Tlx	Dr. C. Schuurmans, University of Calgary	(Stenman et al., 2003b)

For ISH, cryosectioned tissues on microscope slides were fixed in 4% PFA in PBS for 5 minutes then washed in PBS/DEPC ddH$_2$O 3 times for 5 min each. Slides were treated with a Proteinase K solution (1µg/µl Proteinase K, 100mM Tris-HCl pH 8.0, 50mM EDTA) for 15 min, then with an acetylation solution (DEPC ddH$_2$O, triethanolamine, acetic anhydride; solution pH 8.0) for 10 min, and were finally washed in DEPC ddH$_2$O. Slides were then transferred to a 3x SSC/50% formamide solution for between 15 min to 2 hours (time not critical), and then placed in a humidity chamber and 50 µl of hybridization solution containing the riboprobe of interest was added. The hybridization solution was comprised of 50% formamide, 1x Denhardt's solution, 3xSSC, 10mM EDTA, 10% dextran sulphate, 500µg/ml yeast tRNA, 500µg/ml salmon sperm DNA, and a 1:100 dilution of the DIG-UTP cRNA probe. Slides were incubated for 16-20 hours at 55°C. After incubation, slides were washed in 5xSSC (5 min, 55°C), 2xSSC/50% formamide (45 min, 55°C), NTE (10mM Tris-HCl, 1mM EDTA, 500mM NaCl; 15 min, 37°C), NTE and 20µg/ml RNAse A (30 min, 37°C), NTE (15 min, 37°C),

0.5xSSC/50% formamide (45 min, 55°C), 0.5xSSC (5 min, room temperature).

Following these steps the slides were transferred to chambers containing TBS (0.1 M

Tris-HCl pH 7.6, 150mM NaCl) 3 times for 5 min each. The slides were again placed in a

humidity chamber and TBS/10% fetal bovine serum (GIBCO) was added onto the

sections for 60 min at RT as a block. The TBS/10% FBS was poured off and new

TBS/10% FBS with a 1:2000 dilution of anti-digoxigenin-Fab-AP fragments (Roche,

Mississauga, ON) was added, and incubated at 4°C overnight. Slides were then washed in

TBS (3x 10 min) then developed in Buffer 3 (100mM Tris-HCl pH 9.5, 50mM $MgCl_2$,

100mM NaCl, 0.5mg/ml Levamisole, 200μl/10ml NBT/BCIP (Roche). The reaction was

stopped in TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA) and the slides were then

post-fixed in 4% PFA/PBS solution, washed in 1x PBS, transferred through graded

alcohols, Xylene, washed with water, and then mounted with Permount (Fisher

Chemicals, Nepean, ON). For combined immunohistochemistry and *in situ* hybridization,

immunohistochemistry was performed as described above on slides following the post-fix

in 4% PFA and without the previous treatment of proteinase K. All chemicals and

reagents were obtained from Sigma (St. Louis, MO) unless otherwise stated.

3.9 Reverse transcription polymerase chain reaction (RT-PCR) and

semiquantitative Real-Time polymerase chain reaction (Real Time-PCR)

Total RNA of mouse E13.5 striatal tissue was extracted using RNA-Bee reagent

(TEL-TEST INC, Friendswood, TX). 1 μg of total RNA was used as a template to

synthesize the cDNA. Prior to RT, 1ul of DNase I (Sigma) was added to the sample

for 15 minute at 37°C to digest the genomic DNA, then 1 ul of stop solution was added

to inactivate the DNase I. In order to denature the DNase I and to linearize the RNA, the

reaction was treated at 70°C for 10 minutes and then put on ice immediately. RT was performed for 5 minutes at 25°C, 30 minutes at 42°C, and later 5 minutes at 85°C with iScriptTM Reverse Transcriptase (Bio-RAD, Mississauga, ON). The cDNA was used as a template for both subsequent PCR and real-time PCR.

Real-time PCR was carried out using the IQ SYBR Green Supermix (Bio-RAD) and 1ul of the cDNA, 5 pmol of each primer (see list below) for each sample in a 25 µl reaction on an Icycler iQTM Multi-Color Real-Time PCR Detection System. PCR was performed at 95°C for 3 minutes, 95°C for 15 seconds, 57°C for 15 seconds, and 72°C for 30 seconds; for 40 cycles; then 95°C for 1 minute, 57°C for 1 minute, 57°C for 10 seconds; for 77 cycles in total with increasing set-point temperature after cycle 2 by 0.5°C per cycle. For real-time PCR, accumulation of the product was measured as an increase in SYBR green fluorescence and analyzed by the Icycler software. Standard curves relating initial template copy number to fluorescence and amplification cycle were generated using plasmid DNA as a template (standard), and were then used to calculate the mRNA copy number in each sample. The ratio of the intensities of the *Gad* and *Gapdh* (internal control) signals was considered to be a relative measure of the *Gad* mRNA level in each sample.

*Gad*2   Forward primer: 5'-CAAGATAAGCACTATGACCTGTCC -3' Tm=57°C

Reverse primer: 5'-CTCTGCTAGCTCCAAACACTTATC -3'

*Gad*1   Forward primer: 5'-TGACACCCAGCACGTACTC-3'   Tm=57°C

Reverse primer: 5'-CCAGTTTTCTGGTGCATCC-3'

Gapdh   Forward primer: 5'- TTGCCATCAATGACCCCTTCA-3'   Tm=57°C

          Reverse primer: 5'- CGCCCCACTTGATTTTGGA-3'

3.10 BrdU labelling of embryonic forebrain

Timed pregnant animals were injected with Bromodeoxyuridine (BrdU) (5mg/µl). For pulse labelling experiments, animals were sacrificed after 1 hour. Timed-pregnant CD-1 mice were pulsed at E13.5, E16.5, and E18.5. Sections were treated with 50% formamide/2xSSC at 65°C for 2 hours, 2xSSC at 65°C for 5 min, 2N HCl at 37°C for 30 min followed by 0.1M Boric acid pH 8.5 at RT for 10 min. Immunofluorescent detection of BrdU was performed as previously described (de Melo et al., 2005) with a mouse anti-BrdU antibody (Chemicon).

3.11 High Performance Liquid Chromatography (HPLC)

HPLC was performed in collaboration with Dr. Jerry Vriend, Department of Anatomy, University of Manitoba.

3.11.1 HPLC system

The HPLC system consisted of a Beckman model 116 solvent delivery system controlled by Beckman System Gold software, a Beckman model 210A sample injection valve, and a coulometric electrochemical detector (ESA Coulochem II, Model 5200, ESA Inc, Chelmsford, MA) with a model 5011A high-sensitivity analytical cell set at a oxidative potential of 400 mV. The mobile phase consisted of 0.1M Na_2HP0_4, 0.13mM Na_2EDTA, 28% methanol, adjusted to a pH of 6.0 (Donzanti and Yamamoto, 1988). The pump flow rate was 1 ml/min. Standards and samples were separated using a 3 µm HR-80 (4.6mm x 80mm i.d., ESA Inc) reverse phase column. The detector signal was converted from analog to digital using a Beckman model 406 analog interface

module. Concentrations of unknowns were calculated from standard curves made using the Beckman System Gold software. Homoserine was used as an internal standard.

3.11.2 Tissue preparation

E18.5 embryos were dissected and their forebrains were removed (excluding olfactory bulb) and immediately frozen in liquid nitrogen. The tissues were individually weighed, frozen, then homogenized and extracted in the mobile phase to a final concentration of 25mg of frozen weight per 1ml of mobile phase to adjust for the weight difference (Vriend et al., 1993). The extracts were separated by centrifugation for 5 min at 10,000g. The supernatants of extracts were filtered and diluted 1/10 for injection.

3.11.3 Derivatization procedure

The derivatization reagent was *o*-phthaldialdehyde (OPA) / 2-mercaptoethanol (βME) solution (Sigma, Oakville, ON). Manual pre-column amino acid derivatization was performed by mixing 20μl of the working derivatizing reagent with 40μl of diluted tissue extract. Derivatization was allowed to proceed for 2 minutes before injection onto the analytical system.

3.11.4 Standard solutions

Amino acid stock solutions containing glutamate (GLU), glutamine (GLN), gamma-aminobutyric acid (GABA), and homoserine (HSER) were prepared by dissolving 100μg of each powder (Sigma) in 1ml of mobile phase. Working standard solutions were prepared by diluting the stock solutions to GABA (50, 100, 200, 400

ng/ml), Glutamate and Glutamine (250, 500, 1000, 2000 ng/ml), and Homoserine (200, 400, 800, 1600 ng/ml). These standards were derivatized as described above.

3.11.5 Data analysis

Amino acid content was determined by comparing the peak areas of sample chromatograms to a standard curve prepared from the peak areas obtained for the external control chromatograms. To account for sample loss during the extraction procedure, an internal control (HSER) was used in tissue content for balancing the experimental analysis. In addition, each sample was measured for total protein concentration (Bradford method) (Bradford, 1976) and amino acid contents were re-calculated accordingly. Amino acid tissue contents were expressed as ng/injection (average ± standard deviation).

3.12 DLX2 knockdown in primary cell culture using small interfering RNA (siRNA) or small hairpin RNA (shRNA)

3.12.1 Generation of primary embryonic striatal or neocortical cell cultures:

E16.5 and E18.5 CD-1 embryos were used to produce primary cultures. On day 1, neocortex or striatum were dissected and collected in Hank's Balanced Salt solution (HBSS) (Invitrogen). The tissue was incubated for 10 min at room temperature with 0.05mg/ml trypsin (Invitrogen). The cells were then pelleted by centrifugation (1200 rpm, 5 min), resuspended, and gently triturated to single cell suspension in HBSS containing 100µg/ml DNase I (Sigma). The cell suspension was transferred to a tube containing Neurobasal medium with B-27 (Invitrogen) and penicillin-streptomycin-fungizone (100U/ml) (Invitrogen). Cells were counted and 3×10^7 cells were plated per

well using poly-D-lysine coated 24-well plates (Fisher Scientific, Ottawa, ON) and cultured at 37^oC with 5% CO_2 overnight.

3.12.2 Transfection of primary cultures with siRNA

Duplex siRNAs were designed by Shunzhen Zhang, Manitoba Institute of Cell Biology, University of Manitoba, and ordered from Invitrogen. The siRNAs were targeted to the exon 2 coding sequence of *Dlx*2 (Ensemble sequence ENSMUST00000024159). Two different siRNA duplexes and one non-silencing control siRNA were used for the experiment. Transient transfection using Lipofectamine 2000 (Invitrogen) was carried out on culture day 2. siRNAs to a final concentration of 40nM in Neurobasal medium with B-27 (Invitrogen) (following Qiagen RNAifect transfection protocol) were used. 48 hours after transfection, cells were washed twice with cold PBS, then subjected to the immunofluorescence protocol described in Section 3.7 and expression was quantified.

3.12.3 Transfection of primary cultures with Lentiviral shRNA

The viral shRNA production was done by Shunzen Zhang, Manitoba Institute of Cell Biology, University of Manitoba). For Lentivirus generation, HEK293 cells at 50-70% confluence in 100mm culture plates were transfected with 3μg ENV plasmid (VSV-G envelope), 5μg pMDLg/pRRE plasmid (gag/pol elements), 2.5μg pRSV-REV plasmid, and 10μg IRF-1/*Dlx*2 expression plasmid or 10μg of control GFP plasmid using FUGENE6 (Roche Diagnostics). 2 days after transfection, supernatants were cleared by centrifugation at 1,000g for 10min and viruses were pelleted at 50,000g for 90min at 4^oC. Viruses were then suspended overnight at 4^oC in 100μl of 50mM Tris-HCl, pH 7.8, 130mM NaCl, and 1mM EDTA. Infection of adherent cells using Polybrene

infection reagent (Millipore, Billerica, MA) was carried out on culture day 2. Neurobasal medium with B-27 (Invitrogen) was removed prior to addition of 250μl of virus in the presence of 5μg/ml of polybrene. Cells were incubated for 2-3 hours at 37°C with 5% CO_2. 750μl of Neurobasal with B-27 were subsequently added. 48 hours after infection, cells were washed twice with cold PBS, and subjected to the immunofluorescence described in Section 3.7 above.

3.13 Organotypic culture

Organotypic slice cultures of embryonic mouse forebrain were prepared as previously described (Anderson et al., 1997b), with several modifications. Briefly, embryos were placed in ice cold Krebs buffer containing 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.1 mM $CaCl_2$, 11 mM glucose, and 25 mM $NaHC0_3$. Brains were removed and embedded in 5% low-melt agarose (Invitrogen). 250 μm thick coronal sections were then cut on a tissue oscillator (EM Sciences) into cold Krebs buffer, and the sections were transferred to sterile Krebs (buffer pH 7.4, filtered Krebs with 10 mM HEPES, penicillin and gentamicin) on ice. After 15 min, the sections were transferred to polycarbonate culture membranes (13 mm diameter; pore size 8 μm) (Costar, Cambridge, MA) in Falcon organ tissue culture dishes containing of 1 ml of medium (MEM with glutamine, 10% fetal calf serum, 50% glucose, and pen/strep antibiotics) (Invitrogen). They were subsequently placed in a sterile incubator (5% CO2, 37°C) for 1 hr, after which the medium was changed to Neurobasal/B-27 (plus 1x glutamine, 50% glucose, and pen/strep antibiotics) (Invitrogen). DiI crystals (MolecularProbes, Invitrogen) were placed using an insect pin and a dissection stereomicroscope. After incubation for 24-48 hours, the slices were fixed in 4% PFA/PBS

for 1 hour, and mounted on glass slides with Vectashield mounting medium (Vector Laboratories). Cells were visualized on an Olympus BX51 fluorescent compound microscope using rhodamine fluorescence filters.

3.14 Cell counting and statistical analysis

The cell counting was done with the help of Tracie Parkinson, Manitoba Institute of Cell Biology, University of Manitoba. For quantification of cell populations in 4 possible genotypes (wild-type, *Nrp*2 single mutant, *Dlx*1/2 double mutant, *Dlx*1/2/*Nrp*2 triple mutant), pooled counts from a series of matched sections of coronal forebrain of each genotype were taken at regularly spaced intervals to completely survey each forebrain. Two to three sets of brains, each set consisting of one wild-type, one *Nrp*2-/- (single), one *Dlx*1/2-/- (double), one *Dlx*1/2/*Nrp*2-/- (triple) from the same littermates were used for quantification at E13.5, E16.5 and E18.5. A minimum of three littermates of each genotype was used for each experimental protocol. Brains were sectioned at 12μm for IHC, IF, and at 25μm for ISH. Sections were processed by IHC with cell-type specific markers. Sections were then imaged by confocal microscopy. Comparison between sets of count data was made using the student's T-test to determine statistical significance.

For organotypic cultures, coronal slices from each wild-type forebrain were anatomically matched by those from single, double, and triple mutant littermates. Brains were sectioned at 250μm for slice cultures. After DiI placement and subsequent culture, DiI labelled slices were fixed with 4% PFA and DiI labelled cells were quantified using an Olympus BX51 fluorescent compound microscope with rhodamine fluorescence filters. After PFA fixation, DiI tracing was also combined with IHC or ISH, and labelled

cells were then quantified. Comparisons between sets of data were made using the paired
T-test to determine statistical significance.

For DLX2 knock-down experiments using siRNA or shRNA, primary cultures of
neocortex or striatum of each genotype were generated (see **3.12**). After culturing,
primary cells were processed by IHC with cell-type specific markers and with DAPI stain
(Vector Laboratories). Labelled cells were counted using an Olympus BX51 fluorescent
compound microscope. Total cell numbers/section were determined by counting DAPI+
cells, then immunoreactive cells were counted and proportions were determined. The
Student's T-test was utilized to make comparisons between proportions of marker
expression between wild-type and different mutant genotypes.

3.15 Image acquisition and processing

Images were acquired using an Olympus IX70 microscope with Fluoview 2.0
confocal scanning or an Olympus IX81 inverted microscope with Fluoview FV500
confocal laser scanning system (Olympus Optical Co., Tokyo, Japan), an Olympus BX51
fluorescent microscope, or an Olympus SZX12 fluorescent stereomicroscopy system (all
Olympus Optical Co.). The Olympus BX51 and SZX microscopes utilized a SPOT 1.3.0
digital camera for photography (Diagnostic Instruments Inc. Sterling Heights, MI).
Images were processed using Adobe Photoshop 7.0 (Adobe Systems, Mountain View,
CA). Images were formatted, resized, and rotated for the purposes of presentation.

Chapter 4

Transcriptional cross-regulation between *Dlx* genes:

Identification of a direct *Dlx* homeodomain target in the developing mouse forebrain and retina by the optimization of chromatin immunoprecipitation

Rationale: Both *Dlx*1 and *Dlx*2 are highly expressed in the VZ and SVZ of the anlagen of the embryonic striatum which are the MGE and LGE (**Fig. 1**) (Eisenstat et al., 1999; Liu et al., 1997; Porteus et al., 1994). Peak levels of expression occur in forebrain tissues between E12.5 to E13.5 (Eisenstat et al., 1999). *Dlx*5 and *Dlx*6 are expressed in slightly different, but overlapping patterns: *Dlx*5 is expressed strongest in the SVZ, whereas *Dlx*6 is expressed strongest in the mantle zone (MZ) (**Fig. 1**). Neither of the *Dlx*5 nor *Dlx*6 genes are significantly expressed in the VZ (Liu et al., 1997).

Mutant mice that lack both *Dlx1* and *Dlx2* function have a time-dependent block in basal telencephalon differentiation (Anderson et al., 1997b). Although early neurogenesis appears to be normal, later neurogenesis is abnormal. This defective neurogenesis appears to be due to a defect in the production and/or function of commited neural progenitors within the SVZ. Accordingly, in the *Dlx1/2* mutants, *Dlx5* and *Dlx6* expression is not detected in the SVZ of the LGE and MGE, but is maintained in early born mantle cells at E12.5 (Anderson et al., 1997b). Therefore, it is plausible that the DLX1 or DLX2 proteins might be, at least in part, responsible for *Dlx*5 and *Dlx*6 expression via the mouse *Dlx*5/6 intergenic enhancer, which is highly active in the SVZ of the basal telencephalon (Zerucha et al., 2000).

4.1 DLX proteins bind to the *Dlx*5/6 intergenic enhancer (MI56)

Using an optimized ChIP procedure (Zhou et al., 2004) and specific polyclonal

Dorsal

Ventral

Pallium

Subpallium

NCx

VZ

SVZ

M

LGE

MGE
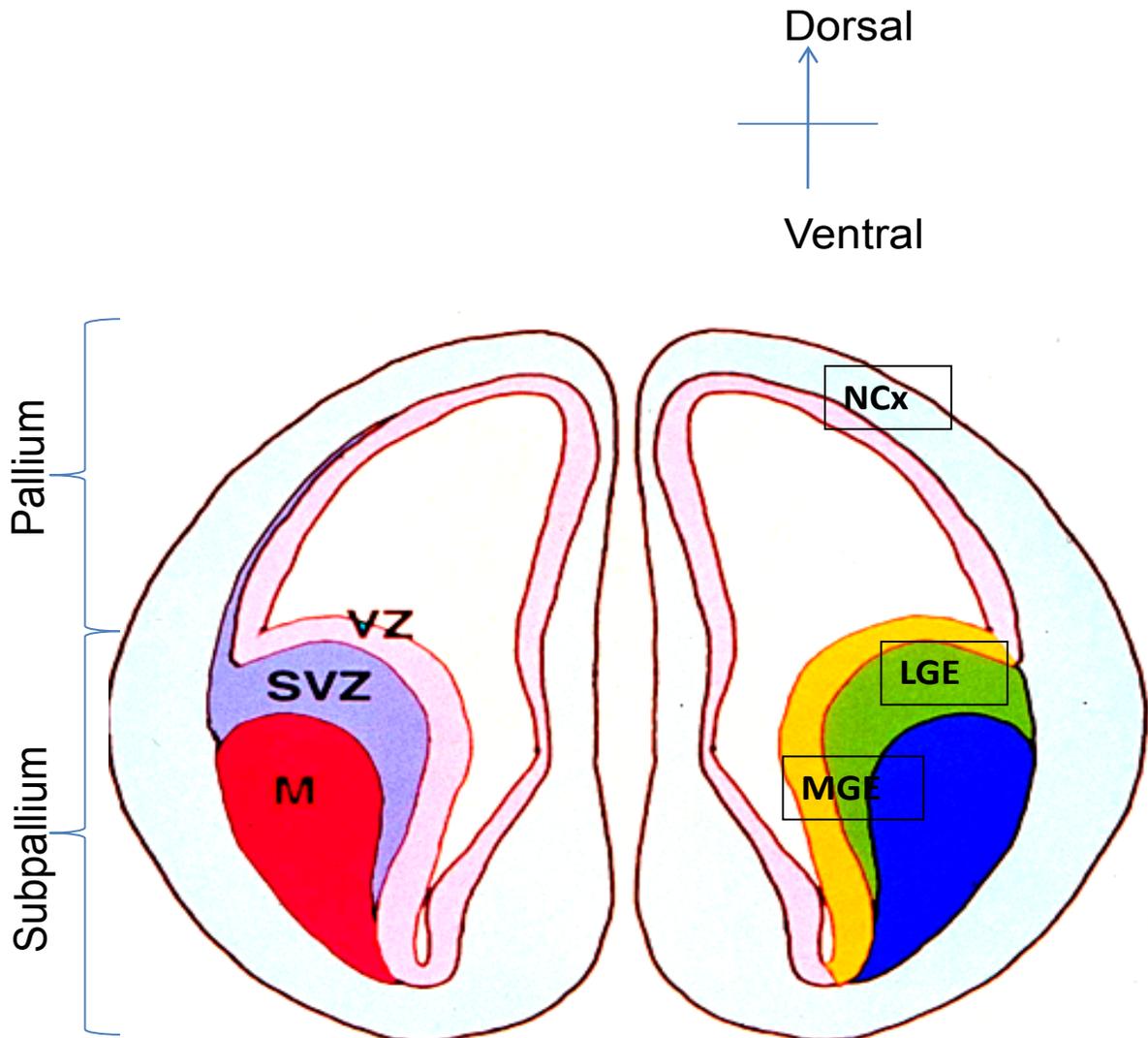
Dlx-1, Dlx-2

Dlx-1, Dlx-2, Dlx-5

Dlx-5, Dlx-6

Ventricular zone
Subventricular zone
Mantle zone

Figure 1. Expression domains of *Dlx*1*, Dlx*2*, Dlx*5 *and Dlx*6 during mouse brain development. This schematic diagram of a coronal section of the E13.5 mouse telencephalon shows the expression domains of the four *Dlx* transcripts. The coloured regions on the right (yellow, green, blue) are used to show the expression of *Dlx*1*, Dlx*2*, Dlx*5 *and Dlx*6*.* Telencephalic regions are as follows. Pallium: neocortex (NCx). Subpallium: lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE). Stages of differentiation are coloured (pink, purple, red): ventricular zone (VZ - pink); subventricular zone (SVZ - purple); mantle zone (MZ - red). Modified from Dr. John Rubenstein's adaptation of: Lumsden A and Gulisano M. Neocortical Neurons: Where do they come from? Science 1997, vol 278:5337, pg 402-403.

antibodies to DLX1 and DLX2 (Eisenstat et al., 1999), DLX1- and DLX2-bound DNA sequences were isolated. Following sonication, the average fragment size, representing candidate regulatory sequences, was 300 bp (Tomotsune et al., 1993). The intergenic enhancer region (MI56) between *Dlx*5 and *Dlx*6 was examined based on previous studies (**Fig. 2A and B**) (Zerucha et al., 2000). MI56 has two candidate homeodomain binding regions based upon groups of putative TAAT/ATTA DNA binding motifs: the I56i region (nucleotides 8641-9039) and I56ii (6543--6840); both have 10 motifs (Zerucha et al., 2000). We designed oligonucleotide primers to encompass both MI56 regions and performed PCR after ChIP of E13.5 GE. Both DLX1 and DLX2 bound to the I56i but not the I56ii region in E13.5 GE (**Fig. 3A).** Mouse genomic DNA was used as a positive control for PCR. Negative controls included omission of the specific antibody from the immunoprecipitation (**Fig. 3A**) and use of embryonic hindbrain, a tissue that does not express any of the *Dlx* genes (**Fig. 3B**). Bands obtained from PCR were subcloned and verified by sequencing.

4.2 Endogenous DLX1 and DLX2 are bound to specific homeodomain-DNA-binding motifs of the *Dlx*5/*Dlx*6 intergenic enhancer

EMSA were used to provide *in vitro* evidence for the binding of DLX1 and/or DLX2 to MI56 enhancer sequences isolated by ChIP *in situ*. We demonstrated specific DLX1- and DLX2-oligonucleotide complexes *in vitro* similar to the DLX3-oligonucleotide complexes shown by (Feledy et al., 1999b). Recombinant DLX1 and DLX2 bind to the I56i region and specific supershifted bands resulted from the addition of specific DLX1 or DLX2 antibodies (**Fig. 4**). The addition of a rabbit anti-Human Secretory Component (an irrelevant polyclonal primary antibody) did not result in a

A
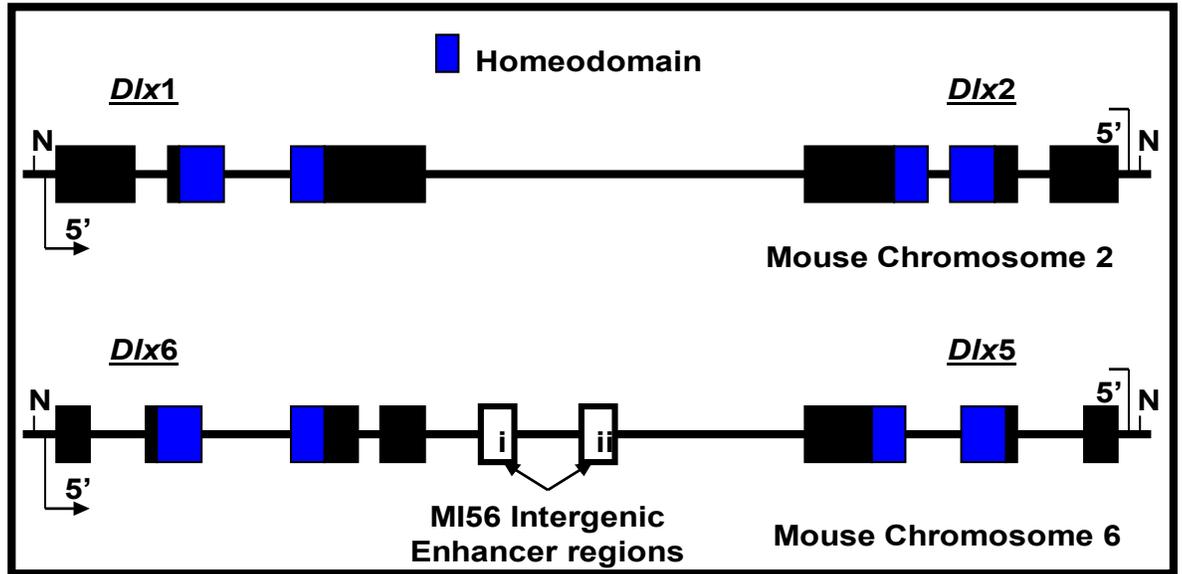
B

Dlx5/Dlx6 intergenic enhancer

I56i (nt 8641-9039)

```
8581 gaataattca gcaaagccct actaccagct gtacttctgc agcctcttcc attctttcca
8641 gcattataat tttggttaat tttcaatttt aggtcctacg tctctgcaat ttgtgtatga
8701 ataacagaat aatttccctc ttttgtttcg cctttcctgt tcctgaatct aaataaagat
8761 ggcttttag tattaaaagt ggaagaaat tacagg taat tatctttgac ggtaaaaacg
8821 ctgtaatcag cgggctacat gaaaattac tctaattatg gctgcattta agagaatgga
8881 aaaaaacctt cttgtggata aaaaccttaa attgtcccca atgtctgctt caaattggat
8941 ggcactgcag ctggaggctt tgttcagaat tgatcctggg gagctacgaa cccaaagttt
9001 cacagtagga aggggaaaa aagaagaaa acatttttcc taatgtaaca atgcgaatgc
9061 tagaaaatga caagactgat cggtttttaaa ccattctgaa
```

I56ii (nt 6543-6840)

```
6481 ttgagtgccc tgtgaaatta caatcgtaca ttttcaactc agcaacccat ttgcagtaca
6541 aaaaataggg tctaaataat ggctgaatta gccctactgg acagtttcag atgtaacact
6601 ctgtataatt atattgcagg ctggattagg atgctattat cataatctgg acgtttacaa
6661 ttatctgtaa tttgcaaaga tgcgccaggt cttgattaca gcagcttttt tttttttttt
6721 tttttttttt tttgtatcac gctaaccatc actaaacagt gacagtaata acagctaatt
6781 ttgctggcaa tataagaggt gctggggtgt gcaaacaatt tcacacctgg atgtgctcac
6841 tcaaccaaga atatagagac agagcctctg ccctgagact canagaaacg ctctcctgtg
```

Figure 2. (A) Genomic organization of the mouse *Dlx*1/*Dlx*2 and *Dlx*5/*Dlx*6 bigenic

clusters. *Hox* genes are sequentially oriented in a 3'-5' direction from anterior to

posterior, whereas the *Dlx* gene family is organized into three bigenic clusters with the

genes facing one another as 5'-3' and 3'-5' pairs. The *Dlx*5/*Dlx*6 intergenic region MI56

contains two enhancer regions, I56i and I56ii (Zerucha et al., 2000). The 60 amino acid

homeodomain (blue boxes) is highly conserved across *Dlx* family members. The

*Dlx*3/*Dlx*7 cluster is not included (Sumiyama et al., 2002). (B) Sequence and organization

of candidate homeodomain DNA binding motifs in the *Dlx*5/*Dlx*6 intergenic enhancer.

The MI56 sequence (GenBank, AY168010) contains the two enhancer elements, I56i and

i56ii, initially described by (Zerucha et al., 2000). Putative TAAT/ATTA homeodomain

DNA binding motifs are highlighted in bold italics. The two underlined I56i sequences

represent the 6th and 9th motifs critical for DLX1 and/or DLX2 binding (**Fig. 4B)** and

transcriptional activation (**Fig. 5**).

A

E13 striatum

gDNA No-Ab αDLX1   gDNA No-Ab αDLX2

I56i

I56ii

B

E13 hindbrain

No-Ab gDNA αDLX1 αDLX2

I56i

I56ii
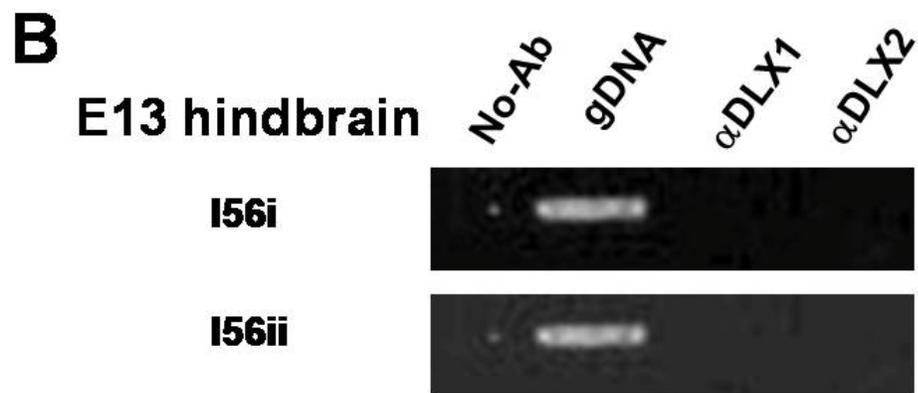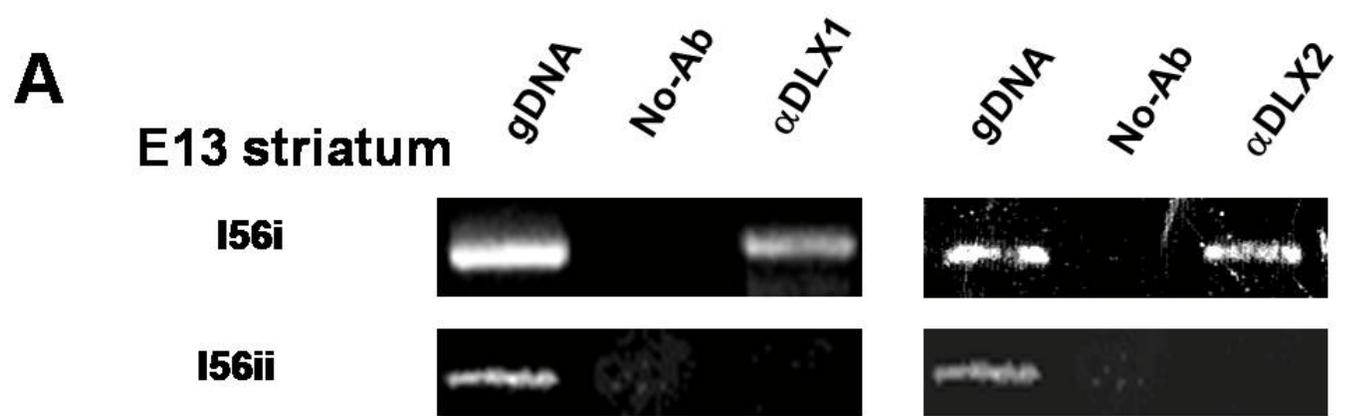
Figure 3. (A) Chromatin IP: DLX1 and DLX2 bind to the *Dlx*5/6 intergenic enhancer in embryonic striatum *in situ*. E13.5 GE was cross-linked with 1% PFA and immunoprecipitated with antibodies specific to either DLX1 or DLX2. After isolation of genomic DNA fragments bound to DLX1 or DLX2 homeoproteins, PCR using oligonucleotide primers to two regions (I56i and I56ii) of the *Dlx*5/6 intergenic region was performed. Total genomic DNA was used as a positive control. Both DLX1 and DLX2 bind to I56i but not to I56ii *in situ*. PCR products were subcloned and sequence-verified. (B) DLX1 and DLX2 antibodies do not isolate the Dlx5/6 intergenic enhancer from embryonic hindbrain. E13.5 hindbrain was selected as a negative tissue control for ChIP with specific DLX1 or DLX2 antibodies. In embryonic hindbrain, neither DLX1 nor DLX2 is expressed. PCR bands were subcloned and sequenced for verification. This figure is contributed by Q. Zhou as a co-author from the Eisenstat laboroatry, Manitoba Institute of Cell Biology, University of Manitoba.

supershift, indicating that the supershifts we did see are specific for the DLX1 or DLX2 antibodies (**Fig. 4A**). We then developed EMSA for nuclear extracts enriched for DLX1 and DLX2 expression obtained from E13.5 GE tissues. Several of the 10 TAAT/ATTA DNA-binding motifs located in the I56i DNA binding region (**Fig. 2B**) were previously shown by (Zerucha et al., 2000) to bind DLX2 *in vitro*; DLX1 binding to this region was not previously assessed. We sequentially deleted each region and determined that only the ninth (**Fig. 4B**, panel A) and sixth (**Fig. 4B**, panel B) homeodomain binding motifs were required for binding both DLX1 and DLX2 in embryonic striatum *in vitro* corroborating the findings of (Zerucha et al., 2000). Supershift experiments confirmed the specificity of each DLX protein-DNA complex. We conclude from these experiments that both recombinant DLX1 and DLX2 and embryonic striatal nuclear extracts enriched for DLX1 and DLX2 expression bind to region 1 of the *Dlx*5/6 intergenic enhancer (I56i) *in vitro*.

4.3 The DLX2 homeoprotein preferentially activates reporter gene activity when bound to its target sequences in the *Dlx*5/*Dlx*6 intergenic enhancer

Although the ChIP assay was used to isolate mouse I56i as a DLX1 and DLX2 DNA target and EMSA confirmed specific protein-DNA interactions *in vitro*, it was important to demonstrate the functional significance of DLX1 or DLX2 binding to this enhancer element. P19 embryonal carcinoma cells are pluripotent cells that can differentiate into neuronal cells under specific conditions, such as treatment with retinoic acid (Berg and McBurney, 1990). Transfection of DLX2, but not DLX1, activated the expression of a co-transfected luciferase reporter gene construct (**Fig. 5**). Western blotting confirmed that recombinant DLX1 and DLX2 proteins are both expressed in the

A

Oligonucleotide primer design for ChIP PCR

[Primers are **bold underlined** and putative homeodomain DNA binding sites *(TAAT/ATTA)* are in bold italics].

Mouse Glutamic acid decarboxylase (GAD) 65 promoter:

GAD65i (region i)

5'-**tttctg ggtggctcac agt**ggacaag gaatagg*taa t*cccaccaaa ccttgaggtg ggctaaactg gagattttgg tatgggtctc ctactgtaaa gagaaagcct tccgggctaa cggtattcct ttcgcagtct actgcctcct ttgtgaac*ta at*gactg*taa ttatta*cctc ccagagctct **tttgttatca acaacccgga**-3' (nt1425-1630)

GAD65ii (region ii)

5'-**aaaagggaaa cagaaagga**t tttttcattc cttt*atta*ga aaacacaaac gaaactactt ctttctgggc tgggaaattg ccactgttgt gttcctaggc acctgctgat ggcaagaact cctcttggga agggacgagg tcagtgggtt gagtatccct gaaaagatgg gagtgccttt cctgggatt tggaaaaact gattcgctta ctttgagact ccctgaatcc tgtgtatgtg tcagctctca cccactccaa gaagtttctg ttcgttttat ttatt*taat*t tttctcagcc tgaggtcctc agtgatagat tccagcgtgg atttt*taat*tg c**ttcaatcag cagcctttct**-3' (nt2761-3120)

Mouse Glutamic acid decarboxylase (GAD) 67 promoter

GAD67i (region i)

5'-**cgcc ctctggtgga aatttt**ttcc aataccgcct ttttatggtg ggggacttca ccccgctcag *taat*gtgctt aaatattgcc aagagagaat cattgaacag cgtgaaactg aaaagaagcg aggagagcgg gccaagacac ggagtgccgt cctggcttcc cggagccacc ctccatgctc tgctgcccca ggcgtgtgga gcggcactcg tgcgtgtt*at ta*accttcag acatgatca*a tta*cgaagat **ttcttttacc cctctccagg**-3' (nt187-460)

GAD67ii (region ii)

5'-**gatacggg atggagggct** aagaagaggg gaaggaagaa tcacccagtg agacaaaatt cttcgtagga *atta*tatttt cccttgccct cacccaacat cgcctatctc aaaaat*aat*t taaaacaaa aaacaaacaa acaaacaaaa aacagagcgt gctgagtgca ttctgg*atta* ctcataggac tttgtcacac acaccctcct ttctggtcgc aaacccgtga gctggattta *taat*cgccct **acaaagctcc agaggcagtc**-3' (nt743-1010)

B

Striatum   **Hindbrain**

Figure 4. (A) Electrophoretic mobility shift assays (EMSA) demonstrate that recombinant DLX1 and DLX2 bind to the *Dlx*5/*Dlx*6 intergenic enhancer *in vitro*. EMSA were performed using recombinant DLX1 or DLX2 proteins (rDLX) and a radiolabeled I56i oligonucleotide probe, with unlabeled competition using excess unlabelled probe (lanes 3 and 7) and specific DLX antibody 'supershift' assays (lanes 4 and 8). Lane 1: labelled I56i alone. Lanes 2-5: labelled I56i probes were incubated with rDLX1 (2), with rDLX1 and unlabeled I56i (3), with rDLX1 and anti-DLX1 (4), or with rDLX1 and a rabbit anti-Human Secretory Component (an irrelevant antibody) (5). Lanes 6-9: labelled I56i probes were incubated with rDLX2 (6), with rDLX2 and unlabeled I56i (7), with rDLX2 and anti-DLX2 (8), or with rDLX2 and irrelevant antibody (9) (r: recombinant; 1: DLX1/anti-DLX1; 2: DLX2/anti-DLX2; I: irrelevant antibody). (B) EMSA of embryonic striatum demonstrates DLX1 and DLX2 binding to specific homeodomain binding motifs within the *Dlx*5/6 intergenic enhancer *in situ*. EMSA was performed using E13.5 GE tissue nuclear extracts and oligonucleotides for four TAAT/ATTA DNA binding motifs contained within I56i. Only the sixth and ninth motifs were bound by E13.5 GE. (Panel A) EMSA for homeodomain binding motif #9 (position 8853-8860 bp, lanes 1-6). (Panel B) EMSA for motif #6 (position 8797-8804 bp, lanes 7-12). Unlabeled competition assays are shown in lanes 3 and 9. Supershift assays with specific DLX antibodies are seen in lanes 4 and 10 (αDLX1) and lanes 5 and 11 (αDLX2).
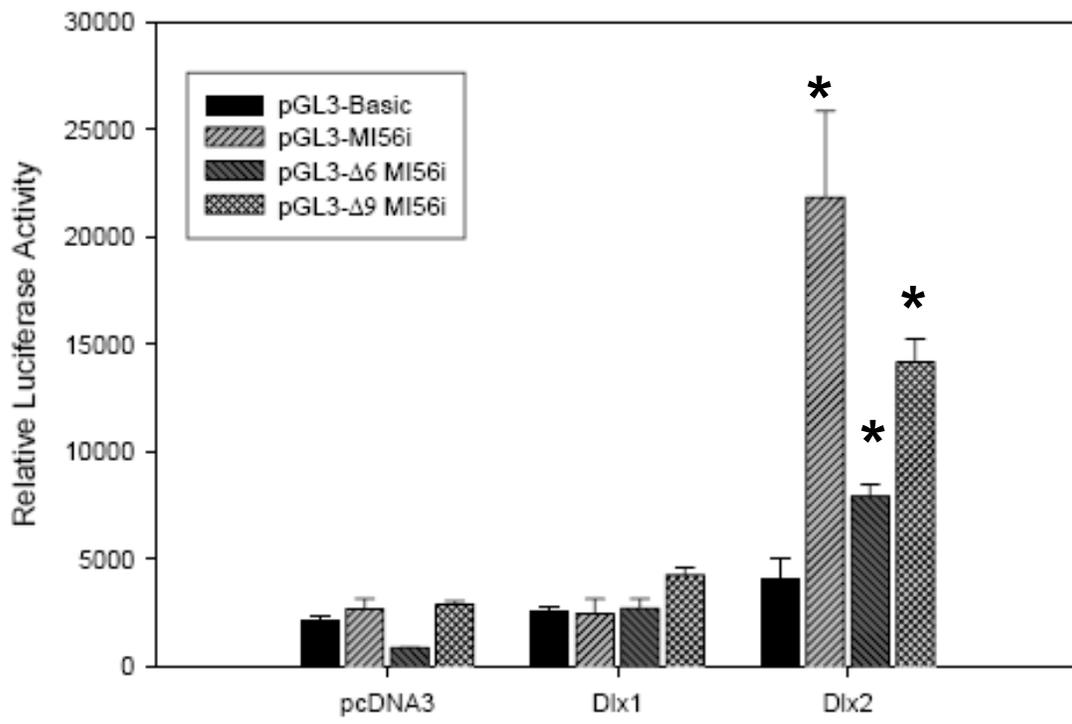
Figure 5. DLX2 expression activates transcription of a luciferase reporter gene containing the *Dlx*5/6 intergenic enhancer *in vitro*. In P19 embryonic carcinoma cells, a luciferase gene reporter construct containing I56i or I56i with or without mutations of either the 6th or 9th homeodomain DNA binding motifs, was co-transfected with expression constructs for DLX1 or DLX2. DLX2, but not DLX1, co-transfection activated reporter gene expression. Site-directed mutagenesis of the sixth TAAT motif significantly reduced reporter gene activity for DLX2 co-transfection. Data shown are relative luciferase activity units (average ± standard deviation) of at least three trials. All luciferase activities are normalized to the activity level of the internal control β-gal. Error bars represent standard error of measurement. * denotes p<0.05. This figure is contributed by Q. Zhou as a co-author, Eisenstat laboratory, Manitoba Institute of Cell Biology, University of Manitoba

transfected P19 cells (data not shown). Site-directed mutagenesis of the sixth or ninth

TAAT/ATTA motifs was completed prior to cotransfection with DLX expression

constructs. Mutation of the sixth motif significantly reduced reporter gene activity

mediated by DLX2, whereas mutation of the ninth motif reduced luciferase activity, but

to a lesser extent, following DLX2 cotransfection (**Fig. 5**). These results are similar to

those obtained by (Zerucha et al., 2000) using the zebrafish *dlx*4/*dlx*6 intergenic

enhancer. Unlike DLX2, reporter gene expression was not affected by DLX1 co-

transfection studies with the intact I56i enhancer element or mutated I56i enhancers

containing mutations of the sixth and ninth TAAT/ATTA motifs. Co-transfection of both

DLX1 and DLX2 did not result in any additive or synergistic effects as compared with

co-transfection of DLX2 alone (data not shown). This study provides further

confirmation of the cross-regulatory roles between *Dlx* homeobox genes as well as the

overlapping and distinct transcriptional roles of DLX1 and DLX2.

Chapter 5

*Dlx*1/2 genes and differentiation of GABAergic interneurons:

GABAergic interneuron differentiation in the basal forebrain is mediated by direct regulation of Glutamic Acid Decarboxylase isoforms by *Dlx* homeobox genes

Rationale: Most cortical GABA (gamma-aminobutyric acid)-producing interneurons are born in the subpallial telencephalon and migrate tangentially to the neocortex in several migratory streams (Anderson et al., 1997a; Anderson et al., 1997b; Anderson et al., 2002; Marin et al., 2000). Genetic analysis of the embryonic basal telencephalon reveals nearly overlapping expression of *Dlx*1 and *Dlx*2 primarily in regional restricted domains including the VZ and SVZ (Eisenstat et al., 1999; Liu et al., 1997). Previous studies showed that in mice lacking both *Dlx*1 and *Dlx*2, there is no detectable tangential interneuronal migration from the subcortical telencephalon to the neocortex and resulting in few GABA-expressing cells residing in the neocortex (Anderson et al., 1997a; Anderson et al., 1997b). Ectopic expression of the *Dlx* genes through electroporation gain-of-function assays in slice cultures of the embryonic mouse forebrain can induce cortical cells to express the glutamic acid decarboxylase (*Gad*) genes (Stuhmer et al., 2002a). However, the prior studies have not demonstrated that the transcriptional association of *Dlx* genes with GABA expression derives from the direct activation of transcription of *Gad*1 and *Gad*2 by DLX1 and DLX2 through binding of these homeodomain proteins to *Gad* isoform promoters.

5.1 DLX1 and DLX2 homeodomain proteins are bound to the *Gad*1 and *Gad*2 promoters *in situ*

We utilized the ChIP technique (Zhou et al., 2004) to identify the binding of DLX1 and/or DLX2 to the *Gad*1 (formerly *Gad*67) and *Gad*2 (formerly *Gad*65) promoters in embryonic striatum *in situ*. ChIP was performed as outlined above. We then used PCR to amplify two candidate homeodomain-binding regions in each of the *Gad*1 and *Gad*2 loci. These regions were chosen based on the presence of TAAT/ATTA homeodomain DNA binding motifs, and were designated GAD65 (region i, nucleotides 1425-1630; region ii, nucleotides 2761-3120, GenBank: AB032757) and GAD67 (region i, nucleotides 187-460; region ii, nucleotides 743-1010, GenBank: Z49978) (**Fig. 6A**). Of interest, the ChIP assay showed that both DLX1 and DLX2 bind to both regions i and ii in the promoter regions of both GAD65 and GAD67 *in situ* (**Fig. 6B**). Mouse genomic DNA was used as a positive control for PCR. Omission of the specific antibody served as a negative control for immunoprecipitation, whereas embryonic hindbrain tissues that do not express *Dlx* genes served as a negative tissue control for the ChIP assay. The resulting ChIP-PCR products of the identified promoter regions were subcloned and sequenced to verify DLX targeting of *Gad*1 and *Gad*2 promoters and for subsequent biochemical analyses.

5.2 DLX1 and DLX2 specifically bind to the *Gad* promoters *in vitro*

To determine the specificity of binding of DLX1 and DLX2 to both regions i and ii of each of the *Gad*65 and *Gad*67 promoters *in vitro*, we used recombinant DLX1 and DLX2 proteins and radioactive α^{32}P-dATP labeled *Gad*65 or *Gad*67 promoter oligonucleotides for each region isolated from the ChIP assay *in situ*. EMSAs demonstrated

A

Oligonucleotide primer design for ChIP PCR

[Primers are **<u>bold underlined</u>** and putative homeodomain DNA binding sites *(TAAT/ATTA)* are in bold italics].

Mouse Glutamic acid decarboxylase (GAD) 65 promoter:

GAD65i (region i)

5'-**<u>tttctg ggtggctcac agt</u>**ggacaag gaatag*gtaa t*cccaccaaa ccttgaggtg ggctaaactg gagattttgg tatgggtctc ctactgtaaa gagaaagcct tccgggctaa cggtattcct ttcgcagtct actgcctcct ttgtgaac*ta at*gactg*taa ttatta*cctc ccagagctct **<u>tttgttatca acaacccgga</u>**-3' (nt1425-1630)

GAD65ii (region ii)

5'-**<u>aaaagggaaa cagaaagga</u>**t tttttcattc cttt*atta*ga aaacacaaac gaaactactt ctttctgggc tgggaaattg ccactgttgt gttcctaggc acctgctgat ggcaagaact cctcttggga agggacgagg tcagtgggtt gagtatccct gaaaagatgg gagtgccttt cctgggatt tggaaaaact gattcgctta ctttgagact ccctgaatcc tgtgtatgtg tcagctctca cccactccaa gaagtttctg ttcgttttat ttattt*taatt* tttctcagcc tgaggtcctc agtgatagat tccagcgtgg atttt*taatt*g c**<u>ttcaatcag cagcctttct</u>**-3' (nt2761-3120)

Mouse Glutamic acid decarboxylase (GAD) 67 promoter

GAD67i (region i)

5'-**<u>cgcc ctctggtgga aatttt</u>**ttcc aataccgcct ttttatggtg ggggacttca ccccgctcag *taat*gtgctt aaatattgcc aagagagaat cattgaacag cgtgaaactg aaaagaagcg aggagagcgg gccaagacac ggagtgccgt cctggcttcc cggagccacc ctccatgctc tgctgcccca ggcgtgtgga gcggcactcg tgcgtgtt*at ta*accttcag acatgatca*a tta*cgaagat **<u>ttcttttacc cctctccagg</u>**-3' (nt187-460)

GAD67ii (region ii)

5'-**<u>gatacggg atggagggct</u>** aagaagaggg gaaggaagaa tcacccagtg agacaaaatt cttcgtagga *atta*tatttt cccttgccct cacccaacat cgcctatctc aaaaa*taat*t taaaacaaa aaacaaacaa acaaacaaaa aacagagcgt gctgagtgca ttctgg*atta* ctcataggac tttgtcacac acaccctcct ttctggtcgc aaacccgtga gctggattta *taat*cgccct **<u>acaaagctcc agaggcagtc</u>**-3' (nt743-1010)

B

Striatum   Hindbrain

Figure 6: DLX proteins associate with *Gad*1 (GAD67) and *Gad*2 (GAD65) through the regulatory homeodomain binding motifs of the *Gad* promoters. (A) The sequences of regulatory elements within the mouse GAD65 (GenBank AB032757) and GAD67 (Genbank Z49978) promoters, designated GAD65i/67i and GAD65ii/67ii, contain putative homeodomain DNA-binding sites (*TAAT/ATTA*), in italics. Oligonucleotide primers used for PCR are bolded and underlined and the TAAT motifs critical for binding to DLX1 and DLX2 are in bold and are italicized. (B) DLX1 and DLX2 target *Gad*1 and *Gad*2 promoter in striatal E13.5 tissues using chromatin immunoprecipitation. ChIP assays of GAD65 and GAD67 promoter on E13 striatal tissue shows that DLX1 and DLX2 bind to both regions (region i and region ii) of each *Gad* gene promoter using specific DLX1 and DLX2 antibodies. Negative controls utilized no addition of antibodies and/or E13.5 hindbrain tissues. The positive control was E13.5 genomic DNA as well as I56i previously validated in our laboratory as a DLX1 and DLX2 transcriptional target. PCR bands were subcloned and sequenced for confirmation. Figure 6B is contributed by Q. Zhou as a co-author from the Eisenstat laboratory, Manitoba Institute of Cell Biology, University of Manitoba.

specific binding of both DLX1 and DLX2 to the *Gad*65 promoter for both regions i and ii (**Fig. 7A**, lanes 2, 6; **Fig. 7B**, lane 2, 6) that can be competitively inhibited by unlabelled *Gad*65 probes (**Fig. 7A**, lanes 3, 7; **Fig. 7B**, lane 3, 7). Moreover, the addition of specific anti-DLX1 or anti-DLX2 antibodies to the protein–DNA complexes resulted in significant "supershifts" (**Fig. 7A** and **7B**, lanes 4, 8), while a nonspecific polyclonal antibody failed to produce such a "supershift" (**Fig. 7A** and **7B**, lanes 5, 9). Within the *Gad*65 regions i and ii, there are homeodomain binding motifs that are necessary for the binding of DLX proteins to these regions (**Fig. 6A**, bold and italicized TAAT/ATTA motifs). Recombinant DLX1 and DLX2 proteins and E13.5 GE nucleoprotein extracts both specifically bind to oligonucleotides containing 3 consecutive TAAT/ATTA motifs (nucleotides 1571-1603) of the *Gad*65 region i, and to oligonucleotides containing the third TAAT/ATTA motif (nucleotides 3086-3107) of *Gad*65 region ii (data not shown). Other candidate homeodomain binding motifs in the *Gad*65 promoter were not bound to recombinant DLX1 or DLX2 (data not shown).

EMSA experiments also demonstrated that DLX1 and DLX2 specifically bind to *Gad*67 promoter regions i and ii (**Fig. 8A** and **8B**, lanes 2, 6) and that this binding can be competitively inhibited by unlabeled *Gad*67 probes (**Fig. 8A** and **8B**, lanes 3, 7). Moreover, the addition of specific anti-DLX1 or anti-DLX2 antibodies to the protein–DNA complexes resulted in significant band mobility shifts (**Fig. 8A** and **8B**, lanes 4, 8), while a nonspecific polyclonal antibody failed to produce such a "supershift" (**Fig. 8A** and **8B**, lanes 5, 9). Within the *Gad*67 regions i and ii, there are homeodomain binding motifs necessary for the binding of DLX proteins to the *Gad*67 promoter (**Fig. 6A**, bold and italicized TAAT/ATTA motifs). Recombinant DLX1 and DLX2 proteins and E13.5

A

Antibody	–	–	–	1	I	–	–	2	I
rDLX	–	1	1	1	1	2	2	2	2
Unlabelled probe	–	–	+	–	–	–	+	–	–
Labelled probe	+	+	+	+	+	+	+	+	+
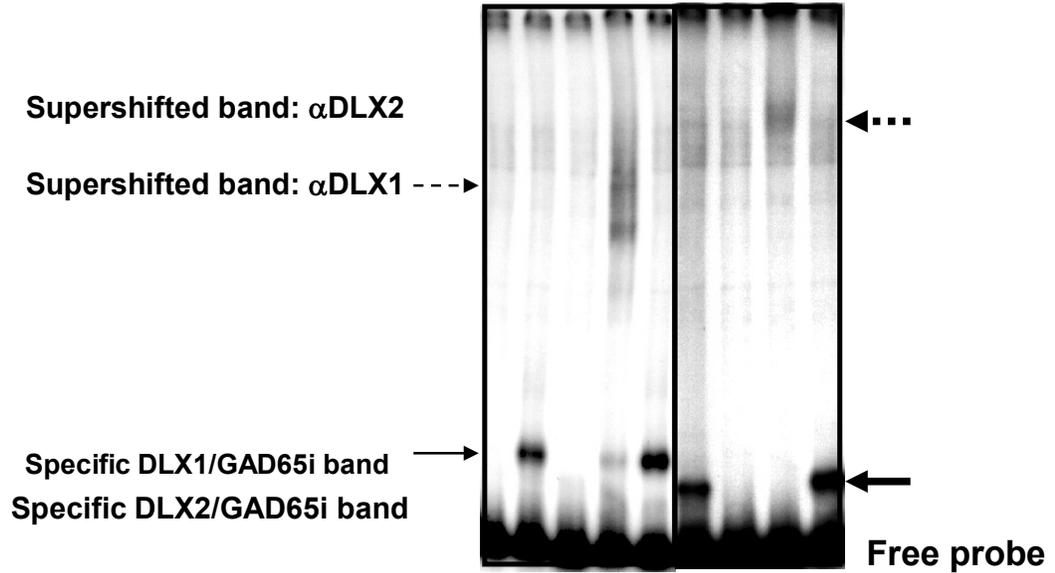
1   2   3   4   5   6   7   8   9

Supershifted band: αDLX2

Supershifted band: αDLX1 - - - →

Specific DLX1/GAD65i band

Specific DLX2/GAD65i band

Free probe

B

Antibody	–	–	–	1	I	–	–	2	I
rDLX	–	1	1	1	1	2	2	2	2
Unlabelled probe	–	–	+	–	–	–	+	–	–
Labelled probe	+	+	+	+	+	+	+	+	+

1   2   3   4   5   6   7   8   9

Supershifted band: αDLX2

Supershifted band: αDLX1 - - - →

Specific DLX1/GAD65ii band

Specific DLX2/GAD65ii band

Free probe

Figure 7: DLX1 and DLX2 proteins demonstrate specific binding to *Gad*65 promoter regions i and ii *in vitro*. EMSA shows recombinant DLX1 or DLX2 binding to *Gad*65 promoter region i (A) and region ii (B) oligonucleotides containing homeodomain binding sites. Labelled *Gad*65i or ii oligonucleotides were incubated alone (Lane 1), with DLX1 proteins (Lane 2-5), with DLX2 proteins (Lane 6-9), using unlabeled competition (Lane 3, 7), with specific DLX antibodies (Lane 4, 8), and with non-specific antibodies (Lane 5, 9). Binding of DLX proteins to specific *Gad*65 oligonucleotide sequences results in gel shifts, indicated by solid arrows. Supershifts with specific DLX antibodies are indicated by broken arrows (DLX1: unbold, DLX2: bold). [r: recombinant; 1: DLX1/anti-DLX1; 2: DLX2/anti-DLX2; I: irrelevant/nonspecific antibody].

A

Antibody	–	–	–	1	l	–	–	2	l
rDLX	–	1	1	1	1	2	2	2	2
Unlabelled probe	–	–	+	–	–	–	+	–	–
Labelled probe	+	+	+	+	+	+	+	+	+

1 2 3 4 5 6 7 8 9

Supershifted band: αDLX2 ◄····

Supershifted band: αDLX1 ---►

Specific DLX1/GAD67i band →

Specific DLX2/GAD67i band →

Free probe

B

Antibody	–	–	–	1	l	–	–	2	l
rDLX	–	1	1	1	1	2	2	2	2
Unlabelled probe	–	–	+	–	–	–	+	–	–
Labelled probe	+	+	+	+	+	+	+	+	+

1 2 3 4 5 6 7 8 9

Supershifted band: αDLX2 ◄····

Supershifted band: αDLX1 ---►

Specific DLX1/GAD67ii band →

Specific DLX2/GAD67ii band →

Free probe
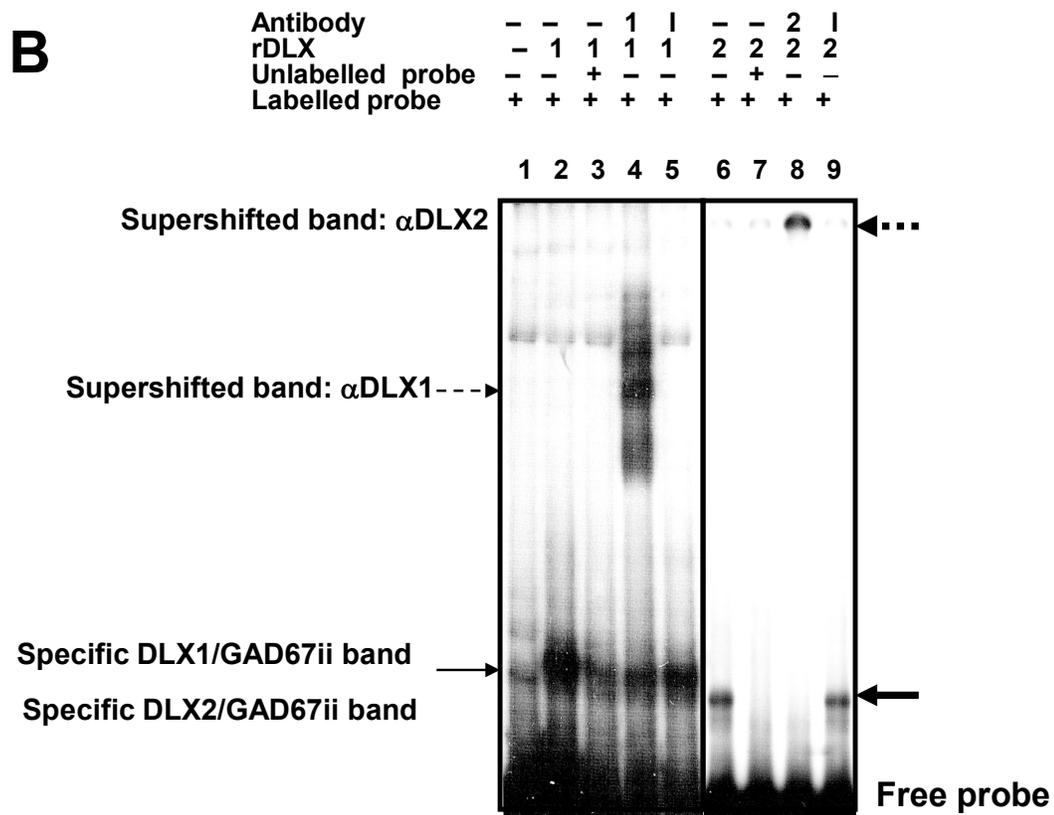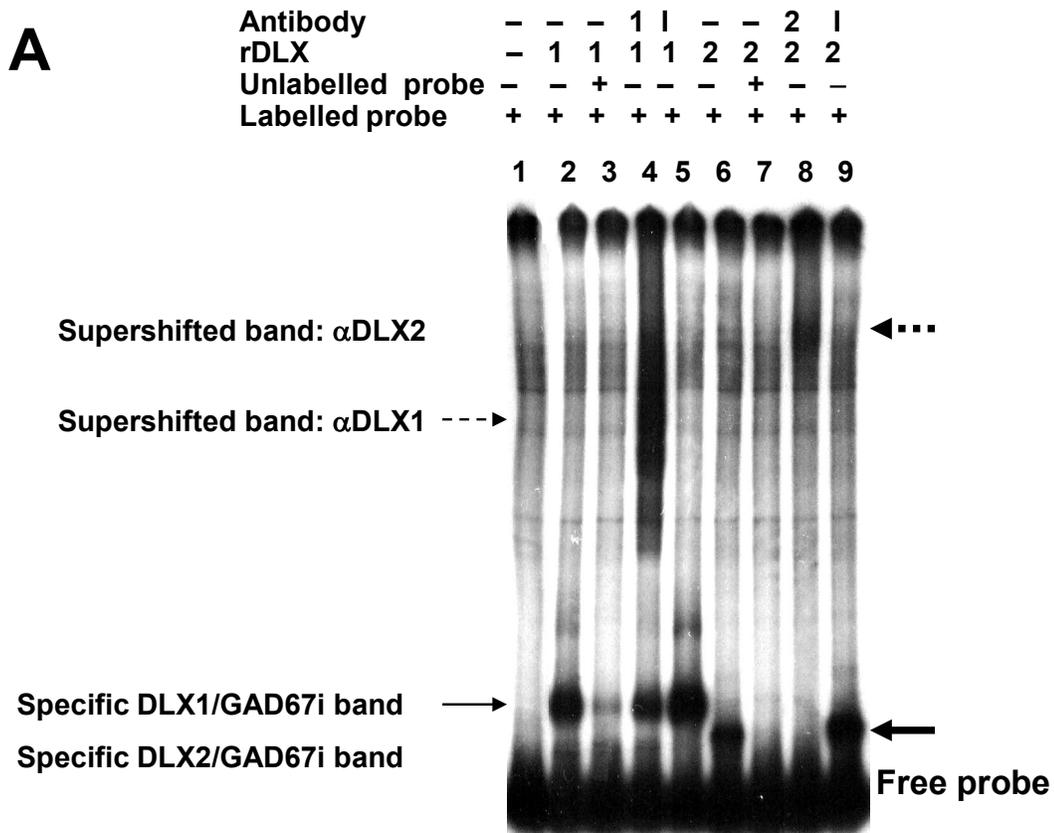
Figure 8: DLX1 and DLX2 proteins demonstrate specifical binding to the *Gad*67 promoter regions i and ii *in vitro*. EMSA shows recombinant DLX1 or DLX2 binding to *Gad*67 promoter region i (A) and region ii (B) oligonucleotides containing homeodomain binding sites. Labelled *Gad*67i or ii oligonucleotides were incubated alone (Lane 1), with DLX1 proteins (Lane 2-5), with DLX2 proteins (Lane 6-9), using unlabeled competition (Lane 3, 7), with specific DLX antibodies (Lane 4, 8), and with non-specific antibodies (Lane 5, 9). Binding of DLX proteins to specific *Gad*67 oligonucleotide sequences results in gel shifts, indicated by solid arrows. Supershifts with specific DLX antibodies are indicated by broken arrows (DLX1: unbold, DLX2: bold). [r: recombinant; 1: DLX1/anti-DLX1; 2: DLX2/anti-DLX2; I: irrelevant/nonspecific antibody].

GE nuclear extracts enriched for these homeodomain proteins both bind to oligonucleotides containing the 2nd and 3rd TAAT/ATTA motifs (nucleotides 400- 420 and 421- 440) of region i, and to oligonucleotides containing the fourth TAAT/ATTA motif (nucleotides 971-990) of region ii (data not shown). Other homeodomain binding motifs in the *Gad*67 promoter were not significantly bound to recombinant DLX1 and DLX2 (data not shown).

5.3 DLX1 and DLX2 activate transcription of *Gad* promoters *in vitro*

The functional significance of DLX1 and DLX2 binding to the *Gad* promoters was assessed using luciferase reporter gene assays. We co-transfected C6 rat glioma cells (or P19 embryonal carcinoma cells, data not shown) with an expression vector encoding either DLX1 or DLX2 and a luciferase vector into which regions i or ii of the *Gad*65 promoter (1425-1630nt and 2761-3120nt, respectively) were subcloned. For maximal activity of *Gad*67 promoter, we cloned the full-length ~1.3kb promoter using a standard PCR protocol with flanking primers designed according to the mouse *Gad*67 promoter sequence (Genbank accession number Z49978). Co-transfection with either wild-type *Dlx*1 or *Dlx*2 expression constructs resulted in significant increases of luciferase activity, compared with controls, for the *Gad*65 reporter constructs (region i: ~7 fold for DLX1 and ~21 fold for DLX2; region ii: ~2 fold for DLX1 and ~11 fold for DLX2, **Fig. 9A**). Wild-type *Dlx*1 and *Dlx*2 also activate *Gad67* reporter gene expression (~3 fold for DLX1 and ~13 fold for DLX2, **Fig. 9B**) in the same manner as was observed for *Gad*65. The increase in reporter gene activity driven by co-expression of either DLX1 or DLX2 proteins indicates that DLX homeodomain proteins act as transcriptional activators of *Gad*1 and *Gad*2 promoter expression *in vitro*.

A
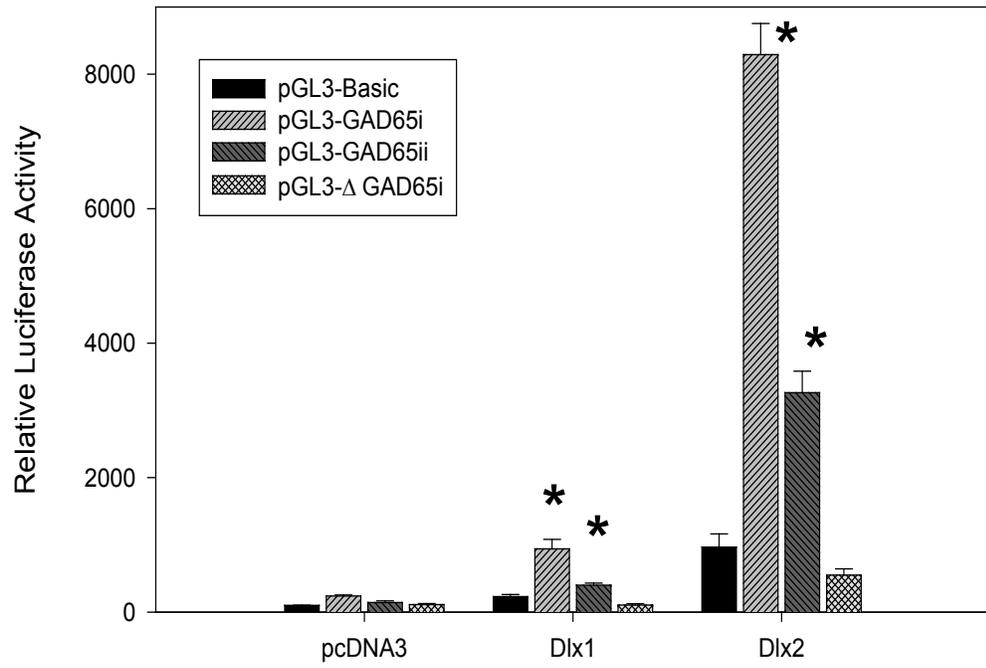
B
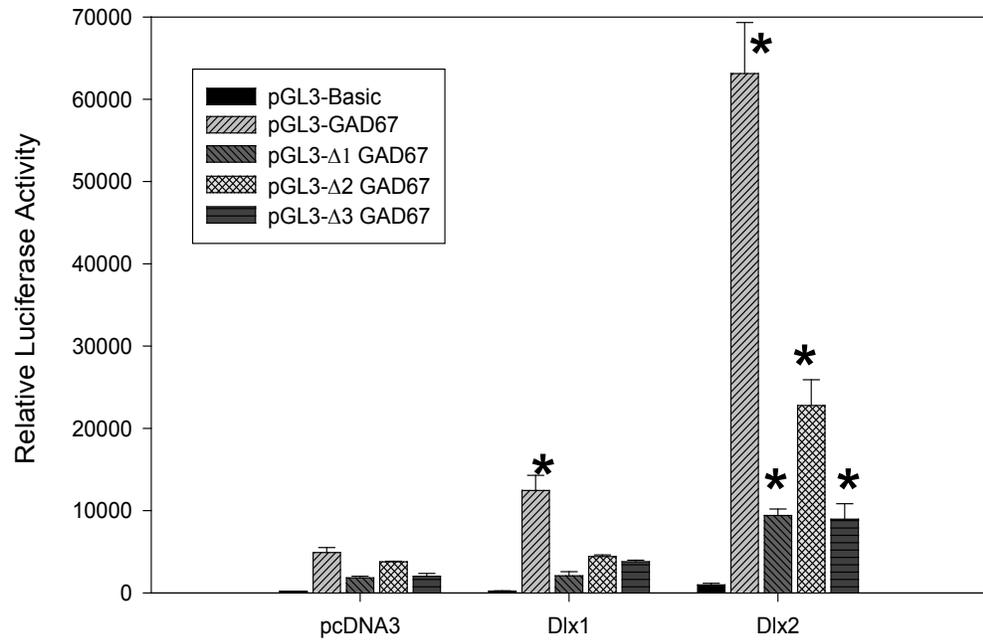
Figure 9: *Dlx*1 and *Dlx*2 activate transcription of *Gad*1 (*Gad*67) and *Gad*2 (*Gad*65) reporter constructs *in vitro*. Transient transfection assays in C6 glioma cells with (A) *Gad*65 promoter regions i and ii, and (B) *Gad*67 1.3kb promoter constructs, containing homeodomain binding sites cloned into a pGL3-Luciferase reporter construct, in the absence or presence of expression of *Dlx*1 or *Dlx*2. *Dlx*1 and *Dlx*2 activate transcription of the reporter genes for the *Gad*65 and *Gad*67 promoters, with DLX2 as a more robust activator. Mutations of specific TAAT/ATTA binding motifs within these promoter sequences lead to the significant reduction of transcriptional activation of these reporter gene constructs. Data shown are relative luciferase activity units (average ± standard deviation) of at least three different experiments. All luciferase activities are normalized to the activity level of the internal control β-gal. Error bars represent standard error of measurement. * denotes $p<0.05$. [ΔGAD65i: mutation of 3 TAATs of *Gad*65 region i, Δ1GAD67: mutation of the second TAAT of *Gad*67 region i, Δ2GAD67: mutation of the third TAAT of *Gad*67 region i, Δ3GAD67: mutation of the fourth TAAT of *Gad*67 region ii]. This figure is contributed by Q. Zhou as a co-author from the Eisenstat laboratory, Manitoba Institute of Cell Biology, University of Manitoba.

To establish the functional significance of specific TAAT/ATTA DNA binding motifs for *Dlx* regulation of *Gad* transcription, we performed site-directed mutagenesis of the *Gad*65 and *Gad*67 promoters to eliminate these binding motifs, then co-transfected the mutated constructs with expression vector encoding wild-type DLX1 or DLX2 into C6 glioma cells (or P19 embryonal carcinoma cells , data not shown). A *Gad*65 promoter region i with 3 TAAT/ATTA motifs mutated was not activated by DLX1 or DLX2 co-expression as compared with that of controls (**Fig. 9A**), indicating that these 3 motifs localized within *Gad*65 region i might constitute an important binding site for DLX1 and/or DLX2. Co-transfection of the *Gad*67 promoter with the 2nd or 3rd TAAT/ATTA motif of region i mutated, or the 4th motif of region ii mutated, resulted in a significant reduction of transcriptional activation mediated by DLX1, indicating that for DLX1 these binding sites are necessary for transcriptional activation of the *Gad*67 promoter (**Fig. 9B**). However, DLX2 co-expression still activated these mutated reporter constructs, although with significantly decreased activity compared to wild-type controls. Of note, mutation of the 3rd TAAT/ATTA motif (region i) resulted in less reduction of luciferase activity than either mutation of the 2nd motif (region i) and the 4th motif (region ii) localized to the *Gad*67 promoter (**Fig. 9B**).

5.4 DLX1 or DLX2 are co-expressed with GAD65, GAD67, or GABA in the developing forebrain

Expression of DLX1 and DLX2 was closely examined and compared with the expression of GABA, GAD65 and GAD67 in the subpallial telencephalon. DLX1 and DLX2 expression becomes well-established at E13.5 in the MGE and LGE, as well as the anterior entopeduncular (AEP) area (**Figs. 10, 11A, 11B**, panels a, d). These DLX

homeoproteins are expressed predominantly in the VZ and SVZ of these regions with lower levels of expression in the MZ of the basal forebrain. GABA expression is mainly in the SVZ and MZ of the LGE and AEP (**Fig. 10**, panels b, e). GABA expression partially overlaps with the expression of its synthetic enzymes: GAD65 (**Fig. 11A**, panels b, e) and GAD67 (**Fig. 11B**, panels b and e). More importantly, GABA, GAD65, or GAD67 expression overlaps with DLX1 or DLX2 expression in the SVZ, especially the LGE and AEP (**Fig. 10, Figs. 11A, 11B**, panels c and f). Co-expression studies of DLX1 or DLX2 with GABA, GAD65 or GAD67 at later embryonic stages, E14.5 (data not shown) and E16.5 (**Appendix Figs. 1A,B** (GABA)**; 2A,B** (GAD65)**; 3A,B** (GAD67), consistently demonstrated overlapping patterns of expression with co-expression of DLX1 or DLX2 and either GABA or GAD65/67 in the basal telencephalon (**Figs. 10, 11A, 11B** inserts). These results support the role of DLX proteins as important transcriptional regulators of *Gad*1 and *Gad*2 gene and GABA expression in the developing mouse forebrain.

5.5 GAD65 and GAD67 expression is reduced concomitant with decreased GABA neurotransmitter expression in the basal forebrain of *Dlx*1/2 double null mice

Whereas expression of GAD65 and GAD67 is strongly maintained in the wild-type basal telencephalon, this robust level of expression is diminished significantly in the *Dlx*1/2 double knockout mice. In the wild-type mouse, GAD65 and GAD67 are highly expressed in the SVZ and MZ of the LGE and AEP (**Figs. 11A, B**; **Fig. 12**, panels c, e), overlapping with expression of DLX1 and DLX2 in the SVZ (**Figs. 11A, B**, panels c, f).

| DLX1	DLX2	
a	**d**	
GABA	**GABA**	**E13.5**
b	**e**	
Merge	**Merge**	
c	**f**	

NCx

LGE H

Str MGE

PCx

POa
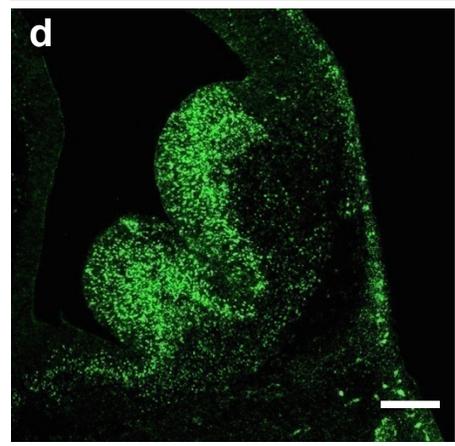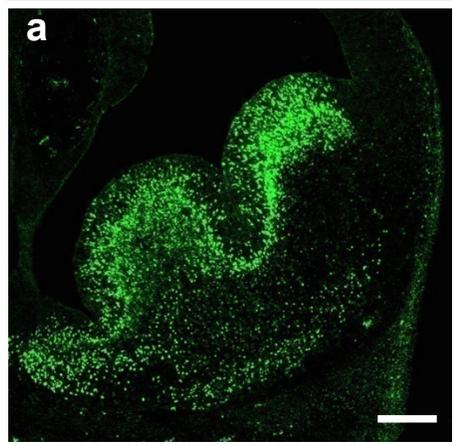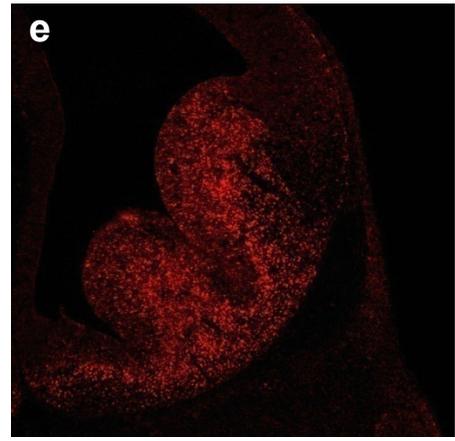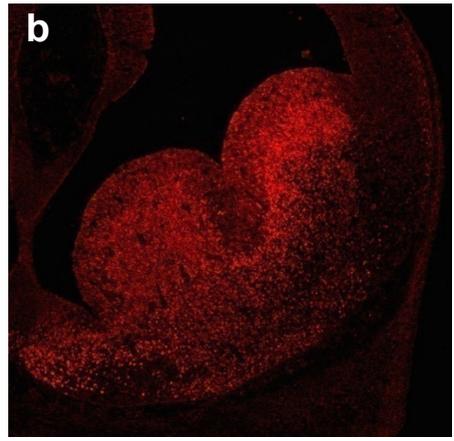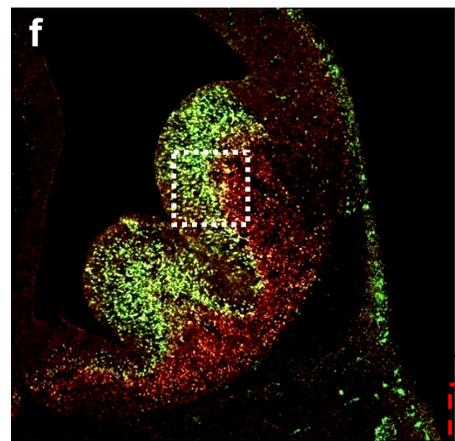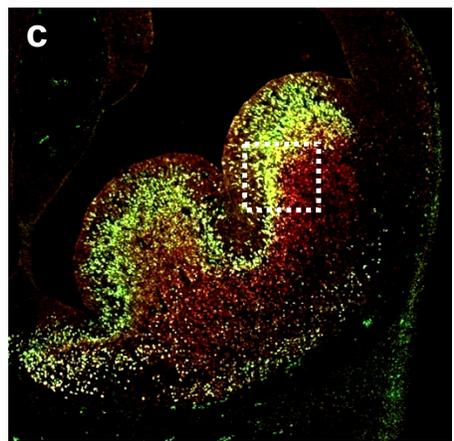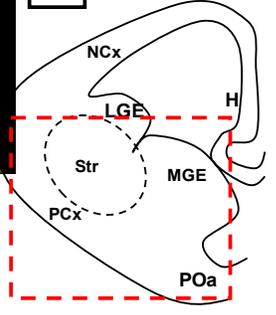
Figure 10: Co-expression of DLX homeodomain proteins and the GABA neurotransmitter in wild-type E13.5 forebrain. Sections were double-labelled with specific antibodies against DLX1 (a), DLX2 (d), GABA (b, e) of E13.5 ganglionic eminences. The top panels (a, d) show DLX1- or DLX2-positive cells (green) in the VZ and SVZ of the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE) and anterior entopeduncular area (AEP). The center panels (b, e) show GABAergic cells (red) in the same tissue sections throughout the basal telencephalon, predominantly in SVZ and mantle zone (MZ) of the LGE and AEP. The bottom panels show the overlay of the two images with GABA co-expressed with DLX proteins in most SVZ interneurons. Coronal sections. Scale bar, 200 µm. Inserts in the Merge row represent a 10x enlargement. [H, Hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; PCx, paleocortex; POa, anterior preoptic area; Str, striatum].

A

DLX1	DLX2
a	d

GAD65	GAD65
b	e

Merge	Merge
c	f

E13.5

NCx

LGE

H

Str

MGE

PCx

POa

B

DLX1	DLX2

a

d

GAD67	GAD67

b

e

Merge	Merge

c

f

E13.5

NCx

LGE

H

Str

MGE

PCx

POa
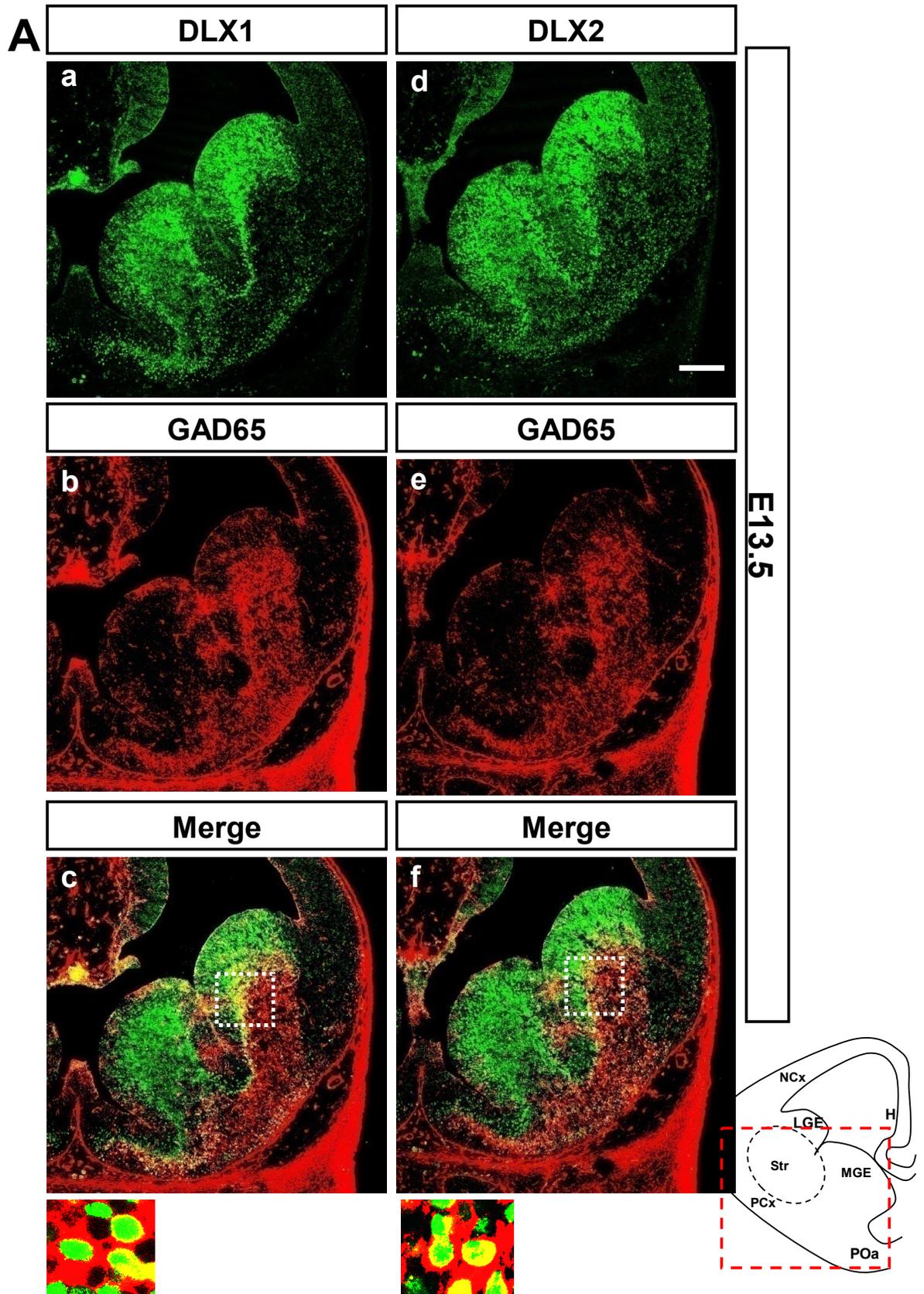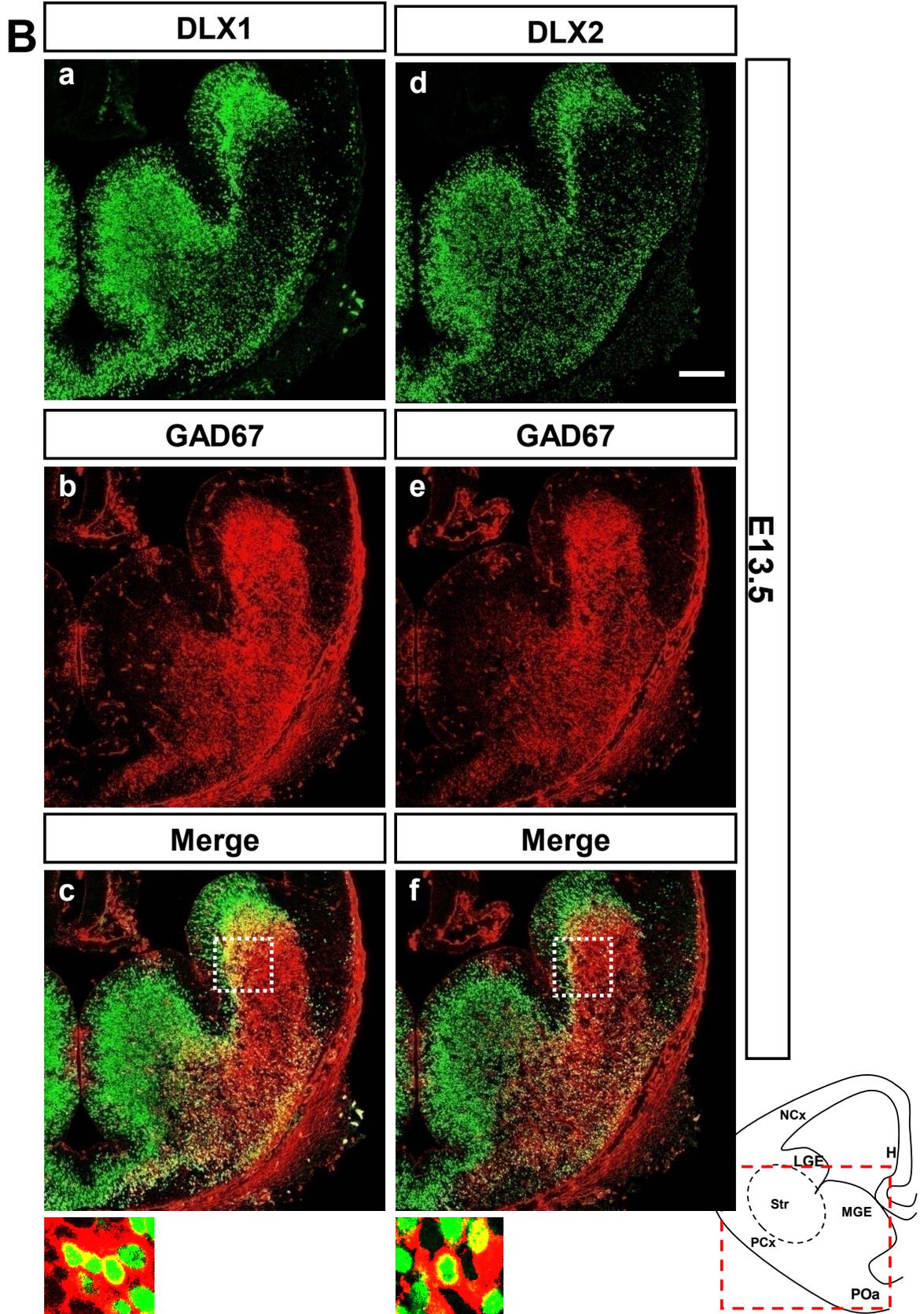
Figure 11: Co-expression of DLX homeodomain proteins and GAD65- (A) or GAD67-(B) positive interneurons in wild-type E13.5 forebrain. Sections were double-labelled with specific antibodies against DLX1 (a), DLX2 (d), in both A and B; and GAD65 or GAD67 (b, e) in A or B, respectively. The top panels (a, d) show DLX1- or DLX2-positive cells (green) in the VZ and SVZ of the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE) and anterior entopeduncular area (AEP). The center panels (b, e) show GAD65- or GAD67-positive cells (red) in the same sections in LGE and AEP, predominantly. The bottom panels show the overlay of the two images with GAD65 or GAD67 co-expressed with DLX proteins in the same SVZ interneurons. Coronal sections. Scale bar, 200 µm. [H, Hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; PCx, paleocortex; POa, anterior preoptic area; Str, striatum].

In the absence of *Dlx*1 and *Dlx*2 function, GAD65 and GAD67 expression levels are significantly reduced in the SVZ, with unchanged expression in the MZ where DLX1 and DLX2 are not strongly expressed normally (**Fig. 12**, panels d, f). Of significance, GAD65 and GAD67 expression is almost abolished in the AEP where DLX1 and DLX2 are normally expressed. Concomitantly, GABA expression is also reduced in the SVZ of the LGE and embryonic striatum with a loss of GABAergic expressing cells at the pallial/subpallial boundary in the double mutant compared to wild-type (**Fig. 12**, panels a, b, white arrow). The reduction of GABA, GAD65, and GAD67 protein expression in the *Dlx*1/2 double knockout mice was detected as early as E13.5 (**Fig. 12**) and later throughout embryogenesis, including E16.5 (**Appendix Fig. 4A** (GABA), **B** (GAD65) **, C** (GAD67) and E18.5 (data not shown). It is important to note that GAD65 and GAD67 protein expression still persists in the mantle zone of the basal ganglia at E13.5 (**Fig. 12**, panels d, f). Furthermore, GABA level is relatively unchanged in the mantle zone with ectopic accumulation in the striatum (**Fig. 12**, panel b, white arrow). This observation may be explained by a defect in tangential migration (Chapter 6), and/or regulatory control of GABA production by other glutamic acid decarboxylase isoforms (Martin and Rimvall, 1993; Pinal and Tobin, 1998), and/or other pathways independent of *Dlx*1/2 transcriptional regulation (Soudijn and van Wijngaarden, 2000). However, these results suggest that *Dlx*1 and *Dlx*2 genes are transcriptionally important for the expression of GAD65 and GAD67 protein levels, and consequently of GABA levels in the basal telencephalon.

5.6 Measurement of GABA level in the mouse forebrain using High Performance Liquid Chromatography (HPLC)

Although the reduction of GABA expression in the neocortex is evident in the E13.5 forebrain along with the reduction of GAD65 and GAD67 levels, it is still difficult to quantify and compare the GABA concentrations, i.e. the neurotransmitter level, in the forebrains of *Dlx*1/2 knockout mice compared to wild-type. In part, this is due to accumulation of GABA-expressing interneurons as ectopias in the basal telencephalon from E13.5 to birth in the double knockout mice (**Fig. 12** and **Appendix Fig. 4A**). This abnormal accumulation of GABAergic interneurons in the basal ganglia, concurrent with the defect in tangential migration of these interneurons to the neocortex, may have masked the underlying reduction of GABA levels in the basal telencephalon and underestimated the important role of *Dlx*1 and *Dlx*2 genes in the terminal differentiation of these interneurons. Hence, to determine the direct role of *Dlx*1/2 genes in the production of GABA in the entire forebrain (excluding the olfactory bulbs), we have used HPLC to measure GABA levels in whole forebrain extracts of wild-type and *Dlx*1/2 double knockout mice. At E13.5 and E16.5, GABA levels are comparable between wild-type and knockout littermates (data not shown). However, at E18.5 we detected a ~26% reduction of GABA level in the entire forebrain of the double knockout compared to the wild-type mice (**Fig. 13B**, $p < 0.02$, n=6). Concentrations of the GABA precursors glutamate (Glu) and glutamine (Gln) were unchanged between wild-type and double knockout mice (**Fig. 13A**, $p < 0.3$ and $p < 0.2$, respectively, n=6). The amino acid concentrations in extracts of whole forebrain were adjusted to the frozen weight, and to the total protein concentration of individual forebrain samples, and to the concentration of an internal control, homoserine.

	Dlx1/2+/+	*Dlx1/2-/-*
GABA	a	b
GAD65	c	d
GAD67	e	f

E13.5

NCx

LGE

H

Str

MGE

PCx

POa
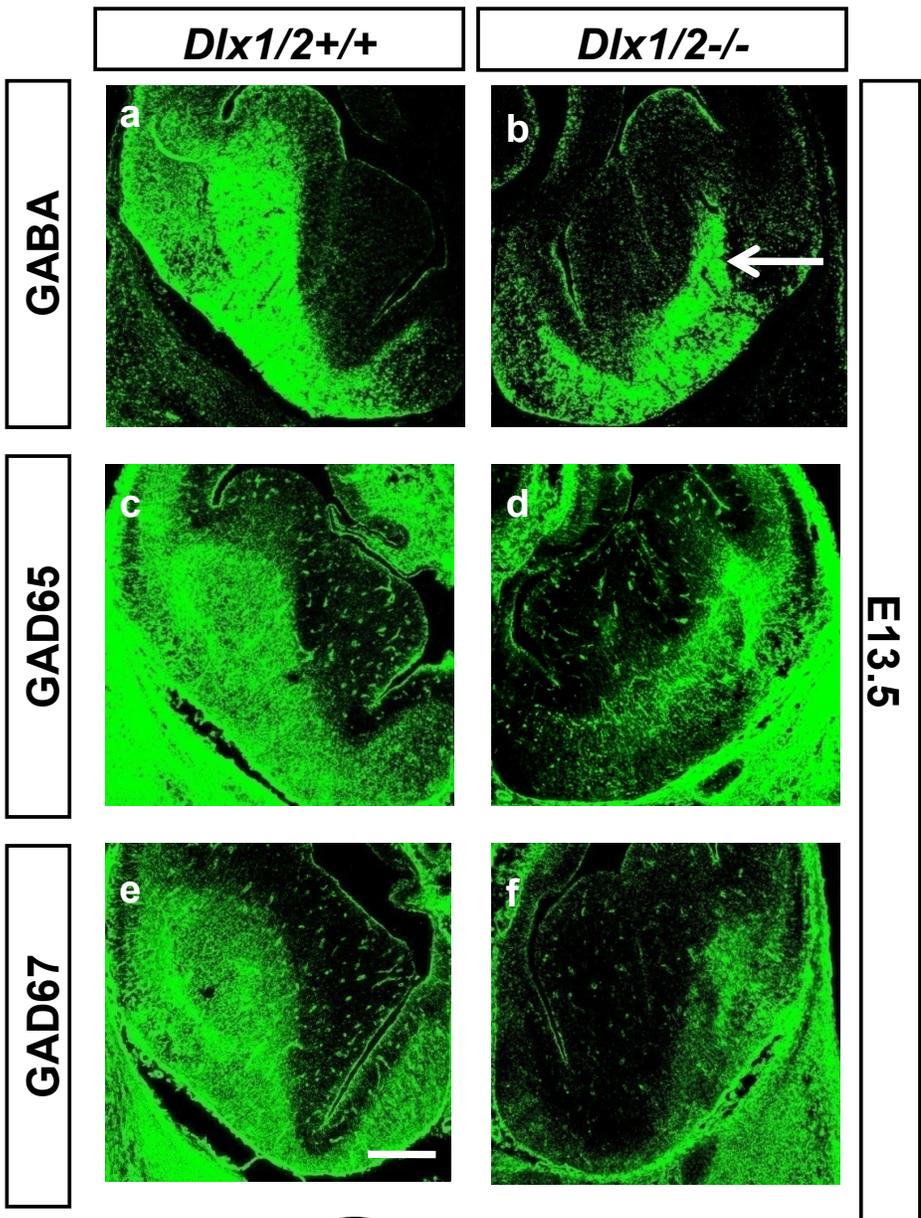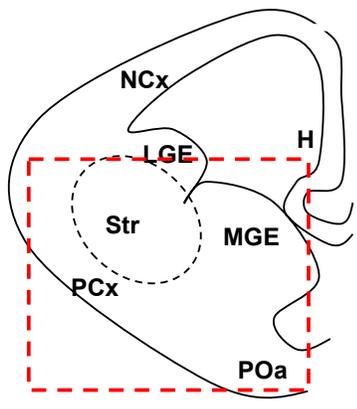
Figure 12: GABA expression in E13.5 basal telencephalon in *Dlx*1/2 wild-type compared to *Dlx*1/2 double knockout mice. In the E13.5 wild-type, GABA (a), GAD65 (c), and GAD67 (e) expression is predominantly localized to the SVZ and MZ of the AEP and LGE. In the absence of *Dlx*1 and *Dlx*2 function, expression of GAD65 (d), GAD67 (f) is severely reduced in the LGE with concomitant loss of GABA expression (b), primarily in the LGE. Coronal sections. Scale bars, 200 μm. [H, Hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; PCx, paleocortex; POa, anterior preoptic area; Str, striatum].

E18.5

A

B
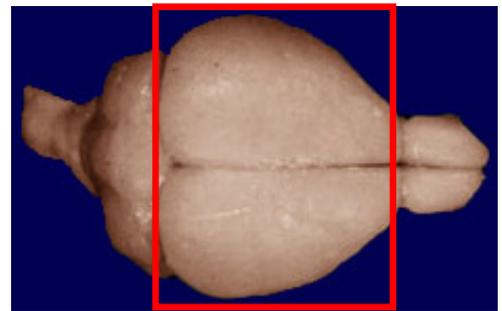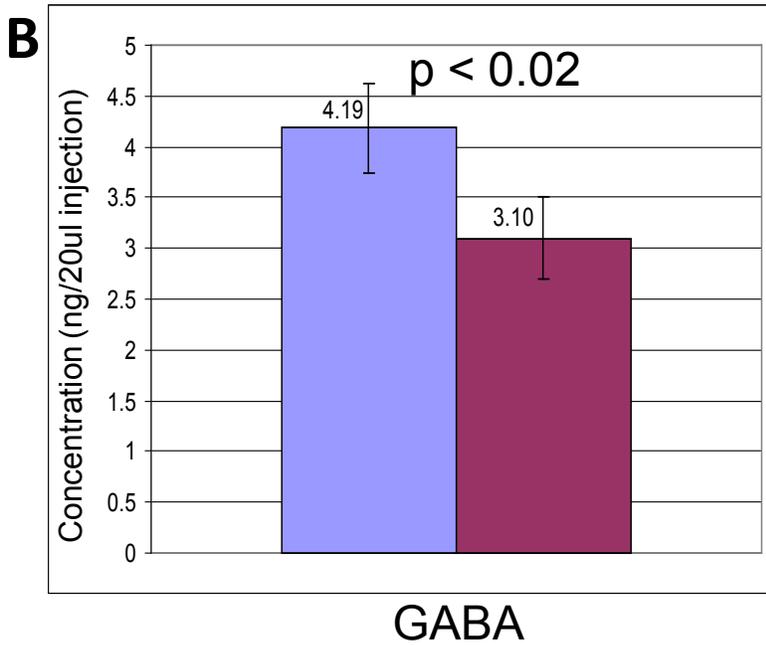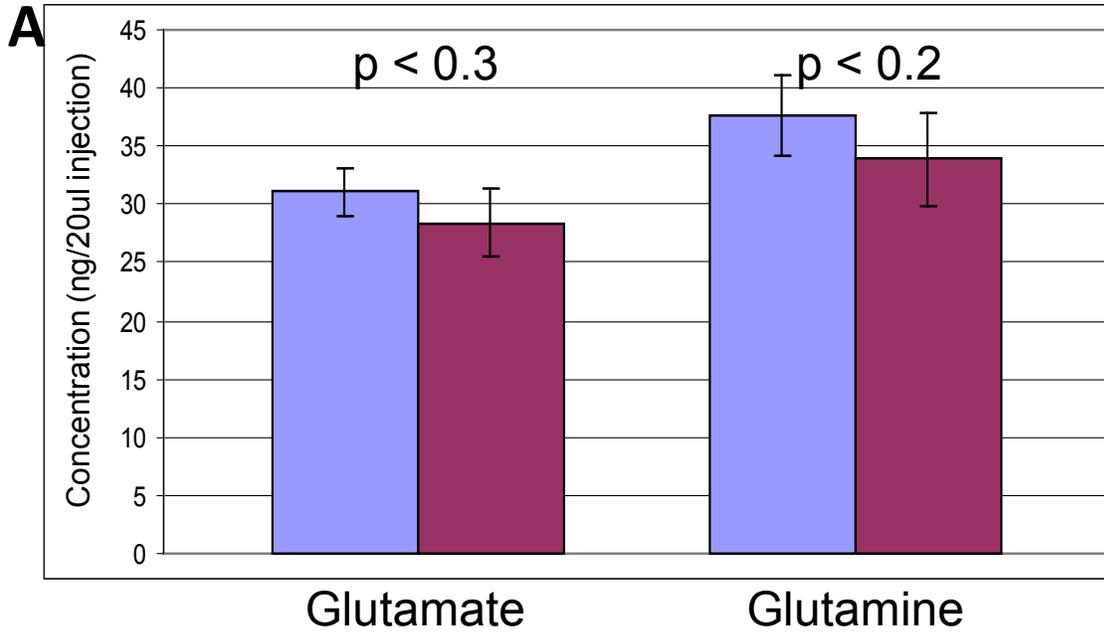
Dlx1/2+/+
Dlx1/2-/-

Figure 13: GABA levels are reduced in the *Dlx*1/2 null forebrains using High Performance Liquid Chromatography. (A) In the E18.5 forebrain, glutamate and glutamine, precursors of GABA, levels do not change when comparing wild-type and *Dlx*1/2 knockout forebrain (excluding the olfactory bulbs). (B) GABA neurotransmitter levels are decreased, by approximately 26%, in the *Dlx*1/2 knockout as compared to wild-type forebrain. Forebrains were dissected as depicted in the red box of the diagram, then frozen, and homogenized. Homoserine was added and used as internal control for the experimental procedure. Sample concentrations were measured from standard curves and expressed as nanogram per 20µl injection into the HPLC apparatus. Error bars represent standard error of measurement. The HPLC experiment was achieved in collaboration with Dr. J. Vriend, Department of Anatomy, University of Manitoba.

5.7 *Gad*65 and *Gad*67 mRNA levels are reduced in the basal telencephalon of *Dlx*1/2 null mice as demonstrated by *in situ* hybridization and Real-Time PCR

In the basal ganglia of the wild-type mouse, *Gad*65 and *Gad*67 RNAs were highly expressed throughout the embryonic forebrain from rostral to caudal regions at different embryonic ages, as early as E12.5 (**Fig. 14i** upper panels **& Fig. 15i**, left panels), based on *in situ* hybridization experiments. Patterns of RNA expression were consistent with the corresponding protein expression domains in the SVZ and MZ of the basal telencephalon, including the AEP (**Figs. 11A, B**; **Fig. 12**, panels c, e), and overlapping with DLX1 and DLX2 expression in the SVZ (**Figs. 11A, B**, panels c, f). In the absence of *Dlx*1 and *Dlx*2 function, *Gad*65 and *Gad*67 RNA expression levels were significantly lower in the basal telencephalon and diencephalon (**Fig. 14i,** lower panels and **Fig. 15i,** right panels). In addition, interneurons that still expressed GAD mRNAs accumulated as ectopias in the striatum (**Figs. 14i, 15i**, black arrows).

Real-Time PCR was performed on three different E13.5 litters, each of which contained at least 6 pairs of wild-type and mutant embryos from which embryonic striatal tissues were dissected and total RNA was subsequently extracted. The results demonstrated an approximate ~75% reduction of *Gad*65 mRNA and a similar ~75% reduction of *Gad*67 mRNA expression in the developing E13.5 mutant striatum (**Figs. 14ii, 15ii**). The reduction of *Gad*65 and *Gad*67 mRNA expression in the *Dlx*1/*Dlx*2 double knockout mice was statistically significant ($p < 0.01$, n=6). These results suggest that *Dlx*1 and *Dlx*2 are transcriptionally important for the expression of *Gad*65 and *Gad*67 mRNA, in order to maintain GABA-producing neurons throughout the basal ganglia as early as E12.5.

i

A Cx Se ST

B Cx ST PAL

C Cx HP VLGN RN ZI ST A

Dlx1/2+/+

D

E

F

Dlx1/2−/−

E16.5

ii

P<0.01

WT   MT

E13.5
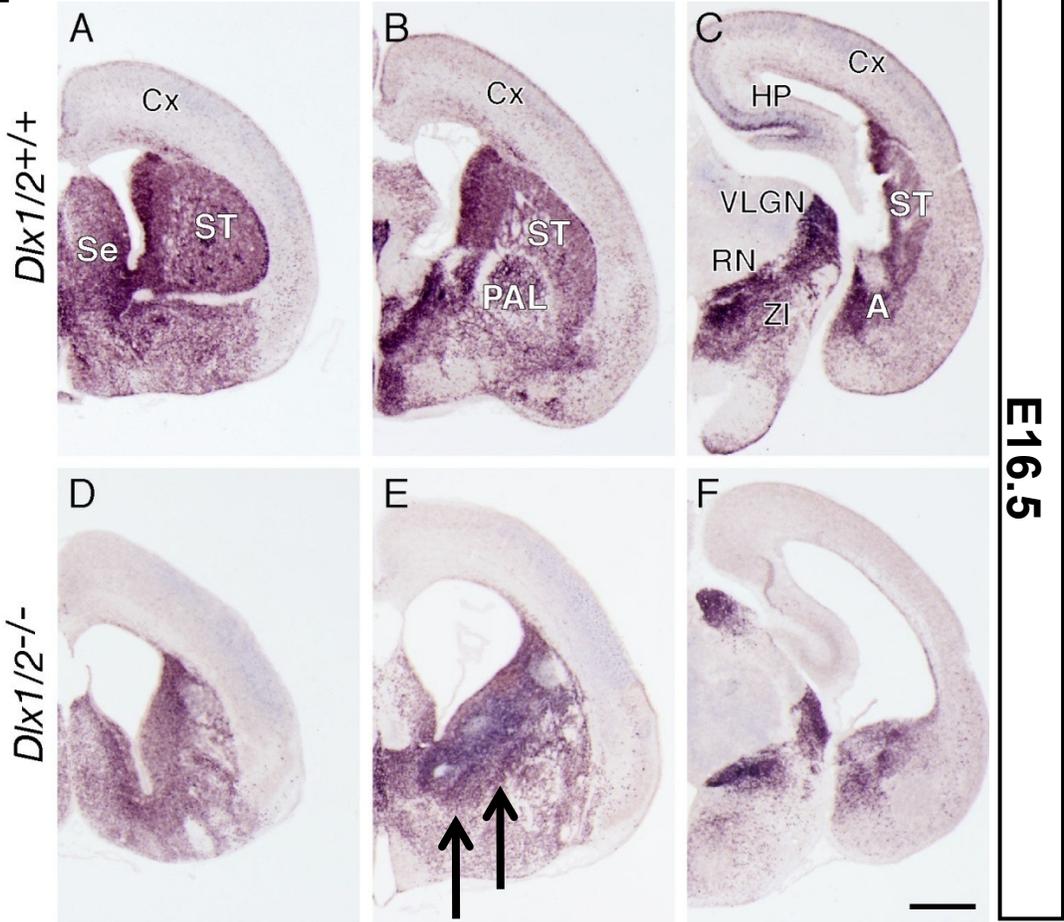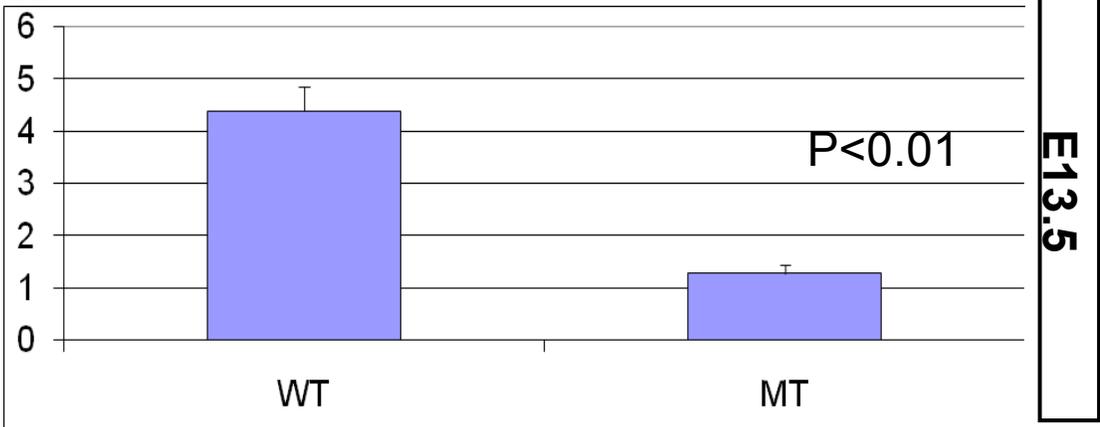
Figure 14: *Gad*65 isoform mRNA expression in the rostral and caudal forebrain in the wild-type and *Dlx*1/2 double knockout demonstrated by digoxigenin *in situ* hybridization and quantitative Real Time PCR. (i) *Gad*65 mRNA is expressed throughout the basal forebrain in a diffuse pattern in the wild-type forebrain (A, B, C – from rostral to caudal, upper panels). In the *Dlx*1/2 knockout forebrain, *Gad*65 RNA expression is severely reduced, with relative accumulation in the embryonic striatum (D, E, F, and black arrows – from rostral to causal, lower panels). (ii) *Gad*65 mRNA levels were examined by Real-Time PCR in the basal ganglia between wild-type and double knockout littermates at E13.5. The results were normalized using the house keeping gene GAPDH as an internal control. Error bars represent standard error of measurement. Cx: cortex, hp: hippocampus, st: striatum, pal: pallidum, se: septum, vlgn: ventral lateral geniculate nucleus, zi: zona incerta nucleus, rn: red nucleus. Scale bar: 400μm. Figure 14i is provided courtesy of I. Cobos and J.L.R. Rubenstein, University of California, San Francisco. Figure 14ii is provided in collaboration with S. Zhang, Eisenstat laboratory, Manitoba Institute of Cell Biology, University of Manitoba.

i

E12.5

a +/ -/-
LGE LGE
Se Se

b +/ -/-
LGE LGE
MGE MGE
Se Se

c +/ -/-
CGE VTH VTH CGE
AH AH

d +/ -/-
CGE VTH VTH CGE
DMH DMH

E16.5

e +/ -/-
LGE LGE
ST ST
Se Se
OT OT

f +/ -/-
LGE LGE
ST MGE
MGE ST
OT VP VP
PO PO OT

g +/ -/-
CGE VLGN VLGN CGE
ST VLGN ST
RN RN
ICA ZI ZI CA
mCA DMH DMH

ii

E13.5

	Mutant	Wild type
1.400		
1.200		
1.000		
0.800		
0.600	p<0.01	
0.400		
0.200		
0.000		
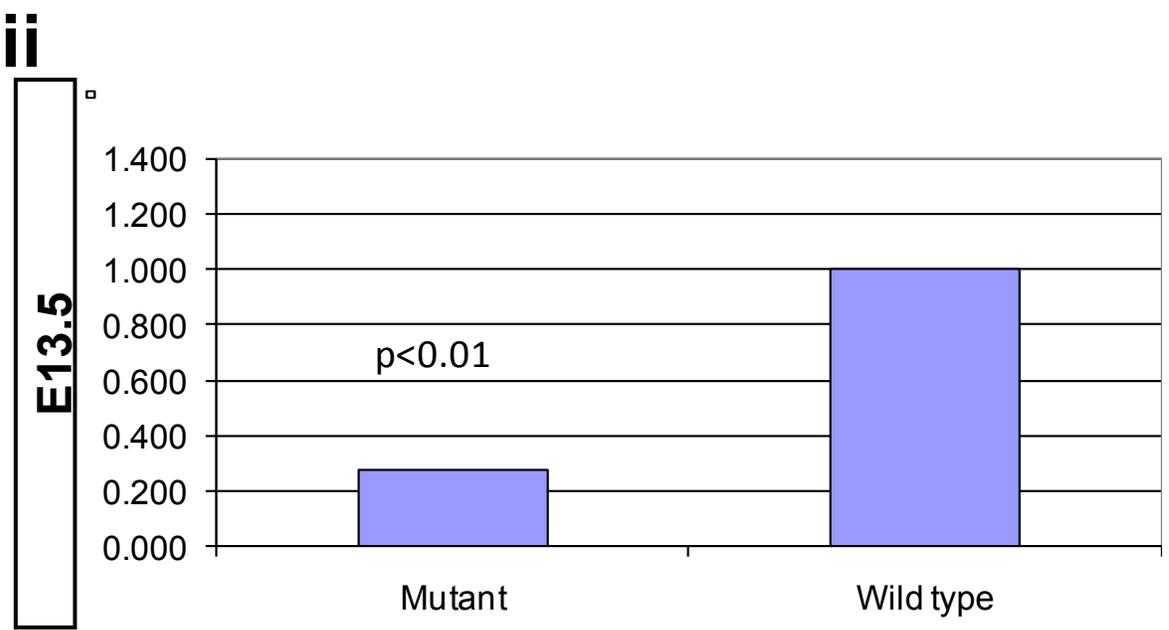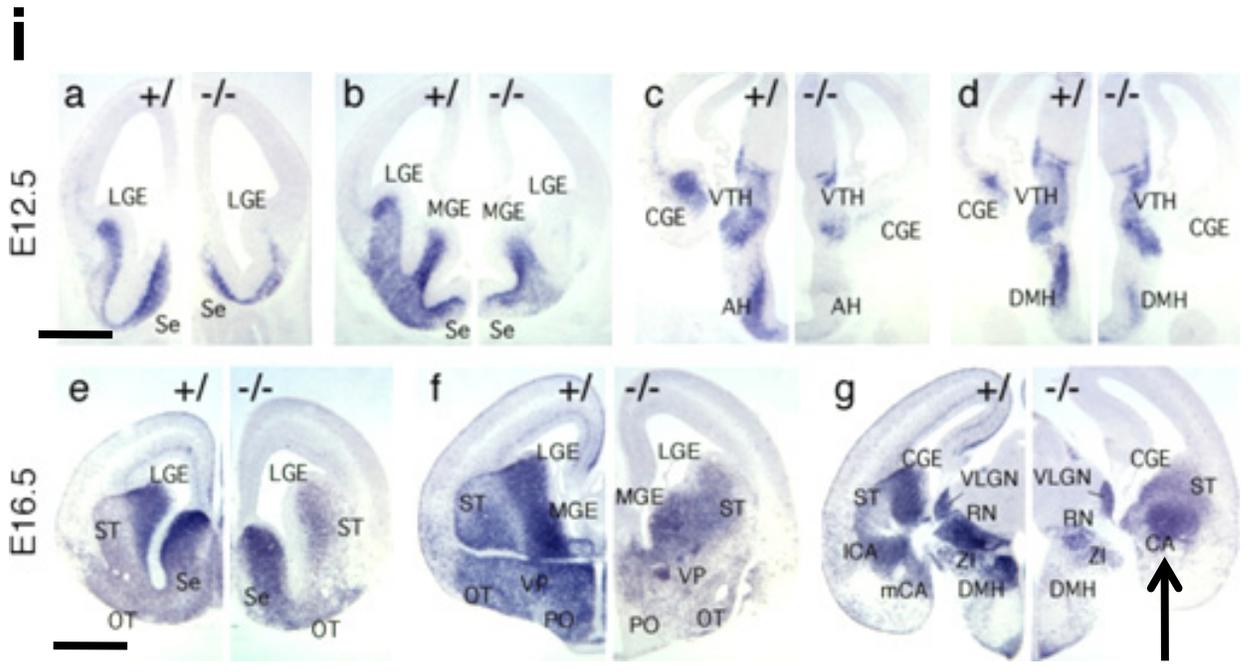
Figure 15: *Gad*67 isoform mRNA expression in the rostral and caudal forebrain in the wild-type and *Dlx*1/2 double knockout demonstrated by digoxigenin *in situ* hybridization and quantitative Real Time PCR. (i) *Gad*67 RNA is expressed throughout the basal forebrain in a diffuse pattern in the wild-type forebrain (a-g – from rostral to caudal, left panels). In the *Dlx*1/2 knockout forebrain, *Gad*67 RNA levels are severely reduced, and its expression relatively accumulates in the embryonic striatum at E16.5 (a-g – from rostral to caudal, right panels and black arrows). (ii) *Gad*67 mRNA levels were examined by quantitative Real-Time PCR comparing wild-type and double knockout littermates at E13.5, and the results were normalized using the house keeping gene GAPDH as an internal control. CGE: caudal ganglionic eminence, LGE: lateral ganglionic eminence, MGE: medial ganglionic eminence, st: striatum, vth: ventral thalamus, ah: anterior hypothalamic nucleus, dmh: dorsal medial nucleus, ot: olfactory tract, se: septum, vp: ventral pallidum, po: preoptic area, vlgn: ventral lateral geniculate nucleus, zi: zona incerta nucleus, rn: red nucleus. Scale bar: 200μm (a-d), 400μm (e-g). Figure 15i is provided courtesy of I. Cobos and J.L.R. Rubenstein, University of California, San Francisco. Figure 15ii is provided in collaboration with S. Zhang, Eisenstat Laboratory, Manitoba Institute of Cell Biology, University of Manitoba.

5.8 Knockdown of DLX2 expression in primary embryonic striatal and neocortical cultures results in decreased GABA expression

Our previous studies have shown that transcriptional regulation by DLX2 is more potent than DLX1, in regards to induction the expression of GABA *in vitro* (**Fig. 9**), and the lack of both *Dlx*1 and *Dlx*2 function reduces GABA expression levels in the embryonic forebrain (**Figs. 12, 13**). We also investigated the potential effects of inhibition of *Dlx*2 expression on GABA expression in primary embryonic forebrain cultures. Western blot analysis of HEK293 cells transfected with interfering RNA constructs demonstrated reduced DLX2 expression, indicating that our siRNA and shRNA constructs were working properly (data not shown). Primary cultures of E16.5 neocortex (**Fig. 16A, B**), E16.5 striatum (**Fig. 16C, D**), E18.5 neocortex (**Appendix Fig. 11A, B**), and E18.5 striatum (**Appendix Fig. 11C, D**) were transfected with either scrambled control shRNA or shRNA targeting DLX2 expression. siRNA methods were also used to demonstrate similar results at E16.5 and E18.5 (data not shown). Overall, shRNA was more efficient than siRNA in transfection of primary cultures, and hence in knocking down DLX2 expression in both neocortical and striatal primary neurons. When DLX2 expression was knocked down in embryonic neocortical (**Fig. 16A**) and striatal cultures (**Fig. 16C**), GABA expression was correspondingly reduced in these neocortical (**Fig. 16B**) and striatal cultures (**Fig. 16D**), respectively. The reduction of GABA expression by the shRNA targeting *Dlx*2 was consistent at both E16.5 (**Fig. 16A-D**) and E18.5 (data not shown). For both E16.5 (**Fig. 17**) and E18.5 (data not shown) neocortical and striatal cultures, the results were quantified as the proportion of DLX2- or GABA- positive cells.

A

	shRNA Control	*shRNA Dlx2*	
Dlx2	a	b	
DAPI	c	d	**E16.5 neocortex**
Overlay	e	f	

Scale bars: 200 µm

B

	shRNA Control	*shRNA Dlx2*
GABA	a	b
DAPI	c	d
Overlay	e	f

E16.5 neocortex

C

	shRNA Control	*shRNA Dlx2*
Dlx2	a	b
DAPI	c	d
Overlay	e	f

E16.5 striatum

D

	shRNA Control	*shRNA Dlx2*
GABA	a	b
DAPI	c	d
Overlay	e	f
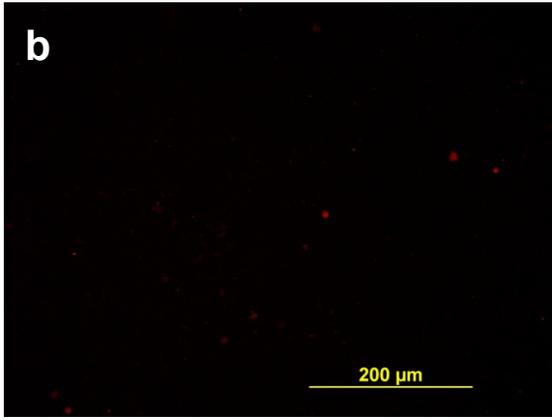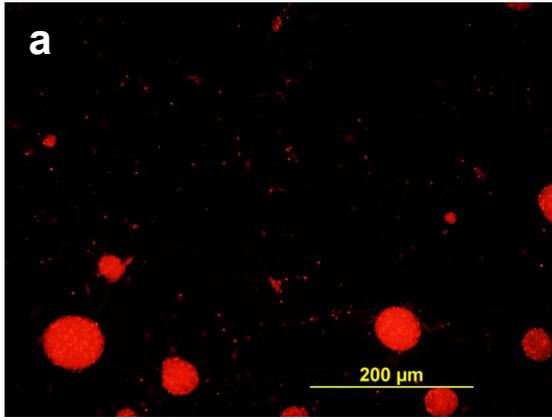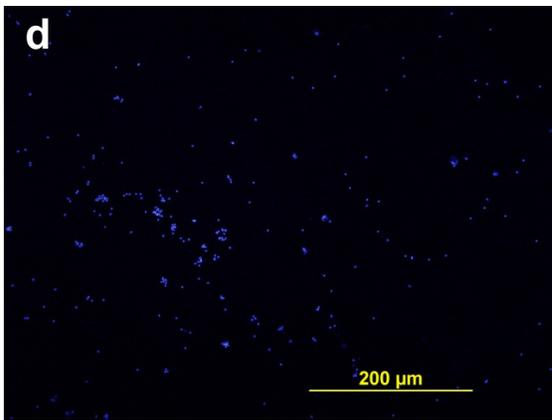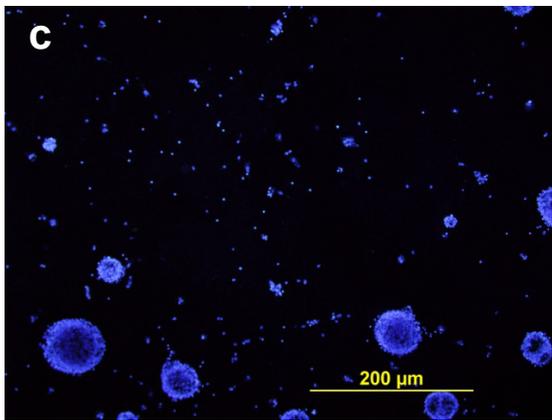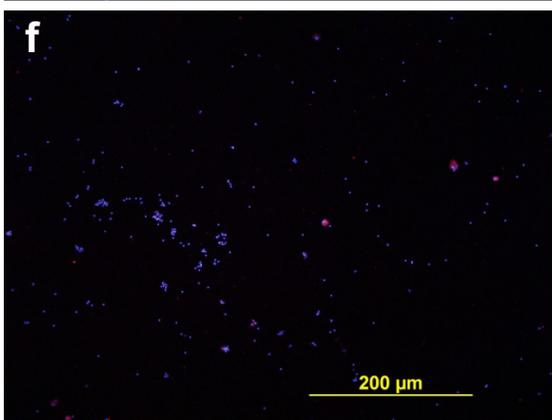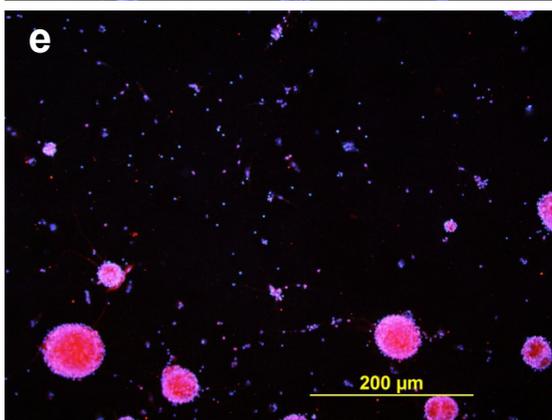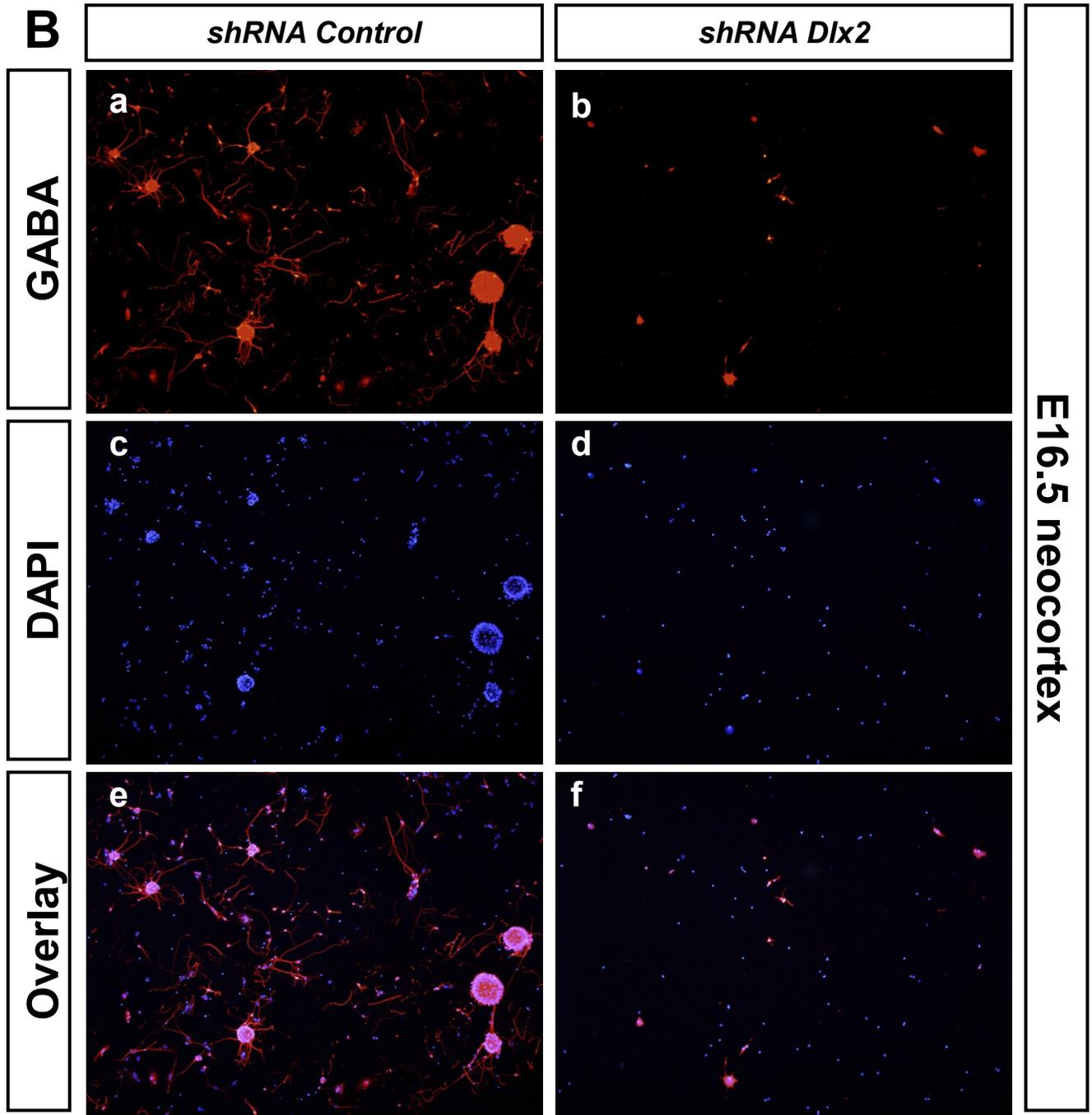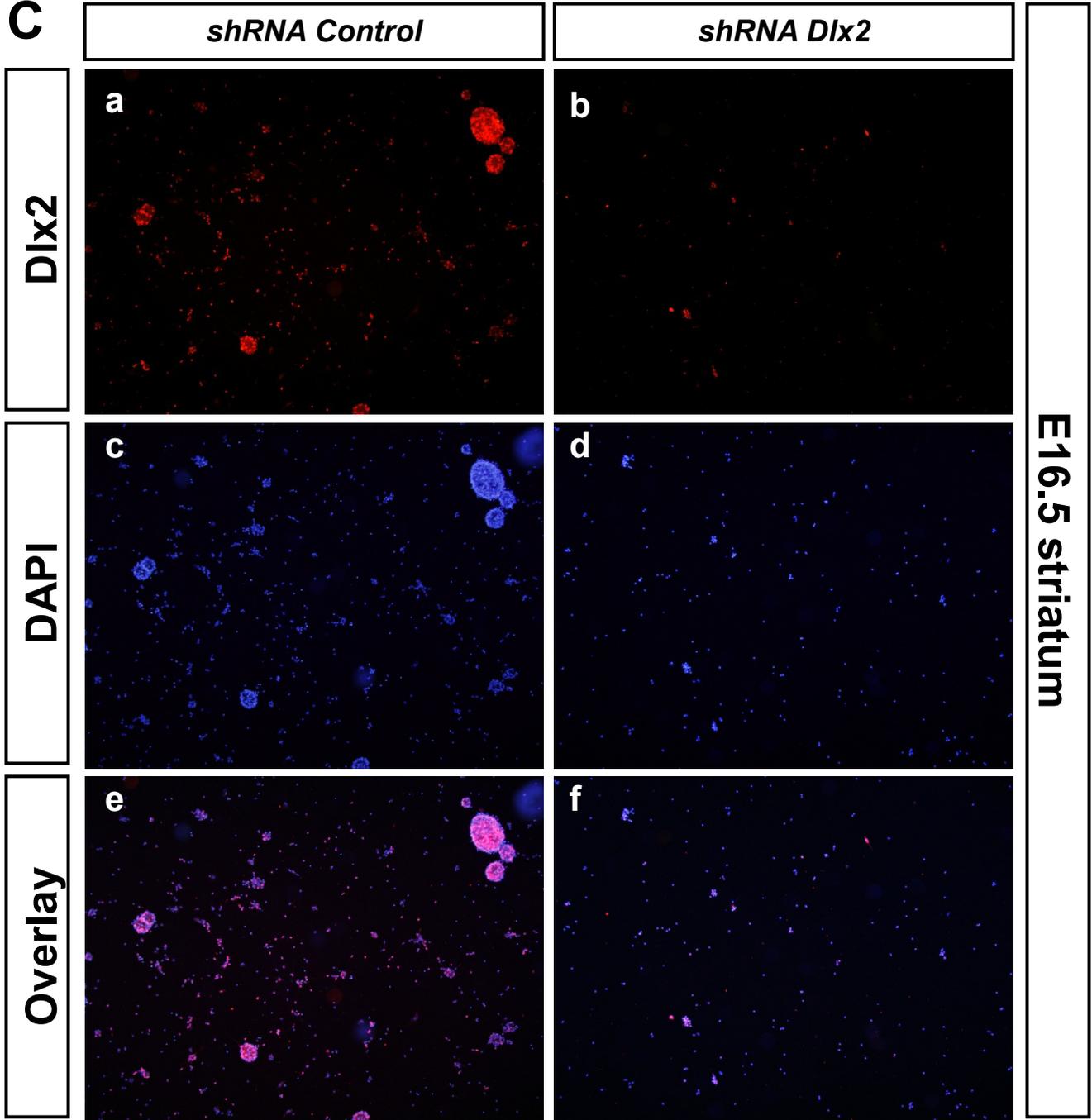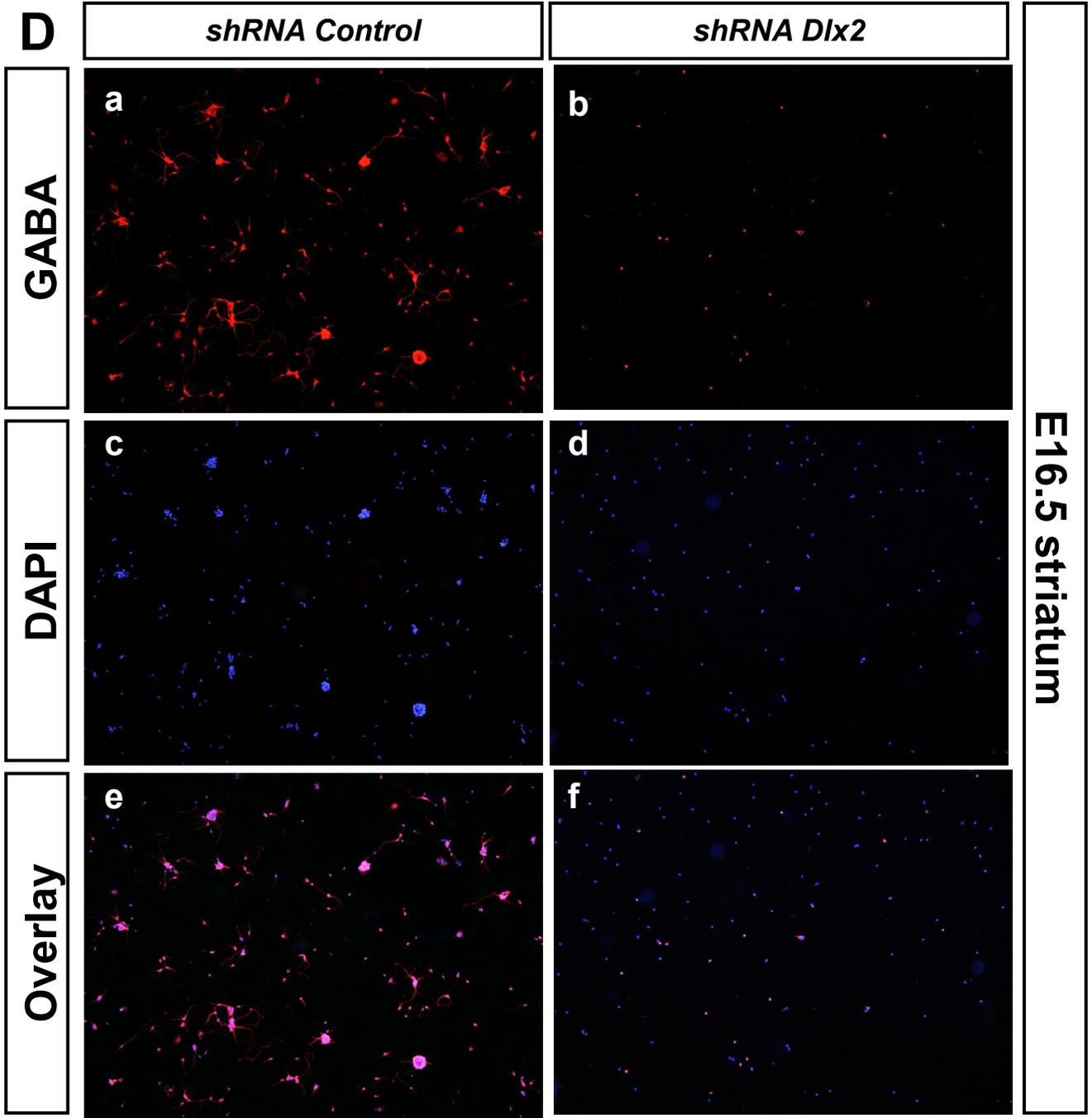
E16.5 striatum

Figure 16. Knockdown of *Dlx*2 expression by shRNA in E16.5 primary embryonic striatal and neocortical cultures. Primary dissociated forebrain cultures of striatum (A, B) and neocortex (C, D) at E16.5 were transduced with lentivirus expressing shRNA targeting *Dlx2* coding sequences. Lentiviral shRNA transduction reduced DLX2 expression (red) (A, C, panel b) and concomitantly decreased GABA expression (red) (B, D, panel b) in both embryonic striatal and neocortical primary cultures when compared to control shRNA-transfected forebrain cells (A, B, C, D, panel a). Cellular nuclei were stained with DAPI (blue) for quantification (A, B, C, D, panels c, d). Merged images show primary cells expressing the specific markers (A, B, C, D, panels e, f). Scale bars 100µm. The viral shRNA production was done by S. Zhang, Eisenstat laboratory, Manitoba Institute of Cell Biology, University of Manitoba.
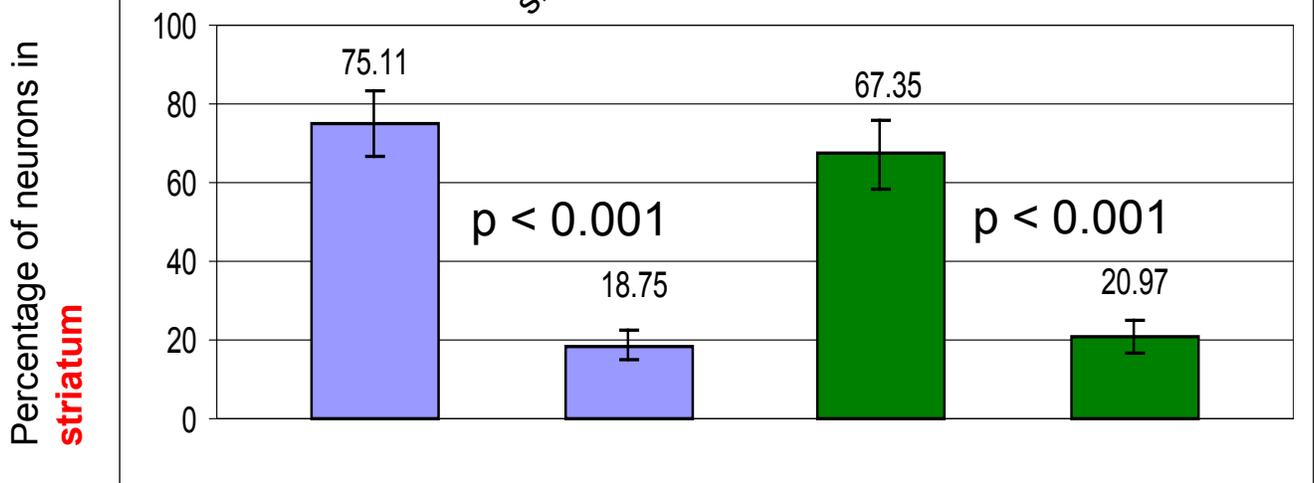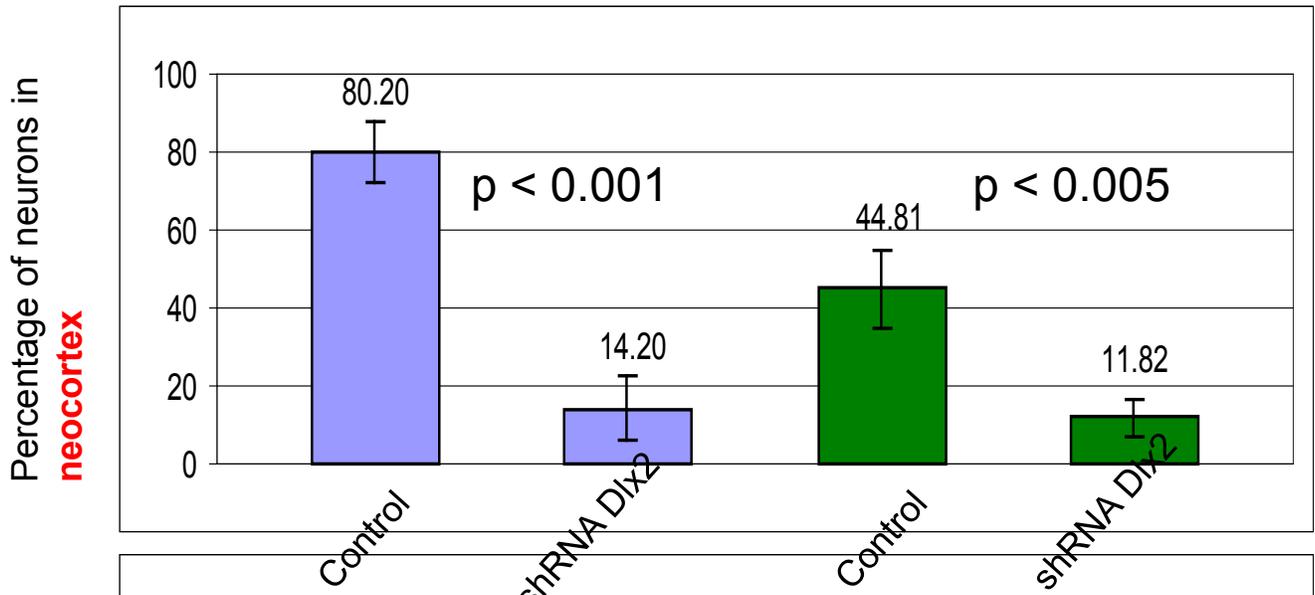
Figure 17. Quantification of DLX2- and GABA-positive cells following reduction of DLX2 expression mediated by shRNA for *Dlx*2 in E16.5 primary embryonic striatal and neocortical cultures. Primary dissociated forebrain cultures of neocortex (upper panel) and striatum (lower panel) at E16.5 were transduced with lentivirus expressing shRNA targeting *Dlx2* coding sequences, then labelled with either DLX2 or GABA antibodies. DLX2- or GABA- positive cells were counted and normalized with the total of number of DAPI-positive cells. shRNA transduction reduced DLX2 expression significantly by approximately 5 fold in neocortex and 4 fold in striatum. As a result of the *Dlx*2 knockdown by shRNA, there was a significant decrease in GABA expression (green) by 4 fold in neocortex and 3 fold in striatum when compared to control scrambled shRNA-transduced forebrain cells. All comparisons were performed in at least 3 trials with significant and reproducible results using the T-test (N=3, $p<0.001$ to 0.005). Error bars represent the standard error of measurements.

DLX2- (blue column) or GABA- (green column) positive cells were labelled, counted, and compared to DAPI-positive total cells. In E16.5 neocortical cultures (**Fig. 17**, upper panel), there was an approximately 5.7 fold reduction ($p < 0.001$, n=6) of DLX2-positive cells and a corresponding 4 fold reduction ($p < 0.005$, n=6) of GABA-positive cells. In the E16.5 striatal cultures (**Fig. 17**, lower panel), there was an approximately 4 fold reduction ($p < 0.001$, n=6) of DLX2-positive cells and 3 fold reduction ($p < 0.001$, n=6) of GABA-positive cells. Similar and statistically significant results were also obtained from E18.5 neocortical and striatal primary cultures (data not shown). This shRNA knockdown study supports a direct transcriptional role of the DLX2 homeodomain protein in the regulation of the key enzymes required for GABA synthesis, and subsequently, GABAergic interneuron differentiation. More importantly, this study has demonstrated that *Dlx* genes directly regulate the transcription of *Gad* genes, and are necessary to induce the fundamental phenotype of GABAergic neurons in the developing forebrain.

Chapter 6

*Dlx*1/*Dlx*2 genes and migration of GABAergic interneurons:

Dlx homeobox genes promote cortical interneuron migration from the basal forebrain by direct repression of the Semaphorin receptor Neuropilin-2

Rationale: In the absence of both *Dlx*1 and *Dlx*2 function, there is abnormal development of the SVZ of the ganglionic eminences. There is an almost complete loss of tangential migration of GABAergic interneurons from the MGE to the neocortex and from the LGE to the olfactory bulb (Anderson et al., 1997a; Bulfone et al., 1993; Long et al., 2007). These migrations comprise the major source of cortical inhibitory interneurons in the murine telencephalon (Anderson et al., 1997a; Letinic et al., 2002; Marin et al., 2001). The semaphorins (Sema) 3A and 3F, by binding to their receptors Neuropilin-1 and Neuropilin-2, respectively, provide strong repulsive guidance cues, and mediate sorting of tangentially migrating interneurons from the ganglionic eminences to the cortex and striatum (Marin et al., 2001). Evidence of ectopic expression of Neuropilin2 (*Nrp*2) in the *Dlx*1/2 mutant forebrain (Marin et al., 2001) provided a rationale to test whether DLX1 and/or DLX2 directly regulate Neuropilin2 expression. Herein, using ChIP assays of E13.5 ganglionic eminences, we demonstrate that DLX1 and DLX2 bind directly to a specific region of the *Nrp*2 but not to the *Nrp*1 promoter *in vivo*. Furthermore, both DLX1 and DLX2 inhibit transcription of *Nrp*2 *in vitro* (Le et al., 2007).

6.1 DLX1 and DLX2 homeobox proteins bind to a Neuropilin2 promoter region in embryonic forebrain *in vivo*

The promoters of both Neuropilin1 (*Nrp*1) and Neuropilin2 (*Nrp*2) genes contain

A **_Mus musculus_ Neuropilin promoters:**

Nrp-1 (region i)

5'- <u>ggaaccg gactacatgg</u> ggggaggggga ggcactgggc
agacaccagg gacgcacctc gtaagtgggt ggcgtatgag
gctggaggat cagtagacac aattgcctg*t aat*ctaaacc
tggcactagg agacccttgc aaagatcatc ctgtcaccct
cacaag<u>gggt cgttgccagt ctt</u> – 3'(nt104-283)

Nrp-1 (region ii)

5'- <u>aacctcaggc tgacacc</u>cag gaagtgaaag cggggactga
cagcgcgatc cacgcctccg ggggcgccgg tacctaggg*a
tta*ggagttc cgtgaagtag cagctaaccc cggtctctcg
gaactccttg actccaggat cctcgcctac acgagcagcc
tagttcagtt ctg<u>gggtgca gagatctcgt</u> – 3'(nt711-900)

Nrp-2 (region i)

5'- <u>gag atcacacagc tgcc</u>tagggg ccgtgtgatg cccagggcaa
ttcttggctt tgattttt*at tattatta*ct *atta*ttttgc
gttcagcttt cgggaaac<u>cc tcgtgatgtt gtagg</u> – 3'(nt138-
255)

Nrp-2 (region ii)

5'- <u>cgttgat cgttagagac c</u>tttgcagaa gacaccacca
ggaggaaa*at ta*gagaggaa aaacacaaag aca*taatta*t
aggagatccc acaaacctag cccgg<u>gagag agcctctctg tc</u> –
3'(nt444-562)

B

Striatum   Hindbrain
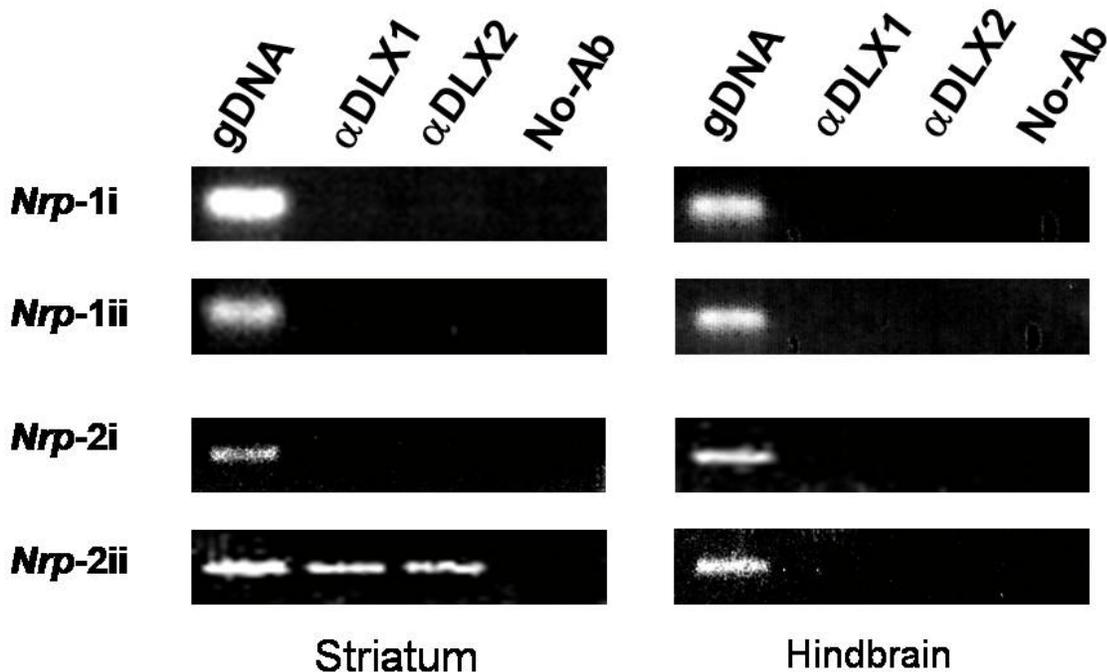
Figure 18. Neuropilin2 but not Neuropilin1 is a DLX homeoprotein target *in vivo*. (A) The sequences of candidate regulatory elements within the mouse Neuropilin1 (GenBank AF482432) and Neuropilin2 (GenBank AF022854) promoters, designated *Nrp1i, Nrp1ii, Nrp2i* and *Nrp2ii*, respectively, contain putative homeodomain DNA binding sites (*TAAT/ATTA*) in red italics. Oligonucleotide primers used for PCR are underlined and the TAAT motif located in *Nrp2ii* required for binding to DLX1 and DLX2 is boxed. (B) Chromatin immunoprecipitation (ChIP) assays were performed on E13.5 mouse forebrain tissues using affinity-purified polyclonal DLX1 and DLX2 antibodies following cross-linking of protein-DNA complexes with 1% paraformaldehyde. Specific bands were evident for *Nrp*2ii but not *Nrp*1i, *Nrp*1ii, *or Nrp*2i (left panel). Negative controls included performing ChIP without the addition of either primary antibody, or the use of E13.5 hindbrain, a tissue that does not express *Dlx* genes (right panel). Positive controls were mouse genomic DNA (gDNA) and primer pairs for the *Dlx*5/*Dlx*6 intergenic enhancer (data not shown; (Zhou et al., 2004)). PCR bands were subcloned and their identity confirmed by DNA sequencing.

putative TAAT/ATTA homeodomain DNA binding sites. Hence, we considered *Nrp*1

and *Nrp*2 as candidate transcriptional targets of several homeobox genes regionally

expressed in the subcortical telencephalon, including members of the *Dlx* gene family. To

test this hypothesis, we assayed for the direct binding to both *Nrp* promoters by DLX1

and/or DLX2 *in vivo*. E13.5 GEs were treated with PFA to cross-link protein-DNA

complexes. Using a modified ChIP procedure, soluble nucleoprotein complexes of ~100-

300 base pair fragments were immunoprecipitated using anti-DLX1 and/or DLX2

antibodies. We then used PCR to amplify candidate homeodomain-binding regions in the

*Nrp*1 and *Nrp*2 loci. These regions were chosen based on the presence of consensus

homeodomain binding motifs, and were designated *Nrp1i* (nucleotides 104-283)*, Nrp1ii*

(nucleotides 711-900)*, Nrp2i* (nucleotides 138-255) and *Nrp2ii* (nucleotides 444-562)

(**Fig. 18A**). Notably, ChIP assays revealed that both DLX1 and DLX2 bind only to the

*Nrp*2 promoter region 2 (*Nrp2ii*) in embryonic striatum but there was no evidence for

binding at the other promoter regions *in vivo*, i.e. *Nrp2i, Nrp1i, Nrp1ii* (**Fig. 18B**). The

resulting amplicons were subcloned and sequenced to verify their identity and for

subsequent biochemical analyses. As expected, control ChIP assays performed without

antibody or with anti-DLX antibodies and chromatin derived from embryonic hindbrain

tissues where *Dlx* genes are not expressed, were negative. For further controls using

genes that have not been known to be downstream targets of DLX proteins, we assessed

both the promoter of *Wnt*7B (GenBank#AF456420.1) and 5' flanking sequence that is

upstream to *Mash*1 coding sequence (GenBank#NM_008553). As expected, ChIP

experiments showed that DLX proteins do not bind to these sequences (**Appendix Fig.**

5).

6.2 DLX1 and DLX2 bind to the Neuropilin2 promoter *in vitro*

To determine whether DLX1 and DLX2 specifically bind to the *Nrp*2 promoter region ii *in vitro*, we used recombinant DLX1 and DLX2 proteins and radiolabelled *Nrp2ii* isolated from the ChIP assay. EMSA showed binding of both DLX1 and DLX2 to *Nrp2ii* (**Fig. 19A**, *lanes* 2,6) that was competitively inhibited by unlabelled *Nrp2ii* probe (**Fig. 19A**, *lanes* 3,7). Moreover, the addition of specific anti-DLX1 or anti-DLX2 antibodies to the protein–DNA complex resulted in significant band mobility shifts (**Fig. 19A**, *lanes* 4,8), while a nonspecific polyclonal antibody failed to produce such a "supershift" (**Fig. 19A**, *lanes* 5,9). Within the region, neither recombinant DLX1 nor DLX2 bind to the 1st TAAT motif, but both DLX proteins bind to the 2nd motif of the *Nrp2ii in vitro* (**Fig. 18A**, *box*; and data not shown). Mutagenesis of individual motifs further demonstrated specific binding of DLX2 to the 2nd motifs of *Nrp2ii* (data not shown). These experiments demonstrate that DLX1 and DLX2 bind specifically to the *Nrp*2 promoter region ii *in vitro*.

EMSAs with nuclear extracts derived from embryonic ganglionic eminences showed that endogenous DLX1 and DLX2 proteins can bind to the *Nrp*2 promoter (**Fig. 19B**, *lane* 2). Unlabelled probe can compete with radiolabelled oligonucleotides for both proteins (**Fig. 19B**, *lane* 3). Experiments using specific DLX1 or DLX2 antibodies and a control antibody confirmed the identity of the DLX complexes (**Fig. 19B**, *lanes* 4, 5, 6). The recombinant and endogenous DLX proteins do not have identical molecular weights, perhaps reflecting different binding partners or post-translational modifications, such as phosphorylation, *in vivo* (Park et al., 2001), (Qiu & Eisenstat, observations). These experiments demonstrate that DLX1 and DLX2 specifically bind to the *Nrp2ii in vitro*.

A

Antibody	–	–	–	1	I	–	–	2	I
rDLX	–	1	1	1	1	2	2	2	2
Unlabeled probe	–	–	+	–	–	–	+	–	–
Labeled probe	+	+	+	+	+	+	+	+	+
	1	2	3	4	5	6	7	8	9

Supershifted band: αDLX2 ← – –

Supershifted band: αDLX1 – –►

Specific DLX1/*Nrp*-2ii band →

Specific DLX2/*Nrp*-2ii band →

Free probe

B

Antibody	–	–	–	1	2	I
Nuclear Extract	–	+	+	+	+	+
Unlabelled probe	–	–	+	–	–	–
Labelled probe	+	+	+	+	+	+
	1	2	3	4	5	6

Supershifted band: αDLX2 ← – –

Supershifted band: αDLX1 – –►

Specific DLX2/*Nrp*-2ii band →

Specific DLX1/*Nrp*-2ii band →

Free probe

Figure 19. DLX1 and DLX2 proteins specifically bind to a regulatory element within the *Neuropilin*2 promoter *in vitro* and *in vivo*. (A) Electrophoretic mobility shift assays (EMSA) show binding of recombinant DLX1 or DLX2 to *Nrp*2 promoter region ii oligonucleotides containing homeodomain binding sites *in vitro*. Radiolabelled *Nrp*2ii oligonucleotide probes were incubated alone (*lane* 1), with recombinant DLX1 protein (*lanes* 2-5), with recombinant DLX2 protein (*lanes* 6-9), with unlabelled *Nrp*2ii probes (*lane* 3, 7), with affinity-purifed DLX antibodies (*lane* 4, 8), and with non-specific antibodies (*lane* 5, 9). (B) EMSA using embryonic forebrain demonstrates that endogenous DLX1 and DLX2 proteins from nuclear extracts bind to *Nrp*2ii *in vivo*. Radiolabelled *Nrp*2ii oligonucleotides were incubated alone (*lane* 1), with nucleoprotein extracts from E13.5 ganglionic eminences (*lanes* 2-6), with unlabelled *Nrp*2ii probe (*lane* 3), with affinity-purified DLX antibodies (*lane* 4, 5), and with non-specific antibodies (*lane* 6). Gel shifts, denoting specific binding of DLX proteins to DNA, are indicated with solid arrows. Supershifts with specific DLX antibodies are indicated by broken arrows. [*Nrp-2*: Neuropilin-2; r: recombinant; 1: DLX1/anti-DLX1; 2: DLX2/anti-DLX2; I: irrelevant/non-specific polyclonal antibody].

6.3 DLX1 and DLX2 repress Neuropilin2 promoter activity *in vitro*

The functional significance of DLX1 and DLX2 binding to the *Nrp*2 promoter was assessed using luciferase reporter gene experiments. We co-transfected HEK293 cells or P19 embryonal carcinoma cells (data not shown) with an expression vector encoding either DLX1 or DLX2 and a vector in which region ii of the *Nrp*2 promoter (444-562 nt) drives luciferase expression. Co-transfection with either wild-type DLX1 or DLX2 expression constructs resulted in significant reductions of luciferase activity compared with controls (~1.7 fold for Dlx1, p<0.001; and ~6 fold for Dlx2, p<0.001; **Fig. 21A**), indicating that both DLX1 and DLX2 proteins can act as transcriptional repressors. We generated several different mutant constructs of DLX1 and DLX2 homeodomain proteins to explore the consequences of modifying specific DLX protein domains on their ability to repress transcription in this system (**Fig. 20A**). The first set of constructs included a mutation that converts glutamine (Q) to glutamate (E) at amino acid position 50 (Q50E) of the DLX homeodomain. This Q50E mutant homeodomain is predicted to abrogate DNA binding (**Fig. 20B**, right panel) and the mutant protein may act in a dominant negative fashion (Gehring et al., 1994). Western blot confirmed that the Q50E mutants are adequately expressed in a cell line (**Fig. 20B**, left panel). In co-transfection assays, mutant DLX2 (Q50E) constructs were unable to repress luciferase gene expression when compared to the wild-type DLX2 expression constructs (**Fig. 21A**). The DNA binding mutants (DLX1/2 Q50E) reduce transcriptional activity for the luciferase reporter to a similar extent, although not significantly; however, since repression of the *Nrp*2 promoter by DLX1 is less than that by DLX2, the difference in DLX1 rescue (from

A

Dlx1 wt

Dlx2 wt

VP16-Dlx

Eng-Dlx

$Q \to E$

VP16-Dlx DB

$Q \to E$

Eng-Dlx DB

$Q \to E$

Dlx DB

| N terminal | Homeobox | C terminal |
| Nuclear localization sequence | Engrailed | VP16 |

B

DLX1 →

β-actin →

DLX1   DLX1 Q50E   DLX2   DLX2 Q50E

← DLX2

DLX1   DLX1 Q50E   DLX2   DLX2 Q50E

Free probe
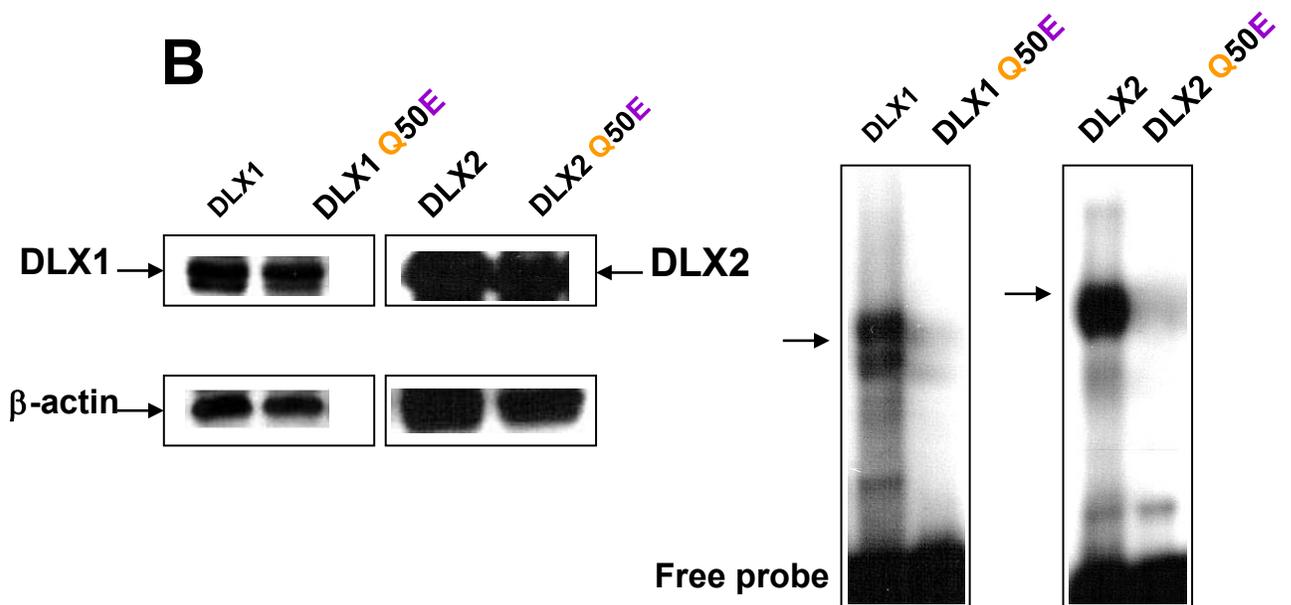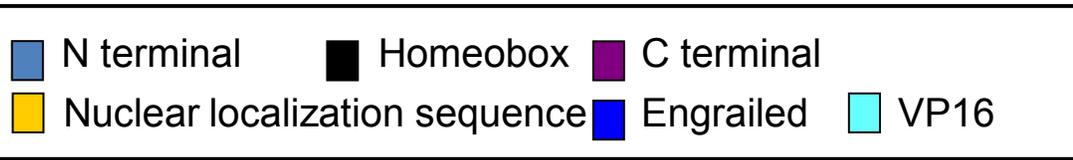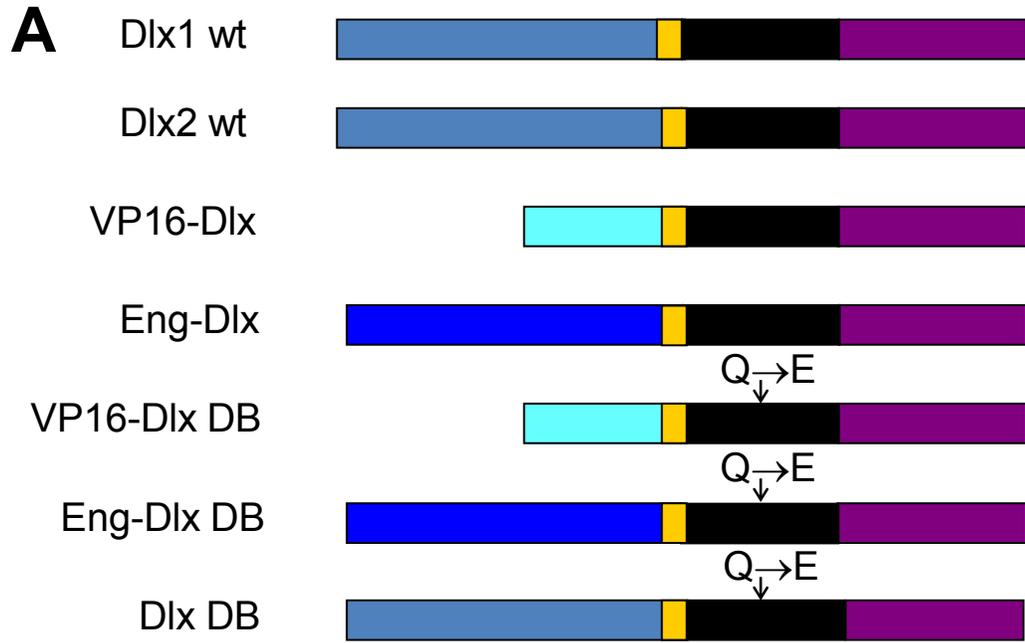
Figure 20. Modifications of DLX structures to generate chimeric proteins for reporter gene assays. (A) The schematic diagram of DLX fusion protein constructs was provided by Dr. J. Wigle, University of Manitoba. The N-terminal domains of wild-type (wt) DLX1 or DLX2, leaving the nuclear localization sequence intact, were replaced with the transactivation domain of Herpes Simplex Virus VP16 (VP16-*Dlx* construct), or the transcriptional repression domain of *D. Melanogaster* Engrailed (Eng-*Dlx* construct, (Kessler, 1997)). In addition, the glutamine residue at amino acid position 50 of the homeodomain, critical for binding of the homeodomain to DNA (Gehring et al., 1994), was mutated to glutamate (Q50E) in the full-length wild-type and fusion protein constructs (DB: DNA-binding mutant). (B) Left panel: Western analysis of total HEK293 cellular extract post-transfection of control DLX constructs or mutant DLX Q50E constructs. Subsequent immunoblotting was performed with DLX1 or DLX2 antibodies to assess level of expression for both control (DLX1 or 2) and mutant (DLX1 or 2 Q50E) proteins. β-actin was used as control for semi-quantitative analysis of protein levels. Right panel: EMSA experiment was performed with total HEK293 cellular extract post-transfection of control DLX constructs or mutant DLX Q50E constructs. Radiolabelled *Nrp*2ii oligonucleotides were incubated with HEK293 cellular protein extracts from control DLX transfection (*first lanes*), or from mutant DLX Q50E transfection (*second lanes*). Arrows indicate specific DLX1 or DLX2/*Nrp*2ii complexes.

A

Relative luciferase activity (fold)

- Nrp-2ii + pcDNA3
- Nrp-2ii + Dlx1 *
- Nrp-2ii + Dlx2 *
- Nrp-2ii + Dlx1 Q50E
- Nrp-2ii + Dlx2 Q50E

B

Relative luciferase activity (fold)

- Nrp-2ii + pcDNA3
- Nrp-2ii + Dlx1 *
- Nrp-2ii + ENG-Dlx1 *
- Nrp-2ii + VP16-Dlx1 *
- Nrp-2ii + pcDNA3
- Nrp-2ii + Dlx2 *
- Nrp-2ii + ENG-Dlx2 *
- Nrp-2ii + VP16-Dlx2 *
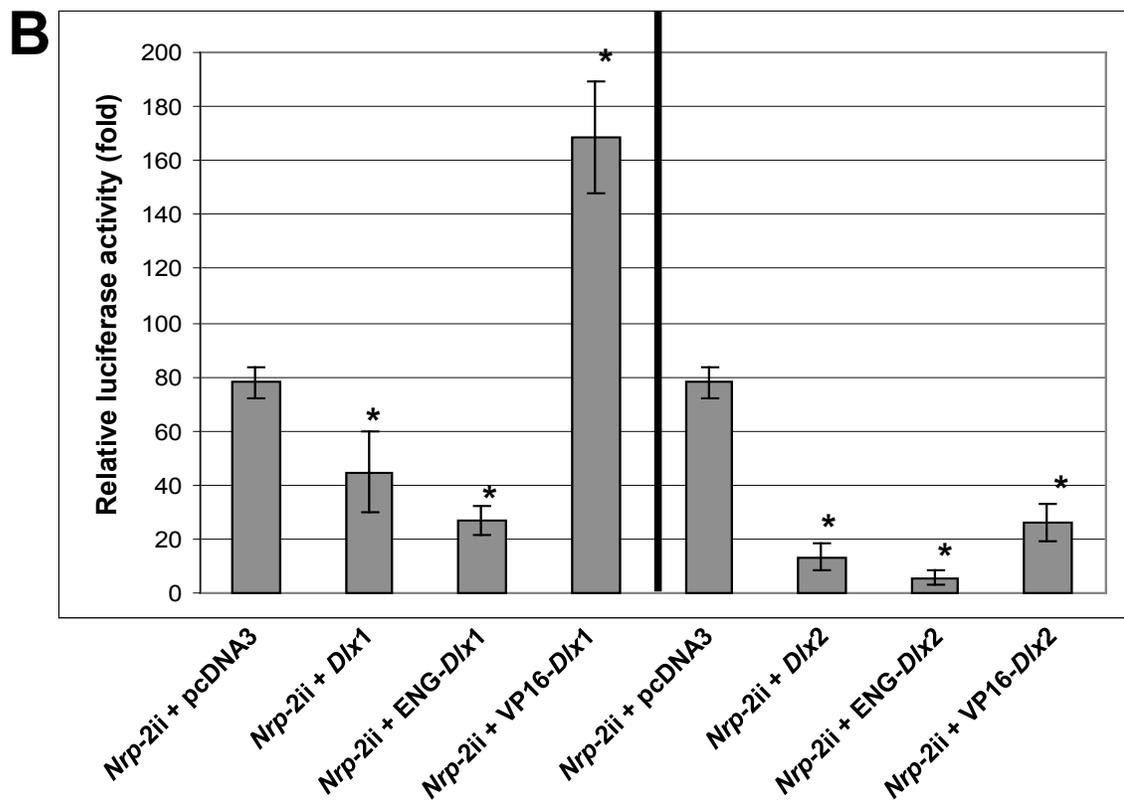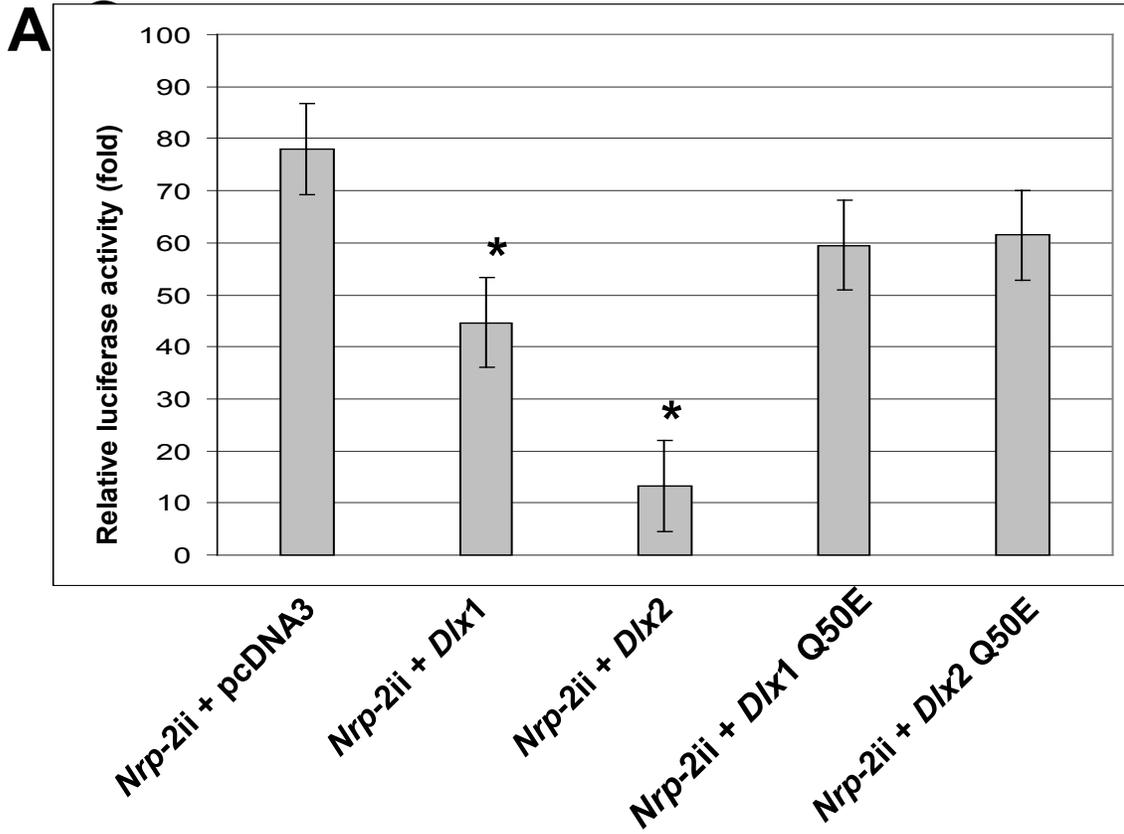
Figure 21. Transcriptional repression of Neuropilin-2 expression by DLX1 or DLX2 *in vitro*. (A) Reporter gene assays following transient co-transfections with *Nrp*2ii DNA sequences cloned into a pGL3-Luciferase reporter, in the absence or presence of DLX1 or DLX2 expression in HEK293 cells. Q50E mutations of the DLX homeodomain resulted in a loss of transcriptional repression of the *Nrp*2 promoter evident with wild-type *Dlx* constructs. (B) DLX homeodomains fused with N-terminal Engrailed repressor or VP16 activator domains modify transcriptional activity of the *Neuropilin*2 promoter. Compared to wild-type DLX proteins, transcriptional activity of *Nrp*2ii was further repressed by Eng-DLX fusion proteins but was reversed by using VP16-DLX fusion proteins (more so for DLX1 than DLX2; refer to Discussion Chapter). Here for both A and B, the data shown are the mean ± standard deviation of at least three trials. β-gal activity was used as an internal control. Since each construct has a different basal level of reporter gene expression, luciferase activity of each experiment (protein construct-pcDNA3 + *Nrp*2ii) was normalized with transfection of empty pcDNA3 + *Nrp*2ii). [* denotes a p value < 0.001 when compared to normal luciferase activity driven by *Nrp*2ii only with no additive protein construct].

repression) is not as significant as in DLX2 rescue by the Q50E DNA binding mutants.
The second group of constructs included an N-terminal domain exchange of DLX1 and
DLX2 with either a VP16 activator domain or an Engrailed repressor domain (Kessler,
1997). N-terminal Engrailed-DLX1 and Engrailed-DLX2 chimeric fusion proteins further
repressed transcription of the *Nrp*2 promoter region ii as compared to the wild-type DLX
constructs. Conversely, N-terminal VP16-DLX1 and VP16-DLX2 chimeric fusion
proteins activated transcription of the *Nrp*2 promoter region ii, overcoming all or some of
the transcriptional repression resulting from the wild-type DLX constructs, by
approximately 3.7 and 1.7-fold, respectively (p<0.001, **Fig. 21B**). In this assay, DLX2 is
a stronger repressor; hence this function is not completely alleviated by placement of the
VP16 domain at its N-terminus. Hence, from these data we can conclude that the direct
interaction of DLX1 or DLX2 proteins with the *Nrp*2 promoter results in the repression
of Neuropilin2 transcription.

**6.4 Complementary DLX1 or DLX2 and Neuropilin2 expression patterns in the
developing forebrain**

    Dlx homeobox genes are expressed in interneurons that express GABA in the
embryonic rostral forebrain (Anderson et al., 1997a; Anderson et al., 1997b; Anderson et
al., 2002; Marin et al., 2000). During the early stages of embryonic telencephalon
development, the subpallium expresses *Dlx*1 and *Dlx*2 primarily in the VZ and SVZ
(Anderson et al., 2001; Anderson et al., 2002; Eisenstat et al., 1999; Liu et al., 1997) with
a clear limitation of expression at the LGE/neocortex (subpallial-pallial) boundary (**Fig.
22** panels a, d). DLX1 and DLX2 expression are both well-established by E10.5
(Eisenstat et al., 1999). Expression of NRP2 is reported to be primarily restricted to the

DLX1	NRP-2	Merge

DLX2	NRP-2	Merge
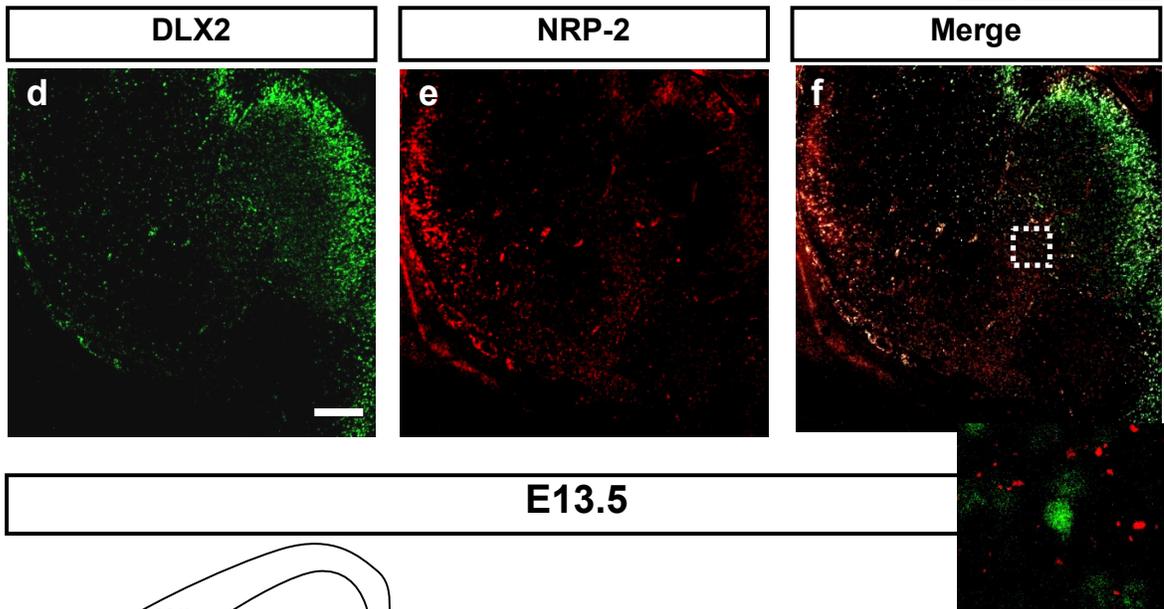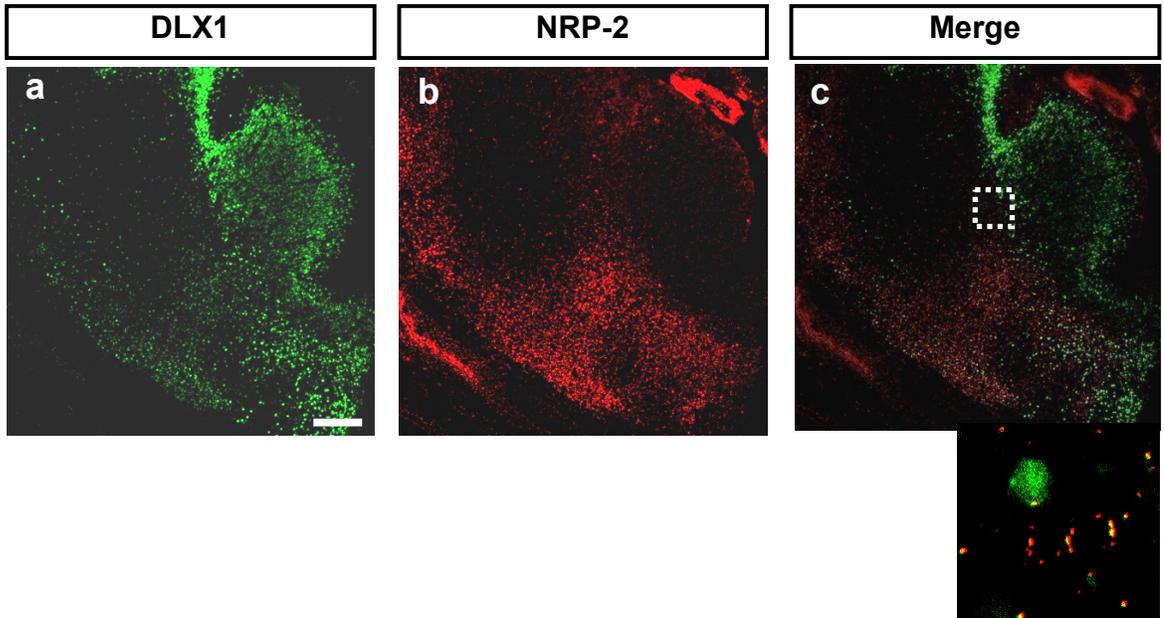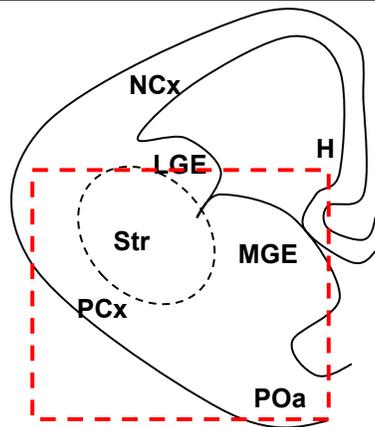
E13.5

Figure 22. Patterns of DLX1 or DLX2 and Neuropilin2 expression in the basal telencephalon. Cryosections of E13.5 forebrain were labelled with specific antibodies against DLX1 (a), DLX2 (d) and NRP2 (b, e). The left panels (a, d) show DLX1- or DLX2-positive cells (green) in lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE) and the anterior preoptic area (POa). The center panels (b, e) show NRP2-positive cells (red) within the mantle zone of the pallidal anlagen. In the right panels, image overlays demonstrate minimal overlap of DLX1 or DLX2 with NRP2 expression. The overlay indicates the majority of DLX1/DLX2 positive cells are not expressed with NRP2 positive cells. Scale bar, 200 μm. Inserts in the Merge column represent a 10x enlargement. [H, Hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; PCx, paleocortex; POa, anterior preoptic area; Str, striatum].

mantle zone of the MGE as well as the olfactory cortex (Marin et al., 2001). We found that endogenous expression of NRP2 becomes well-established from E13.5 in the mantle zone of the subcortical telencephalon using *in situ* hybridization (**Appendix Fig. 6**). Furthermore, we also detected NRP2 protein expression as early as E13.5 using immunofluorescence (**Fig. 22** panels b, e; **Fig. 23A** panel c). NRP2 expression becomes more restricted to the mantle zone of the MGE and paleocortex at age E16.5 (**Fig. 23B** panel c; **Appendix Fig. 6B** panels b, e) and E18.5 (**Fig. 23C** panel c). Co-expression studies of DLX1 or DLX2 and NRP2 at E13.5 (**Fig. 22** panels c, f and inserts), and at E16.5 and E18.5 (data not shown) show only a minimal overlapping pattern between the major populations of DLX1/DLX2 and NRP2 single positive cells in the basal telencephalon. Combined ISH (*Nrp*2) and IHC (DLX1 or DLX2) experiments at E13.5 and E16.5 confirmed that only a few cells co-expressed NRP2 and DLX1/DLX2 within this neuroanatomic region (**Appendix Fig. 6**). Since most cells do not co-express DLX and NRP2, these results are consistent with the potential role of DLX proteins as repressors of Neuropilin2 expression in Dlx-containing cells in the developing forebrain.

6.5 Neuropilin2 is ectopically expressed in the absence of *Dlx*1 & *Dlx*2 gene function

The above assays show that DLX proteins bind *Nrp*2 *in vivo* and *in vitro*, that they repress the *Nrp*2 promoter *in vitro* and that DLX1/DLX2 and NRP2 are primarily expressed in separate neuronal populations *in vivo*. However, these experiments do not reveal whether DLX proteins are required to repress *Nrp*2 *in vivo*. To address this key issue, we determined the expression patterns of *Nrp*1 RNA, NRP2 protein and *Nrp*2 RNA in the forebrains of *Dlx*1/2 double mutant mice. No differences in *Nrp*2 expression were

A

	Dlx1/Dlx2+/+	*Dlx1/Dlx2-/-*

NRP-2

E13.5

a

b

c

d

NCx

LGE

Str

PCx

MGE

H

POa

a,b

c,d

B

Dlx1/Dlx2+/+	*Dlx1/Dlx2-/-*
a	b
c	d
e	f

NRP-2

E16.5

NCx
H
a,b
LGE
MGE
Str
PCx
POa
c,d
e,f

C

Dlx1/Dlx2+/+   **Dlx1/Dlx2-/-**

NRP-2

E18.5

a   b

c   d

e   f

NCx

H

LGE

a,b

Str

MGE

c,d

POa

e,f
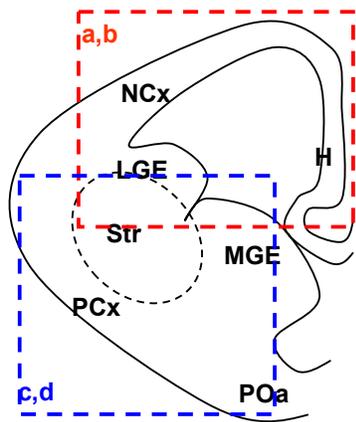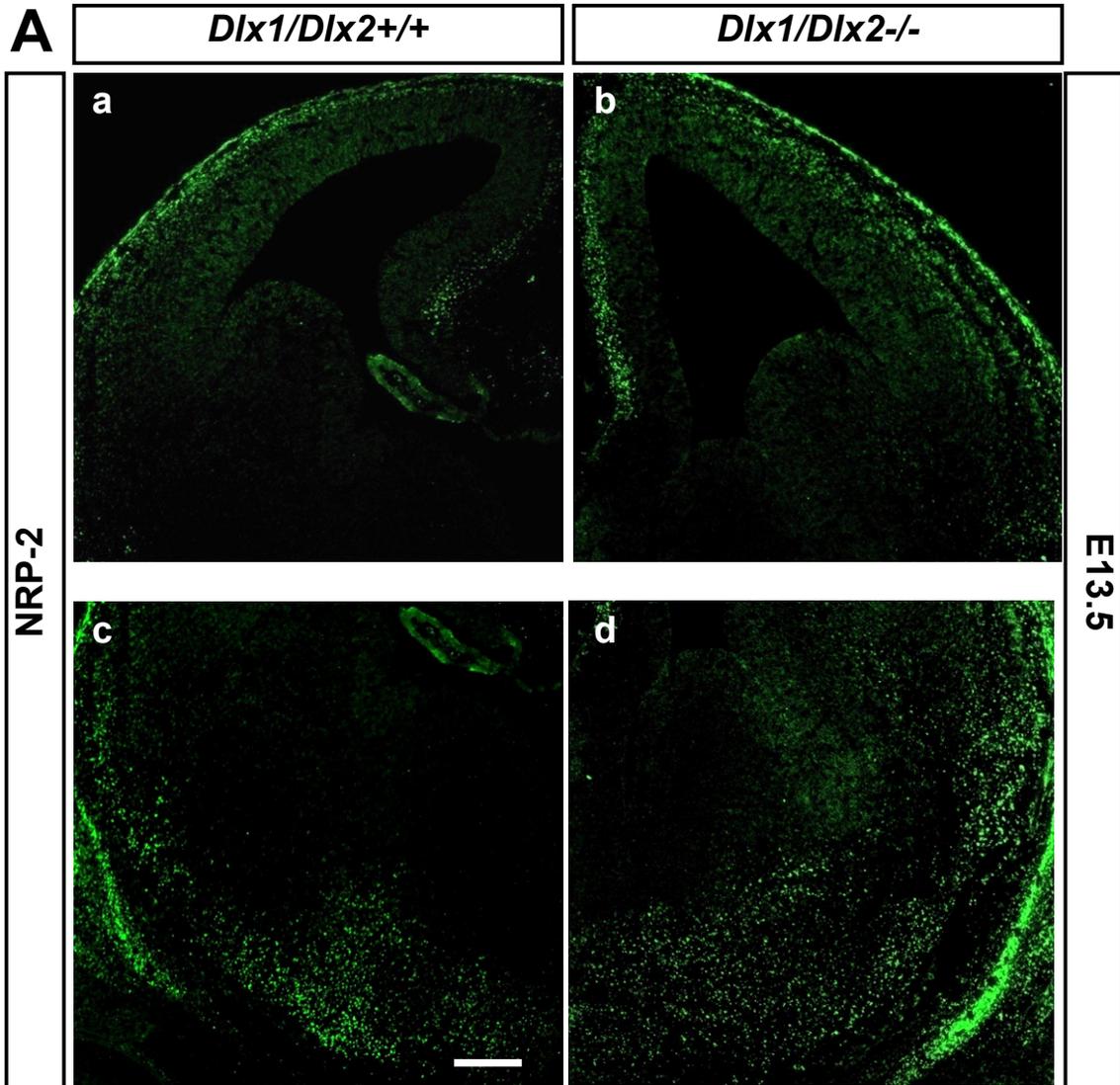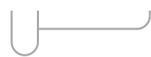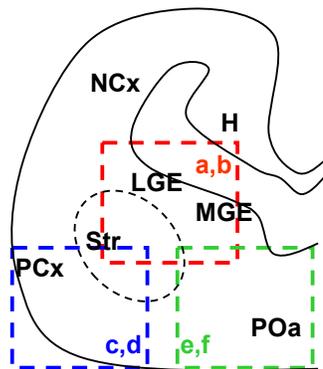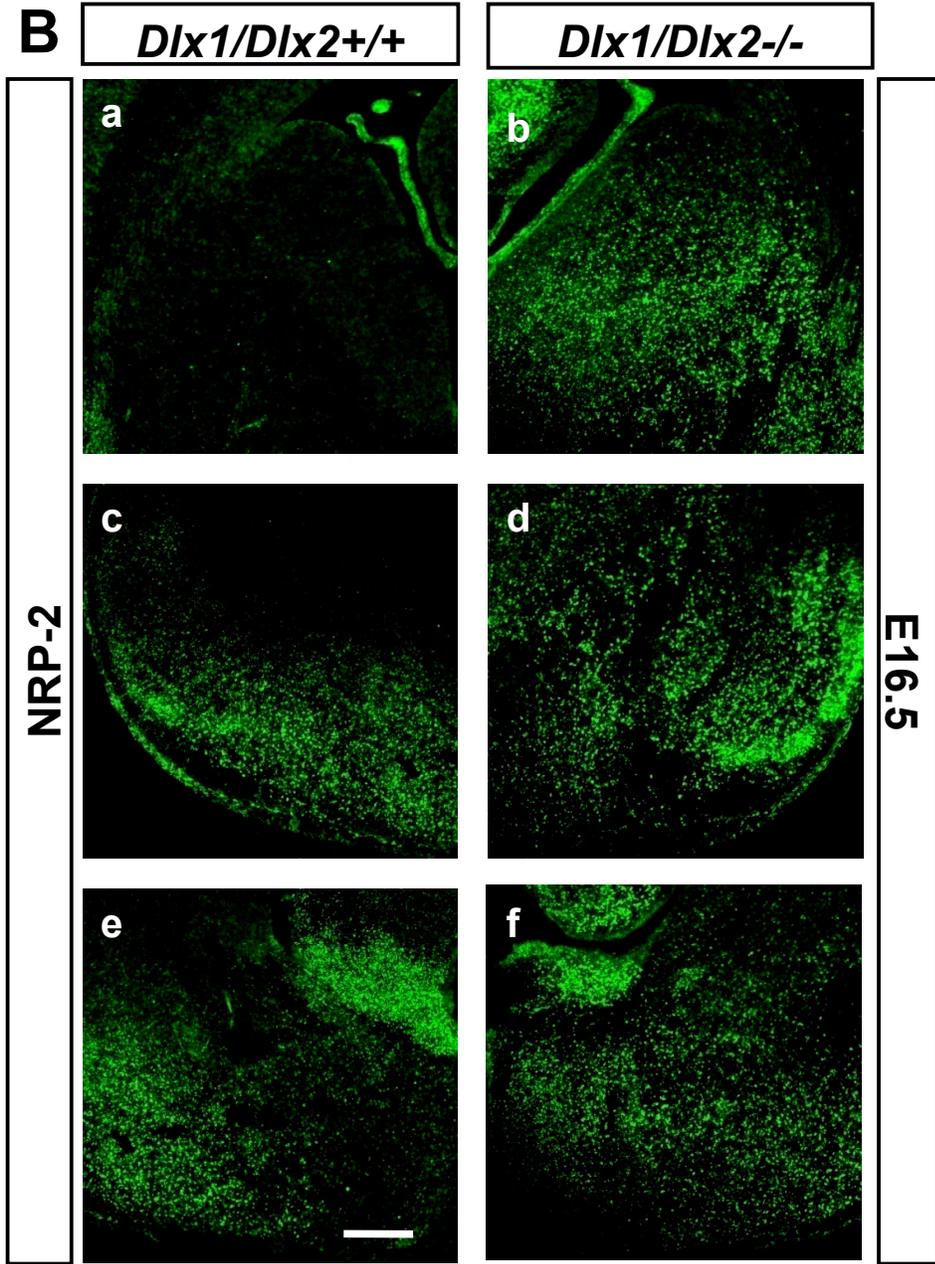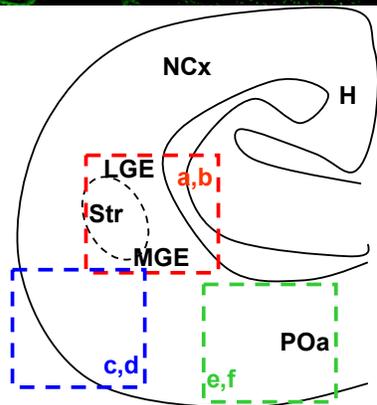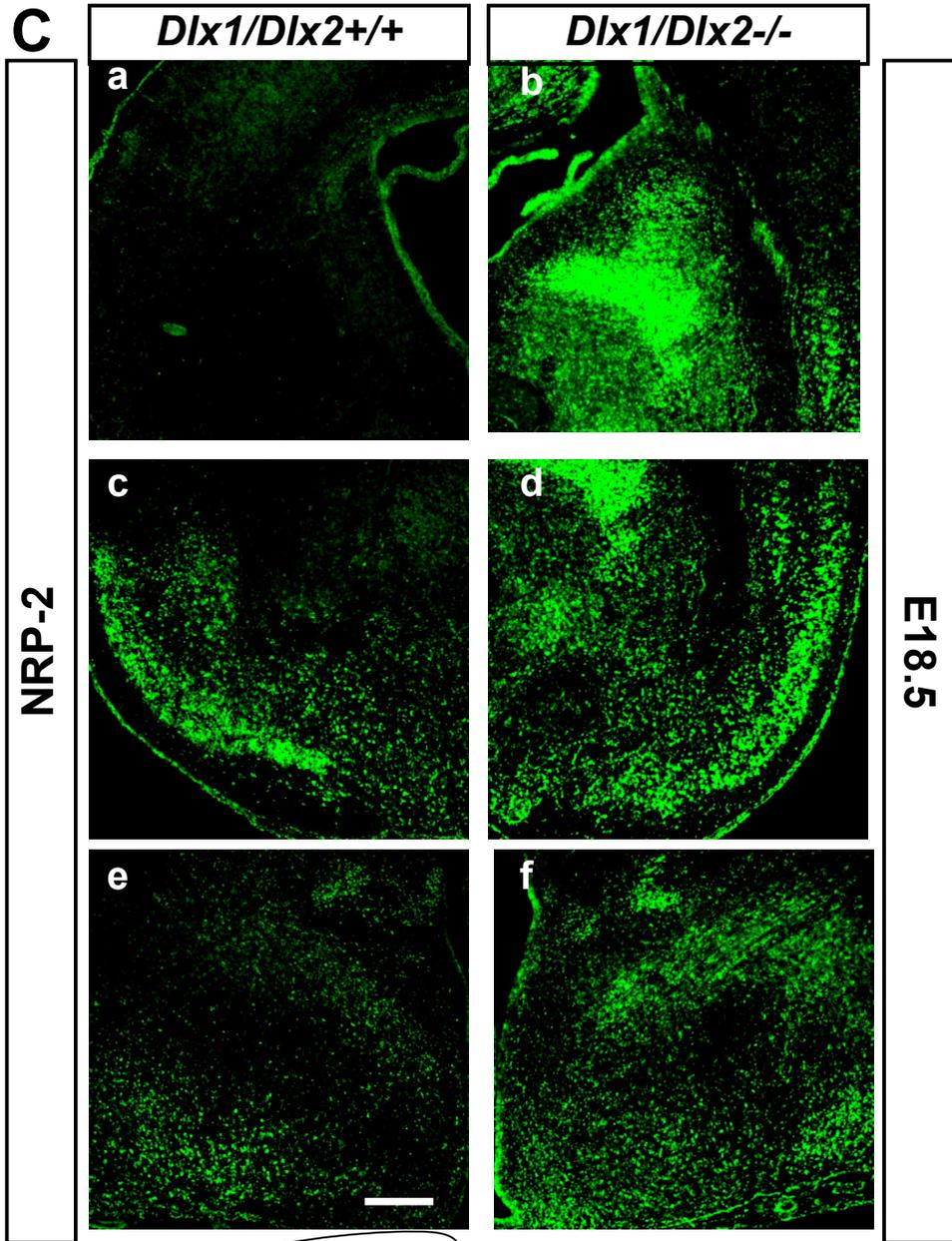
Figure 23. Neuropilin2-expressing interneurons accumulate in the striatal anlagen in the *Dlx*1/*Dlx*2 double mutant. (A) At E13.5, NRP2 expression remains unchanged in the mantle zone of the embryonic striatum when comparing wild-type and *Dlx*1/*Dlx*2 double-mutant littermates. At E16.5 (B) and E18.5 (C), in the absence of *Dlx*1 and *Dlx*2 gene function, there is increased NRP2 expression with extension in the mutant ganglionic eminences and accumulation within the striatal anlagen. Coronal sections; scale bars, 200 μm (A), 400μm (B), 500μm (C). [H, hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; PCx, paleocortex; POa, anterior preoptic area; Str, striatum].

Nrp-2

Dlx1/Dlx2+/+ *Dlx1/Dlx2-/-*

NCx
H
LGE
MGE
Str
PCx
POa

NCx
H
LGE
Str
MGE
POa

a b E16.5
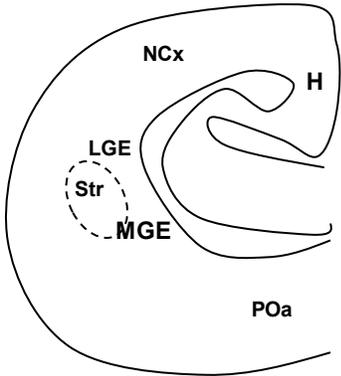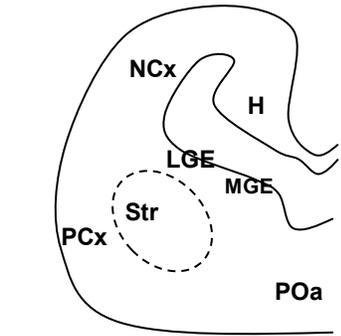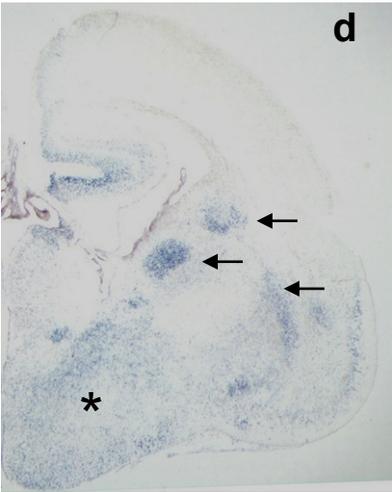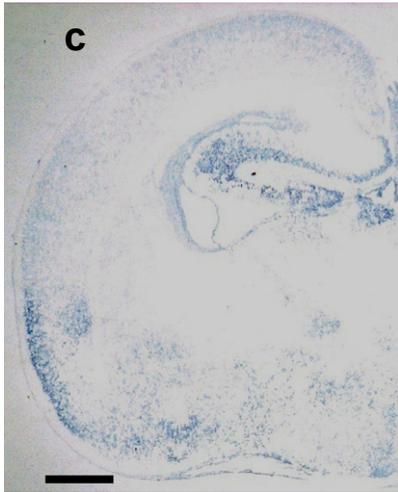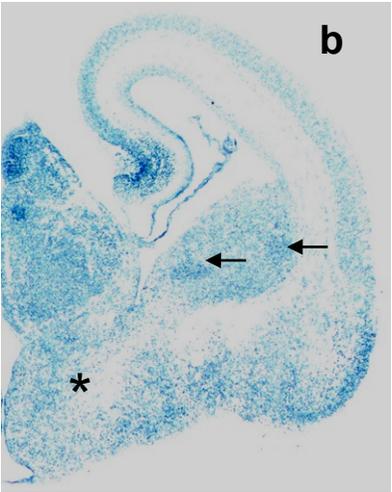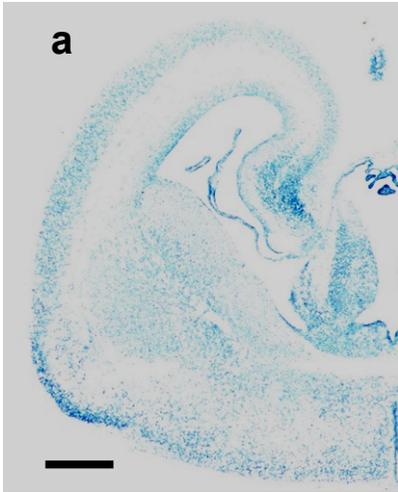
c d E18.5

Figure 24. Interneurons in the *Dlx1/Dlx2* double mutant express ectopic Neuropilin-2 in the SVZ of the basal telencephalon. Digoxigenin *in situ* RNA hybridization confirms ectopic expression of NRP2 in the *Dlx*1/*Dlx*2 null ganglionic eminences at E16.5 (b) and E18.5 (d) compared to matched wild-type tissue sections (a, c), respectively. The asterisk in (b) denotes the AEP located in this more caudal cryosection. Arrows demarcate small "collections" of interneurons expressing ectopic NRP2 that resemble periventricular heterotopias or ectopias most evident at E18.5 (b, d). Coronal sections. Scale bars: (a, b) 400 μm; (c, d) 500 μm. [AEP, anterior entopeduncular area; H, hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; PCx, paleocortex; POa, anterior preoptic area; Str, striatum]. The experiment was done with the help of Dr. G. Du, Eisenstat laboratory, Manitoba Institute of Cell Biology, University of Manitoba.

observed between wild-type and *Dlx1/2* mutant telencephalon at E13.5 (**Fig. 23A**).

Similarly, the expression pattern of *Nrp*1 was unaffected throughout development of the

embryonic forebrain to P0 (**Appendix Fig. 7C**). *Mash*1, a proneural gene encoding a

basic-helix-loop-helix (bHLH) transcription factor, had been implicated in the

differentiation of early- and not late-born neurons in the striatum in a temporally defined

manner (Casarosa et al., 1999; Yun et al., 2002). *Wnt*7b, wingless-type MMTV

integration site family member 7B, had been implicated in oncogenesis and in several

developmental processes, including regulation of cell fate and patterning during

embryogenesis (Abu-Khalil et al., 2004; Wang et al., 2005b). DLX proteins were not

found to bind to either *Mash*1 5' upstream sequences or *Wnt*7b promoter (**Appendix Fig.
5**). These genes were compared between wild-type and *Dlx*1/2 mutant to show no

changes in expression of these specific markers in the basal telencephalon (**Appendix

Fig. 7A, B**), further confirming these negative ChIP results. Significantly,

immunofluorescence and *in situ* RNA hybridization experiments demonstrate that there is

progressive accumulation of Neuropilin-2 expressing cells in the SVZ of the LGE and

MGE after E13.5 through to birth (Marin et al., 2001) when the mutant mice die (**Figs.
23B, C; 24**). These results are consistent with the hypothesis that with the absence of

*Dlx*1 and *Dlx*2 function there is loss of transcriptional repression by DLX1 or DLX2

leading to the aberrant expression of NRP2, resulting in the formation of SVZ ectopias in

subcortical cells of the mutant GEs (**Fig. 24**, *arrows*, panels b, d). More importantly, this

study is the first to report that *Dlx* transcription factors may function as transcriptional

repressors *in vivo* and their downstream targets play a significant role in tangential

migration of interneurons during forebrain development.

Chapter 7

Partial rescue of tangential migration in *Dlx*1/*Dlx*2/*Nrp*2 triple mutant mice:

Inhibition of Semaphorin signalling by *Dlx* homeobox genes promotes tangential

migration of interneurons to the neocortex

Rationale: *Dlx* genes are expressed in the ganglionic eminences (GE) of the developing

forebrain (Panganiban and Rubenstein, 2002). *Dlx*1/2 double knockout mice die at birth

with abnormal cortical development, including loss of tangential migration of

GABAergic inhibitory interneurons to the neocortex (Anderson et al., 1997a; Anderson et

al., 1997b). Through confirmation by ChIP and subsequent molecular analyses, we have

identified Neuropilin2 to be a direct downstream target for DLX1 and DLX2 *in vivo* (Le

et al., 2007). Furthermore, reporter assays demonstrated that both DLX1 and DLX2

repress *Nrp*2 expression, while lack of *Dlx*1/2 function leads to increased and ectopic

NRP2 expression in the basal telencephalon. Hence, without transcriptional repression of

Neuropilin2 by DLX, NRP2 is aberrantly expressed and might explain why these

interneurons cannot migrate to the neocortex due to Semaphorin-Neuropilin interactions

(**Fig. 25**). We have generated a *Dlx*1/*Dlx*2/*Nrp*2 triple KO mouse by crossing *Dlx*1/*Dlx*2

heterozygotes with *Nrp*2 null mutants. By removing Neuropilin2 expression in the

accumulated interneurons in the basal forebrain of *Dlx*1/2 mutants, we hypothesized that

some of these interneurons could migrate to the neocortex by removing the

chemorepulsion mediated by Semaphorin signalling. The neocortex of the triple KO

compared to the *Dlx*1/2 double KO demonstrated a partial restoration of GABA and

calbindin-expressing interneurons in the neocortex by E18.5 ($p < 0.05$), and a complete

MODEL: DLX1 & DLX2 ⊣ NRP2

a +/+

NCx

LGE

MGE

Str

POa

b *Dlx1/2-/-*

NCx

LGE

MGE

Str*

POa
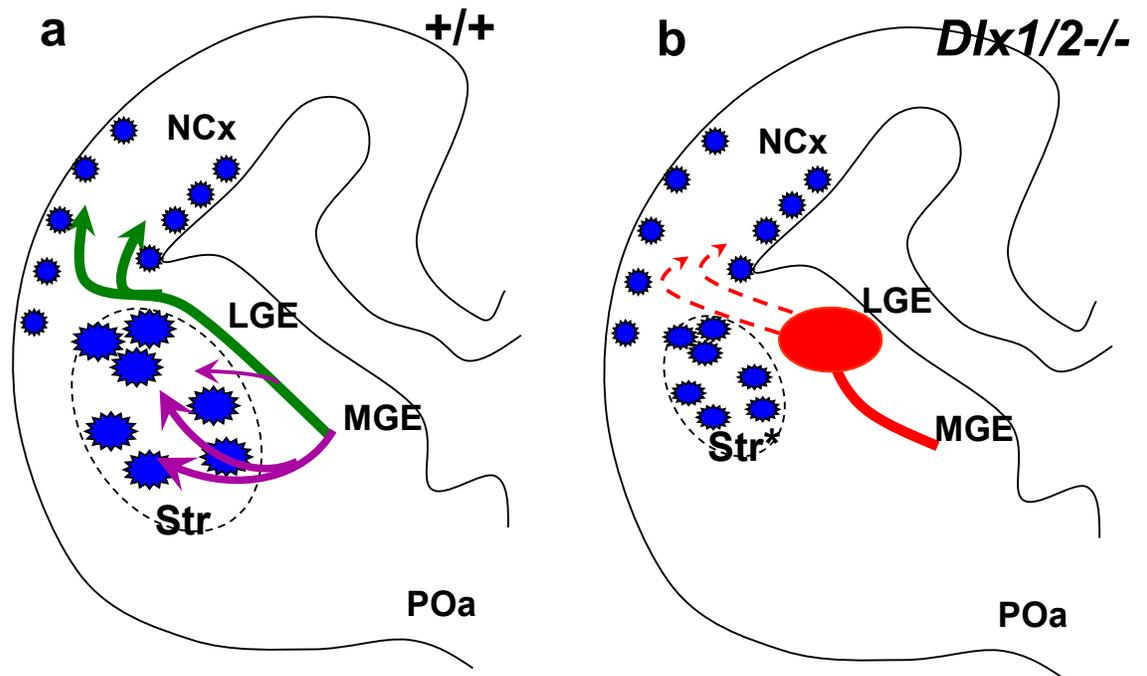
→ *Dlx*1/2 on, *Nkx*2.1 off, NRP2 low

→ *Dlx*1/2 on, *Nkx*2.1 on, NRP2 off

→ *Dlx*1/2 off, NRP2 high

✹ Sem3F

Figure 25. Schematic model of a mode of tangential migration regulated by *Dlx*, *Nrp*2, and *Sema*3A/3F. Class 3 semaphorin proteins are chemorepellents for growing axons (Raper, 2000; Tamagnone and Comoglio, 2000), allowing them to avoid specific regions in the striatum and channelling them into appropriate locations in fiber tracts in the neocortex (Zou et al., 2000). During the period of interneuron migration (E12.5 to E16.5), semaphorins 3A and 3F (Sema3A and Sema3F) (blue) are expressed in the embryonic striatum and neocortex but are excluded from regions surrounding it. The high-affinity semaphorin receptor Neuropilin2 is required for mediating the repulsive effect of class 3 semaphorins on axons (Raper, 2000; Tamagnone and Comoglio, 2000). During the period of interneuron migration, Neuropilin2 receptors (red) are expressed in the basal telencephalon in a pattern complementary to that of Sema3A and Sema3F—that is, in cells located either superficial or deep to the striatum but excluded from striatal cells. (a) In the wild-type forebrain, tangentially migrating interneurons are expressing *Dlx* genes which down-regulate NRP2 expression. These interneurons (green) can migrate past striatal Semaphorin-expressing populations towards the neocortex, because they are not being repelled by Semaphorin-Neuropilin interactions. (b) In the *Dlx*1/2 double knockout forebrain, absence of the function of *Dlx* genes will allow Neuropilin2 to be highly expressed due to absence of *Dlx* mediated repression. These interneurons (red) cannot migrate past the striatal Semaphorin-expressing populations toward the neocortex, due to repulsion facilitated by Semaphorin-Neuropilin interactions. This defect in tangential migration leads to an accumulation of GABAergic interneurons in the basal ganglia as periventricular ectopias (**Fig. 24** panel d). [MGE/LGE, medial/lateral ganglionic eminence; NCx, neocortex; POa, anterior preoptic area; Str, striatum]

restoration of Somatostatin co-expressing interneurons in the neocortex by E18.5

(p<0.02).

7.1 Comparative analysis of layer-specific genes in cortical lamination

Examples of cortical layer-specific genes included, among many others: *Math*2, marker of developing cortical plate of later-born neurons (Bartholoma and Nave, 1994; Guo et al., 2002); *Cux*2, marker of layers II/III to IV (Nieto et al., 2004; Zimmer et al., 2004); *ROR*β, marker of layer IV (Schaeren-Wiemers et al., 1997; Schuurmans et al., 2004); *ER*81, marker in cortical and subcortical projection neurons of layer V (Arber et al., 2000; Hevner et al., 2003); *Tbr*1, marker of neurons of layer VI (Englund et al., 2005; Hevner et al., 2001); *Lhx*2, marker of neocortical VZ (Bulchand et al., 2001; Monuki et al., 2001); and *Ngn*2, a proneural bHLH gene that is required to specify the identities of early born, deep-layer neurons (Britz et al., 2006; Gradwohl et al., 1996). These genes are expressed in one specific neuronal type within a layer or across layers, among a large number of genes that exhibit varying degrees of restricted expression (Arlotta et al., 2005). Consistent with the observations of other groups (Giger et al., 2000), many *Nrp*2 single knockout mice were viable and their external appearance was indistinguishable from wild-type and heterozygous littermates. Molecular analyses, showed no obvious abnormalities in neuronal differentiation and migration. *ROR*β, which encodes an orphan nuclear receptor, is specifically expressed in layer-IV neurons (Schaeren-Wiemers et al., 1997). *ROR*β expression was greatly reduced in the dorsal neocortex of the double and triple knockout, compared to the wild-type and single knockout cortices (**Fig. 26**, panels a-d). More importantly, the reduction in dorsal expression of *ROR*β in the double knockout neocortex remained the same in the triple knockout, with only ventral

expression unaffected in the pallial/subpallial zone of the telencephalon. *ER*81 is a transcription factor of the ETS family (de Launoit et al., 1997). It is expressed almost exclusively in a subset of cortical layer V projection neurons across various cortical areas (Hevner et al., 2003; Xu et al., 2000). *ER*81 expression was more restricted to layer-V of the neocortex, and its level of expression remained unchanged in all 4 genotypes (**Fig. 26**, panels e-h). *ER*81 was also expressed in neuronal populations of the SVZ of LGE, the RMS, remnant of MGE, and in differentiating pallidum (Stenman et al., 2003) of both wild-type and the single Nrp2 knockout. However, *ER*81-expressing interneurons were severely reduced in all areas of the double knockout LGE, including in the RMS similar to (Long et al., 2007). Yet surprisingly, the *ER*81-expressing population in the ventral differentiating pallidum was present in the triple knockout. *Neurogenins* are specifically expressed in cortical but not subcortical progenitors, where they specify the regional identity of the earliest-born preplate neurons in the neocortex of the telencephalon (Bertrand et al., 2002; Fode et al., 2000). *Ngn*2 expression was found in VZ progenitors of both wild-type and single knockout cortices (**Fig. 26**, panels i-l; (Britz et al., 2006)). Interestingly, in the double knockout cortex, *Ngn*2-expressing cells were also found in the cortical plate and/or mantle zone as ectopias. The results indicated that these cells are either terminally differentiated cells expressing *Ngn*2 abnormally, or differentiating progenitor cells with aberrant migration to outer layers of neocortex. However, in the triple knockout neocortex, *Ngn*2-expressing cells could not be found outside of their usual VZ/SVZ domains in the embryonic forebrain. These data suggest that any abnormality in expression of cortical *Ngn*2 in the double knockout was not present in the triple knockout neocortex.

| | *Dlx1/2+/- Nrp2+/-* | *Dlx1/2+/- Nrp2-/-* | *Dlx1/2-/- Nrp2+/-* | *Dlx1/2-/- Nrp2-/-* |

RORβ — a, b, c, d

ER81 — e, f, g, h

Ngn2 — i, j, k, l

E18.5

NCx, H, LGE, Str, MGE, Thal, Amy, AEP
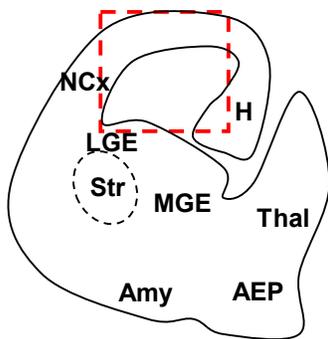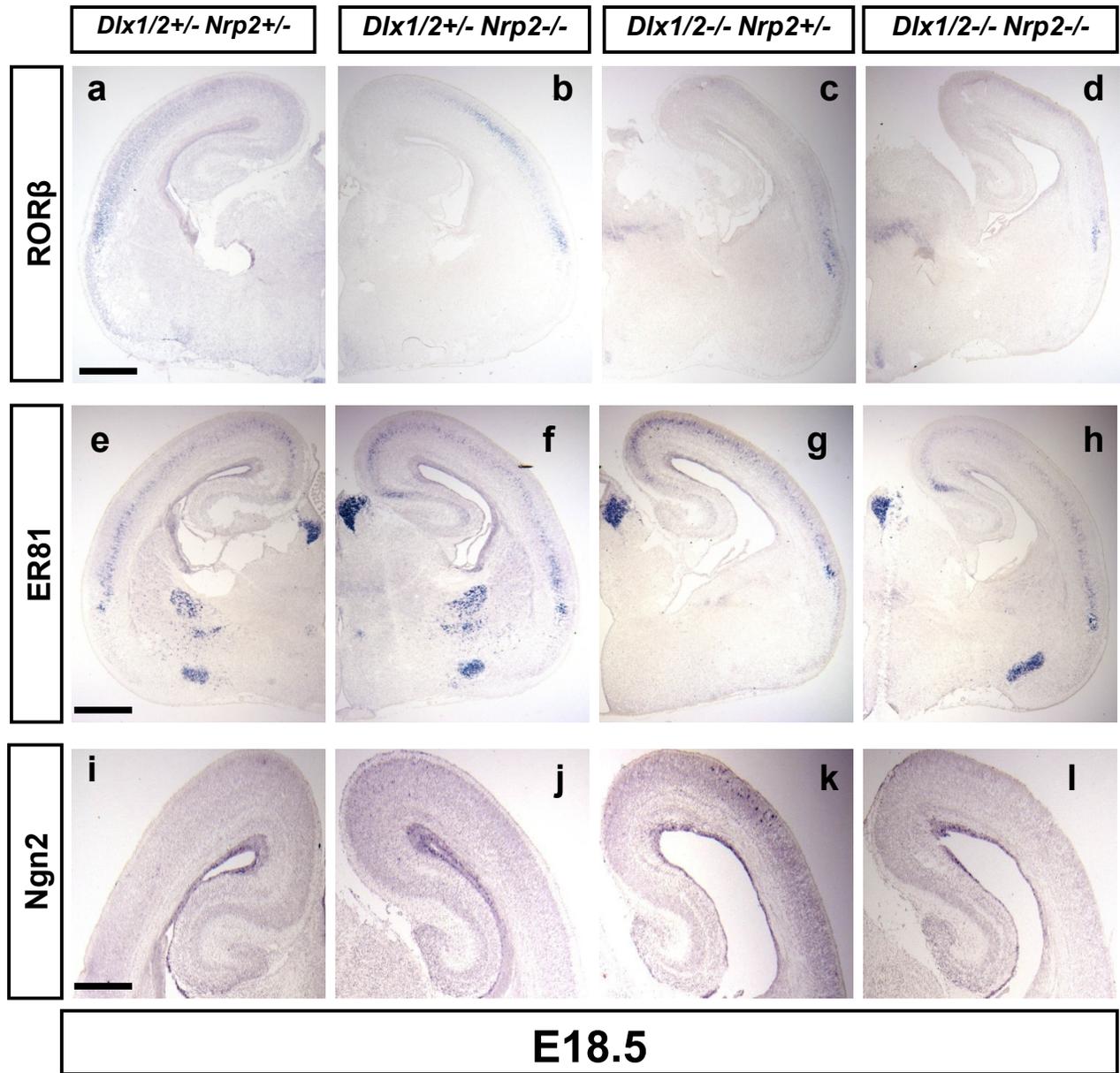
Figure 26: Changes in expression patterns of the specific markers for neocortex between four genotypes at E18.5. *In situ* hybridizations using *ROR*β (top row), *ER*81 (middle row), and *Ngn*2 (bottom row) riboprobes were performed on coronal sections of E18.5 wild-type, single, double and triple knockout forebrains. *ROR*β expression in layer-IV neurons was unchanged between wild-type (a) and single (b) knockout cortices, but there was a much-reduced expression domain dorsally in double (c) and triple (d) knockout cortices. *ER*81 expression is more restricted to layer-V of the neocortex, and its level of expression remains unchanged in all 4 genotypes. However, striatal *ER*81-expressing interneurons were severely reduced in the double (g) knockout compared to wild-type (e) and single (f) knockout striata. An *ER*81-expressing population in the mantle zone was re-established in the triple (h) knockout. *Ngn*2 is a proneural gene that is expressed in the VZ of wild-type (i) and single (j) knockout cortices. However, *Ngn*2-expressing cells can be found in outer cortical layers as ectopias in the double (k) knockout cortex, but these cells were not found in the triple (l) knockout cortex. Coronal sections; scale bars, 500 μm. [H, hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Amyl, amygdala; Str, striatum; Thal, thalamus; AEP: anterior entopeduncular area]. The experiement was done with the help of Tracie Parkinson, Eisenstat laboratory, Manitoba Institute of Cell Biology, University of Manitoba.

Using *in situ* hybridization, other cortical layer-specific markers (*Math*2, *Cux*2,

*Tbr*1, *Lhx*2) were tested and were not significantly altered among all 4 genotypes

(**Appendix Fig. 8**). Other layer-non-specific markers for neocortex were also tested and

found to be similar when comparing all 4 genotypes using immunofluorescence

(**Appendix Fig. 9**). These markers included: glutamate, an excitatory neurotransmitter

expressed in pyramidal neurons (Hill et al., 2000; Ottersen and Storm-Mathisen, 1984);

microtubule-associated protein 2 (MAP2), a marker of neuronal morphogenesis,

cytoskeleton dynamics and organelle trafficking in axons and dendrites (Dehmelt and

Halpain, 2004; Sanchez et al., 2000); the vertebrate muscle segment homeobox (*Msx*),

homeodomain transcription factors characterized as transcriptional repressors (Davidson,

1995; Ramos and Robert, 2005); Tyrosine kinase receptor, type B (*Trk*B), the primary

receptor for the neurotrophin brain-derived neurotrophic factor (BDNF) and

neurotrophin-mediated neuronal survival and migration (Bibel and Barde, 2000; Medina

et al., 2004); and T box family *reeler*-like (*Tbr*1), a marker of differentiating cortical

progenitors and/or early-born neurons of the preplate and layer VI (Bulfone et al., 1995;

Hevner et al., 2001).

7.2 Comparative analysis of striatal-specific genes

Tyrosine hydroxylase (TH) is the rate-limiting enzyme involved in the synthesis

of catecholaminergic neurotransmitters; it is expressed cytosolically in medium-sized

projection neurons which are enriched in the extrastriosomal matrix (Graybiel, 1990;

Johnston et al., 1990; van der Kooy, 1984). TH is also a faithful marker of dopaminergic

neurons which are morphologically similar to the medium-sized aspiny striatal

interneurons that use GABA as their principal neurotransmitter (Cicchetti et al., 2000).

TH expression was abundant in the striatum of wild-type and single knockout mice, with distribution of expression from LGE to MGE to the preoptic area (POa), marking the location of spiny and aspiny dopaminergic interneurons (**Fig. 27A,** panels a, b, e, f, i, j). However, TH expression was severely down-regulated in both double and triple knockout striata. Furthermore, TH-positive striatal interneurons accumulated as ectopias in both double and triple knockout striata (**Fig. 27A**, panels c, d, g, h, k, l; white arrows). The results suggest a differentiation defect in extrastriosomal matrix development in both double and triple knockout mice.

Dopamine- and cyclic AMP-regulated phosphoprotein (DARPP32) is a D1-dopamine-receptor associated signalling protein found in striatal projection neurons in the striosomes at E18.5, specifically medium-sized spiny striatal interneurons that express substance P and/or enkephalin (Foster et al., 1987; Huot and Parent, 2007). DARPP32-labelled striatal interneurons were clearly present in the wild-type and single knockout striatum, including both MGE and LGE (**Fig. 27B**, panels a, b, e, f). However, DARPP32 expression was diminished in both double and triple knockout striata with only few positive cells in the mantle zone of the basal telencephalon (**Fig. 27B**, panels c, d, g, h). These data indicate that there is a differentiation defect in striosomal development and loss of striatal interneurons in both the double and triple knockout.

The paired box gene 6 (*Pax*6) is a member of the Paired box gene family of tissue-specific transcription factors containing a paired class homeodomain as well as a paired domain (Stoykova et al., 1997). *Pax*6 shows a rostro-caudal and dorsal-ventral gradient of expression in the VZ of the neocortex (Puelles et al., 1999). *Pax6* is also

A

	Dlx1/2+/- Nrp2+/-	*Dlx1/2+/- Nrp2-/-*	*Dlx1/2-/- Nrp2+/-*	*Dlx1/2-/- Nrp2-/-*

TH

a   b   c   d

e   f   g   h

i   j   k   l

E18.5

NCx
H
a,b,c,d LGE
Str   MGE
Thal
e,f,g,h
AEP
i,j,k,l

B

| *Dlx1/2+/- Nrp2+/-* | *Dlx1/2+/- Nrp2-/-* | *Dlx1/2-/- Nrp2+/-* | *Dlx1/2-/- Nrp2-/-* |

DARPP-32: a, b, c, d, e, f, g, h

Pax6: i, j, k, l, m, n, o, p

E18.5

NCx, H, LGE, Str, MGE, Thal, AEP

i,j,k,l
a,b,c,d
m,n,o,p
e,f,g,h
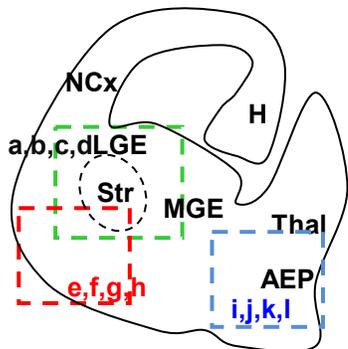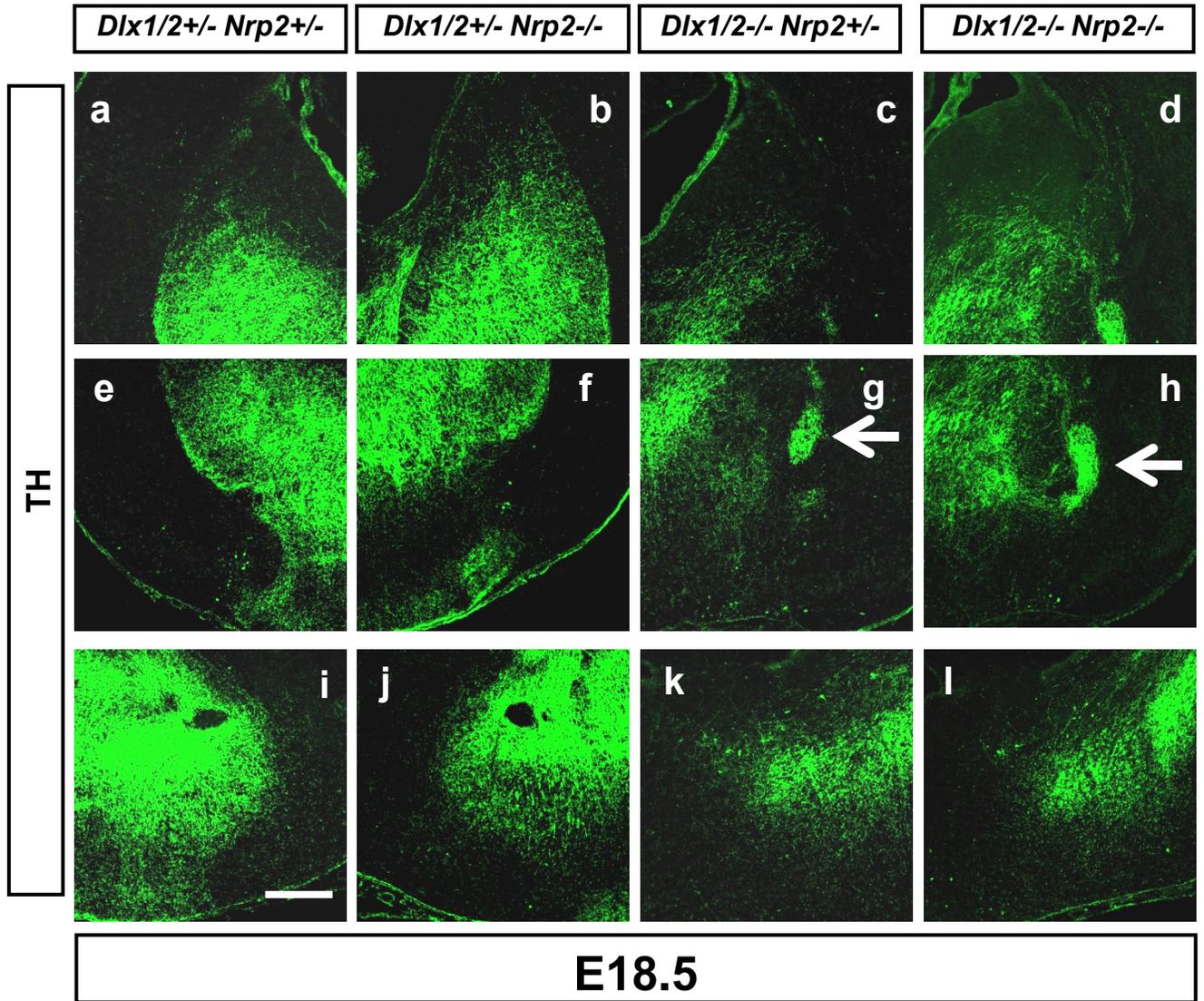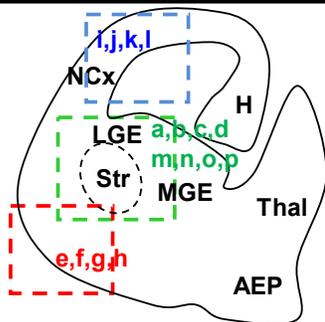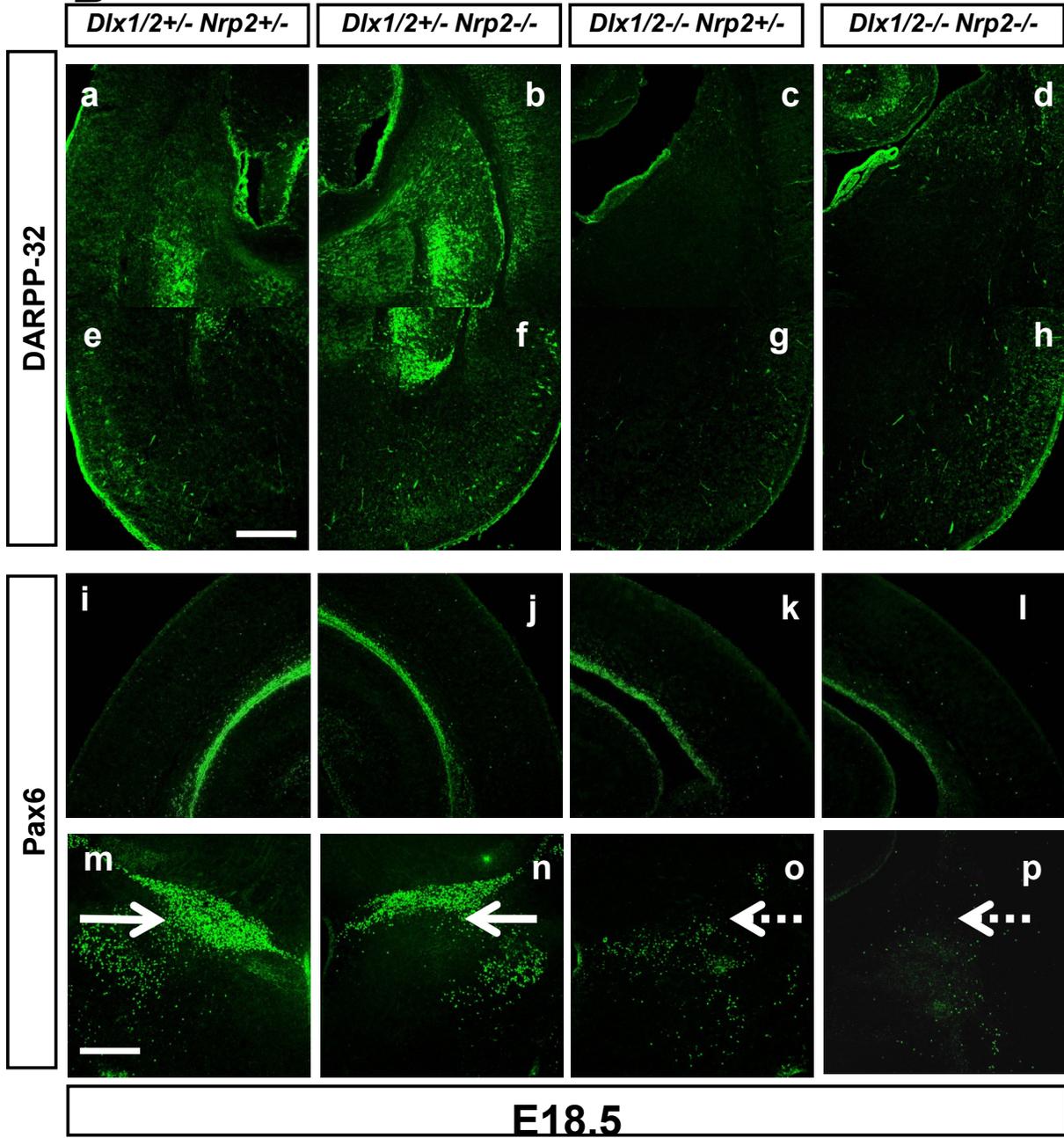
Figure 27. Changes in expression patterns of specific markers for striatum between four genotypes at E18.5. (A) Immunofluorescence experiments showed Tyrosine hydroxylase (TH) expression predominantly in the striatum of the wild-type (a, e, i) and single (b, f, j) knockouts. Dopaminergic neurons are reduced in both double (c, g, k) and triple (d, h, l) mutants. Arrows indicate TH-positive neurons accumulated as ectopias. (B) Immunofluorescence experiments showed DARPP32-labelled striatal projection neurons in the wild-type (a, e) and single (b, f) knockout but its expression is reduced in both double (c, g) and triple (d, h) mutants. Immunofluorescence experiments showed PAX6 expression mainly in the ventricular zone of the neocortex (i-l). PAX6 labels a region of the dorsal MGE of the wild-type (m) and single (n) knockout; this expression domain is almost absent in the striatum of the double (o) and triple (p) knockout. Coronal sections; scale bars, 200 μm. [H, hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str, striatum; Thal, thalamus; AEP: anterior entopeduncular area].

expressed in the VZ of the LGE, although at a very low level (Hallonet et al., 1998). *Pax*6 expression, predominantly in the neocortex, was also present in the dorsal MGE at low levels and in a restricted pattern in both wild-type and single knockout basal ganglia (**Fig. 27B**, panels i, j, m, n; solid arrows). However, this MGE domain of expression of *Pax*6 was severely reduced, and there were only few scattered immunoreactive cells remaining in the MGE of the double and triple knockout basal ganglia (**Fig. 27B**, panels k, l, o, p; dashed arrows). These results suggest that in the absence of *Dlx*1&2, later-born neurons of the developing striatum are unable to migrate out of the proliferative region. Instead they accumulate within the ganglionic eminences, where they partially differentiate (eg. and express MAP2 but not DARPP-32, TH or PAX6) (Anderson et al., 1997b).

7.3 Characterization of cellular proliferation and apoptosis

To assess cellular proliferation in the basal ganglia, BrdU-labelling experiments were conducted to label cells in the S-phase of E18.5 forebrains (de Melo et al., 2005). There were no significant differences demonstrated between the populations of S-phase cells in double and triple mutants compared to wild-type and single knockout (data not shown). As well, experiments using an antibody to phosphohistone H3, an M-phase marker (Ajiro et al., 1996), suggested a slight increase in M-phase labelled cells in the proliferative zone of LGE of the double mutant (de Melo et al., 2005), and triple mutant basal telencephalon (**Fig. 28**, panels a-h). To assess apoptosis, antibodies specific to activated caspase-3, an effector caspase, were then utilized to quantify apoptosis. Double and triple mutant LGE demonstrated a small increase in numbers of activated caspase-3 positive cells at E18.5 (**Fig. 28**, panels i-l). The increase in the cell-proliferation marker (phosphohistone H3) along with the increase in the apoptosis marker (caspase-3) may

	Dlx1/2+/- Nrp2+/-	*Dlx1/2+/- Nrp2-/-*	*Dlx1/2-/- Nrp2+/-*	*Dlx1/2-/- Nrp2-/-*

Phospho histone H3

a b c d

e f g h

Caspase3

i j k l

E18.5

NCx H LGE Str MGE Thal AEP

a,b,c,d

e,f,g,h
i,j,k,l
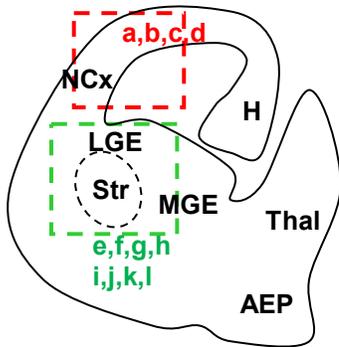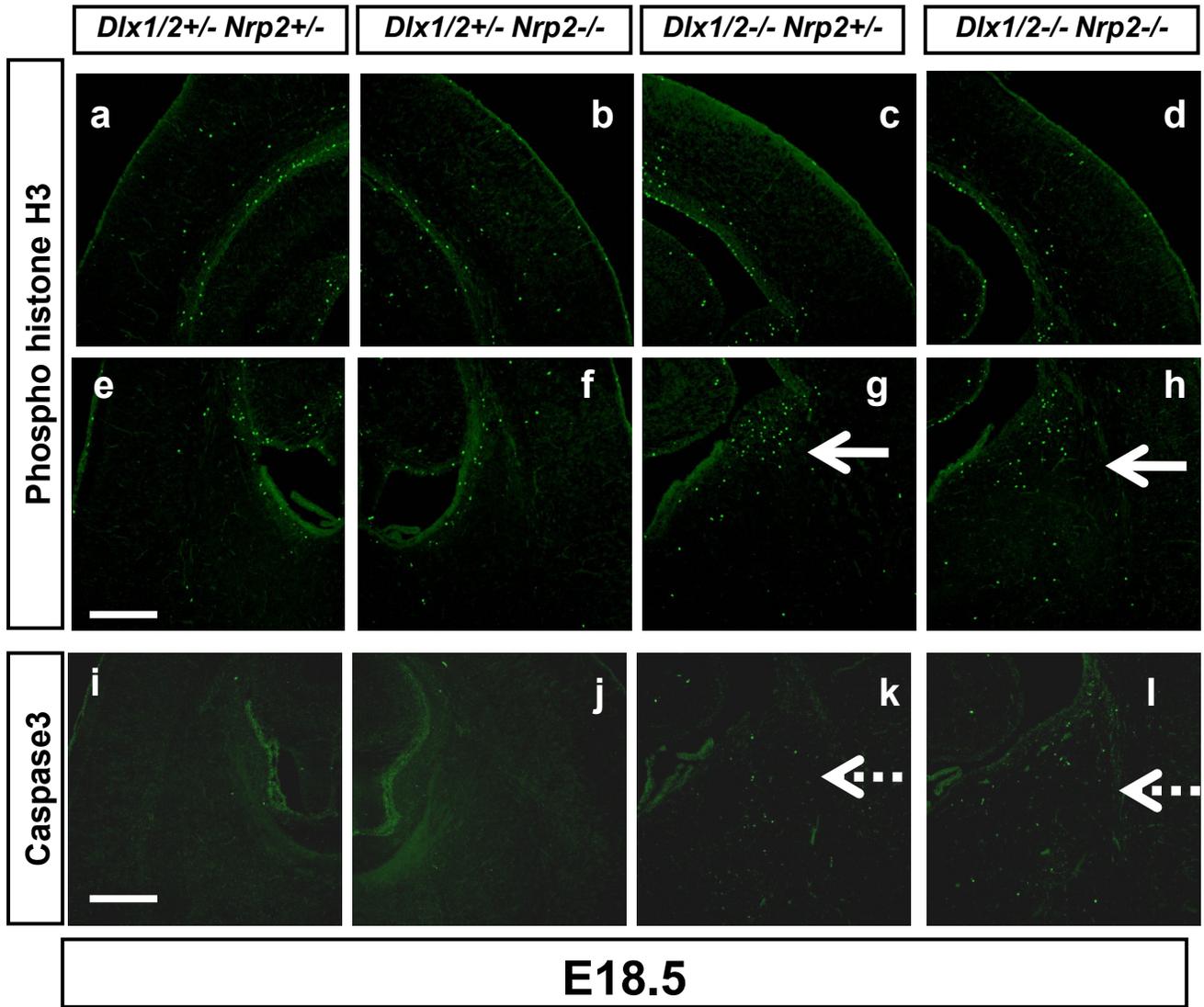
Figure 28: Qualitative characterization of cellular proliferation and apoptosis in basal ganglia between 4 genotypes at E18.5. Immunofluorescence experiments showed phosphohistone H3 (PH3) labelling in the upper 2 rows (a-h) at E18.5. The solid arrows indicate an increase in PH3-positive cells in the LGE of the double (g) and triple (h) knockouts. Immunofluorescence experiments also showed activated caspase-3 labelling on the bottom row (i-l) at E18.5. The dashed arrows indicate the increased caspase-3 positive cells in the LGE of the double (k) and triple (l) knockouts. Coronal sections; scale bars, 200 μm. [H, hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str, striatum; Thal, thalamus; AEP: anterior entopeduncular area].

signify a slight increase in cellular turnover in the double and triple mutants compared to the wild-type and single mutant.

7.4 Characterization of markers for neuronal migration

In addition to controlling the ventral to dorsal direction of tangential or non-radial migration, guidance cues are also required to distribute interneurons to different telencephalic structures. Sorting of interneurons destined for the cerebral cortex or the striatum appears to be mediated by Neuropilin/Semaphorin interactions (Marin et al., 2001). Migration of cortical interneurons responds to a chemorepulsive activity localized to the striatal mantle, of which the class 3 semaphorins are components (**Fig. 29B,** panels a, b; (Marin et al., 2001)). Of note, Sema3A expression is down-regulated in the striatum of the double and triple knockout (**Fig 29B**, panels c, d). In the subpallium, *Neuropilin*1 and *Neuropilin*2 are expressed by interneurons that migrate to the cortex but not by interneurons that invade the developing striatum. *Neuropilin*1 and *Neuropilin*2 expression patterns were mainly found in the hippocampus and the mantle zone of the wild-type and single knockout basal telencephalon, and not found in the striatum (**Figs. 29A**, panels a, b, e, f, i, j; **22,** panels b, e; **23A**, panels a, c; **23B**, panels a, c, e; **23C**, panels a, c, e; **24**, panels a, c; **Appendix Fig. 7C**). Loss of *Dlx*1/2 function increased Neuropilin2-expressing cells accumulating as periventricular dorsal ectopias of MGE and LGE (adjacent to the striatum) and decreased the number of labelled interneurons in the embryonic cortex (**Fig. 29A**, panels g, k; solid arrow; **23B**, panels b, d, f; **23C,** panels b, d, f; **24,** panels b, d; (Marin et al., 2001). Due to the ability of the NRP2 antibody to recognize the C-terminal domain of the truncated NRP2 protein, we still observe a persistent increase in NRP2 expression in the basal ganglia of the triple knockout. However, the ectopias are much less severe, and there is an increased number of

A

| *Dlx1/2+/- Nrp2+/-* | *Dlx1/2+/- Nrp2-/-* | *Dlx1/2-/- Nrp2+/-* | *Dlx1/2-/- Nrp2-/-* |

NRP1

a b c d

NRP2

e f g h

i j k l

E18.5

NCx

H

LGE

Str

MGE

Thal

e,f,g,h

i,j,k,l

AEP

B

Dlx1/2+/- Nrp2+/-	*Dlx1/2+/- Nrp2-/-*	*Dlx1/2-/- Nrp2+/-*	*Dlx1/2-/- Nrp2-/-*

Sema3A

a   b   c   d

E18.5

NCx
H
LGE
Str   MGE
Thal
AEP
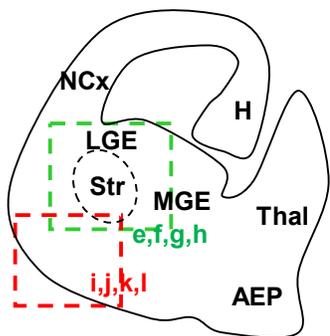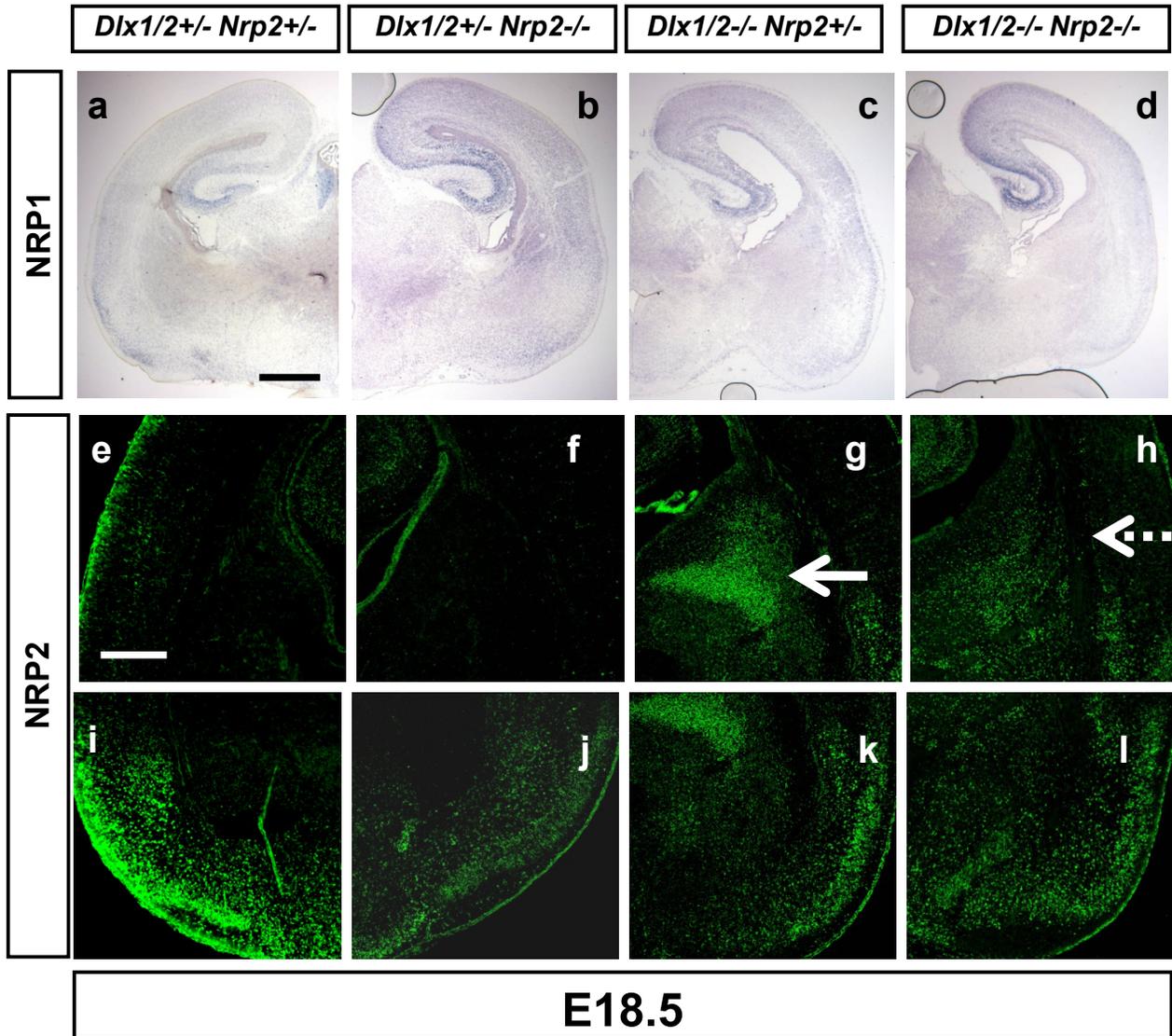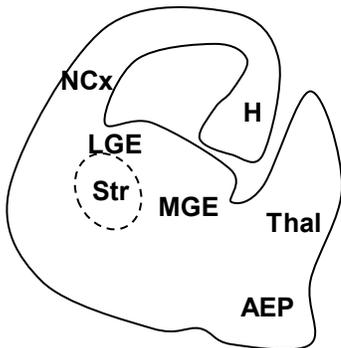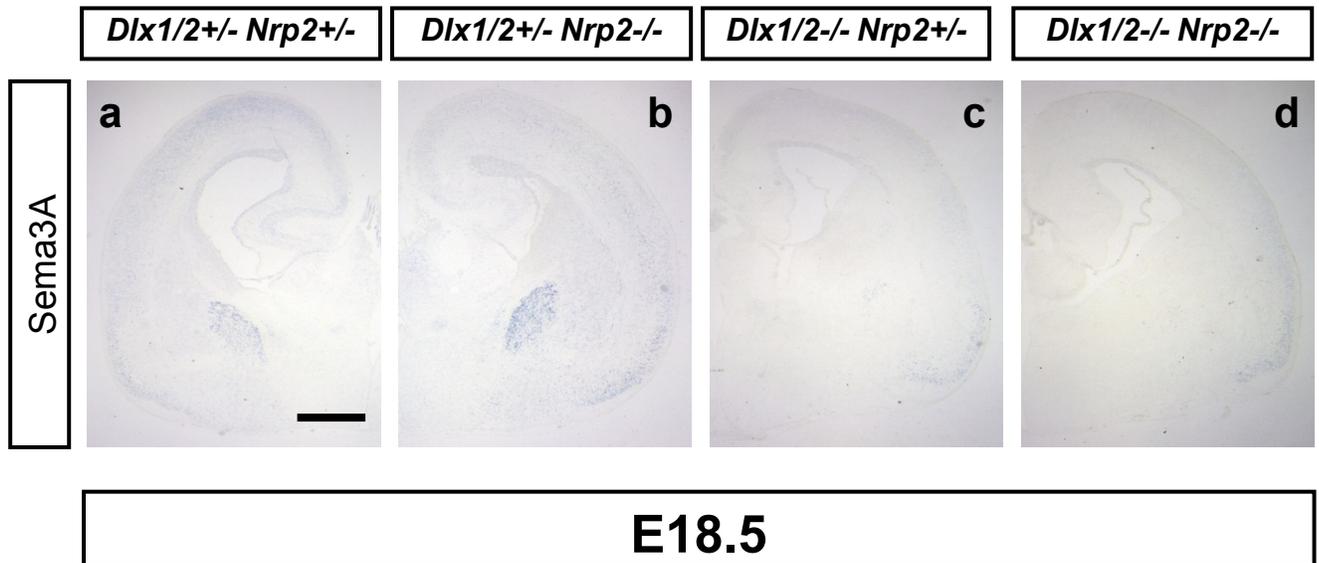
Figure 29. Qualitative characterization of specific markers for neuronal migration between four genotypes at E18.5. (A) *In situ* hybridization of Neuropilin1 (*Nrp*1) showed transcript expression predominantly in the hippocampus and in the mantle zone of the basal telencepahlon (a-d). Immunofluorescence experiements also showed Neuropilin2 (*Nrp*2) expression in the hippocampus and in the deeper layers of the striatal anlagen, but not in the striatum of both wild-type (e, i) and single (f, j) knockouts. *Nrp*2 expression was increased in the accumulating neurons in the MGE and LGE of the double (g, k; solid arrow) knockouts. The *Nrp*2 single mutant was generated by removing exon1 as part of the N-terminal of the protein. The NRP2 antibody recognizes the C-terminal domain of the truncated NRP2 protein; hence, it is useful in visualizing the mutant (truncated) protein in *Nrp*2-/- and *Dlx*1/2-/-; *Nrp*2-/- mice. Ectopic *Nrp*2-expressing cells no longer accumulate in the ganglionic eminences, but appear to be migrating toward the striatum and neocortex in the triple knockout (h, l; dashed arrow). Coronal sections; scale bars, 500 μm for a-d, 200μm for e-l). (B) *In situ* hybridization of Semaphorin3A (Sema3A) labels striatal neurons in the wild-type (a) and single (b) knockout but its expression is diminished in both double (c) and triple (d) mutants. Coronal sections; scale bars, 500 μm. [H, Hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str, striatum; Thal, thalamus; AEP: anterior entopeduncular area].

interneurons migrating to the striatum and subpallial-pallial boundary (**Fig. 29A**, panels h, l; dashed arrow). Hence, by eliminating Semaphorin-Neuropilin interactions in the triple knockout basal ganglia, tangential migration of interneurons can potentially be restored. Of note, there is no difference in *Neuropilin*1 expression throughout four genotypes (**Fig. 29A**, panels a-d), consistent with previous results (**Fig. 18B**, **Appendix Fig. 7C**).

7.5 Partial restoration of GABAergic interneurons in the neocortex of the triple mutant mice

The subpallial telencephalon (the LGE, MGE, CGE, septum and anterior entopeduncular region) is the origin of multiple streams of tangentially migrating interneurons that express *Dlx* genes. These migratory pathways are temporally and spatially distinct, and give rise to a variety of GABA containing interneurons in the neocortex (Anderson et al., 1997a; Lavdas et al., 1999; Stuhmer et al., 2002a; Sussel et al., 1999; Wichterle et al., 1999). Loss of *Dlx*1 and *Dlx*2 function blocks the differentiation of late-born subpallial telencephalic neurons (Chapter 4 and 5; Marin and Rubenstein, 2003). Furthermore, in *Dlx*1/2 double-mutant mice, partially differentiated interneurons (that is, cells that are able to express GABA) fail to migrate, and collect as periventricular ectopias (Anderson et al., 1997b; Marin et al., 2000). Consequently, the number of GABA expressing neurons found in the cortex, olfactory bulb and hippocampus at the time of birth is severely reduced compared with that of wild-type littermates (Anderson et al., 1997a; Bulfone et al., 1998; Pleasure et al., 2000). GABA expression was found mainly in the intermediate zone of the neocortex and throughout the basal telencephalon of both wild-type and single mutants, and there was no difference

| Dlx1/2+/- Nrp2+/- | Dlx1/2+/- Nrp2-/- | Dlx1/2-/- Nrp2+/- | Dlx1/2-/- Nrp2-/- |

GABA

E18.5

NCx

H

LGE

Str

MGE

POa

e,f,g,h

i,j,k,l
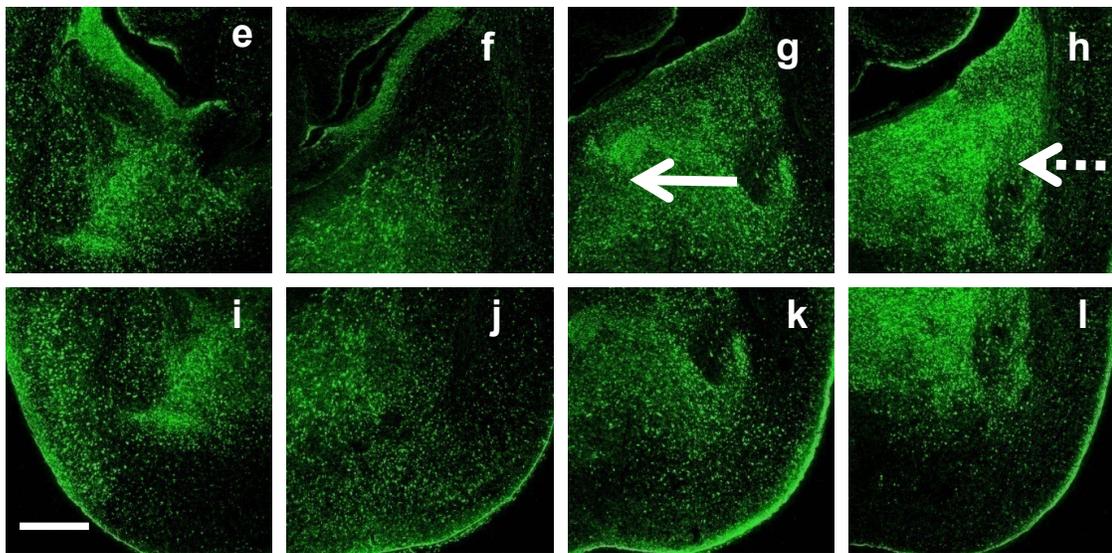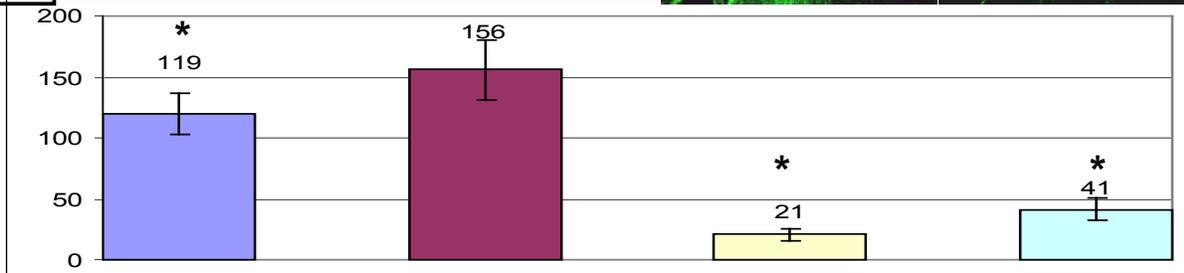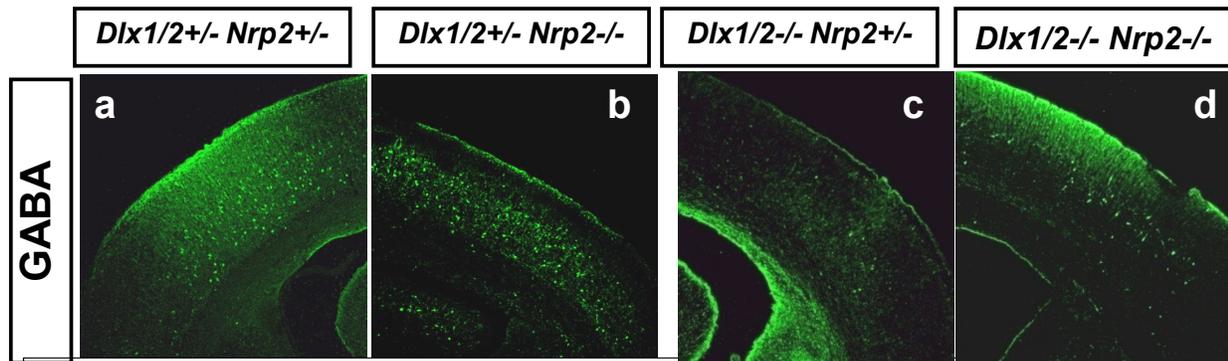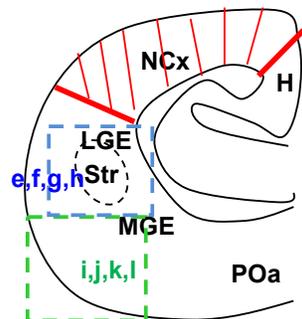
Figure 30. Partial restoration of GABA expression in the neocortex of the triple mutant at E18.5. Immunofluorescence experiments showed GABA expression predominantly localized to the intermediate zone of the neocortex of the wild-type (a) and single (b) mutants. GABA is also expressed in a diffuse pattern in the basal telencephalon of the wild-type (e, i) and single (f, j) mutants. In the double knockout, GABA expression is severely reduced in the neocortex (c), and accumulates in the ganglionic eminences (g, k; solid arrow). In the triple knockout, GABA expression is partially restored in the neocortex (d) with less accumulation in the ganglionic eminences (h, l; dashed arrow). Quantitative cell counts were performed for the entire neocortex (labelled area in red of the schematic diagram). [* denotes statistically significant]. Cell counting was reproducible and performed at least five times (N=5). Error bars represent standard error of measurements. Coronal sections; scale bars, 500 μm. [H, Hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str, striatum; POa, preoptic area]. The cell counting was done with the help of Tracie Parkinson, Eisenstat laboratory, Manitoba Institute of Cell Biology, University of Manitoba.

statistically (**Fig. 30**, panels a, b, e, f, i, j). However, in the double mutant, neocortical GABA was significantly reduced compared to wild-type (N=5, p<0.001), and GABA-expressing cells were found to accumulate in the MGE and LGE as ectopias (**Fig. 30**, panels c, g, k, solid arrow). The total level of GABA was also reduced in the entire forebrain of the double mutant, which was previously demonstrated by HPLC (**Fig. 13B**). The presence of residual neocortical GABA-expressing cells in the double mutants suggests that neocortical interneurons are derived from multiple spatially distinct sources. Interestingly, in the triple mutant, neocortical GABA expression was increased compared to double mutant, and there was reduced accumulation of GABA-expressing cells in the ganglionic eminences, as there appeared to be more widespread expression within the striatum and subpallial-pallial boundary (**Fig. 30**, panels d, h, l, dashed arrow). Quantitative analysis was performed by manual cell counting of the entire neocortical area. The results showed a two fold increase in the number of GABA-expressing cells in the neocortex of the triple mutant compared to the double mutant (**Fig. 30**, p<0.001, n=6).

Calcium-binding proteins (CBPs) are a family of proteins found in a variety of tissues across many different species (Christakos et al., 1989). In the CNS, the most well described CBPs include parvalbumin (PV), calbindin-D28K (CB), calretinin (CR), calmodulin, calcineurin, and the S100 family. In the cerebral cortex, PV, CB and CR play a vital role in calcium homeostasis and are discrete markers for select subclasses of GABAergic interneurons (e.g. PV is found in chandelier and basket cells, CB in double-bouquet neurons, CR in bipolar and bitufted neurons (Conde et al., 1994; Lewis, 1998)). Collectively, CBP-containing interneurons make up 90% of all GABAergic neurons in the cerebral cortex (Lund and Lewis, 1993). To confirm the partial restoration of GABA

calbindin

| *Dlx1/2+/- Nrp2+/-* | *Dlx1/2+/- Nrp2-/-* | *Dlx1/2-/- Nrp2+/-* | *Dlx1/2-/- Nrp2-/-* |
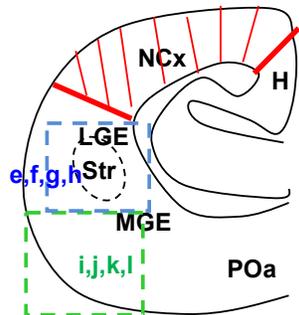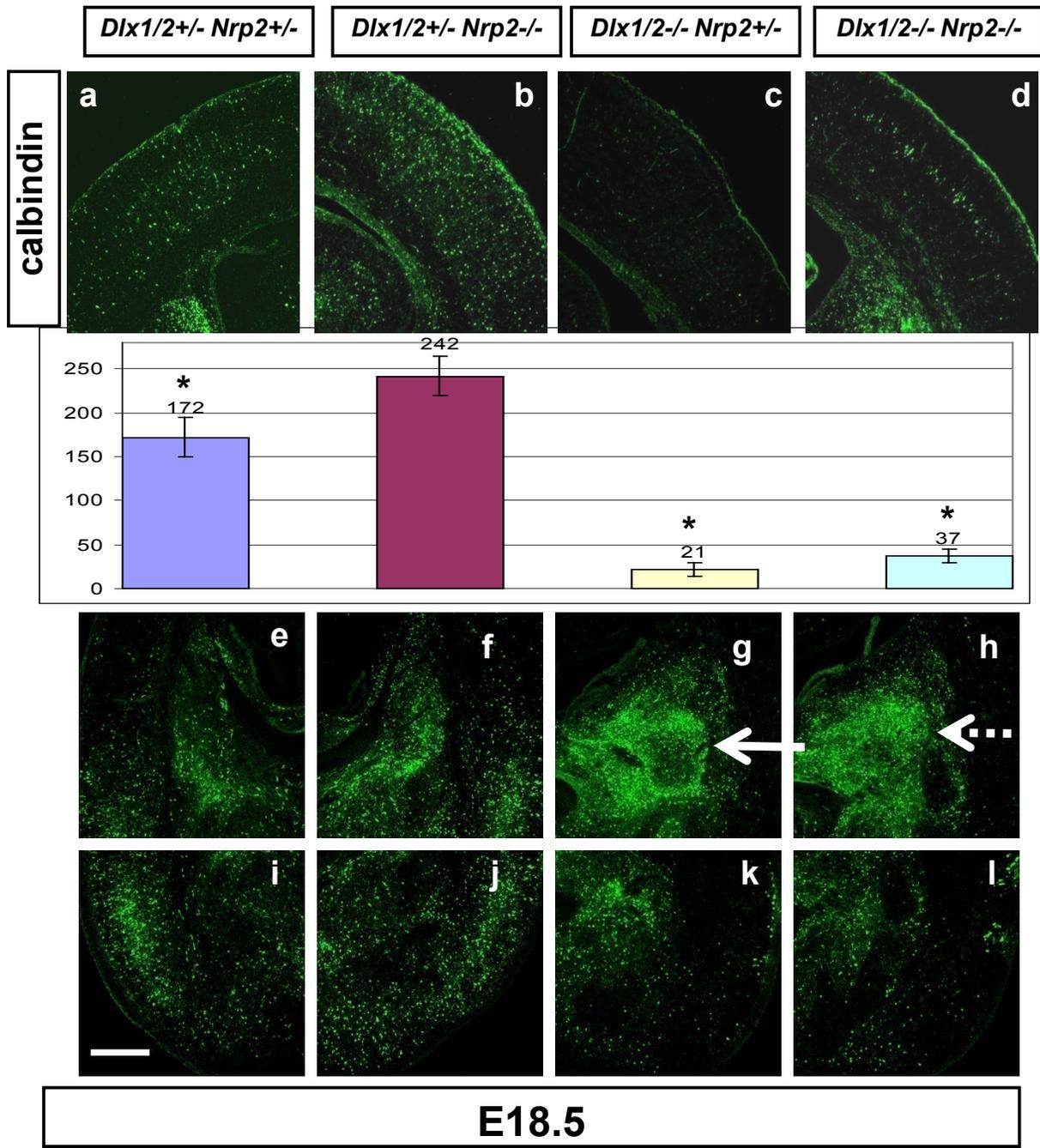
E18.5

Figure 31. Partial restoration of calbindin expression in the neocortex of the triple mutant at E18.5. Immunofluorescence experiments showed Calbindin expressed in the intermediate zone of the neocortex of the wild-type (a) and single (b) mutants. Calbindin is also expressed in a diffuse pattern in the basal telencephalon of the wild-type (e, i) and single (f, j) mutants. In the double knockout, Calbindin expression is severely reduced in the neocortex (c), and accumulates in the ganglionic eminences (g, k; solid arrow). In the triple knockout, Calbindin expression is partially restored in the neocortex (d) and there is less accumulation in the ganglionic eminences (h, l; dashed arrow). Quantitative cell counts were performed for the entire area of neocortex (labelled area in red of the schematic diagram). [* denotes statistical significance]. Cell counting was reproducible and performed at least five times (N=5). Error bars represent standard error of measurements. Coronal sections; scale bars, 500 μm. [H, Hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str, striatum; POa, preoptic area]. The cell counting was done with the help of Tracie Parkinson, Eisenstat laboratory, Manitoba Institute of Cell Biology, University of Manitoba.

in the neocortex, calbindin expression was assessed in the intermediate zone of the neocortex and throughout the basal telencephalon of both wild-type and single mutants. There was an increase in calbindin expression in neocortex of the single knockout, but it was not statistically significant (N=5, p<0.08) (**Fig. 31**, panels a, b, e, f, i, j). As shown for GABA expression, calbindin expression was diminished in the neocortex of the double mutant compared to wild-type (N=5, p<0.001), and calbindin-labelled cells were found to accumulate in the MGE and LGE as ectopias (**Fig. 31**, panels c, g, k, solid arrow). Similarly, in the triple mutant, neocortical calbindin-labelled cells were also partially restored, and there was reduced calbindin-labelled interneuron accumulation in the ganglionic eminences. These cells were presumably migrating towards the striatum and subpallial-pallial boundary (**Fig. 31**, panels d, h, l, dashed arrow). Quantitative cell counting also showed a two fold increase in the number of calbindin-labelled cells in the neocortex of the triple mutant compared to the double mutant (**Fig. 31**, N=5, p<0.004). Other CBPs, such as CR and PV, were tested and found to show similar levels of expression between all four genotypes (data not shown).

To further confirm the partial restoration of GABA-expressing cells in the neocortex, *in situ* hybridization of *Gad*67 RNA was performed. *Gad*67 was expressed uniformly in the intermediate zone of the neocortex and throughout the basal telencephalon similarly between wild-type and single mutants (**Fig. 32,** panels a, b, e, f). However, in the double mutant, neocortical *Gad*67 expression was significantly reduced compared to wild-type (N=5, p<0.001), and *Gad*67-expressing cells were found to accumulate in the MGE and LGE as ectopias (**Fig. 32**, panels c, g, solid arrow). As expected, in the triple mutant, neocortical *Gad*67 expression was partially restored

| *Dlx1/2+/- Nrp2+/-* | *Dlx1/2+/- Nrp2-/-* | *Dlx1/2-/- Nrp2+/-* | *Dlx1/2-/- Nrp2-/-* |

Gad67

a   b   c   d

e   f   g   h

362   373   49   92

E18.5

NCx   H   L-GE   Str   MGE   POa
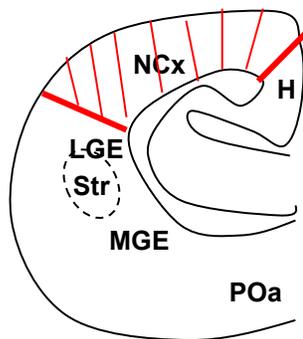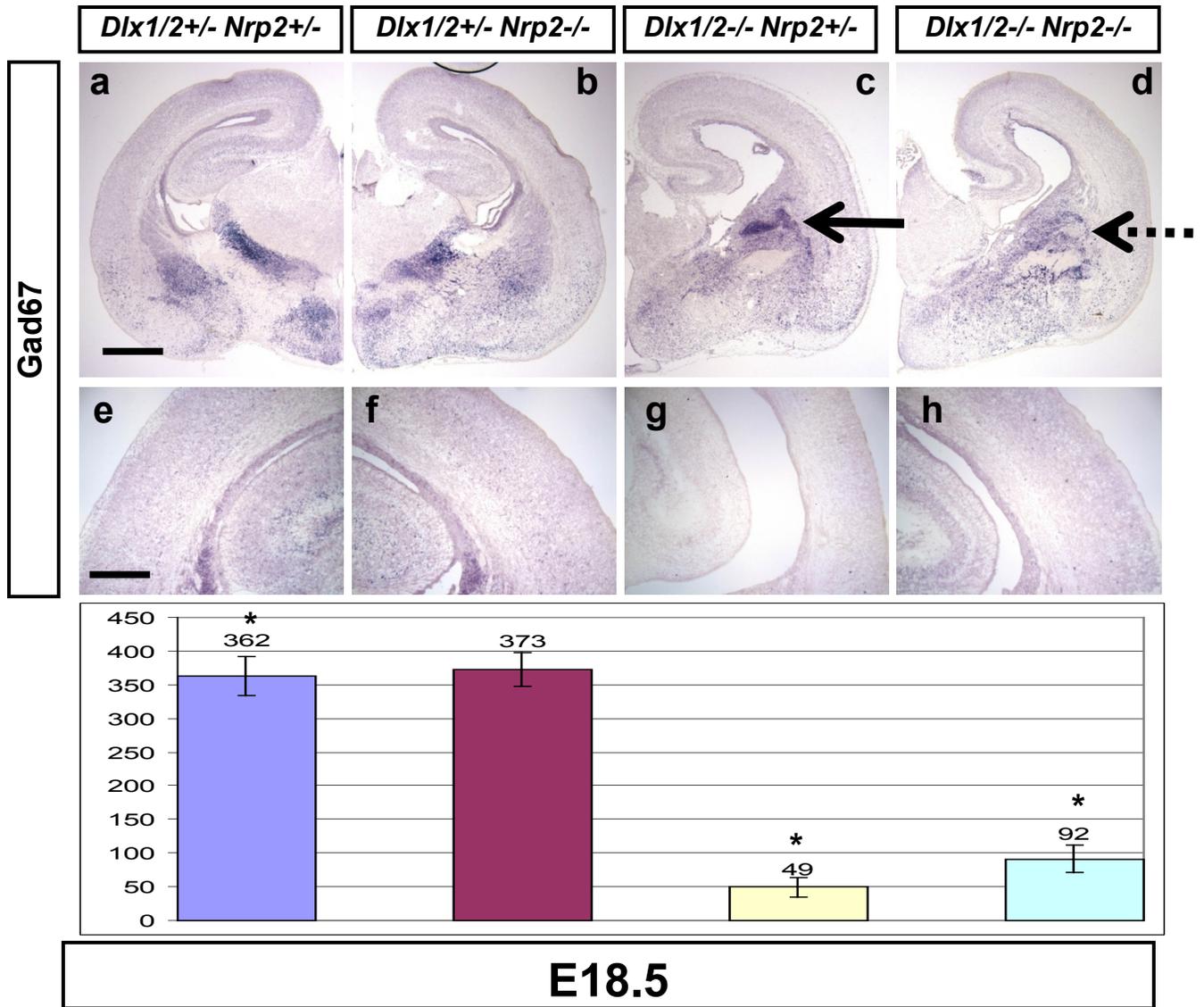
Figure 32. Partial restoration of *Gad*67 RNA expression in the neocortex of the triple

mutant at E18.5. *In situ* hybridization of *Gad*67 transcript showed mRNA expression in

the intermediate zone of the neocortex of the wild-type (e) and single (f) mutants. *Gad*67

is also expressed in a diffuse pattern in the basal telencephalon of the wild-type (a) and

single (b) mutants. In the double knockout, *Gad*67 expression is severely reduced in the

neocortex (g), and accumulates in the ganglionic eminences (c; solid arrow). In the triple

knockout, *Gad*67 expression is partially restored in the neocortex (h) and there is less

accumulation in the ganglionic eminences (d; dashed arrow). Quantitative cell counts

were performed for the entire area of neocortex (labelled in red of the schematic

diagram). [* denotes statistical significance]. Cell counting was reproducible and

performed at least five times (N=5). Error bars represent standard error of measurements.

Coronal sections; scale bars, 500 µm. [H, Hippocampus; LGE, lateral ganglionic

eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str, striatum; POa,

preoptic area]. The cell counting was done with the help of Tracie Parkinson, Eisenstat

laboratory, Manitoba Institute of Cell Biology, University of Manitoba.

compared to the double mutant (N=5, p<0.02), and these cells accumulated less in the ganglionic eminences, supporting the postulate that they could be migrating towards the striatum and subpallial-pallial boundary (**Fig. 32**, panels d, h, dashed arrow). Quantitative cell counting showed a two fold increase in the number of *Gad*67-expressing cells in the neocortex of the triple mutant compared to the double mutant (**Fig. 32**, p<0.02, n=6). Hence, removing *Nrp*2 expression in the double knockout mice reduced Semaphorin-Neuropilin interaction and partially restored GABAergic interneuron migration to the neocortex.

7.6 Complete restoration of Somatostatin (SST)-expressing interneurons in the neocortex of the triple mutant mice

On the basis of their neurotransmitter content, several interneuron subtypes can be distinguished in the cortex, olfactory bulb and striatum. The most common type of telencephalic interneuron contains GABA as its main neurotransmitter. GABAergic interneurons can be further subdivided on the basis of their content of calcium-binding proteins (calbindin, calretinin and parvalbumin), neuropeptides (neuropeptide Y, somatostatin), and other neurotransmitters (NADPH-diaphorase, nitric oxide synthase), (Kawaguchi et al., 1995; Kawaguchi and Kubota, 1997; McBain and Fisahn, 2001, Wonders and Anderson, 2006).

Neuropeptide Y (NPY), a 36 amino acid residue polypeptide, is widely distributed in the mammalian brain where its actions are mediated by at least five NPY receptor subtypes (NPY1-5) that belong to the G-protein coupled receptor superfamily (Michel et al., 1998). NPY is involved in many brain functions, such as regulation of blood pressure, circadian rhythms, feeding, behaviour, anxiety, memory processing, etc. Furthermore, evidence points to an important role for NPY in the regulation of neuronal activity during

| Dlx1/2+/- Nrp2+/- | Dlx1/2+/- Nrp2-/- | Dlx1/2-/- Nrp2+/- | Dlx1/2-/- Nrp2-/- |

NPY

a b c d

810 930 571 674

e f g h

i j k l

E18.5

NCx H
LGE
e,f,g,h Str
MGE
i,j,k,l POa

215
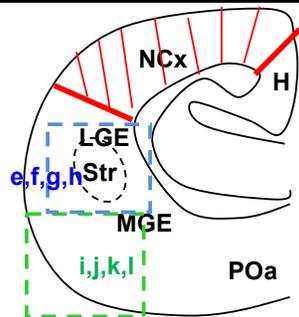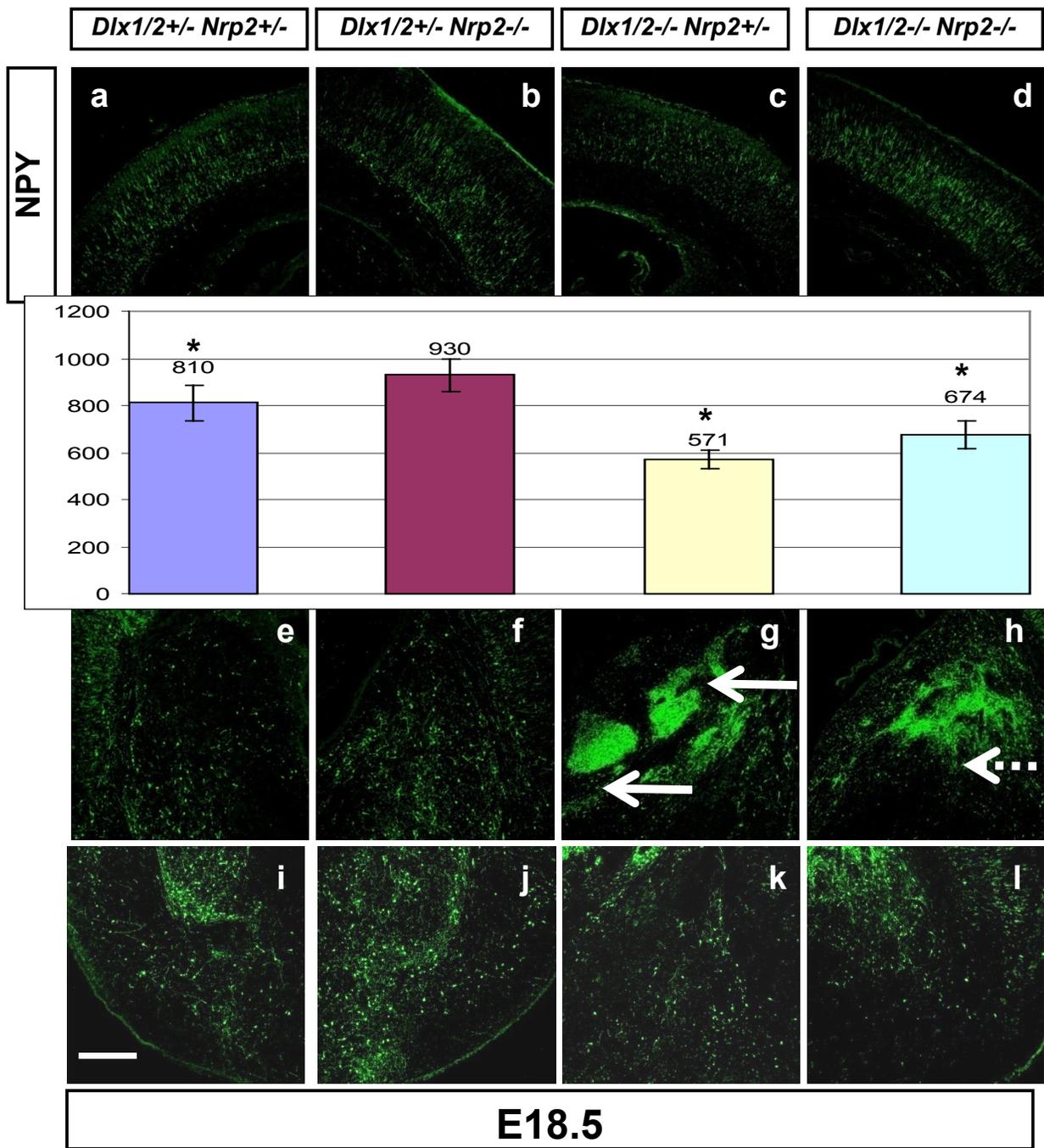
Figure 33. Partial restoration of Neuropeptide Y expression in the neocortex of the triple mutant at E18.5. Immunofluorescence experiments showed NPY expression localized to layers II/III to V of the neocortex of the wild-type (a) and single (b) mutants. NPY is also expressed in a diffuse pattern in the basal telencephalon of the wild-type (e, i) and single (f, j) mutants. In the double knockout, NPY expression is significantly reduced in the neocortex (c), and accumulates in the ganglionic eminences (g, k; solid arrows). In the triple knockout, NPY expression is partially restored in the neocortex (d) and is less accumulated in the ganglionic eminences (h, l; dashed arrow). Quantitative cell counts were performed for the entire area of neocortex (labelled are in red of the schematic diagram). [* denotes statistical significance]. Cell counting was reproducible and performed at least five times (N=5). Error bars represent standard error of measurements. Coronal sections; scale bars, 500 μm. [H, Hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str, striatum; POa, preoptic area]. The cell counting was done with the help of Tracie Parkinson, Eisenstat laboratory, Manitoba Institute of Cell Biology, University of Manitoba.

pathological hyperactivity, such as that occurring during seizures (Klapstein and Colmers, 1997). The pattern of expression of NPY is predominantly in layers II/III to V of the neocortex, and throughout the basal telencephalon of the wild-type, with a slight increase in single knockout, but the difference was not statistically significant (**Fig. 33**, panels a, b, e, f, i, j). However, in the double knockout, NPY expression was diminished in the neocortex compared to wild-type (N=5, p<0.001) (**Fig. 33**, panel c). In the striatum, NPY-expressing cells were found to accumulate in the MGE and LGE as ectopias (**Fig. 33,** panels g, k, solid arrows) (Marin et al., 2000). Interestingly, in the triple mutant, neocortical NPY-expressing cells were increased, and NPY-expressing interneurons accumulated less in the ganglionic eminences (**Fig. 33**, panels d, h, l, dashed arrow). Quantitative cell counting was done for the entire neocortical area, and there was a small but significant increase in the number of NPY-expressing cells in the neocortex of the triple mutant compared to the double mutant (**Fig. 33**, N=5, p<0.02). A marker for another subclass of GABAergic interneurons, neuronal nitric oxide synthase (nNOS), was also tested. nNOS expression level was down-regulated in double and triple knockout basal telencephalon, but there was no difference in the expression pattern between double and triple mutants (data not shown).

Somatostatin is a regulatory neuropeptide hormone that plays an inhibitory role in a variety of organ systems. This cyclic tetradecapeptide inhibits the secretion of many important hormones, including somatotropin (growth hormone), insulin, and glucagon. Somatostatin is found in both the hypothalamus and in the pancreas. Somatostatin-containing interneurons are aspiny cells that can also use nNOS, NPY, and GABA as their neurotransmitter. Somatostatin expression was localized mainly in layers II/III to V

Dlx1/2+/- Nrp2+/- | Dlx1/2+/- Nrp2-/- | Dlx1/2-/- Nrp2+/- | Dlx1/2-/- Nrp2-/-

somatostatin

a   b   c   d

160 *   164   104 *   * 160

e   f   g   h

i   j   k   l

E18.5

NCx   H

LGE

Str

e,f,g,h

MGE

i,j,k,l

POa
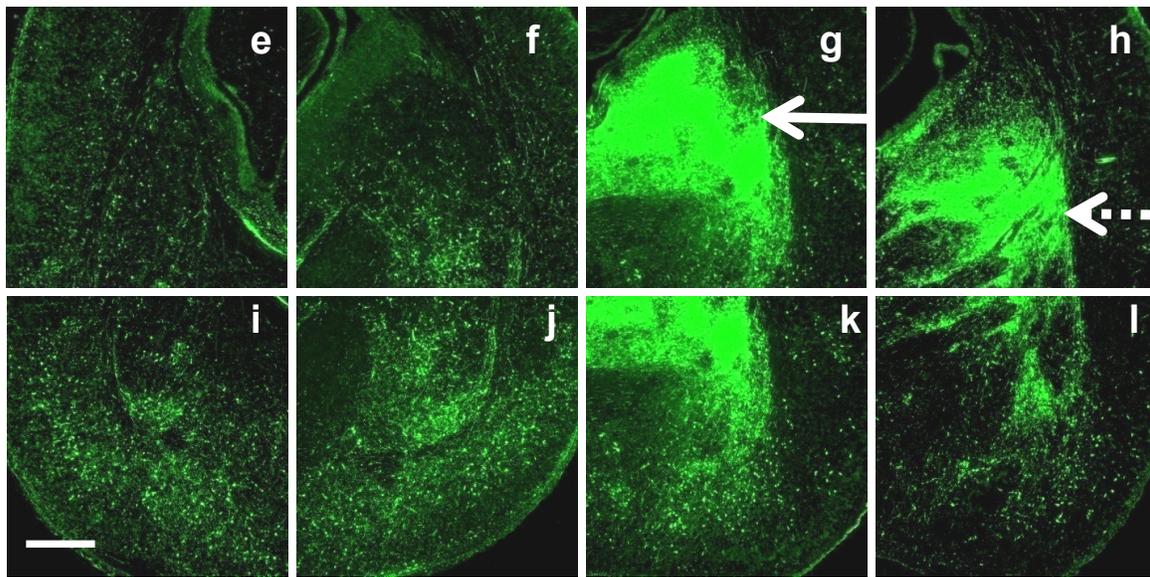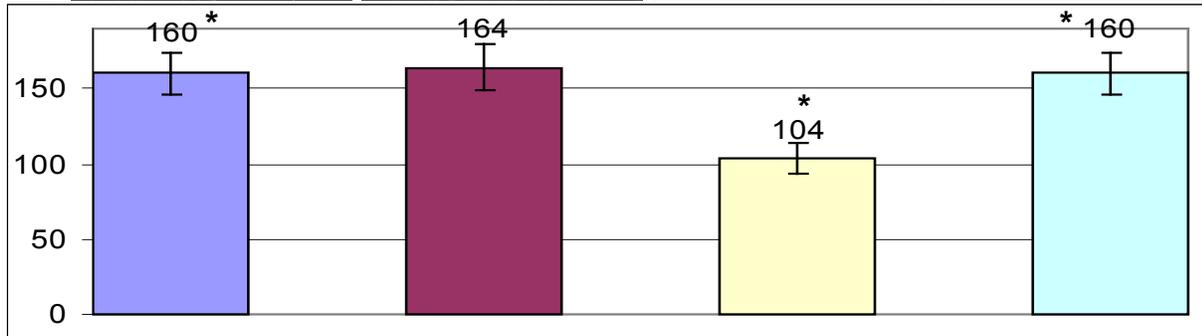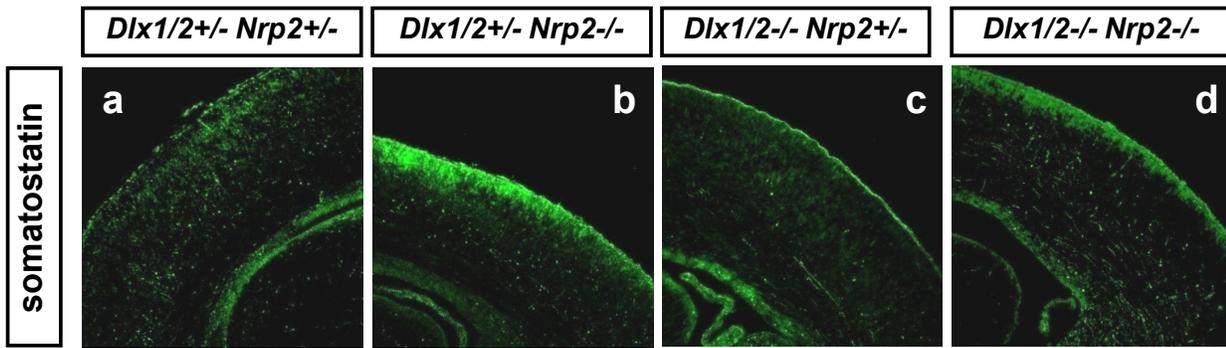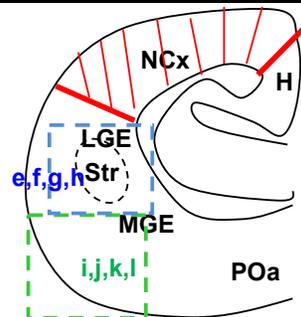
Figure 34. Complete restoration of Somatostatin expression in the neocortex of the triple mutant at E18.5. Immunofluorescence experiements showed Somatostatin is expressed in layers II/III to V of the neocortex of the wild-type (a) and single (b) mutant. Somatostatin is also expressed in a diffuse pattern in the basal telencephalon of the wild-type (e, i) and single (f, j) mutants. In the double knockout, Somatostatin expression is significantly reduced in the neocortex (c), and accumulates in the ganglionic eminences (g, k; solid arrow). In the triple knockout, Somatostatin expression is completely restored in the neocortex (d) and accumulates to a lesser extent in the ganglionic eminences (h, l; dashed arrow). Quantitative cell counts were performed for the entire area of neocortex (labelled area in red of the schematic diagram). [* denotes statistical significance]. Cell counting was reproducible and performed at least five times (N=5). Error bars represent standard error of measurements. Coronal sections; scale bars, 500 μm. [H, Hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str, striatum; POa, preoptic area]. The cell counting was done with the help of Tracie Parkinson, Eisenstat laboratory, Manitoba Institute of Cell Biology, University of Manitoba.

in the intermediate zone of the neocortex and diffusely throughout the basal

telencephalon similarly in both wild-type and single mutants (**Fig. 34**, panels a, b, e, f, i,

j). However, in the double mutant, neocortical Somatostatin expression was significantly

decreased compared to wild-type (N=5, p<0.001), and SST-expressing cells were found

to accumulate in the MGE and LGE as ectopias (**Fig. 34**, panels c, g, k, solid arrow).

Interestingly, in the triple mutant, neocortical SST expression was significantly increased

compared to double mutant (N=6, p<0.001), and SST-expressing cells accumulated less

in the ganglionic eminences, (**Fig. 34**, panels d, h, l, dashed arrow). Quantitative analysis

was done by manual cell counting of the entire neocortical area. There was a significant

increase in the number of SST-expressing cells in the neocortex of the triple mutant and

the cell count were similar between triple mutant and wild-type (**Fig. 34**, N=6, p<0.5).

These results were confirmed by semi-quantitative immunoblotting analysis which

demonstrated a significant increase in SST levels in the triple mutant neocortex, when

compared to the double knockout (data not shown). To further confirm the complete

restoration of cortical SST-expressing cells, quantitative Real-Time PCR was performed

and it showed a statistically significant increase in Somatostatin mRNA levels in the

triple mutant neocortex compared to the double mutant (**Fig. 35**, N=6, p< 0.004). More

importantly, cortical Somatostatin mRNA levels were similar between wild-type and

triple mutant (**Fig. 35**, N=6, p<0.3). Of note, there was no difference between cortical

Somatostatin mRNA level between wild-type and single mutant (N=6, p<0.3). Hence,

removing *Nrp*2 activity in the double mutant completely restored SST-expressing cells in
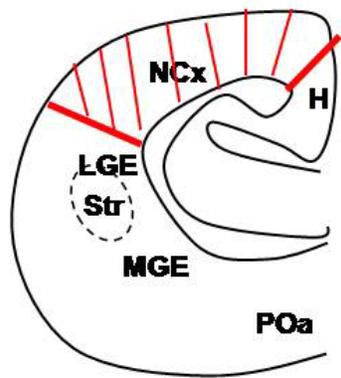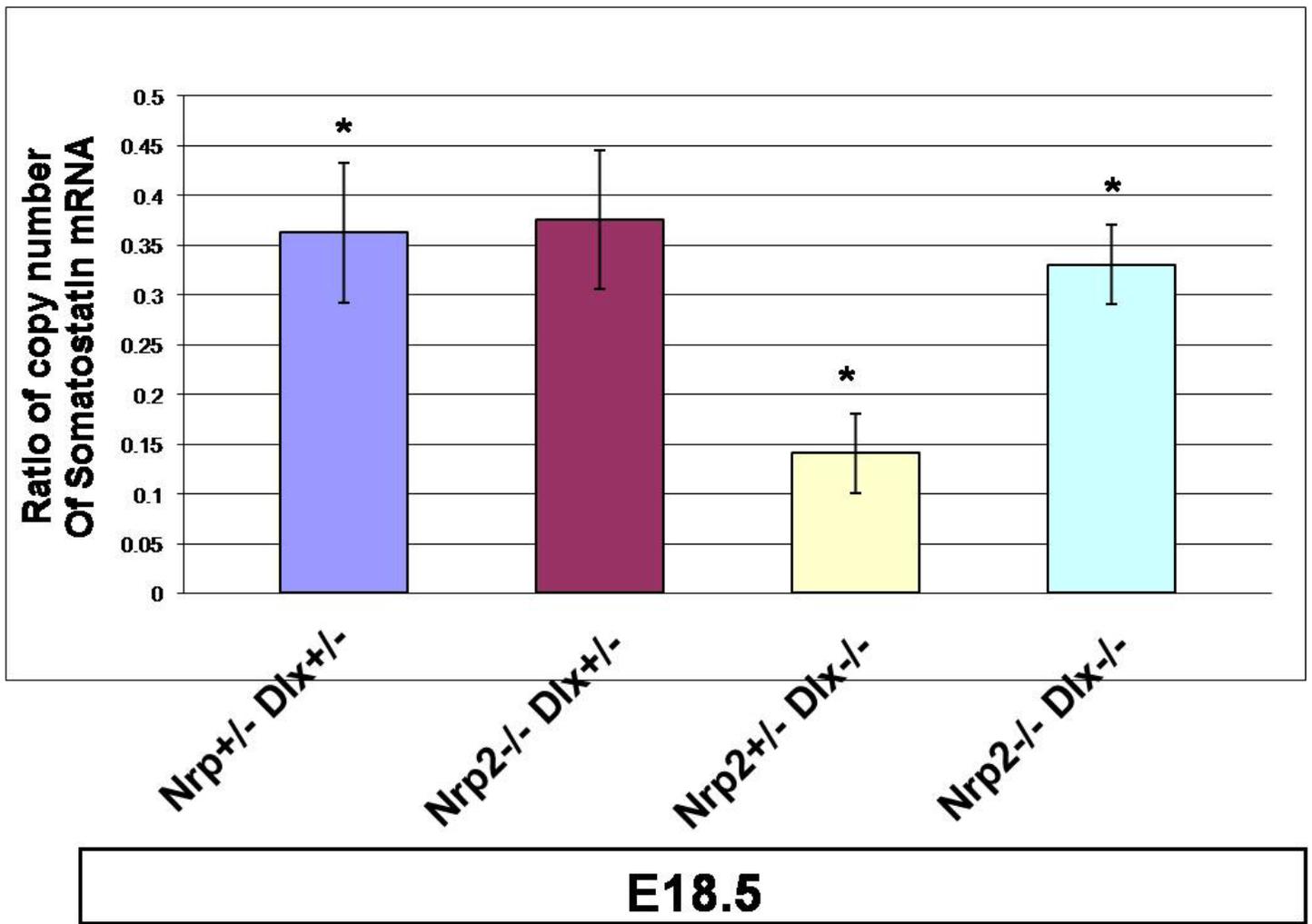
the neocortex.

E18.5

Figure 35. Confirmation of the complete restoration of Somatostatin expression in the neocortex of the triple mutant at E18.5 by quantitative Real-Time PCR. mRNAs were extracted at E18.5 from dissected neocortex (labelled area in red of the diagram) and Somatostatin expression was subjected to a quantitative RT-PCR analysis. Comparative levels between wild-type and single mutants were observed. There was about a 50% decrease in Somatostatin levels in the double mutant compared to wild-type (*). However, there was a significant increase in Somatostatin mRNA levels in the triple mutant when compared to double mutant. The Somatostatin level in the triple mutant is not significantly different from the level found in the wild-type. [* denotes statistical significance]. Comparative experiments were reproducible and performed at least five times (N=5). Error bars represent standard error of measurements. [H, Hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str, striatum; POa, preoptic area].

7.7 Co-localization of GABA expression in Somatostatin-expressing interneurons restored in the neocortex of the triple mice mice

Previous studies have provided evidence that almost all cortical GABA interneurons in the adult brain are derived from cells that express the *Dlx* genes (Stuhmer et al., 2002a). This result is consistent with the hypothesis that most cortical GABA-expressing interneurons are derived from the GEs by migrating through the intermediate zone before dispersal throughout the neocortex (Marin et al., 2001). GABA was expressed diffusely throughout the basal telencephalon and neocortex of the wild-type and single mutants (**Fig. 36A**, panels a, d, g; **Fig. 36B**, panels a, d, g). The expression of GABA overlapped substantially with SST expression (**Fig. 36A**, panels b, e, h; **Fig. 36B**, panels b, e, h) in both the basal telencephalon and neocortex (**Fig. 36A**, panels c, f, i, insert; **Fig. 36B**, panels c, f, i, insert) of both wild-type and single mutants. Analysis of mice with a mutation in both *Dlx*1/2 also showed abnormal migration from the GE, resulting in an accumulation of partially differentiated neurons in the GE, hypoplasia of the striatum, and a marked reduction of cortical GABA and SST expression (**Fig. 36C**, panels a, d, g; **Fig. 36C**, panels b, e, h). There was little detectable SST expression in the neocortex, while the remaining GABA- and Somatostatin- expressing cells accumulated in the basal telencephalon (**Fig. 36C**, panels c, f, i, insert). Of interest, in the triple knockout, there was partial restoration of both GABA-expressing cells and Somatostatin-expressing cells in the neocortex (**Fig. 36D**, panels a, b), and some of these cells were found to express both GABA and SST (**Fig. 36D**, panel c, insert). In the basal telencephalon of the triple mutant, the accumulation of partially differentiated cells was significantly changed, perhaps as cells migrated more towards the striatum and subpallial-pallial boundary (**Fig. 36D**, panels d, e, f, g, h, i). These results support that SST-expressing cells were

A

E18.5 Dlx1/2 +/- Nrp2+/-

GABA	Somatostatin	Merge

a

b

c

d

e

f

g

h

i

NCx

a,b,c

H

LGE d,e,f

Str

MGE

Thal

g,h,i

AEP

B

E18.5 *Dlx*1/2+/- *Nrp2*-/-

GABA	Somatostatin	Merge
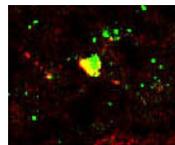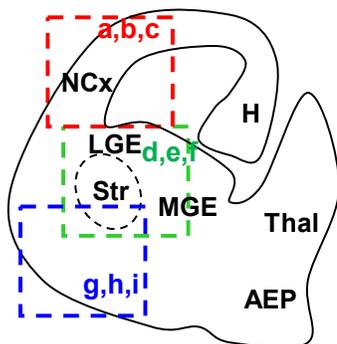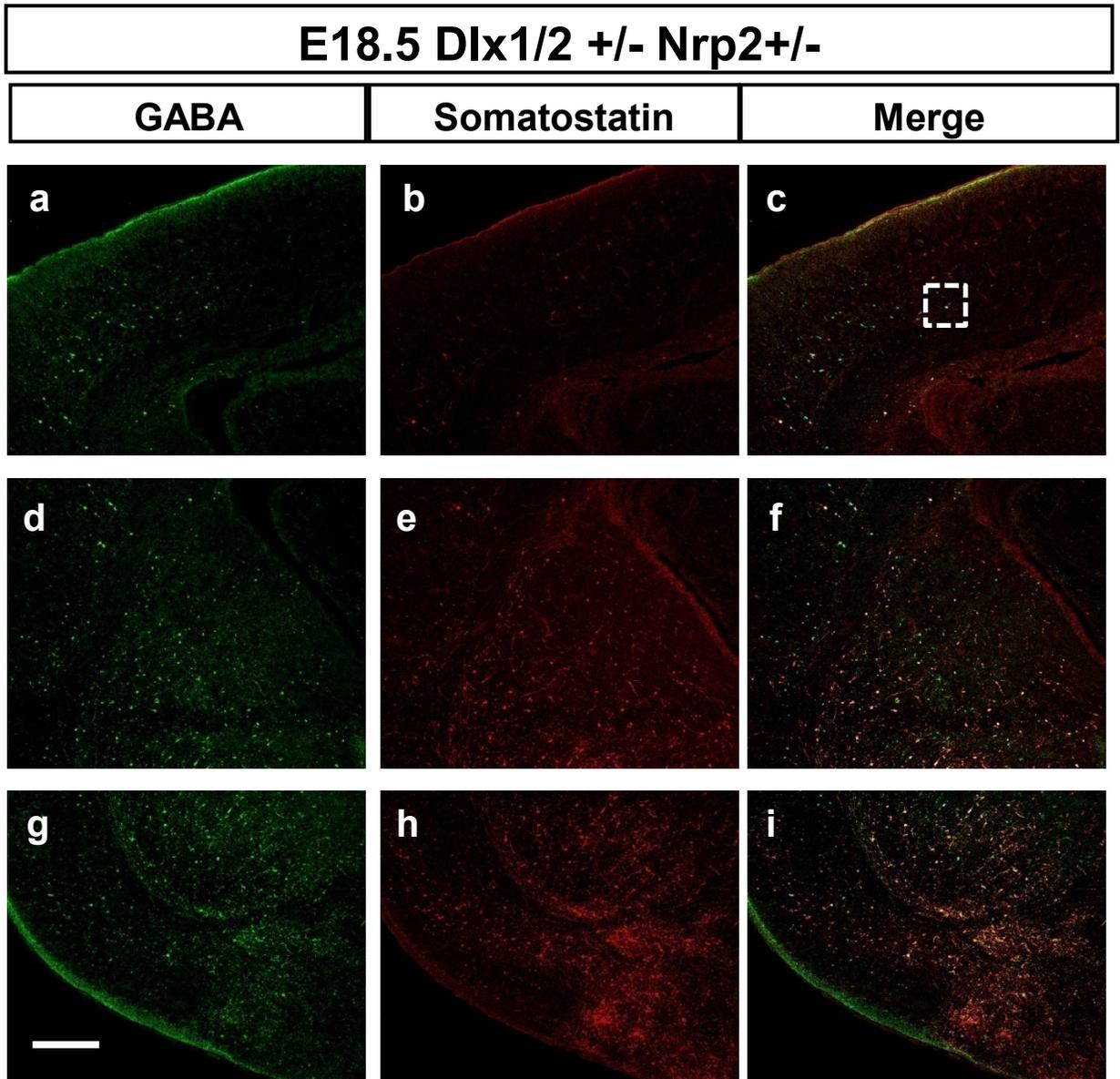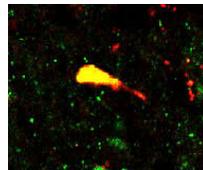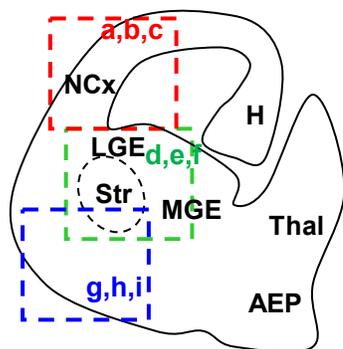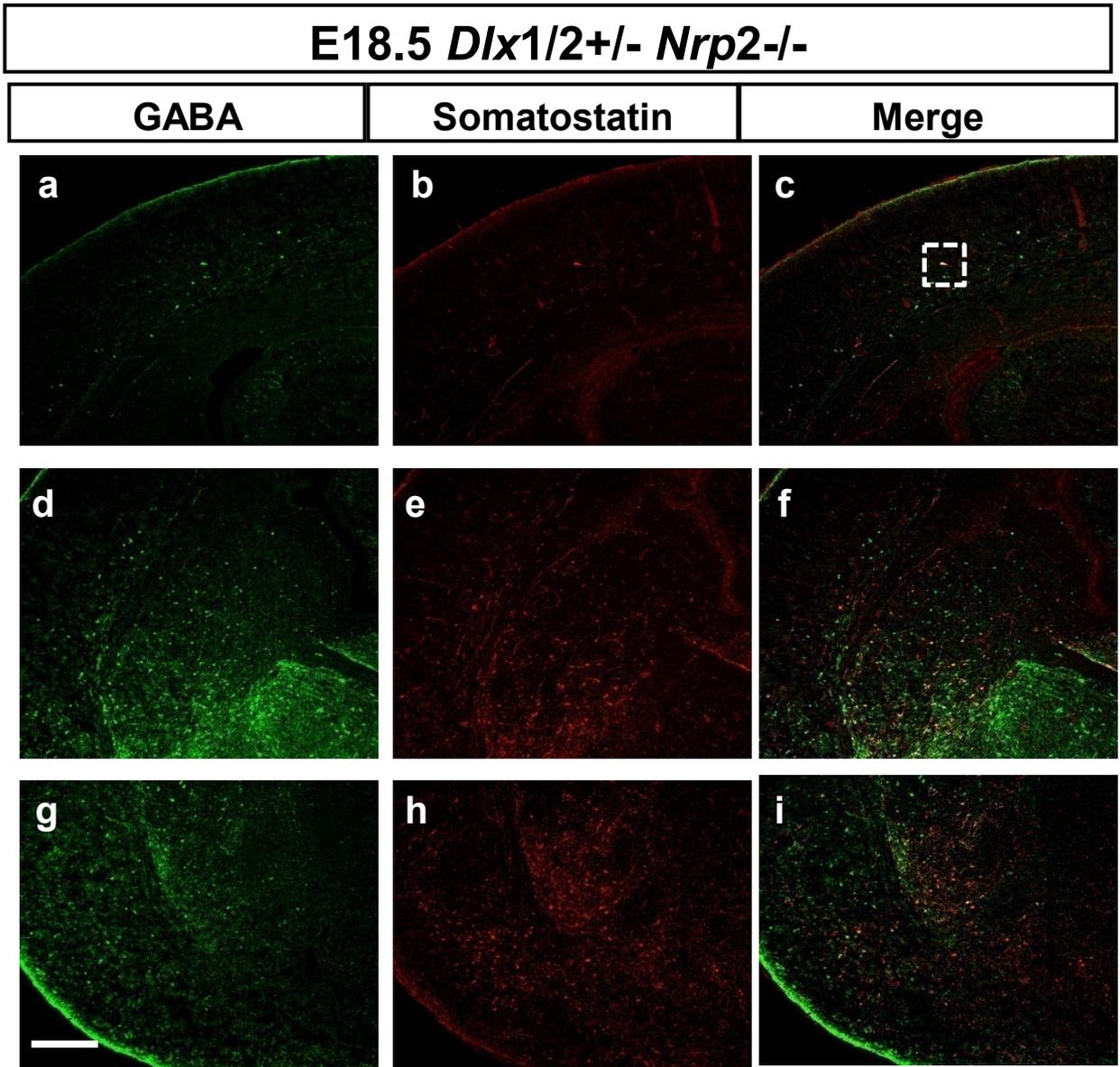
C

	E18.5 *Dlx*1/2-/- *Nrp*2+/-	
GABA	**Somatostatin**	**Merge**
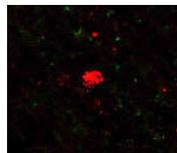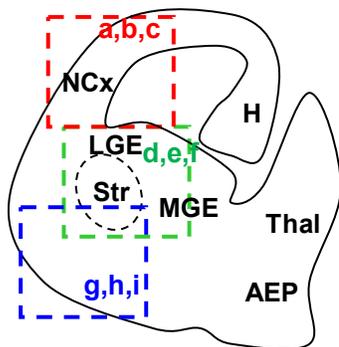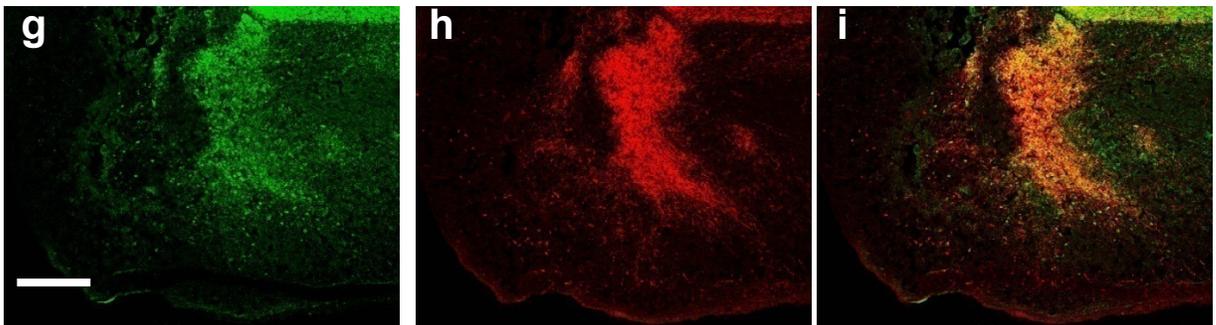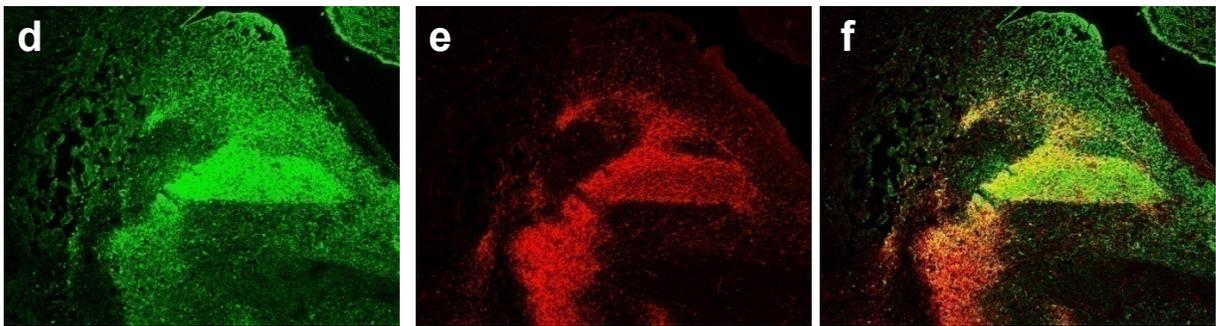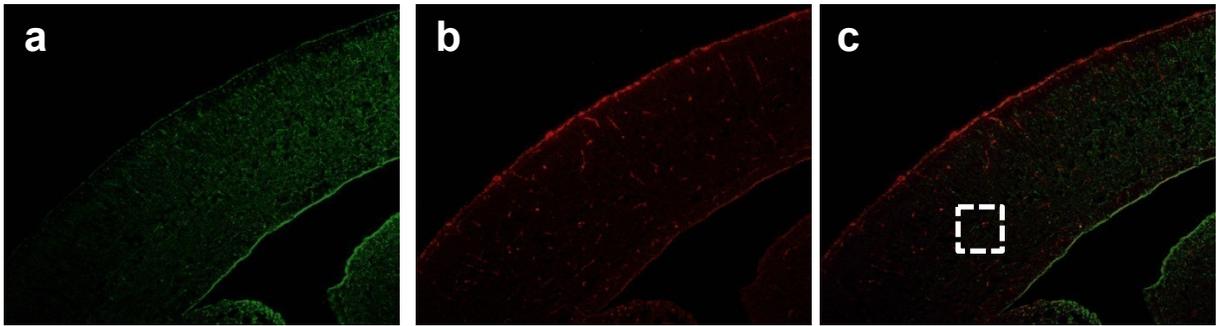
D

E18.5 *Dlx*1/2-/- *Nrp2*-/-		
GABA	Somatostatin	Merge
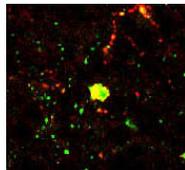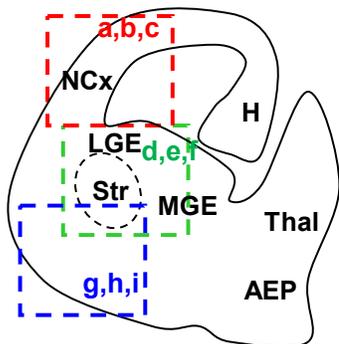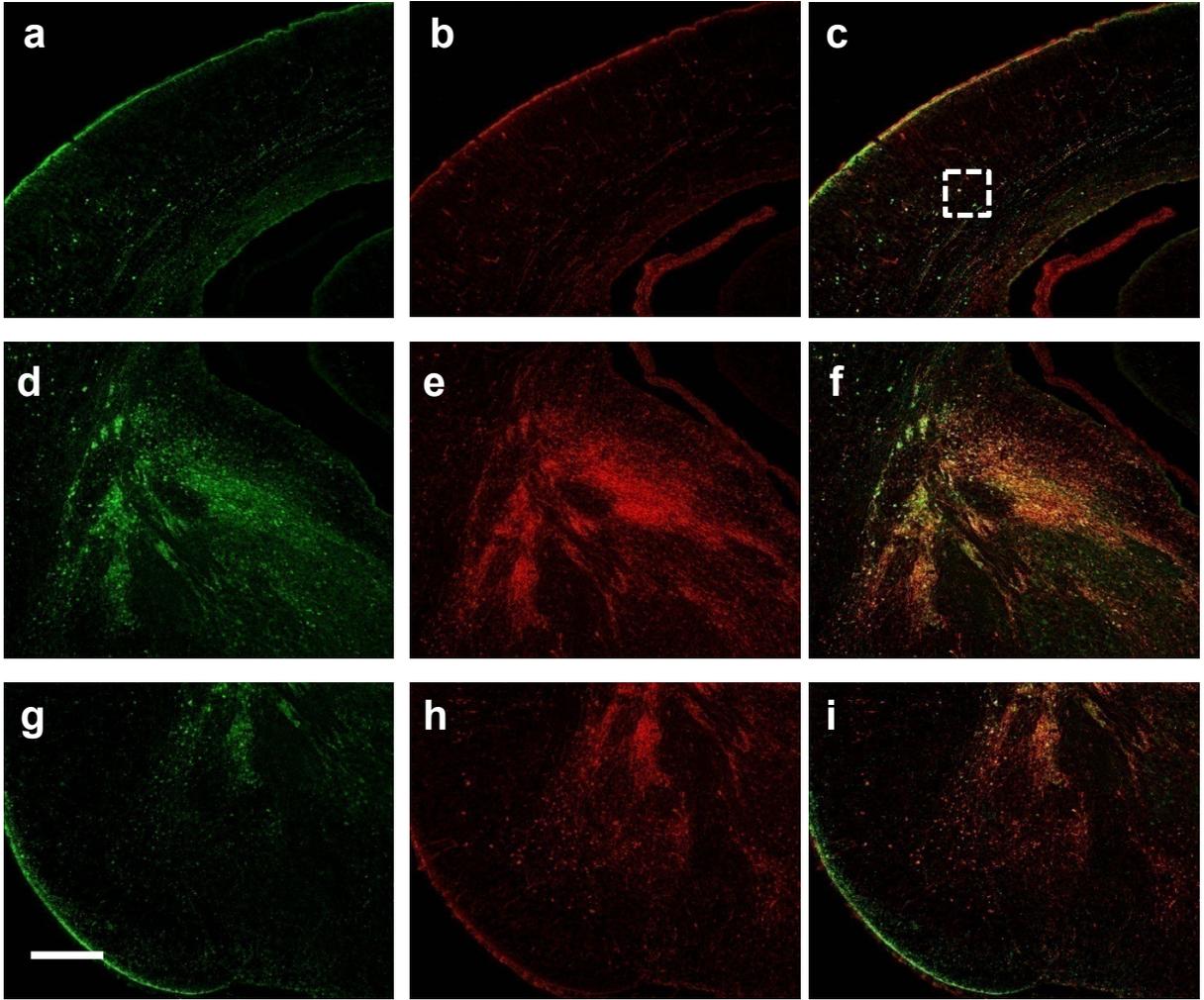
Figure 36. GABA is partially co-expressed in the completely-restored Somatostatin-positive cells in the neocortex of the E18.5 triple mutant. Expression of GABA is shown by immunofluorescent labelling with fluorescein isothiocyanate antibody (FITC – green) (a, d, g in A-D) and expression of Somatostatin is visualized with Texas Red immunolabelling (b, e, h in A-D). In merged images, the yellow colour represent co-localization of GABA in Somatostatin-positive cells (c, f, i, insert). A, wild-type. B, single mutant: $Nrp2-/-$. C, double mutant $Dlx1/2^{-/-}$. D, triple mutant $Dlx1/2^{-/-}$ $Nrp2^{-/-}$. Coronal sections. Scale bar, 200 µm. Inserts in the Merge column represent a 10x enlargement. [H, hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Amyl, amygdala; Str, striatum; Thal, thalamus; AEP: anterior entopeduncular area].

completely restored in the neocortex, and that some of these cells co-expressed GABA as their neurotransmitter.

7.8 Co-localization of NRP2 expression in Somatostatin-expressing interneurons restored in the neocortex of the triple mutant mice

Striatal and specific cortical cells express semaphorins which are predicted to create an exclusion zone for migrating cortical interneurons and channel them into adjacent paths (Marin and Rubenstein, 2003). Interneurons migrating into the neocortex express neuropilins, whereas interneurons migrating into the striatum do not. Neuropilin2 expression was found in some cells migrating tangentially in the neocortex, but not found in SST-expressing cells, presumably due to DLX mediated repression in these GABAergic cells in both wild-type and single knockouts (**Fig. 37A**, panels a-c, insert; **Fig. 37B**, panels a-c, insert). SST expression was also complementary or non-overlapping with Neuropilin2 expression in the basal telencephalon, perhaps due to DLX repression in these SST-positive cells (**Fig. 37A**, panels d-i; **Fig. 37B**, panels d-i). However, in the double mutant, Neuropilin2 was expressed in the SST-positive cells that accumulated in the GE (**Fig. 37C**, panels d-i), as well as in some of the SST-expressing cells in the neocortex (**Fig. 37C**, panels a-c, insert). The normal expression pattern of Neuropilin2 was also noted in the mantle zone of the basal telencephalon (**Fig. 37C**, panels g, i, green labeled cells) since the antibody can recognize the mutant Neuropilin2 protein (Le et al., 2007)(Giger et al., 1998). Of interest, in the triple mutant, there was significant restoration of SST-expressing cells in the neocortex, and some of these cells co-expressed mutant Neuropilin2 supporting their migration from the basal forebrain (**Fig. 37D**, panels a, b, c, insert). In the basal telencephalon of the triple mutant,

A

E18.5 wild-type		
NRP2	Somatostatin	Merge

B

E18.5 *Dlx*1/2+/- *Nrp2*-/-		
NRP2	Somatostatin	Merge

NCx
H
LGE
Str
MGE
Thal
AEP

a,b,c
d,e,f
g,h,i

C

E18.5 *Dlx*1/2-/- *Nrp*2+/-		
NRP2	Somatostatin	Merge

D

E18.5 *Dlx*1/2-/- *Nrp*2-/-		
NRP2	Somatostatin	Merge

NCx
H
LGE
Str
MGE
Thal
AEP
a,b,c
d,e,f
g,h,i
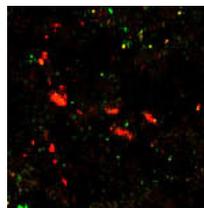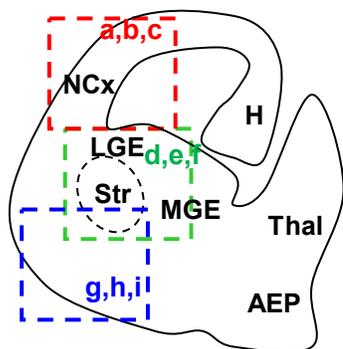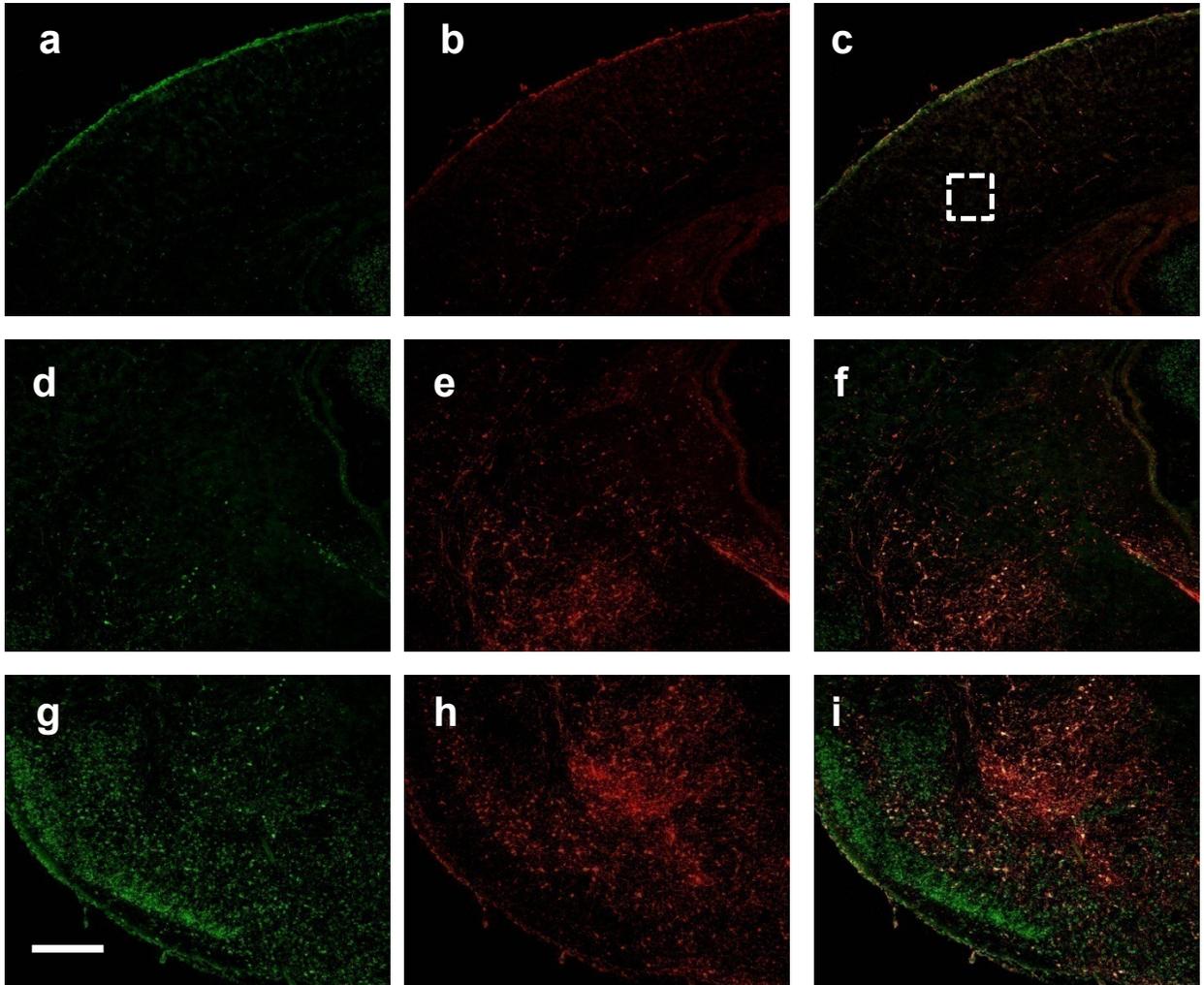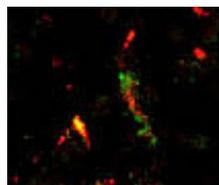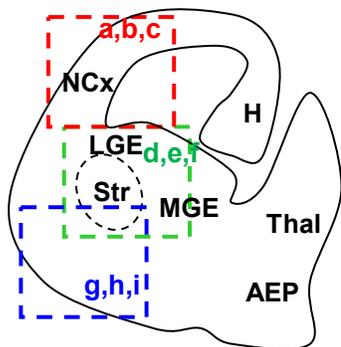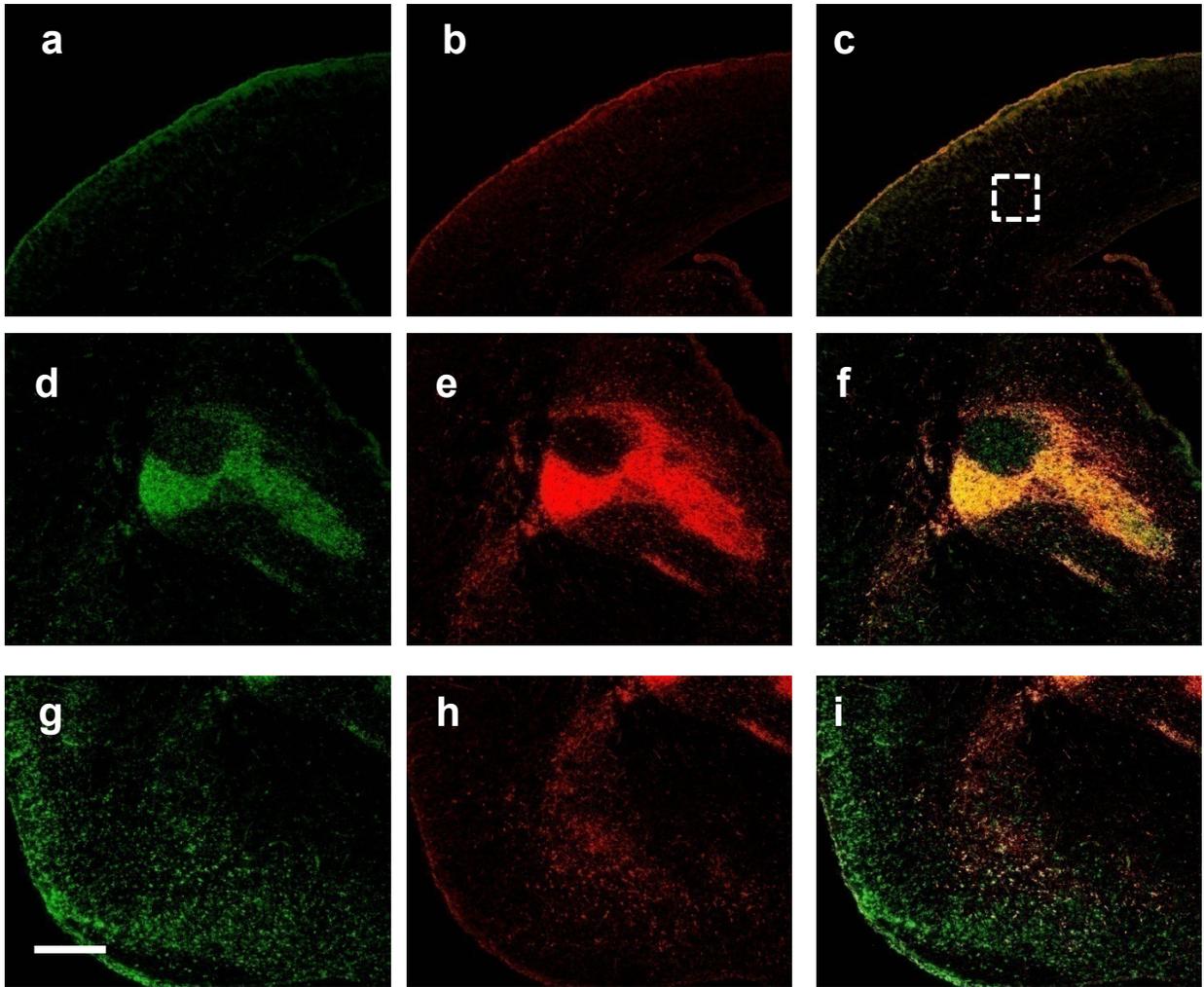
Figure 37. Mutant Neuropilin2 is co-expressed in the completely-restored Somatostatin-positive cells in the neocortex of the E18.5 triple mutant. Expression of NRP2 is shown by immunofluorescent labelling with fluorescein isothiocyanate (FITC – green) (a, d, g in A-D) and expression of Somatostatin is visualized with Texas Red immunolabelling (b, e, h in A-D). In merged images, the yellow colour represent co-localization of NRP2 in Somatostatin-positive cells (c, f, i, insert). A, wild-type. B, single mutant $Nrp2^{-/-}$. C, double mutant $Dlx1/2^{-/-}$. D, triple mutant $Dlx1/2^{-/-} Nrp2^{-/-}$. The $Nrp2$ single mutant was generated by removing exon1 as part of the N-terminal of the protein. The NRP2 antibody recognizes the C-terminal domain of the truncated NRP2 protein; hence, it is useful in visualizing the mutant (truncated) protein in $Nrp2$-/- and $Dlx1/2$-/-; $Nrp2$-/- mice. Coronal sections. Scale bar, 200 μm. Inserts in the Merge column represent a 10x enlargement. [H, hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Amyl, amygdala; Str, striatum; Thal, thalamus; AEP: anterior entopeduncular area].

E16.5

Dlx1/2+/- Nrp2+/-	*Dlx1/2+/- Nrp2-/-*	*Dlx1/2-/- Nrp2+/-*	*Dlx1/2-/- Nrp2-/-*
a	b	c	d

E16.5

2 days later

H

Thal

AEP

NCx

LGE

Str

MGE

H

Thal

AEP
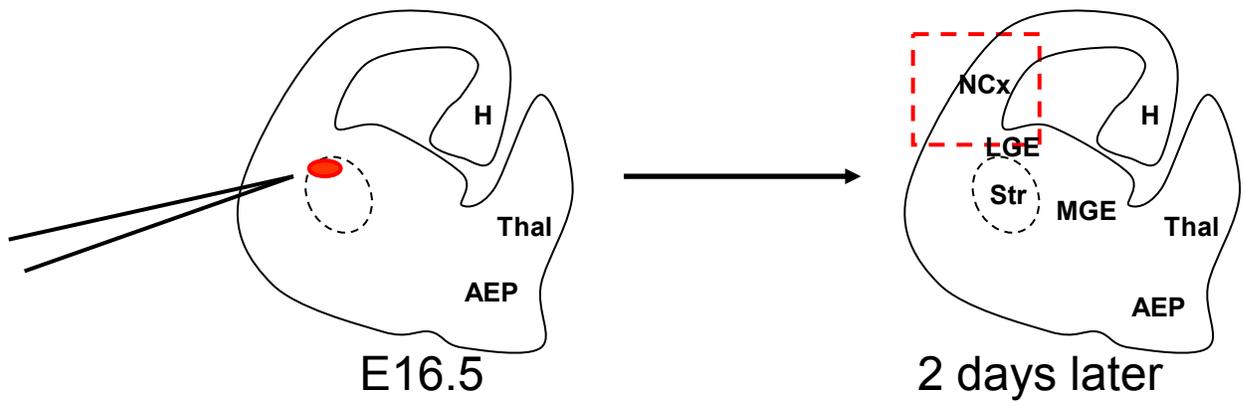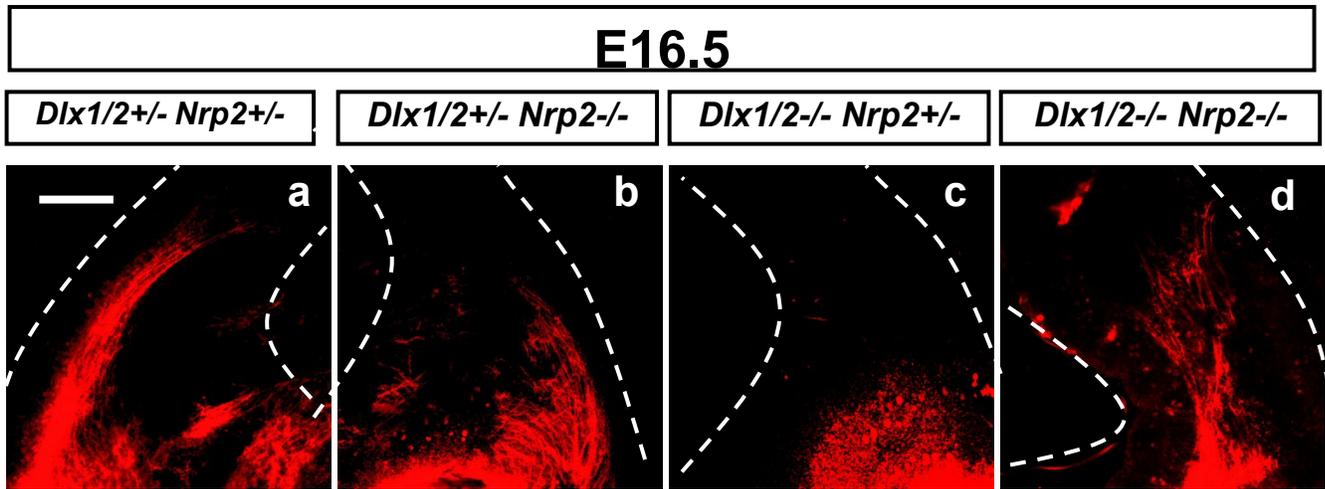
Figure 38. Cell migration from the LGE to the neocortex in E16.5 brain slice cultures of embryonic forebrain. These slices were prepared from an E16.5 embryo using a tissue oscillator. DiI (1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate; the chemical formula is $C_{59}H_{89}ClN_2O_4$) was placed in the LGE of the slices which were then cultured for 48 hours. Many DiI-labelled cells and axonal processes are present in the neocortex of the wild-type (a) and single mutants (b). Analysis of the double mutants (c) shows a reduction in DiI-labelled cells in the neocortex compared to the wild-type. In the triple mutants, DiI-labelled cells and axonal processes are found in the neocortex. Coronal sections. Scale bar, 400 μm. [H, Hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Amyl, amygdala; Str, striatum; Thal, thalamus; AEP: anterior entopeduncular area].

accumulations of partially differentiated cells were reduced (**Fig. 37D**, panels d-i), while co-expressing SST and mutant Neuropilin2. These results support that interneurons that co-express mutant Neuropilin2 and Somatostatin, in fact, might be derived from the basal ganglia of the triple mutants.

7.9 Restoration of tangential neuronal migration of later-born LGE cells in the triple mutant mice

Neuronal differentiation is associated with the migration of postmitotic cells from the proliferative zone to the neocortex. This process can be assessed using vital dye staining with DiI in organotypic brain slice cultures to study the ability of LGE cells to migrate (Tobet et al., 1994). Crystals of DiI were placed into the LGEs of all four genotypes, and the locations of DiI-labelled cells were determined up to 48 hours *in vitro*. At E16.5 (**Fig. 38**, panels a-d) and E18.5 (**Appendix Fig. 10**, panels a-d), there was a marked reduction in tangential migration from the LGE to the neocortex in the double mutant (c) compared to the wild-type (a) and single mutants (b). However, in the triple knockout (d), there were significant neuronal processes evident at the pallial/subpallial border between the LGE and the neocortex. These results suggest that in the absence of *Dlx*1/2 function, later-born cells are unable to migrate from the proliferative region of the LGE SVZ. Instead, they accumulate within the LGE, where they are partially differentiated (**Fig. 37C**, panel e). By removing *Nrp*2 expression from the *Dlx*1/2$^{-/-}$ cells accumulating in the basal ganglia, these interneurons can migrate to the neocortex due to lack of Semaphorin-Neuropilin mediated repression of tangential migration.

Chapter 8

Discussion

8.1 Identification of direct transcriptional targets of DLX homeoproteins from embryonic tissues *in vivo* using Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) technology represented a major technological advance for the identification of direct target genes of specific transcription factors, especially homeobox genes (Gould et al., 1990; Graba et al., 1997; Kuo and Allis, 1999; Orlando et al., 1997). The utilization of biochemical approaches, such as ChIP, rather than genetic approaches, provides several advantages. Identified target genes are directly downstream and derived from physiological homeodomain-DNA complexes obtained *in vivo*. Isolated gDNA fragments may be from regulatory elements of known or novel genes. ChIP may be applied to diverse species, including *Drosophila* and different vertebrates. Cross-linking preserves naturally existing (*in situ*) protein-DNA interactions. Utilizing these methods, homeodomain targets have been isolated, including transcription factors, growth factors, adhesion molecules and secreted proteins (Boudreau and Bissell, 1998; Graba et al., 1997; Mannervik, 1999).

The search for homeobox targets in vertebrates has not yielded as many targets as in *Drosophila*. Although several candidates have been identified using *in vitro* and tissue culture methods, the significance of these interactions *in vivo* remains to be confirmed. ChIP was used to identify a tumor suppressor gene and putative adhesion molecule as a downstream target of *Hox*-C8 in mouse spinal cord, suggesting that it is possible to isolate other direct vertebrate homeobox gene targets (Tomotsune et al., 1993). However, the ChIP methodology used by this group may have presented several technical obstacles

for subsequent isolation of other homeobox gene targets. For example, cross-linking was performed overnight using 4% PFA and the entire spinal cord was used as a tissue source rather than a specific region expressing high levels of *Hox*-C8. It is also unclear whether the specific antibody used had a high affinity for this homeobox protein. Hence, many investigators have eschewed ChIP in favor of cDNA microarray or other techniques to find genes potentially regulated by their transcription factor of interest. Recently, ChIP has been successfully coupled with DNA microarray analysis. Human CpG microarrays, probed with immunoprecipitated chromatin, was used to rapidly identify target promoters for the transcription factor E2F (Weinmann et al., 2002). Hence, combining ChIP and microarray technologies may improve both the efficiency and scale of standard ChIP approaches. We have optimized our ChIP approach by reducing the concentration of the cross-linking reagent PFA and the incubation time to preferentially obtain homeoprotein-genomic DNA complexes from embryonic tissues *in situ*. The polyclonal antisera to DLX1 and DLX2 have been subjected to a rigorous affinity purification process and are sensitive and highly specific (Eisenstat et al., 1999). In addition, chromatin is derived from nuclear extracts derived from tissues where the peak developmental expression of DLX1 and DLX2 occurs in restricted anatomical regions, enriching for the selection of *Dlx*1- and *Dlx*2-specific DNA target fragments. The identification of multiple *Dlx* target sequences *in vivo* will facilitate the elucidation of a consensus DLX DNA-binding-site initially derived *in vitro* for *Dlx*3 by (Feledy et al., 1999b). Finally, the expression of novel *Dlx* gene targets identified by ChIP may be altered in the *Dlx*1/2 null mouse, providing further understanding of how *Dlx* genes function throughout development. By combining genetic and molecular approaches, we have identified several *Dlx*-dependent

target genes that are critical effectors of forebrain development. In addition, this optimized ChIP approach can be used to isolate direct targets of other transcription factors during vertebrate development.

8.2 *Dlx* gene expression domains in the embryonic forebrain

Our initial investigations extended from the analysis of DLX1 and DLX2 transcription factors that are expressed in subpopulations of neurons in the developing forebrain. The restricted domains of expression of these homeodomain proteins define distinct regions of the early forebrain (Bulfone et al., 1993; Porteus et al., 1994; Price, 1993; Tole and Patterson, 1995). *Dlx*1 and *Dlx*2 are expressed in cells of the subcortical telencephalon that migrate across the pallial-subpallial boundary and enter the mantle and subventricular zones of the cerebral cortex from E12.5 mouse embryos (**Fig. 1**). Mice with disrupted *Dlx*1 and *Dlx*2 genes exhibit a reduction in number as well as defective differentiation of both striatal projection neurons and neocortical interneurons (Anderson et al., 1997a; Anderson et al., 1997b). These data support the current hypothesis that cortical projection neurons and interneurons are derived from distinct regions of the telencephalon (Anderson et al., 1999).

The *Dlx*5 and *Dlx*6 genes are expressed in the developing forebrain, with a very similar profile (Simeone et al., 1994). Transcripts are detected early in the primordium of the ganglionic eminence, and in the ventral diencephalon (**Fig. 1**). From E12.5, these genes are expressed in the ventral thalamus, in both the medial and lateral ganglionic eminences, and in the basal telencephalic vesicle anterior to the preoptic area. Several *Dlx* genes are expressed in the primordia of the basal ganglia, in overlapping patterns according to the stage of cell differentiation (Liu et al., 1997). *Dlx*1 and *Dlx*2 are

expressed in the least mature cells both in the VZ and in the SVZ. In contrast, *Dlx*5 is expressed in cells of the SVZ and in post-mitotic cells of the MZ, but not in the VZ, while *Dlx*6 expression is higher in the mantle cells (Liu et al., 1997). Interestingly, expression of *Dlx*5 and *Dlx*6 is reduced in the SVZ, but not in the mantle zone of the double mutants, suggesting that *Dlx*1 and/or *Dlx*2 might be required for the maintenance of *Dlx5/Dlx*6 expression in SVZ cells (Anderson et al., 1997b; Zerucha et al., 2000).

8.3 Cross-regulation of DLX1 and DLX2 homeoproteins, mediated by the intergenic sequences, is essential for *Dlx*5 and *Dlx*6 expression

The four *Dlx* genes expressed in the developing forebrain are organized into two tail-to-tail pairs, *Dlx*1/*Dlx*2 and *Dlx*5/*Dlx*6, a genomic arrangement conserved in distantly related vertebrates such as zebrafish (Ellies et al., 1997b; Zhou et al., 2004). The *Dlx*5/*Dlx*6 intergenic region contains two sequences of a few hundred base pairs, remarkably well conserved between mouse and zebrafish (Akimenko et al., 1994; Stock et al., 1996). Reporter transgenes containing these two sequences are expressed in the forebrain of transgenic mice and zebrafish with patterns highly similar to endogenous *Dlx*5 and *Dlx*6 (Zerucha et al., 2000). The activity of the transgene is drastically reduced in mouse mutants lacking both *Dlx*1 and *Dlx*2, consistent with the decrease in endogenous *Dlx*5 and *Dlx*6 expression. We have optimized the ChIP assay, using our specific DLX1 and DLX2 antibodies, to demonstrate for the first time that both DLX1 and DLX2 bind to the *Dlx*5/*Dlx*6 intergenic enhancer in embryonic forebrain *in vivo* (**Figs. 2, 3**). Our results provide a direct mechanism to explain the marked reduction in both *Dlx*5 and *Dlx*6 expression in the embryonic striatum observed in the *Dlx*1/*Dlx*2 double knockout mouse (Anderson et al., 1997b). In addition, we have demonstrated

similar transcriptional regulation of this enhancer by DLX2 but not DLX1 in the mouse retina, signifying a conservation of function of *Dlx* genes wherever these homeobox genes are expressed (Zhou et al., 2004). The subtle phenotypes observed in the developing forebrain of the *Dlx*1 and *Dlx*2 single knockouts, as well as the overlapping expression domains and co-expression in subsets of embryonic striatum, support functional redundancy of *Dlx*1 and *Dlx*2 (Eisenstat et al., 1999; Liu et al., 1997; Panganiban and Rubenstein, 2002; Qiu et al., 1995; Qiu et al., 1997). DLX1 and DLX2 expression peaks at E13.5 and gradually decreases by late gestation in the embryonic striatum. Hence, we have shown that DLX1 and DLX2 bind to MI56 in E13.5 GE, as demonstrated by ChIP and EMSA assays (**Figs. 3, 4**). The activation of reporter gene expression by *Dlx*2, but not *Dlx*1, co-transfection with MI56 constructs suggests that DLX1 may require additional co-factors not present in the P19 embryonal carcinoma cells *in vitro* (**Fig. 5**). However, luciferase and other reporter assays are *in vitro* "non-chromatin" based methods. Hence, using ChIP, the investigator may detect binding without functional activation/repression of regulatory elements *in vitro*. The lack of demonstrated cooperation of DLX1 with DLX2 to activate the MI56 enhancer reporter gene suggests that DLX1 and DLX2 may have some non-overlapping functions during striatal neuronogenesis. These results support the functional differences observed between *Dlx* paralogs in *Dlx*1, *Dlx*2 and *Dlx*1/2 null mice (Eisenstat et al., 1999; Panganiban and Rubenstein, 2002; Qiu et al., 1997). This study also was the first to confirm direct transcriptional activation of *Dlx*1&2 genes on cross-regulation of *Dlx*5&6 *in vivo* using the chromatin immunoprecipitation assay.

8.4 *Dlx* genes and differentiation of GABAergic interneurons: DLX homeoproteins directly bind to and activate transcription of *Gad*1 & *Gad*2 promoters

Within the forebrain, the expression of the *Dlx* genes coincides with the location of virtually all neurons that use GABA as their neurotransmitter. This suggests that the *Dlx* genes may have a general role in the development of this cell type (Anderson et al., 1997a; Anderson et al., 1997b; Stuhmer et al., 2002b). Indeed, ectopic expression of *Dlx2* or *Dlx5* in cortical neurons using either retroviral vectors or electroporation methodology, induces expression of the GABAergic phenotype (Anderson et al., 1999; Stuhmer et al., 2002a). Mice lacking both *Dlx*1 and *Dlx*2 have striking abnormalities in development of the striatal SVZ, especially in the differentiation of striatal matrix neurons, and in the migration of neocortical GABAergic interneurons from the subcortical telencephalon (Anderson et al., 1997a; Anderson et al., 1997b, Cobos et al., 2005b). Glutamic acid decarboxylase (GAD) synthesizes GABA from glutamic acid. In the embryonic forebrain, the expression pattern of the DLX transcription factors is nearly identical to that of the GAD (GAD65 and GAD67) enzyme isoforms. For example, at early stages of embryonic telencephalon development, unlike the pallium the subpallium expresses *Gad*65 (*Gad*2) and *Gad*67 (*Gad*1); and *Dlx*1, *Dlx*2, *Dlx*5 and *Dlx*6 (Eisenstat et al., 1999; Fode et al., 2000; Katarova et al., 2000; Liu et al., 1997; Porteus et al., 1994; Price, 1993; Robinson et al., 1991; Zerucha and Ekker, 2000). The spatial and temporal coincidental patterns of DLX and GAD expression suggest that the *Dlx* genes may have an important role in regulating GAD expression.

Relatively little is known about the transcriptional regulation of *Gad* genes. The reduction of NMDA receptor-mediated currents is likely to be only one out of several

mechanisms by which decreased neuronal activity results in down-regulation of GAD67 expression in the cortex of schizophrenic patients. For example, there is increasing recognition that brain-derived neurotrophic factor (BDNF) and its high affinity receptor, the receptor tyrosine kinase (TrkB), are key regulators of GABAergic inhibitory interneuron function in cerebral cortex and other brain regions (Marty et al., 1997; Rutherford et al., 1997). Reelin is a large, approximately 420–450 kDa glycoprotein that plays a crucial role for orderly brain development, including positioning of cortical neurons. Importantly, GABAergic interneurons of adult rodent and primate cerebral cortex and hippocampus express Reelin (Pesold et al., 1998; Rodriguez et al., 2002). Notably, a deficit of Reelin in prefrontal, temporal and cerebellar cortex of schizophrenic or bipolar disorder subjects is associated with decreased levels of GAD67 transcript and protein (Fatemi et al., 2005; Guidotti et al., 2000; Impagnatiello et al., 1998). Furthermore, mutant mice expressing approximately 50% of wild-type Reelin levels have decreased GAD67 expression in frontal and parietal cortex (Liu et al., 2001). Presently, the molecular mechanisms linking Reelin expression to the transcription of GAD67 are still unknown.

Herein, utilizing ChIP of embryonic striatal tissue, we have demonstrated for the first time that DLX1 and DLX2 bind directly to specific regions of *Gad*1 and *Gad*2 promoters (**Fig. 6**). Specificity of binding as well as specific homeodomain binding motifs of each region within the *Gad* promoters was confirmed by EMSA assays *in vitro* using both recombinant DLX proteins and endogenous DLX proteins obtained from nuclear extracts (**Figs. 7, 8**; data not shown). Reporter gene assays and site-directed mutagenesis demonstrated the transcriptional activation mediated by DLX1 and DLX2

binding to the *Gad*1 and *Gad*2 promoters *in vitro* (**Fig. 9**). DLX2 acted as a more potent transcriptional activator than DLX1 of both the *Gad*65 and *Gad*67 promoters, signifying a more important role for *Dlx*2 gene transcription in forebrain development. Indeed, mice homozygous for the *Dlx*1 mutation have a milder phenotype than mice homozygous for the *Dlx*2 mutation. *Dlx*1 mutants are viable at birth, dependent on the background mouse strain, but are small and all die within 1 month with an abnormal phenotype including a seizure disorder. *Dlx*2 mutants behave like the *Dlx*1/2 mutants; they all die within a few hours after birth with distinctive craniofacial skeletal deformities. Of note, a 3 grouped TAAT/ATTA homeodomain binding motifs within *Gad*65 promoter region i are crucial for *Dlx*-driven transcription as their mutation abolished *Gad*65 reporter gene activity (**Table 3**; data not shown; **Fig. 9A**). Similarly, all 3 individual TAAT/ATTA motifs spanning both regions of the *Gad*67 promoter are important for *Dlx*-driven transcription based on mutagenesis results (**Table 3**; data not shown; **Fig. 9B**). Hence, we have established the direct transcriptional activation of *Dlx*1&2 to specific DNA-binding-motifs on *Gad* promoters *in vitro* and *in situ*.

8.5 *Dlx* genes and differentiation of GABAergic interneurons: Loss of *Dlx* function affects expression of *Gad* genes and levels of GABA neurotransmitter in the developing forebrain

We have provided evidence that *Dlx* homeobox gene expression marks the vast majority of GABAergic cortical and striatal neurons, from the beginning of their development in the basal telencephalon, through their migration to the neocortex (**Fig. 10, 11, 12**). The results obtained highlight the fact that in the basal ganglia, mainly in the SVZ, the *Dlx* genes are co-expressed with GABA and *Gad*1, *Gad*2 genes, all of which

are hallmarks of an interneuronal phenotype (**Figs.10, 11**; **Appendix Figs. 1-3**). From the outset of their tangential migration from the GEs and through their migrations in the marginal, intermediate and subventricular zones, to their residence in the cortical plate, the distribution of GABA, *Dlx* gene, and *Gad* gene expression shows a remarkable coincidence.

The close link of the *Dlx* and *Gad* genes suggest a functional relationship. There are several lines of evidence indicating that the *Dlx* genes are essential for the development of GABAergic cortical neurons. In the *Dlx*1/2 double mutants, there is an ~80% reduction in neocortical GABAergic neurons (**Fig. 12**; **Appendix Fig. 4A**) (Anderson et al., 1997a). The reduction is primarily due to a block in the tangential migration of these cells from the GEs. However, there is evidence from loss- and gain-of-function experiments that the *Dlx* genes may have additional roles in differentiation of GABAergic cells (Anderson et al., 1999; Stuhmer et al., 2002a). Indeed, GABA, GAD65, and GAD67 expression are severely decreased in the SVZ of the basal telencephalon in the *Dlx*1/2 mutants from E13.5 to birth (**Fig. 12**, **Appendix Fig. 4A-C**). The fact that there is residual GABA and *Gad* expression in the *Dlx*1/2 mutants suggests that there are other transcription factors that can compensate for the absence of these homeobox genes. Of note, *Dlx*1*, Dlx*2*, Dlx*5 and *Dlx*6 are expressed in overlapping sets of cells in the developing forebrain, suggesting potential redundant functions (Bulfone et al., 1993; Eisenstat et al., 1999; Liu et al., 1997). However, since expression of *Dlx*5 and *Dlx*6 is greatly reduced in the *Dlx*1/2 mutants (**Chapter 4**), other genes are more likely to play this role. Candidates include *Mash*1, a bHLH transcription factor, whose expression appears to be upstream of the *Dlx* genes (Fode et al., 2000). Other candidates are *Gsh*1

and *Gsh*2 homeobox genes, whose expression is unaffected in the *Dlx*1/2 mutants (Yun et al., 2003). As well, in the *Dlx*1/2 double mutants, the first wave of neurogenesis (from approximately embryonic days 10-12) appears to be undisturbed, whereas differentiation of later born neurons (E13.5 to birth) is largely affected. This leads us to the primary proliferative population (PPP; located in the VZ) which appears normal in the double mutants, while abnormalities are found in the SVZ that contains the secondary proliferative population (SPP) of neuroblasts (Marin et al., 2000). These studies help to explain the role of *Dlx* genes in the differentiation of later-born interneurons, not early born interneurons, as well as provide a region-specific role of *Dlx* genes in the developing basal telencephalon.

To assess the global transcriptional control of *Dlx* genes on the development of GABAergic interneurons, we quantitatively measured GABA levels by HPLC, as well as determined *Gad*65 and *Gad*67 RNA expression in the embryonic forebrain. In addition to reduced GABAergic interneuron migration to the neocortex, there is a significant reduction (~25%, $p<0.02$, n=6) of GABA expression in the basal telencephalon in the *Dlx*1/2 null mouse compared to wild-type (**Fig. 13**). Of note, overall GABA levels do not decrease significantly until E18.5, signifying the role of *Dlx* genes in the terminal differentiation of later-born neurons. Likewise, both *Gad*65 and *Gad*67 mRNA expression show signs of reduction in the *Dlx*1/2 double mutant as early as E12.5, but the reduction becomes more significant later on (**Figs. 14, 15**).

To further assess DLX2 as a potent regulator of GABA expression, we utilized both shRNA and siRNA technologies to knock down *Dlx*2 expression in primary wild-type embryonic striatal and neocortical cultures. E16.5 and E18.5 primary cultures were

chosen to better represent the regulation of differentiation of later-born GABAergic interneurons of DLX2. siRNAs were able to effectively reduce DLX2 expression and concomitantly decrease GABA expression when compared to control scrambled siRNA (data not shown). Similarly, the more efficient delivery of shRNA specific to *Dlx*2 via lentiviral transduction mediated robust *Dlx*2 knockdown and more significantly reduced GABA expression in both embryonic striatal and neocortical culture systems (**Figs. 16, 17**). In agreement with previous gain-of-function experiments (Stuhmer et al., 2002a, 2002b), we have further demonstrated that *Dlx* genes play a significant role in transcriptional activation of *Gad* genes in the embryonic forebrain by direct quantification of *in vivo* GABA level in whole forebrain, as well as *in situ* loss-of-function experiments. This study establishes that the *Dlx* genes directly bind to and activate transcription of *Gad* promoters, and *Dlx* genes are necessary to maintain the fundamental GABAergic phenotype in later-born interneurons.

8.6 *Dlx* genes and migration of GABAergic interneurons: DLX homeoproteins directly bind and repress transcription of the *Neuropilin*2 promoter

DLX proteins have been previously characterized as transcriptional activators (Zerucha et al., 2000; Zhang et al., 1997; Zhou et al., 2004, Cobos et al., 2005a, Colasante et al., 2008), although it was shown that DLX homeoproteins can repress transcription of several reporter plasmids *in vitro* (Yu et al., 2001). In addition, BP-1, an isoform of DLX7, represses the β-globin gene *in vitro* (Chase et al., 2002). We have demonstrated that both DLX1 and DLX2 bind to specific homeodomain binding motifs in a *cis*-regulatory domain of the *Neuropilin*2 (*Nrp*2) but not the *Neuropilin*1 (*Nrp*1), *Mash*1

or *Wnt*7B promoters *in situ* (**Figs. 18, 19**; **Appendix Figs. 5, 7**) and repress transcription of a reporter vector containing the *Nrp*2 sequence *in vitro* (**Fig. 21**).

We have also confirmed that the homeodomain of DLX1 and DLX2 is necessary for DNA binding and that this binding is essential for *Dlx* mediated repression of *Nrp2* expression. A single base-pair mutation of glutamine (Q) to glutamate (E) at amino acid (a.a.) position 50 (Q50E) of the 60 amino acid homeodomain is sufficient to eliminate the DNA-binding ability of the DLX1/DLX2 homeodomain and subsequent transcriptional repression of a reporter gene *in vitro* (**Figs. 20, 21A**). Residual DNA binding in the Q50E mutant proteins due to other possible DNA binding sites localized within the homeodomain (not detected by EMSA) or by binding of DLX1/2 to other proteins bound to the *Nrp*2 promoter, could account for the ability of *Dlx*1/2Q50E to still marginally repress (although this repression was not statistically significant). Consistent with the evidence that *Dlx*1 and *Dlx*2 function as repressors when bound to the *Nrp*2 promoter is the observation that *Nrp*2 expression is significantly increased in the basal forebrain of *Dlx1/Dlx2* double mutant mice (Marin et al., 2001); (**Figs. 23, 24**).

In contrast to our finding that DLX1 and DLX2 are transcriptional repressors of the *Nrp*2 promoter, DLX1 and DLX2 act as transcriptional activators of specific targets, the *Dlx*5/*Dlx*6 intergenic enhancer *in vitro* and *in vivo* (Zerucha et al., 2000; Zhou et al., 2004) and GnRH regulatory elements *in vitro* (Givens et al., 2005). Furthermore, the *Dlx* family member DLX3 also acts as a transactivator of a model target gene construct, *Dlx3*-CAT (Feledy et al., 1999b) and the osteocalcin gene promoter (Hassan et al., 2004). In support of a repressor function of the *Dlx* genes, DLX1, DLX2 and DLX5 interact with a homeodomain binding site within the *Wnt*1 enhancer *in vitro* (Iler et al., 1995; Liu et al.,

1997). Mutation of this site results in extension of the rostral boundary of *Wnt*1/*lacZ* expression in transgenic animals. The authors suggest that this site may mediate repression of *Wnt*1 expression in the forebrain, although neither DLX1 nor DLX2 have been implicated in transcriptional repression of *Wnt*1 *in vivo* (Iler et al., 1995). Unlike DLX1, DLX2 was a robust inducer of *Dlx*5&6 and *Gad* isoform expression (**Chapters 4 and 5**) and a strong repressor of *Nrp*2 (**Fig. 21**). Based on their amino acid sequences, the *Dlx* genes fall into two major homology groups: Type A (*Dlx2, Dlx3* and *Dlx5*) and Type B (*Dlx1, Dlx6* and *Dlx7*) (Liu et al., 1997; Stock et al., 1996). Our data provide further support for biochemical differences in the functions of A and B *Dlx* gene subtypes. Therefore, our results combined with previous studies suggest both redundant and distinct functions for different members of the *Dlx* gene family in regulating aspects of differentiation related to migration. The hypothesis that Type A and B *Dlx* genes have different functions *in vivo* is consistent with the observation that *Dlx1* and *Dlx2* single mutants have distinct maxillary dysmorphologies, despite their similar expression patterns in the first branchial arch (Qiu et al., 1997).

The amino termini of both DLX1 and DLX2 may mediate, in part, the transcriptional repression activity of these homeodomain proteins, as demonstrated by the results of substituting their N-termini with the Engrailed repressor or VP16 activation domains in the reporter gene assays (**Fig. 20, 21B**). As discussed previously, we have found DLX2 to be a better repressor than DLX1 *in vitro*; therefore the replacement of the DLX1 N-terminal domain with the VP16 activation domain was able to overcome DLX1's weaker repressive function. In contrast, DLX2 is a stronger repressor; hence this function is not completely alleviated by placement of VP16 at its N-terminus. Certainly,

the VP16-DLX2 mutant represses less than the wild-type protein. Perhaps the fusion protein did not sufficiently eliminate all of the repressor domains of DLX2. For example, there may be another repressor domain at the C-terminus of DLX2, given that the chimeric VP16-DLX2 construct was unable to transactivate reporter gene expression to levels seen with the VP16-DLX1 construct. Subsequently, fusion of the DLX1 with VP16 may have been able to activate better than the VP16-DLX2 mutant used in this study. Both the N- and C-termini of DLX3 are required for mediating transcriptional activation *in vivo* using *Xenopus* embryo expression assays (Feledy et al., 1999b). Further analysis will better delineate functional domains of DLX proteins other than the homeodomain that are important for the modulation of transcription.

DLX2 is more robust than DLX1 as a transcriptional activator (Zhou et al., 2004) or transcriptional repressor (**Fig. 21**). Furthermore, co-transfection of *Dlx*1 and *Dlx*2 wild-type or chimeric constructs is neither additive nor synergistic, yielding results which are similar to that of transfection of *Dlx*2 constructs alone (Zhou et al., 2004); (data not shown). This lack of potentiation suggests that there may be greater affinity of DLX2 than DLX1 for binding to their specified homeodomain DNA binding sites and/or slower rates of dissociation. In the embryonic telencephalon, it is also possible that DLX2 interacts with one or more co-repressors or coactivators with which DLX1 does not interact. The Evf-2 non-coding RNA is a recently identified DLX2 transcriptional coactivator transcribed from the *Dlx*5/*Dlx*6 intergenic region (Feng et al., 2006). Although DLX2 and DLX5 may form homodimers and also heterodimerize with MSX repressor proteins *in vitro* and *in vivo* (Zhang et al., 1997), heterodimerization of *Dlx* family members has not been established. The DNA-binding specificity of MSX1 is

determined by its association with cofactors, such as PIAS1, that direct this homeoprotein to subnuclear compartments where its target genes are located (Lee et al., 2006). It is likely that the specificity of transcriptional regulation by *Dlx* genes may involve other factors in unique protein-protein complexes with different transcriptional activities.

Whether DLX proteins function as activators or repressors of target gene expression may depend on cooperation with other transcription factors as demonstrated for other homeobox proteins with paired-type homeoproteins (Mikkola et al., 2001) or the TATA binding protein (Zhang et al., 1996). DLX transcriptional activity may also depend on post-translational modifications, such as phosphorylation as shown for DLX3 (Park et al., 2001), or interaction with proteins such as the PDZ protein GRIP1 (Yu et al., 2001) and the MAGE protein Dlxin (Masuda et al., 2001; Panganiban and Rubenstein, 2002). It has been shown that DLX1 interacts through its homeodomain with Smad4, a co-SMAD, during hematopoietic differentiation (Chiba et al., 2003). It will also be interesting to determine whether DLX1 and DLX2 have non-overlapping sets of downstream gene targets at various developmental time points or in specified tissues where *Dlx* genes are expressed.

Although a consensus DNA-binding sequence has been established for paired-type homeoproteins such as PAX6, 5'-*TAAT(N3)ATTA-3'* (Mikkola et al., 2001), and HOX proteins, 5'-*(C/G)TAATTG-3'* (Pellerin et al., 1994), one specific for *Dlx* family members remains to be established. Previous study identified an 8-base consensus 5'-*(A/C/G)TAATT(G/A)(C/G)-3'* for DLX3 DNA binding sites (Feledy et al., 1999b). This sequence was conserved at specific sites of the *Dlx5/Dlx6* intergenic enhancer bound by DLX1 or DLX2 *in vitro* or *in situ* ((Zerucha et al., 2000; Zhou et al., 2004), although

there was discordance at the most 3' nucleotide. As well, only seven nucleotides match for the region of the *Neuropilin*2 promoter, *ATAATTAT*, bound by DLX1 or DLX2 *in vitro* (**Fig. 18A**). These results suggest that although there are sequence similarities between DLX1, DLX2 and DLX3 binding sites, there are currently insufficient verified DLX targets to establish a consensus DNA-binding site for the *Dlx* gene family. This study is the first to report that *Dlx* transcription factors may function as transcriptional repressors *in vivo*.

8.7 *Dlx* genes and migration of GABAergic interneurons: Loss of DLX function affects expression of the *Nrp*2 gene and distribution of GABAergic interneurons in the developing forebrain

In the developing forebrain, although cell migration along radial glia is predominant, non-radial or tangential migration is also an important means for other sets of differentiating cells to reach their destinations. For instance, immature GABAergic interneurons produced in the ganglionic eminences (subpallium) migrate tangentially to the neocortex and hippocampus (pallium) (Anderson et al., 1997a; Letinic et al., 2002; Marin et al., 2001; Stuhmer et al., 2002b; Wichterle et al., 1999; Wichterle et al., 2001). Interneurons migrating to the cortex arise primarily from the MGE and the AEP and follow two major pathways as they traverse the LGE/striatum: a superficial subpial route and a deep route adjacent to the SVZ (Anderson et al., 2001; Anderson et al., 2002; Marin and Rubenstein, 2003). The molecular mechanisms that guide and sort these migrating cells are being elucidated (Marin and Rubenstein, 2003). Recently, ErbB/neuregulin signalling has been implicated in supporting migration through the LGE towards the cerebral cortex (Flames et al., 2004; Hanashima et al., 2006; Lopez-Bendito

et al., 2006). GDNF signalling via GFRα1 has a role in the differentiation and migration of cortical GABAergic cells from the MGE (Pozas and Ibanez, 2005). Likewise, chemorepellant tissues and factors have been identified that participate in directing these migrations (Nóbrega-Pereira et al., 2008, Marin et al., 2003; Wichterle et al., 2003).

There is evidence that striatal expression of Semaphorin3A and 3F repels tangentially migrating interneurons (Marin et al., 2001). Semaphorin3A can also act as a chemoattractant *in vitro* (Carmeliet and Tessier-Lavigne, 2005; Song and Poo, 2001), whereas another secreted class III semaphorin, semaphorin3B, mediates both attraction and repulsion *in vivo* (Falk et al., 2005). Semaphorins interact with receptor complexes consisting of neuropilins (ligand-binding subunits) and class A plexins (signal transduction subunits) (Garrity, 2005; Guan and Rao, 2003; Pasterkamp and Kolodkin, 2003). *Neuropilin*2 null mice demonstrate an increased numbers of tangentially migrating cells entering the striatum (Marin et al., 2001). Similarly, interneurons that express a dominant negative form of *Neuropilin*1 also aberrantly enter the striatum (Marin et al., 2001). Previous studies demonstrated that *Dlx*1 and *Dlx*2 function is necessary for migration of more than 75% of tangentially migrating interneurons to the murine cortex (Anderson et al., 1997a), olfactory bulb (Bulfone et al., 1998) and hippocampus (Pleasure et al., 2000). Upon loss of *Dlx*1 and *Dlx*2 function, there is ectopic accumulation of cells with molecular properties of cortical interneurons within the SVZ of the ganglionic eminences. These cells, which express high levels of *Nrp*2 (Marin et al., 2001) (**Figs. 23, 24**) are presumed to be collections of interneurons that have failed to migrate to the cortex. Normally, *Neuropilin*2 expression patterns minimally overlap with *Dlx*1 and *Dlx*2 expression (**Fig. 22**; **Appendix Fig. 6**) and with the pathways followed by tangentially

migrating interneurons (Tamamaki et al., 2003a). These data support our hypothesis in which DLX1- and/or DLX2-expressing cells can down-regulate *Nrp*2 expression after E13.5, enabling later-born interneurons, most derived from the MGE and AEP, to take the deep route to the striatum towards the neocortex. Repression of *Nrp*2 expression may therefore allow these interneurons to migrate through Semaphorin expressing cells in the striatum and at the pallial-subpallial interface (Marin and Rubenstein, 2001; Marin et al., 2001; Marin and Rubenstein, 2003). Late-born interneurons which aberrantly express NRP2 in the absence of *Dlx*1 and *Dlx*2 function would fail to migrate to neocortex due to the repulsive guidance cues from Semaphorin3F-expressing cells mediated via NRP2 and accumulate as subventricular ectopias (Marin and Rubenstein, 2001; Marin et al., 2001; Marin and Rubenstein, 2003). In the *Dlx*1/2 mutant, loss of DLX-dependent repression of *Nrp*2 transcription may explain, in part, the ectopic accumulation of GABAergic interneurons in the ganglionic eminences (**Fig. 25**). These studies do not exclude the likely possibility that other molecules, some directly regulated by *Dlx* genes and others independent of *Dlx* function, contribute to the tangential migrations of interneurons especially after they have passed the pallial/subpallial junction (Marin et al., 2001). Other transcriptional factors, in addition to DLX-dependent repression, may contribute to repress *Nrp*2 expression in interneurons entering the striatum, as evident by the presence of Semaphorin expressing domains and the lack of *Neuropilin*2 expression in sorted striatal interneurons (Marin et al., 2001). However, our results support that *Dlx*1- and *Dlx*2 transcriptional repression contributes to the regulation of tangential migration of late-born inhibitory interneurons from the subcortical telencephalon via Semaphorin-Neuropilin interactions.

8.8 Analysis of *Dlx*1/2; *Nrp*2 triple-mutant mice: Intact cortical regional identity and laminar-specific properties of neurons in the cortical plate

There are two broad classes of cortical neurons: interneurons, which make local connections; and projection neurons, which extend axons to distant intracortical, subcortical and subcerebral targets. Projection neurons are glutamatergic neurons characterized by a typical pyramidal morphology that transmit information between different regions of the neocortex and to other regions of the brain (Tan et al., 1998). By contrast, GABA-containing interneurons and Cajal–Retzius cells are generated primarily from progenitors in the ventral telencephalon and cortical hem, respectively, and migrate long distances to their final locations within the neocortex (Anderson et al., 2002). In this manner, multiple progenitor zones contribute to the rich variety of neuronal types found in the neocortex. To assess cortical lamination between the four different genotypes in this study, we first examined several specific markers of different cortical layers. Within the developing neocortex, distinct populations of projection neurons are located in different cortical layers and areas, have unique morphological features, express different complements of transcription factors, and ultimately serve different functions. Different neuronal subtypes of postmitotic projection neurons express subtype-specific markers that allow identification of cortical layers and laminar specification in the neocortex. Examples of layer-specific genes (from layer II-VI, SVZ, VZ) included, among many others: *Math*2, *Cux*2, *ROR*β, *ER*81, *Tbr*1, *Lhx*2, and *Ngn*2. Most of these layer-specific markers (*Math*2, *Cux*2, *Tbr*1, *Lhx*2) for neocortex were tested and there were no significant changes among the various mutants using *in situ* hybridization (**Appendix Fig. 8**). Other layer-non-specific markers (glutamate, MAP2, *Msx*, *Trk*B, *Tbr*1) for

neocortex were also tested with similar findings for all 4 genotypes using immunofluorescence (**Appendix Fig. 9**). The lack of any obvious defects in cortical lamination may be explained by the specific requirement of *Dlx*1 and *Dlx*2 for the development of neocortical GABAergic interneurons, but not for the development of glutamatergic projection neurons which populate most of the neocortex (Marin and Rubenstein, 2003). Furthermore, despite their vital role in corticogenesis and development of cortical circuitry, GABAergic interneurons represent only ~20% of all of the neurons in the mouse cerebral cortex (Tamamaki et al., 2003b). Hence, in the *Dlx*1/2 mutant, a reduction of ~75% of tangential migration of GABAergic interneurons from the GEs to the neocortex (Anderson et al., 1997a) might not be of sufficient significance to alter cortical lamination. There may be other compensating mechanisms including additional interneuron sources within the cortical neuroepithelium (Letinic et al., 2002), or subcortical migrations of early-born interneurons from the MGE mediated by other transcription factors such as *Nkx*2.1 (Sussel et al., 1999), or from the CGE mediated by *Gsh*2 (Corbin et al., 2003) or *ER*81 (Stenman et al., 2003a).

Retinoid-related orphan receptors, *ROR*α, −β, and −γ, are transcription factors belonging to the steroid hormone receptor superfamily. During embryonic development RORs are expressed in a specified spatial and temporal manner and are critical in the regulation of cellular differentiation and the development of several tissues (Michel et al., 1998; Sashihara et al., 1996). In the neocortex, at E12.5, *ROR*β is not detected in the dorsal telencephalon, but by E14.5 high levels of expression are evident in the lateral and rostral parts of the neocortex. By E16.5, levels of *ROR*β mRNA are increased and expressed in a graded fashion: from high (lateral or rostral) to low (medial or caudal)

levels of expression (**Fig. 26** a, b). At birth, high levels of expression were observed in the somatosensory area and putative auditory and visual areas in the caudal neocortex in layer IV and sporadically in layer V (Nakagawa and O'Leary, 2003). $ROR\beta$ expression was significantly reduced in the dorsal neocortex of the double and triple knockout, compared to wild-type and single knockout cortices (**Fig. 26**, a-d). These results indicated that *Dlx*1 and/or *Dlx*2 could regulate $ROR\beta$ expression. Furthermore, GABAergic interneurons may contribute to cortical lamination of layers IV to V.

ER81 is a member of the ETS transcription factor family (de Launoit et al., 1997). It is expressed almost exclusively in a subset of cortical layer V projection neurons across various cortical areas (Hevner et al., 2003; Xu et al., 2000). ER81 also marks a subpopulation of neurons in the SVZ of the LGE, the rostral migratory stream and olfactory bulb interneuron progenitors (**Fig. 26** e,f) (Stenman et al., 2003a), as well as in the subiculum, medial habenular nucleus, and basal amygdaloid nuclei (Yoneshima et al., 2006). In this study, we demonstrated that *Er*81 marked a subpopulation of cortical layer V neurons (**Fig. 26** e-h) which were relatively unchanged between four genotypes. This is not surprising results given that *Dlx* genes have not been known to play a part in transcriptional regulation of glutamatergic neurons in the cortex. However, *Er*81-expressing cells of the dorsal LGE, RMS region, remnant of MGE, and ventral pallidum were severely reduced in the double mutant (**Fig. 26** g), in agreement with the corresponding reduction of dorsal LGE *Pax*6 expression (**Fig. 27** m-p). Both ER81 and Pax6 were found to be severely reduced in prenatal olfactory bulb (Long et al., 2007). PAX6 has been found to be an upstream transcriptional regulator of *Er*81 (Tuoc and Stoykova, 2008). Surprisingly, a domain of *Er*81-positive cells that was "lost" in the

ventral pallidum of the double mutant, was "found" in the triple mutant (**Fig. 26** h). This result indicates that Semaphorin/NRP2 signalling might play a role in migration of *Er*81-positive neurons as part of the rostral migratory stream. Further evidence must be provided to closely assess tangential migration from the CGE or LGE to the olfactory bulb. It would be interesting to know where this restored *Er*81-positive population originated and how Semaphorin-Neuropilin interactions are involved. Further studies of *Nrp*2's role in the migration of projection neurons in the RMS and possibly in radial migration, and between nuclei of the developing basal forebrain will help to elucidate the significance of these results.

Neurogenins are specifically expressed in cortical but not subcortical progenitors, where they specify the regional identity of the earliest-born preplate neurons in the neocortex of the telencephalon (**Fig. 26** i) (Bertrand et al., 2002; Fode et al., 2000). Expression of the *Dlx* gene family is first detected in the ventral telencephalon by E10.5 (Eisenstat et al., 1999; Liu et al., 1997), and its expression is largely complementary to that of the cortically expressed *neurogenins*. The expression patterns of *Ngn*2 and *Dlx*1/2 in the embryonic telencephalon suggests a role for these molecules in the regulation of dorsal-ventral identity. It has been demonstrated that neurogenins are required to repress *Mash1* and *Dlx* gene expression in the developing cerebral cortex, and *Mash1* and *Dlx* genes are ectopically expressed in the neocortex of *neurogenin*1 and *neurogenin*2 mutants (Fode et al., 2000). Conversely, the loss of DLX and MASH1 expression in the *Gsh*2 mutant correlate well with ectopic expression of cortical markers (specifically *Pax*6, *Ngn*1, *Ngn*2) in progenitors of the mutant LGE (Toresson et al., 2000). Interestingly, in our study of the *Dlx*1/2 mutant, a small subset of *Ngn*2-expressing cells

were found ectopically expressed in the cortical plate and/or mantle zone where *Dlx*-positive cells would normally found in the wild-type via tangential migration (**Fig. 26** k). However, in the triple mutant neocortex, the removal of *Nrp*2 expression resulted in a loss of the ectopic *Ngn*2-expressing cells in the neocortex (**Fig. 26** l). At E18.5, *Ngn*2 expression is highly restricted to VZ of neocortex, and the ectopias appeared to be small and insignificant with respect to the number of cells. Hence, even with further study it might be difficult to assess the role of the Neuropilin2 mutation on such a small change in *Ngn*2 gene expression. Yet, these results suggest that further studies of Semaphorin-Neuropilin signalling might yield more interesting insights regarding the migration of neural stem cells during their neurogenesis. Overall, removal of Neuropilin2 expression in *Dlx*1/2 double mutant does not show any significant changes in cortical lamination up to birth.

8.9 Analysis of *Dlx*1/2; *Nrp*2 triple-mutant mice: Abnormalities in development of the striatal subventricular zone and of striatal matrix neurons

The striatum has several levels of organization, including the striosome and matrix domains. Striosome and matrix domains have different patterns of connectivity, and distinct histochemical and adhesive properties (Gerfen, 1984; Graybiel, 1990; Krushel and van der Kooy, 1993). Earlier born neurons form the deeper neocortical layers and striosomal compartment of the striatum, while later born cells form the superficial neocortical layers and the striatal matrix (Huot and Parent, 2007; Kawaguchi et al., 1995). Previous studies provided evidence that *Dlx*1 and *Dlx*2 regulate this process in the striatum (Anderson et al., 1997b). They demonstrated that early (~E10.5-E12.5) striatal histogenesis appears normal, whereas at later times most of the postmitotic

neurons do not exit the proliferative zone. In our study, E18.5 striatal tissue of the *Dlx*1/2 double mutant appeared to be reduced morphologically and there was down-regulation of specific markers of the striosomal compartment (DARPP32) and of extrastriosomal matrix (TH) (**Fig. 27 A, B**). Hence, in addition to the absence of matrix cells (later born neurons) in the poorly developed striatum of the double mutant (Anderson et al., 1997b), there might also be defects in migration and differentiation of some of the striosomal neurons. Overall, defects in differentiation and migration of both striatal neurons and GABAergic interneurons from the ganglionic eminences led to the accumulation of cells as periventricular ectopias within the LGE (**Fig. 23C** b). Some of these cells were partially differentiated, proliferating, or even undergoing apoptosis. Hence we noted increased cellular turnover by BrdU labelling (data not shown), phosphohistone H3, and activated caspase-3 expression (arrows in **Fig. 28**) in the abnormal LGE of the double mutant. As expected, loss of *Nrp*2 expression in the triple mutant did not rescue defects in differentiation of striatal neurons or GABAergic interneurons in the underdeveloped LGE or striatum of the double mutant. However, removal of *Nrp*2 expression in the triple mutant resulted in the observation that the ectopic accumulations in the LGE/striatum became partially dispersed (**Fig. 29A** e-l). *Nrp*1 expression was not under transcriptional control of *Dlx* genes (**Fig. 18B**), hence its pattern of expression remained unchanged in all mutants examined (**Fig. 29A** a-d). Of note, *Sema*3A expression in the LGE/striatum was diminished in both double and triple mutant, indicating that Sema3A expression might be downstream of *Dlx* gene regulation. More importantly, lack of Sema3A expression excluded its role as an active chemorepellent to accumulating interneurons in the basal ganglia of the double mutant. The expression of Sema3F is currently being

investigated as an active repellent in the double mutant basal ganglia. Our results further support the important role of *Dlx*1 and *Dlx*2 genes in specification of the basal ganglia, specifically the striatum. However, removal of *Nrp*2 expression in *Dlx*1/2 double mutant showed no significant changes in neuronal makeup or deficits that were previously caused by lack of *Dlx*1/2 gene function.

8.10 Analysis of *Dlx*1/2; *Nrp*2 triple-mutant mice: Partial restoration of tangential migration of GABAergic interneuron from the basal ganglia to the neocortex

A number of recent studies suggest that most telencephalic inhibitory interneurons are derived from progenitors in the subcortical telencephalon. There are at least two principal subcortical telencephalic sources of these tangentially migrating interneurons. One is in a region that includes the LGE and the septum. This region appears to produce interneurons that migrate rostromediodorsally to populate the olfactory bulb and perhaps the cerebral cortex (Anderson et al., 1999; de Carlos et al., 1996; Luskin and Boone, 1994). The other is from the MGE that produces interneurons that contribute to the striatum and cerebral cortex through a laterodorsal migration (Anderson et al., 2001; Lavdas et al., 1999; Wichterle et al., 2001). Thus, in the *Dlx*1/2 double mutant, there is a massive reduction in the GABAergic interneurons of the cerebral cortex (hippocampal complex, isocortex, olfactory cortex and olfactory bulb (Anderson et al., 1997a; Bulfone et al., 1998; Pleasure et al., 2000). This is due to the lack of tangentially migrating immature interneurons from the subcortical telencephalon into the cerebral cortex (**Fig. 23C**). Neuropilin/semaphorin signaling is implicated in sorting migrating subcortical telencephalic interneurons to distinct target tissues (Marin and Rubenstein, 2001). In the subpallial telencephalon, neuropilins 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 chemorepellent activity in the striatal mantle, of which the Class 3 Semaphorins, Sema3a and Sema3f, are principal components. *Dlx*1/2 double mutants have ectopic accumulations of interneurons in the abnormal LGE that express high levels of *Nrp*2 due to lack of *Dlx* transcriptional repression (**Fig. 23C**; **Chapter 6**). Hence, the accumulation of these neurons and the lack of tangential migration can possibly be explained by Semaphorin chemorepulsion of these *Nrp*2-expressing cells in the basal telencephalon. Loss of *Nrp*2 expression in these cells in the triple mutant likely removes the Semaphorin-mediated chemorepulsion, and allows these cells to take part in any possible migrations from this neuroanatomic region.

*Dlx*1/2 double mutants have abnormal migrations from the LGE resulting in an accumulation of partially differentiated neurons in the LGE, hypoplasia of the striatum, loss of normal olfactory bulb interneurons, and greatly reduced neocortical GABA- and calbindin- expressing cells (Anderson et al., 1997a; Anderson et al., 1997b). In our study, we found that there was up to 75-85% reduction of GABA expression in the neocortex of the double mutant compared to wild-type (**Fig. 30** c; histogram). Interestingly, there was 2-fold increase in GABA expression in the neocortex of the triple mutants compared to the double mutants (**Fig. 30** d; histogram). This was further supported by a similar 2-fold increase in both calbindin and GAD67 expression in the neocortex of the triple mutants compared to the double mutants (**Fig. 31** d; **Fig. 32** d; respective histograms). Of note, partial restoration of GABAergic cells in the neocortex was coincident with a more dispersed pattern of ectopic accumulation in the LGE of the triple mutants compared to double mutants (arrows of **Fig. 30** g, h; **Fig. 31** g, h; **Fig. 32** c, d). The results supported

our hypothesis that further removal of *Nrp*2 expression helped to remove the Semaphorin mediated chemorepulsion from the striatum and neocortex and to allow ectopic accumulation of neurons in the LGE to disperse and possibly migrate.

On the basis of their neurotransmitter content, several interneuron subtypes can be distinguished in the cortex, olfactory bulb and striatum. The most common type of telencephalic interneuron contains GABA as its main neurotransmitter (Martin and Rimvall, 1993). To further delineate the neuronal subtypes of the restored cells observed in the neocortex of the triple mutant, we looked at specific markers of GABAergic interneurons. GABAergic interneurons can be further subdivided on the basis of their content of calcium-binding proteins (calretinin and parvalbumin) and neuropeptides (neuropeptide Y, somatostatin) (DeFelipe, 1997; Kawaguchi et al., 1995; McBain and Fisahn, 2001). Calretinin-containing interneurons primarily originate in the dorsal CGE which strongly expresses different transcription factors: *Gsh*2 and *ER*81 (Corbin et al., 2003; Stenman et al., 2003a), and *Nkx*6.2 (Fogarty et al., 2007). We found that Calretinin expression remained unchanged in all 4 genotypes at E18.5 (data not shown), implicating that *Dlx* genes unlikely take part in transcriptional regulation of Calretinin expression, and Neuropilin2 may not participate in tangential migration from dorsal CGE to cortex.

Neuropeptide Y (NPY)-containing interneurons, as a GABAergic subtype, in part, are affected by *Dlx* gene expression (Anderson et al., 1999; Anderson et al., 2001; Lavdas et al., 1999). NPY expression, overlapping ~50% with somatostatin-containing interneurons in the cortex, are derived from the MGE (Xu et al., 2004), LGE (Butt et al., 2005, Fogarty et al., 2007), and the dorsal CGE and ventral CGE which expresses *Gsh*2/*ER*81 (Corbin et al., 2003; Stenman et al., 2003a) and *Nkx*2.1 (Sussel et al., 1999),

respectively. Hence we found that NPY-containing interneurons only decrease by ~30% in the neocortex of the double mutant (**Fig. 33** c; histogram) compared to wild-type. This NPY reduction is much less severe compared to GABA or calbindin reduction in double mutant neocortex. Several possible explanations for this finding are: (1) *Dlx* genes are important for terminal differentiation of interneurons, i.e. activation of GABA production, but not necessary for partial differentiation of interneurons, i.e. NPY production; (2) other transcription factors regulate NPY production e.g. *Nkx*2.1, *Nkx*6.2, *Lhx*6 (Anderson et al., 2001; Fogarty et al., 2007; Du et al., 2008); (3) there are other more significant sources of NPY-positive cells from LGE, CGE, and septum (Wonders & Anderson, 2005); (4) only a small proportion of total NPY-expressing interneurons is *Dlx*-dependent. Of note, there was a small increase in NPY-containing cells in the neocortex of the triple mutant (**Fig. 33** d; histogram) compared to the double mutant, and this increase still was significantly less than NPY levels in the wild-type. However, one should consider the temporal contribution of the LGE to tangential migration which begins at later stages (~E15.5) as cells follow a deep route to the neocortex (Marin & Rubenstein, 2001). When considering the absolute number of "restored" NPY-expressing cells, there were over $\sim 10^2$ more cells in the neocortex of the triple mutant compared to double mutant. This overall number of NPY+ cells is greater than that of Somatostatin (**Fig 34**; histogram). Unfortunately, the triple mutant also died at birth, som we are unable to assess whether cortical NPY migration persists postnatally. Importantly, along with the small restoration of NPY-containing cells in the neocortex of the triple mutant, there was also a similar reduction of their ectopic accumulation in the GEs (arrow, **Fig. 33** h).

Parvalbumin (PV) and Somatostatin (SST) containing interneurons are derived almost entirely from the MGE, although these are two non-overlapping neurochemical subgroups with distinct physiological characteristics and connectivities (Gonchar and Burkhalter, 1997; Kawaguchi and Kubota, 1997). Furthermore, a strong bias exists for SST+ interneurons being generated by progenitors in the dorsal MGE and PV+ interneurons from the ventral MGE, with high levels of molecular heterogeneity for each subgroup (Wonders et al., 2008). In our study, we also found differences between SST+ and PV+ subgroup expression when examining the four genotypes. PV expression appeared unchanged in all genotypes studied (data not shown), indicating PV might not be under regulation of *Dlx* genes. Unchanged PV expression can also be explained by other sources of transcriptional control by *Nkx*2.1 (Xu et al., 2006), *Vax*1 (Taglialatela et al., 2004), and/or *Lhx*6 (Liodis et al., 2007). Similar results were obtained in the postnatal *Dlx*1 single mutant where parvalbumin+ subpopulations were unaffected (Cobos et al., 2005). On the other hand, SST containing cells were decreased by ~35% in the neocortex of the double mutant, and restored completely in the neocortex of the triple mutant when compared to wild-type (**Fig. 34**). This observation signified the role of *Dlx* genes in migration of SST+ cells from the basal ganglia to the neocortex and implied that there was a subpopulation of SST+ cells that was highly dependent on Semaphorin-Neuropilin interactions for tangential migration. We coincidentally observed a more dispersed pattern of ectopic accumulation of SST-containing cells in the LGE/striatum of the triple mutant compared to the double mutant (arrows of **Fig. 34** g, h). Once *Nrp*2 expression is removed from the double mutant, SST-containing cells might be able to tangentially migrate past the zone of Semaphorin chemorepulsion toward the neocortex, and perhaps

subsequently be facilitated by other potential migratory mechanisms such as netrin/DCC or Slit/Robo signalling pathways (Serafini et al., 1996; Skaliora et al., 1998; Yuan et al., 1999). The complete restoration of SST-expressing cortical interneurons was further confirmed by Western analysis (data not shown) and Real Time-PCR (**Fig. 35**).

We also found that GABA is co-expressed in most of SST-containing interneurons of the neocortex of the wild-type and single mutant, but not in the double mutant (insets of **Fig. 36A-D**), probably due to loss of transcriptional activation of *Gad* isoforms by *Dlx* genes (**Chapter 5**). Most GABA and SST expressing cells remained in the basal ganglia as ectopias due to defective migration in the double mutant. In the neocortex of the triple mutant, some of the "restored" SST-containing cells co-expressed GABA as their neurotransmitter (inset of **Fig. 36D**). However, not all of these "restored" SST-containing cells expressed GABA due to lack of *Dlx* function. In the basal ganglia of the triple mutant, the ectopias are much less severe, and both GABA+ and SST+ cells invaded the striatum and pallial/subpallial boundary (**Fig. 36D** d,e,f).

Since the NRP2 antibody can recognize the truncated NRP2 mutant protein, we were able to examine the expression pattern of (mutant) NRP2 in both single and triple mutants. In contrast to GABA expression, NRP2 co-expression was rarely found in SST-containing interneurons that were tangentially migrating in the neocortex of the wild-type and single mutant, possibly due to transcriptional repression of *Dlx* genes in these cells (insets of **Fig. 37A, B**). However, loss of *Dlx* function permitted NRP2 to be co-expressed with SST in the same cells in both neocortex and within ectopic accumulations in the basal ganglia of the double mutant (inset of **Fig. 37C**). Most of the SST+ cells accumulated as ectopias in the basal ganglia, and they expressed normal NRP2 protein

around the striatum. This result further supported the hypothesis that the defect in migration might be due to Semaphorin-Neuropilin interaction in these cells. As well, due to loss of *Dlx*1/2 function, NRP2 was co-expressed with SST in the same cells in both the "restored" cortical SST+ cells and in the basal ganglia of the triple mutant. However, in this circumstance, Neuropilin2 function is eliminated, the ectopias are much less severe, and SST+ cells invaded the striatum and pallial/subpallial boundary (**Fig. 37D** d,e,f).

Providing further support for partial restoration of tangential migration from the subcortical telencephalon in the triple mutant, we used embryonic slice cultures to demonstrate migration of cells from the LGE to the neocortex at E16.5 (**Fig. 38**) and at E18.5 (**Appendix Fig. 10**). Both wild-type and *Nrp*2 single mutant slice cultures showed normal axonal projections from the LGE to the neocortex, consistent with normal tangential migration at both E16.5 and E18.5 (**Fig. 38** a, b; **Appendix Fig. 10** a,b). *Dlx*1/2 double mutant slice cultures only showed ectopic accumulations of interneurons in the basal ganglia at both embryonic stages (**Fig. 38** c; **Appendix Fig. 10** c). Interestingly, *Dlx*1/2/*Nrp*2 triple mutant slice cultures showed an increase of axonal projections from the basal ganglia toward the neocortex, consistent with partial restoration of tangential migration (**Fig. 38** d; **Appendix Fig. 10** d). However, several studies have indicated that any LGE contribution to the population of cortical interneurons is far smaller than that of the MGE (Wichterle et al., 1999; Wichterle et al., 2001; Anderson et al., 2001). Future studies should aim to assess diI labelling of MGE to evaluate restoration of migration in all four genotypes. In summary, tangential migration of GABAergic interneurons from the basal forebrain to the neocortex, specifically of the SST-containing subtype, can be restored in the *Dlx*1/2 double mutant by removal of Semaphorin-Neuropilin interactions.

Conclusions and Significance

Autism spectrum disorders are significant health problems in the pediatric population, and the incidence of autism is increasing. We are encouraged by the potential implications of our work on *Dlx* genes in forebrain development for the field of neurodevelopmental disorders.

This thesis is concerned with the genetic regulation and determination of cell fate by *Dlx* genes in forebrain development. It is important to note that the search for homeobox targets in vertebrates has not been as productive as in *Drosophila*. Although candidates have been identified *in vitro*, the significance of many of these interactions *in vivo* remains to be confirmed. A major advance towards the identification of direct target genes of specific transcription factors was the development of chromatin immunoprecipitation (ChIP), followed by *in vitro* and *in vivo* target characterizations. Utilizing these methods, homeodomain targets have been isolated, including transcription factors, growth factors, adhesion molecules, and secreted proteins. We have successfully optimized ChIP in embryonic forebrain to establish that DLX1 and DLX2 bind to the *Dlx5/6* intergenic enhancer *in vivo* and directly regulate its expression *in vitro*. We have also determined that the promoters of both *Gad* isoforms are direct *Dlx*1 and *Dlx*2 transcriptional targets. We have also established that Neuropilin-2 expression is under direct transcriptional control of *Dlx*1 and *Dlx*2 genes. The utilization of biochemical approaches such as ChIP provides identification of target genes that are directly downstream and derived from physiologically relevant homeodomain-DNA complexes obtained *in vivo*, and may be applied to diverse species.

Furthermore, we have not only identified the *Dlx*5/6 intergenic enhancer (I56ie), the *Gad* isoforms *Gad*1 and *Gad*2 necessary for GABA synthesis, and the Semaphorin receptor Neuropilin-2, as direct transcriptional targets of DLX1 and/or DLX2, but also how their expression is controlled by *Dlx* genes. These gene targets are either transcriptionally activated (I56ie, *Gad*1/*Gad*2) or repressed (*Nrp*2) and their identification contributes to the molecular mechanisms underlying *Dlx* gene family cross-regulatory interactions, GABA interneuron differentiation and tangential migration, respectively. By turning "on" or "off" specific transcriptional targets in a precise temporal and spatial pattern, *Dlx* homeobox genes, together with other transcriptional factors, regulate GABAergic cell-fate specification in the basal ganglia, as well as cellular migration from the basal ganglia to the neocortex by unlocking important molecular interactions of Semaphorin ligands with their corresponding Neuropilin receptors.

Taken together, our published and preliminary data support a critical role for *Dlx* genes in CNS progenitor cell fate specification, differentiation and migration of GABAergic interneurons in the developing forebrain. Furthermore, identification of DLX-mediated activation and repression of downstream target genes that are key effectors of interneuronal development may provide additional insights into regulation of GABAergic interneuron subtype differentiation and migration to the neocortex. This thesis will advance our understanding of the regulation of forebrain development by *Dlx* genes and may lead to potential targets for treating disorders such as Autistic Spectrum Disorders by restoring the balance of excitation to inhibition that contributes to the pathogenesis of autism, especially in those patients with a concomitant seizure disorder.

Future Directions

While these studies have enriched our understanding of the role of *Dlx*1 and *Dlx*2 in the forebrain development, important questions remain. There is postnatal *Dlx* expression in the cortical SVZ (Stuhmer et al., 2002b; Anderson et al., 2001). However, it is unclear whether this expression arises from cortical progenitors, or whether these *Dlx*-positive cells are introduced into the cortex by tangential migration from the basal telencephalon. Through this latter mechanism, progenitor cells that are specified in the subpallial telencephalon might continue to proliferate after they reach the progenitor zone of the cortex, providing a secondary source of GABAergic interneurons. Future development of a conditional knockout model using *Cre-lox* technology can resolve the functional significance of *Dlx* genes after birth with respect to postnatal forebrain development and other Cre-lines can be used to knockout *Dlx* gene function in the retina and other tissues. This model will advance our understanding of the regulation of cortical interneuron development by *Dlx* genes, and potentially lead to a mouse model for autism, autism spectrum disorders, and/or congenital epilepsies.

Chromatin immunoprecipitation has become a principal tool for understanding transcriptional cascades and deciphering the information encoded in chromatin. Owing to the recent remarkable progress in high-throughput sequencing platforms, ChIP–seq is poised to become the dominant profiling approach for generation of libraries of *Dlx* downstream targets. Thus, it will be important to use this technology in the identification of novel genes or new transcriptional pathways affecting neuronal differentiation and migration in the developing forebrain. Understanding the cellular and molecular

mechanisms that govern neuronal differentiation and migration will reshape our knowledge of forebrain development in normal and pathological situations.

It is also unclear whether the partial restoration of GABA expression and the complete restoration of Somatostatin expression in the neocortex of the triple mutant have any physiological importance for the inhibitory signalling circuitry of the cortex. Further assessment of our genetic models using neurophysiologic techniques, such as measuring inhibitory currents using microelectrode recording from neocortical slice cultures, may help to determine if there is a physiologic increase in inhibition in the neocortex corresponding to the increase in Somatostatin and GABA expression in the *Dlx*1/*Dlx*2/*Neuropilin*-2 triple mutant mice.

Also of interest is the characterization of the consensus binding sequence of *Dlx* genes. A consensus DNA-binding site for the *Xenopus Dlx*3 ortholog, *Xdll*2 used a binding site selection procedure from a random oligonucleotide pool (Feledy et al., 1999). The consensus site identified as (A/C/G)TAATT(G/A)(C/G) was reported in zebrafish (Zerucha et al., 2000). Since the homeodomains of the *Dlx* family are very homologous, it is likely that DLX proteins will recognize a similar sequence. However, there still are major variations in the flanking sequences of the TAATT core motif above. The difference in binding capabilities between members of *Dlx* family may also be due to different transcriptional co-factors of DLX proteins as well as post-translational modifications, such as phosphorylation, which have yet to be identified. Development of *Dlx* libraries of downstream targets will facilitate the future comparision of downstream sequences and identification of the consensus binding sequence.

Appendix A

Supplementary results

A

DLX1	GABA	Merge
a	d	g

DLX1	GABA	Merge
b	e	h

DLX1	GABA	Merge
c	f	i

E16.5

NCx

a,d,g

H

LGE

Str

MGE

Thal

B,e,h

C,f,i

AEP

B

DLX2	GABA	Merge
a	d	g

DLX2	GABA	Merge
b	e	h

DLX2	GABA	Merge
c	f	i

E16.5

NCx

H

LGE

a,d,g

Str

MGE

Thal

B,e,h

AEP

C,f,i
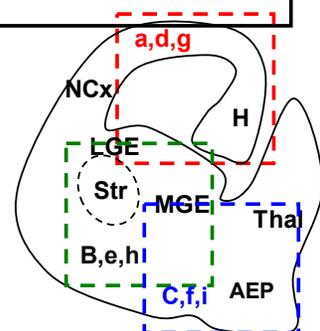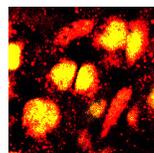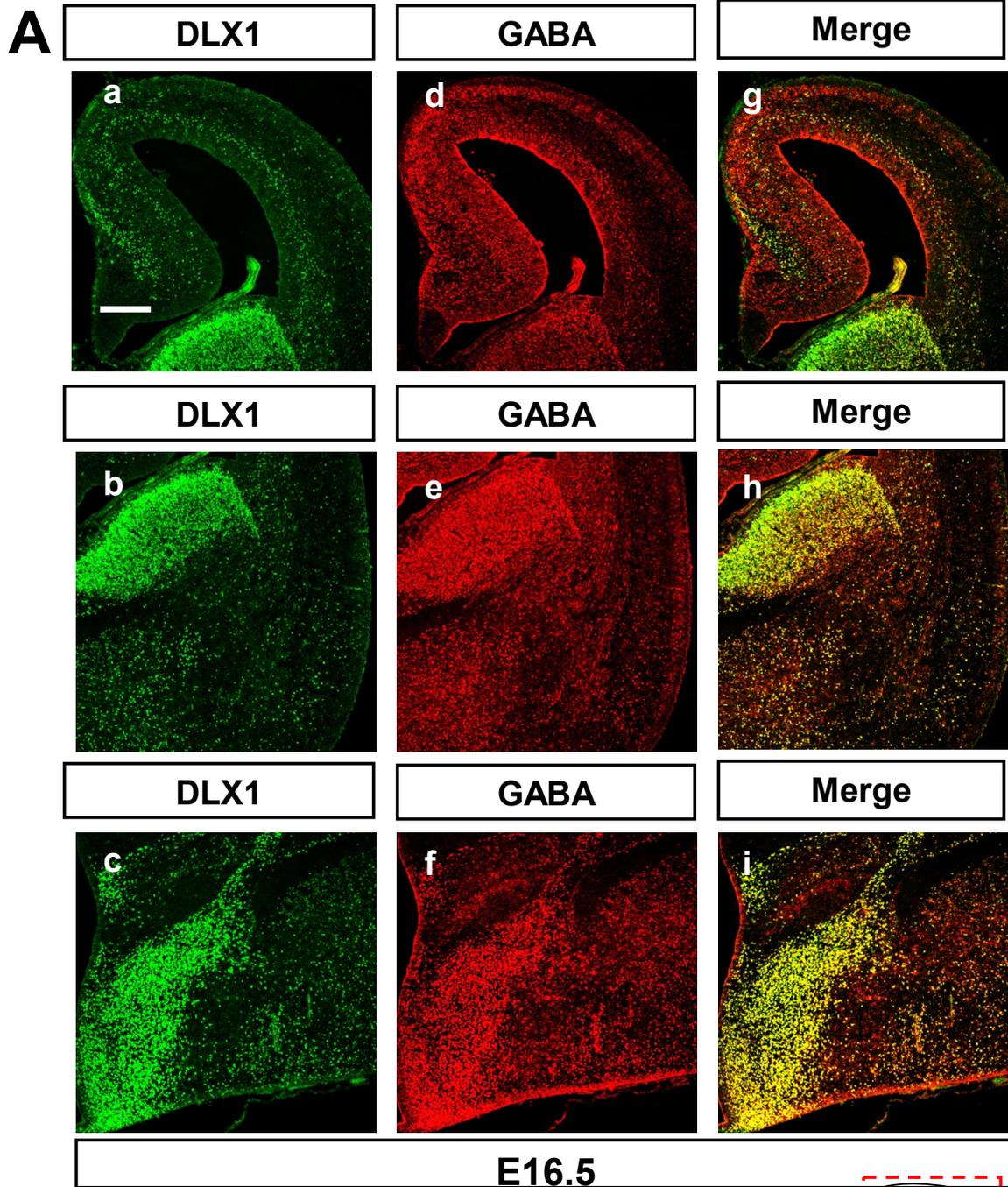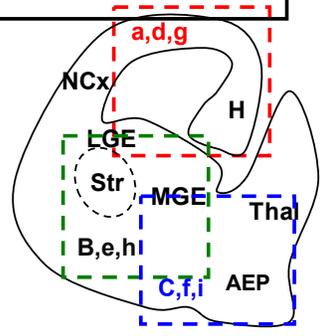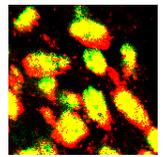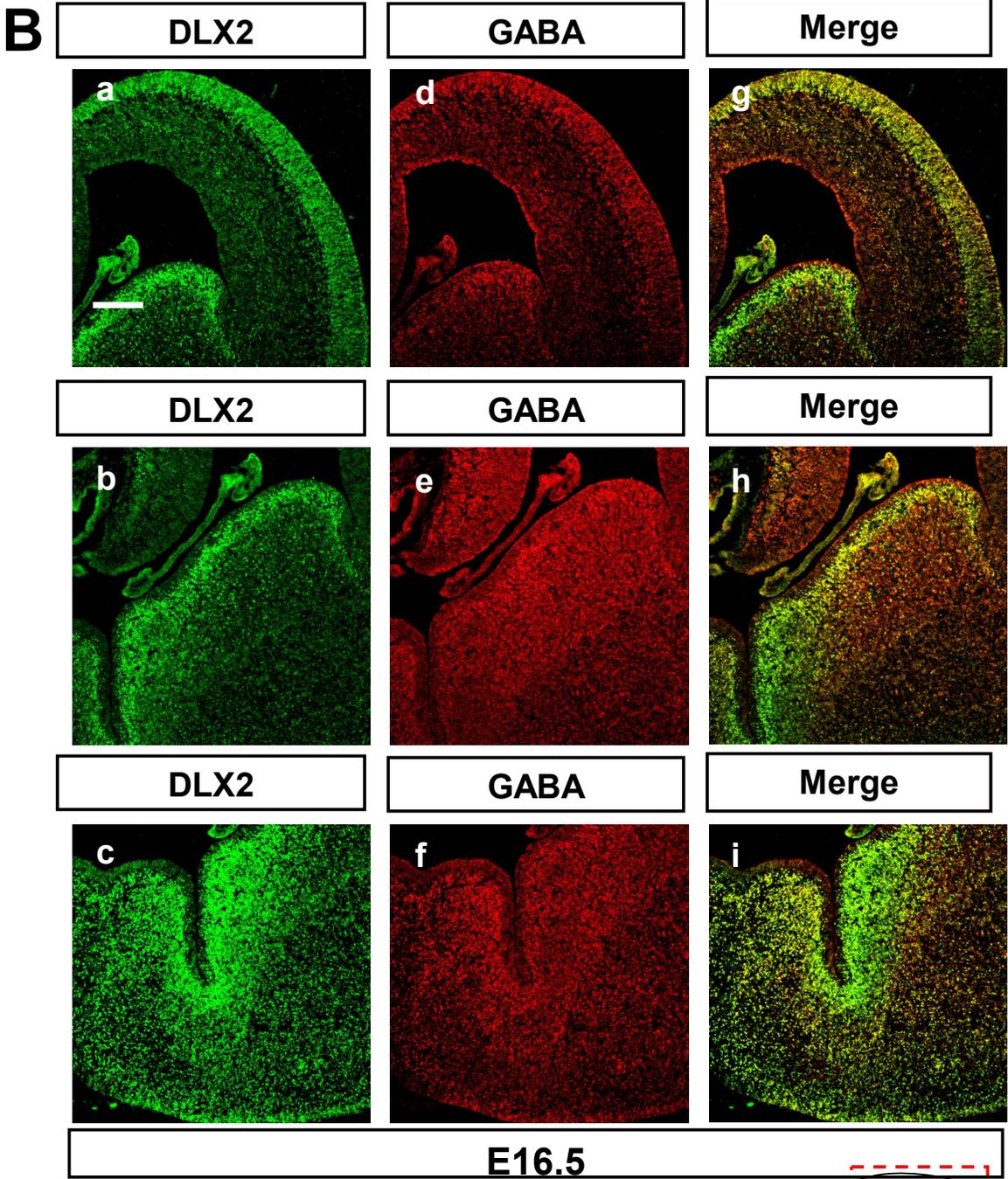
Appendix Figure 1: Co-expression of DLX1 (A) and DLX2 (B) homeodomain proteins and GABA-positive interneurons in the wild-type forebrain at E16.5. Sections were double-labelled with specific antibodies against DLX proteins (a, b, c) or GABA (d, e, f) of E16.5 basal forebrain. In the left column, DLX1- or DLX2-positive cells (green) are found in the ventricular zone (VZ) and subventricular zone (SVZ) of the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE) and anterior entopeduncular area (AEP). The center column shows GABA-positive cells (in red) in LGE and AEP, predominantly, from the same sections. The right column shows the overlay of the two images with GABA co-expressed with DLX proteins in most basal interneurons in the SVZ. Coronal sections. Scale bar, 400 μm. [H, Hippocampus; NCx, neocortex; Str, striatum; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Thal, thalamus; AEP: anterior entopeduncular area]. Inserts in the Merge column represent a 10x enlargement.

A

DLX1	GAD65	Merge
a	d	g

DLX1	GAD65	Merge
b	e	h

DLX1	GAD65	Merge
c	f	i

E16.5

NCx

a,d,g

H

LGE

Str

MGE

Tha

B,e,h

c,f,i

AEP

B

DLX2	GAD65	Merge
a	d	g
DLX2	GAD65	Merge
b	e	h
DLX2	GAD65	Merge
c	f	i
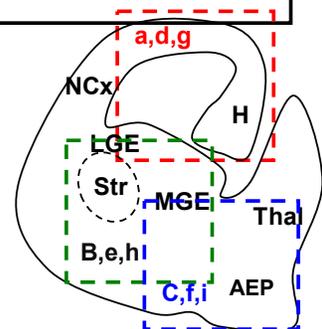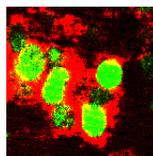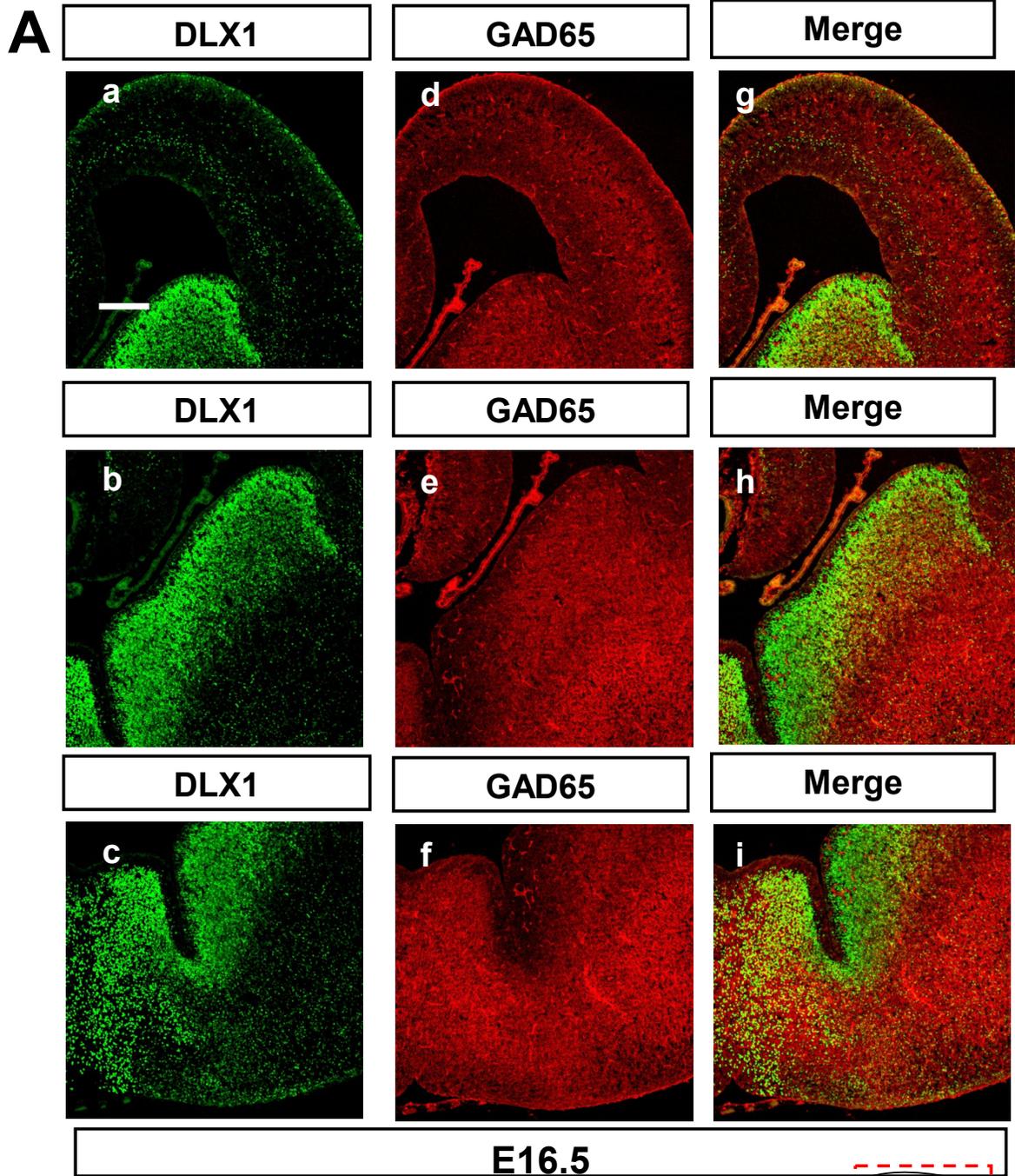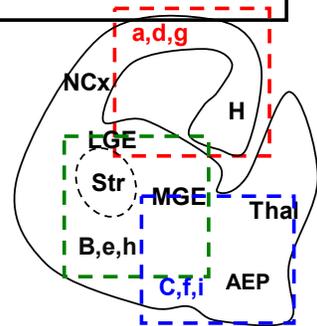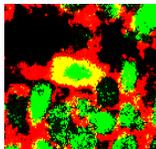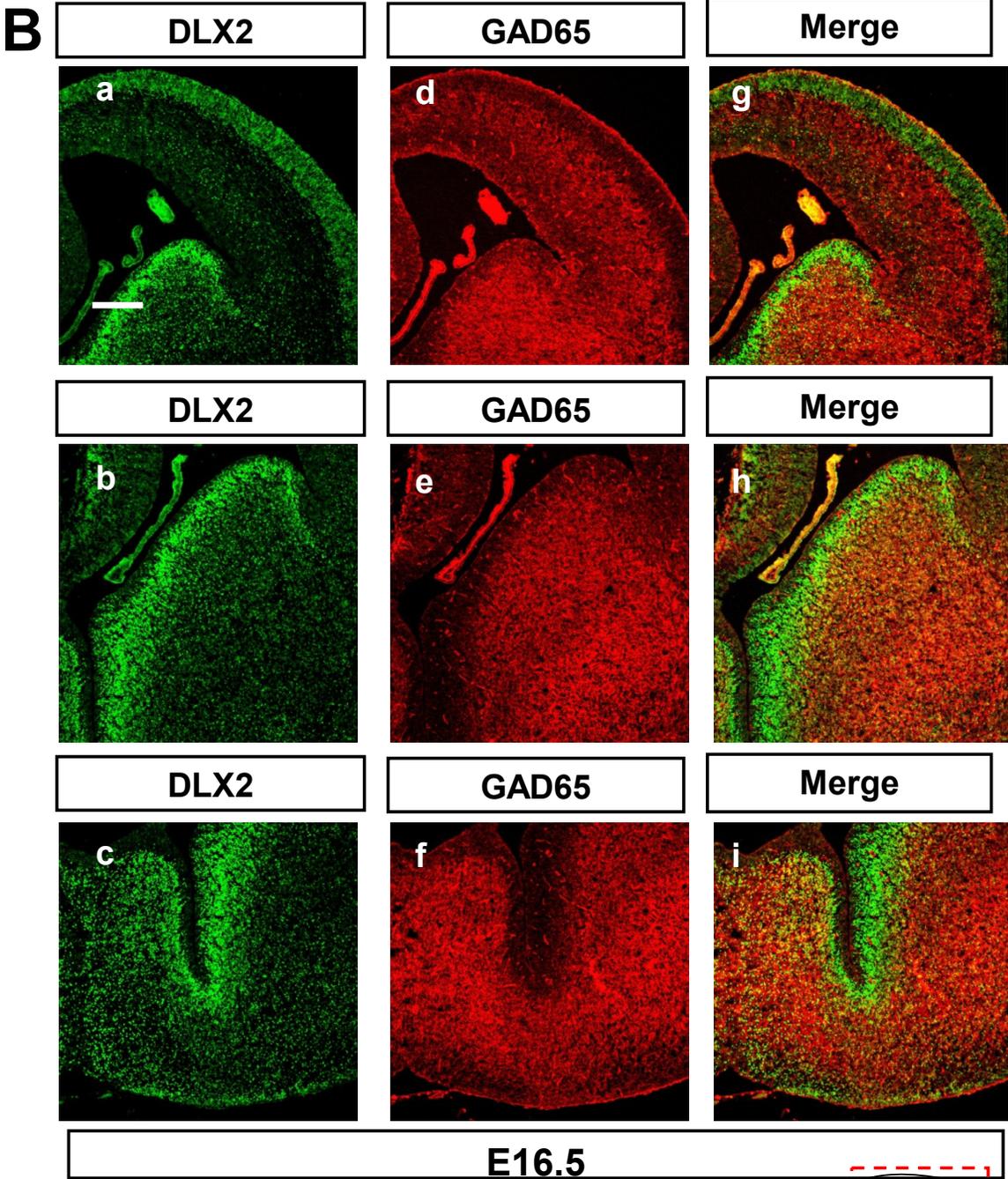
E16.5

Appendix Figure 2: Co-expression of DLX1 (A) and DLX2 (B) homeodomain proteins and GAD65-positive interneurons in the wild-type forebrain at E16.5. Sections were double-labelled with specific antibodies against DLX proteins (a, b, c), or GAD65 (d, e, f) of E16.5 basal forebrain. In the left column, DLX1- or DLX2-positive cells (green) are found in the ventricular zone (VZ) and subventricular zone (SVZ) of the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE) and anterior entopeduncular area (AEP). The center column shows GAD65-positive cells (in red) in the LGE and AEP, predominantly, from the same sections. The right column shows the overlay of the two images with GAD65 co-expressed with DLX proteins in many basal interneurons of the SVZ. Coronal sections. Scale bar, 400 μm. [H, Hippocampus; NCx, neocortex; Str, striatum; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Thal, thalamus; AEP: anterior entopeduncular area]. Inserts in the Merge column represent a 10x enlargement.

A

DLX1	GAD67	Merge
a	d	g
DLX1	GAD67	Merge
b	e	h
DLX1	GAD67	Merge
c	f	i

E16.5

NCx
H
LGE
Str
MGE
Thal
AEP
a,d,g
B,e,h
C,f,i

B

DLX2	GAD67	Merge

a d g

DLX2	GAD67	Merge

b e h

| DLX2	GAD67	Merge

c f i
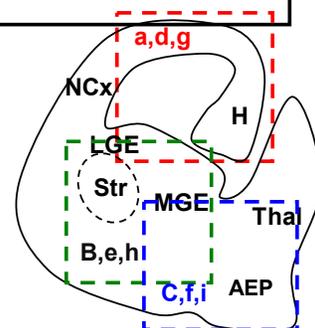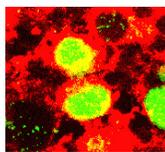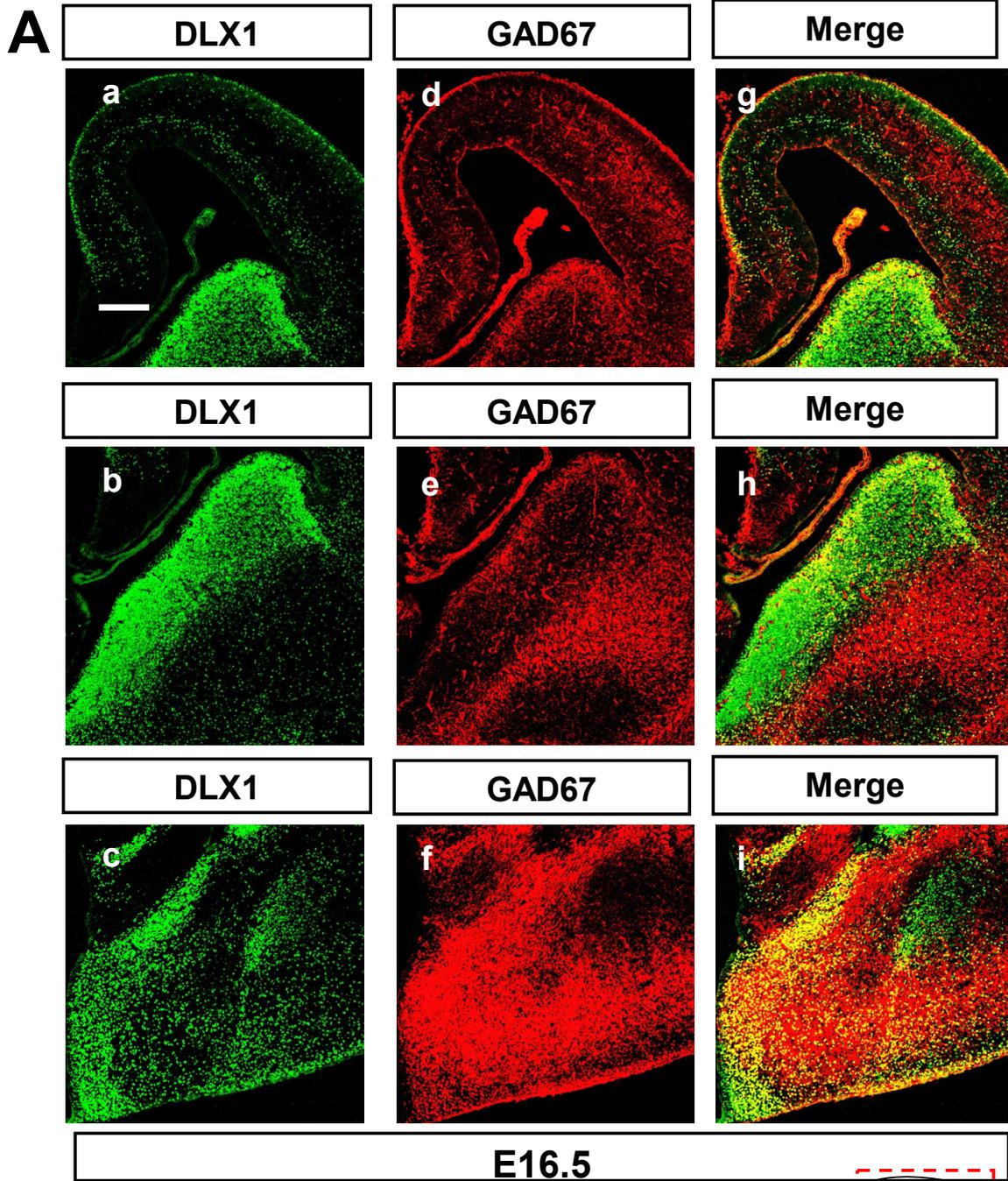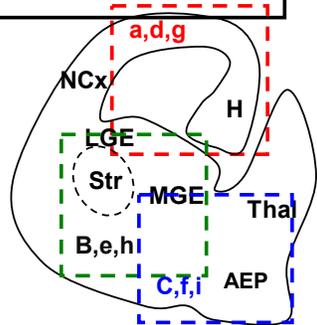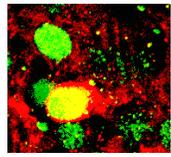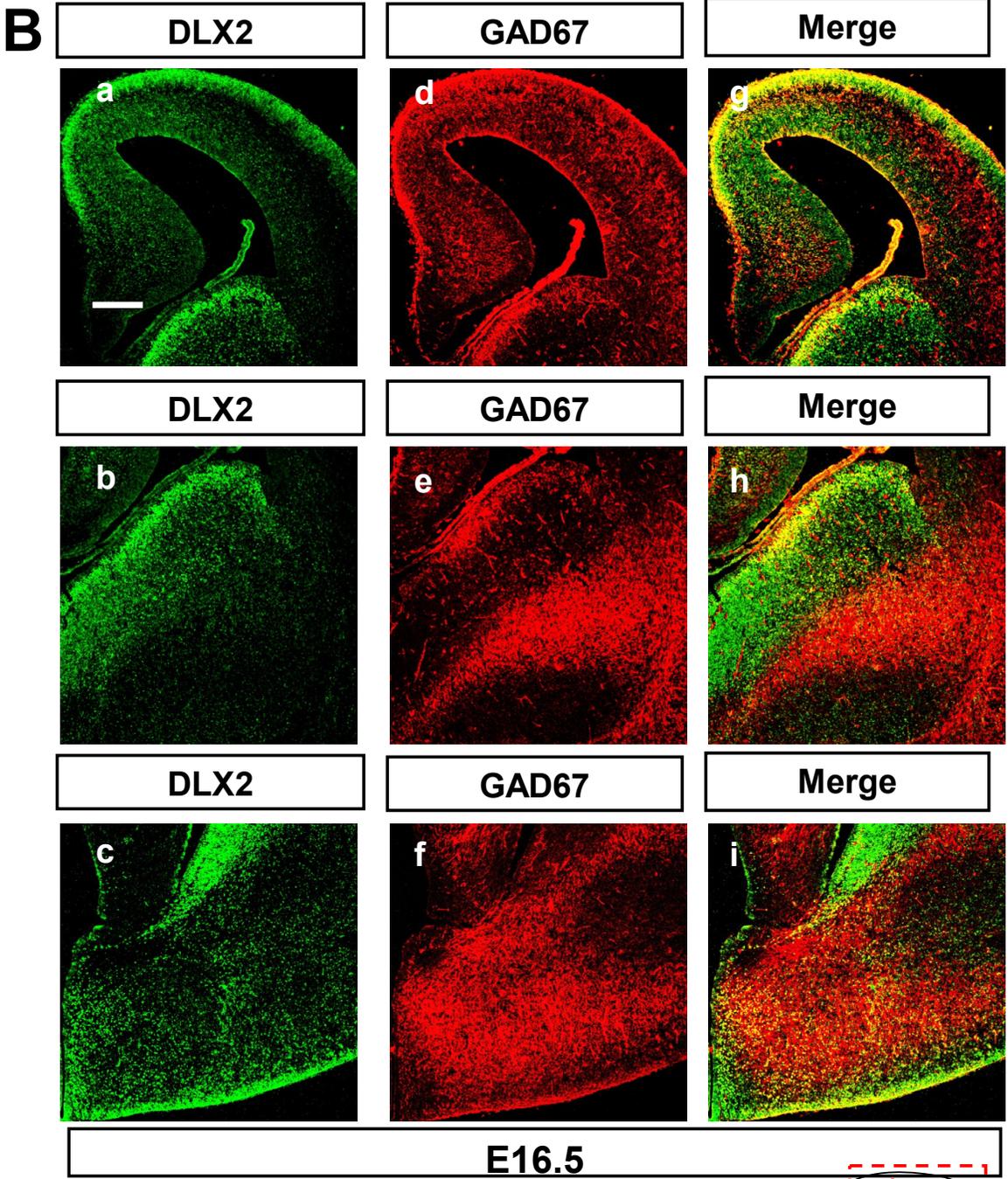
E16.5

Appendix Figure 3: Co-expression of DLX1 (A) and DLX2 (B) homeodomain proteins and GAD67-positive interneurons in the wild-type forebrain at E16.5. Sections were double-labelled with specific antibodies against DLX proteins (a, b, c) or GAD67 (d, e, f) in E16.5 basal forebrain. In the left column, DLX1- or DLX2-positive cells (green) are found in the ventricular zone (VZ) and subventricular zone (SVZ) of the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE) and anterior entopeduncular area (AEP). The center column shows GAD67-positive cells (in red) in LGE and AEP, predominantly, from the same sections. The right column shows the overlay of the two images with GAD67 co-expressed with DLX proteins in many basal interneurons in the SVZ. Coronal sections. Scale bar, 400 μm. [H, Hippocampus; NCx, neocortex; Str, striatum; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Thal, thalamus; AEP: anterior entopeduncular area]. Inserts in the Merge column represent a 10x enlargement.

A

Dlx1/2 +/-   Dlx1/2 -/-

GABA

E16.5

a   e

b   f

c   g

d   h

a,e
NCx   H
b,f
LGE
Str   MGE
c,g   Thal
d,h
AEP

B

	Dlx1/2 +/-	*Dlx1/2 -/-*

GAD65

E16.5

a

e

b

f

c

g

d

h

NCx

H

LGE

Str

MGE

Thal

AEP

a,e

b,f

c,g

d,h

C

	Dlx1/2 +/-	*Dlx1/2 -/-*

GAD67

a e

b f

c g

d h

E16.5

NCx
LGE
Str
MGE
H
Thal
AEP

a,e
b,f
c,g
d,h
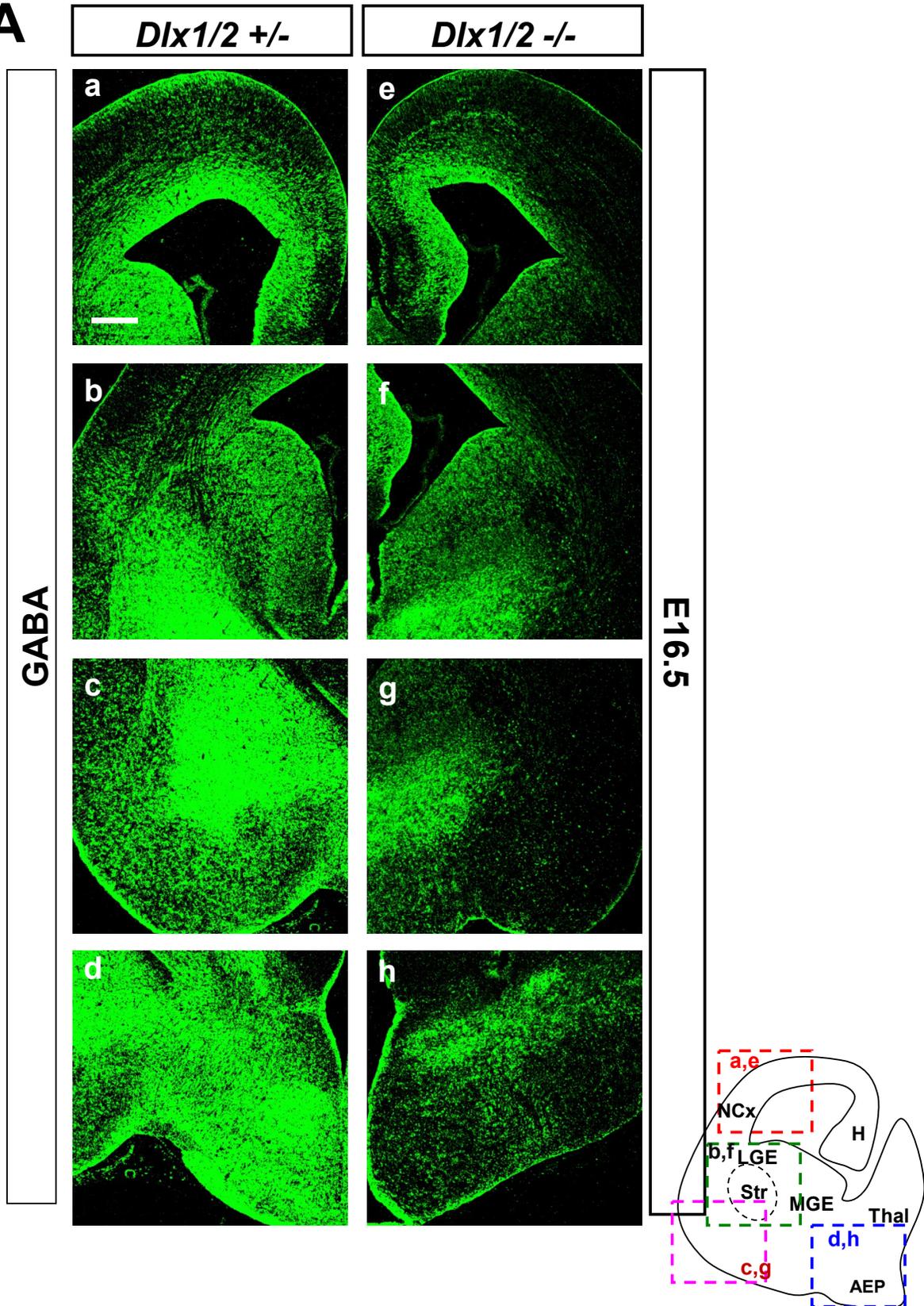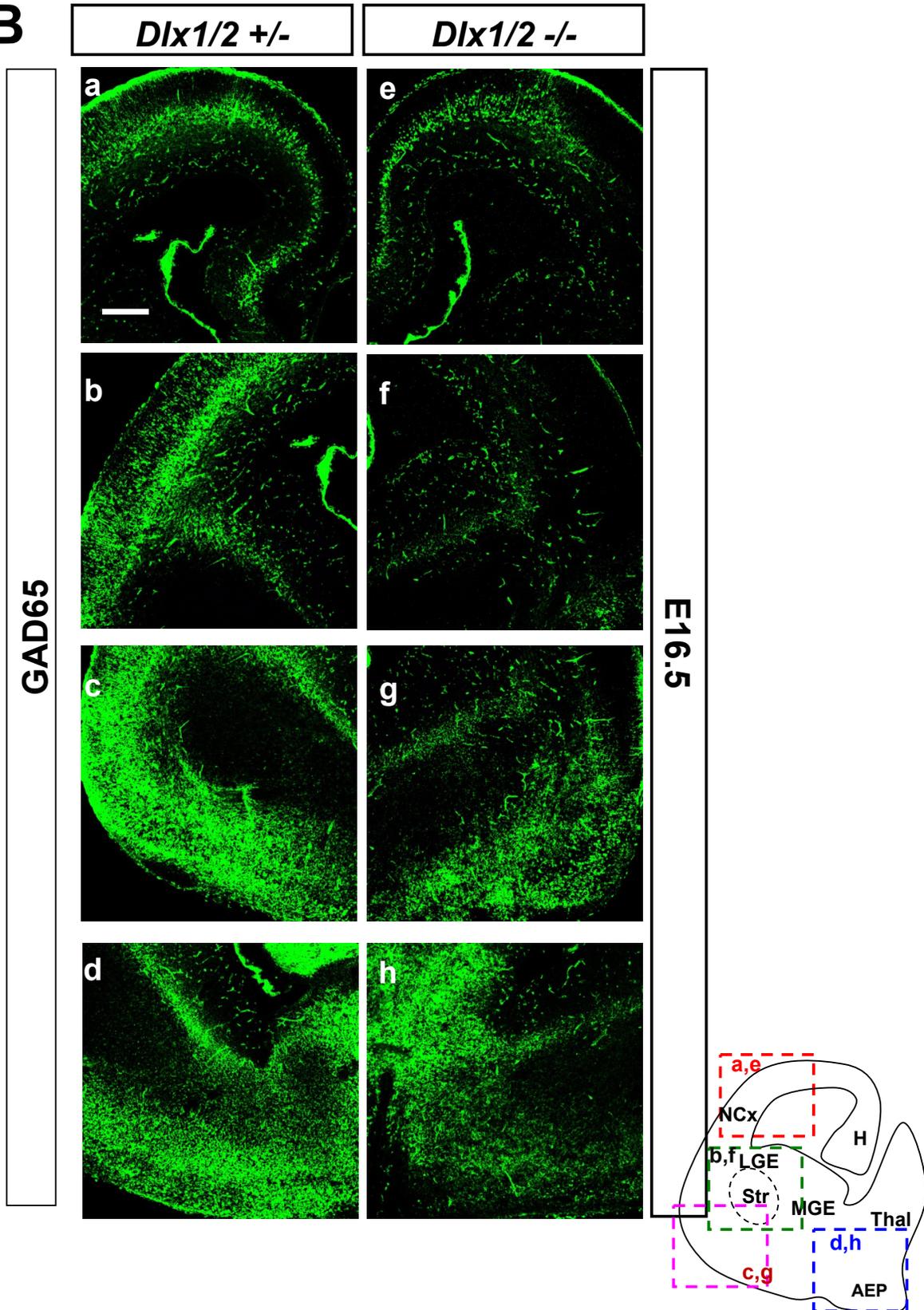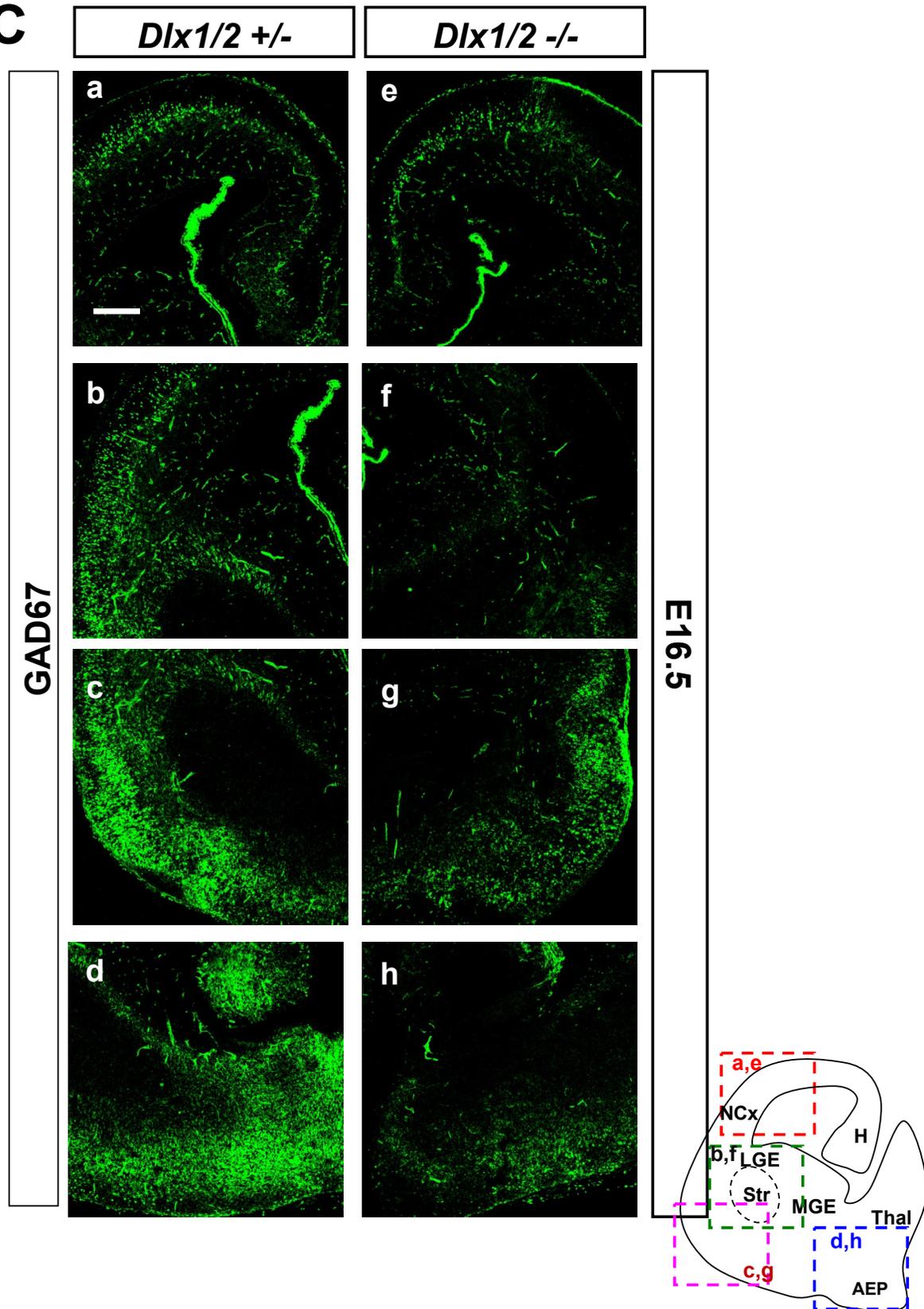
Appendix Figure 4: GABA (A), GAD65 (B), GAD67 (C) expression in E16.5 basal forebrain in wild-type and *Dlx*1/2 double knockout mice. Immunofluorescence experiments showed GABA (A), GAD65 (B), GAD67 (C) expression predominantly in the SVZ of the neocortex, and in the SVZ and MZ of the basal telencephalon (specifically the striatum for GABA), and in the AEP. In the absence of DLX1 and DLX2 function, expression of GABA (A), GAD65 (B), and GAD67 (C) is severely down-regulated in the neocortex, basal ganglia, and also the AEP. Coronal sections. Scale bars, 400 μm. [H, Hippocampus; NCx, neocortex; Str, striatum; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Thal, thalamus; AEP: anterior entopeduncular area].

Nrp-2ii

Mash1

Wnt7b

gDNA αDLX2 No-Ab gDNA αDLX2 No-Ab

Striatum   Hindbrain
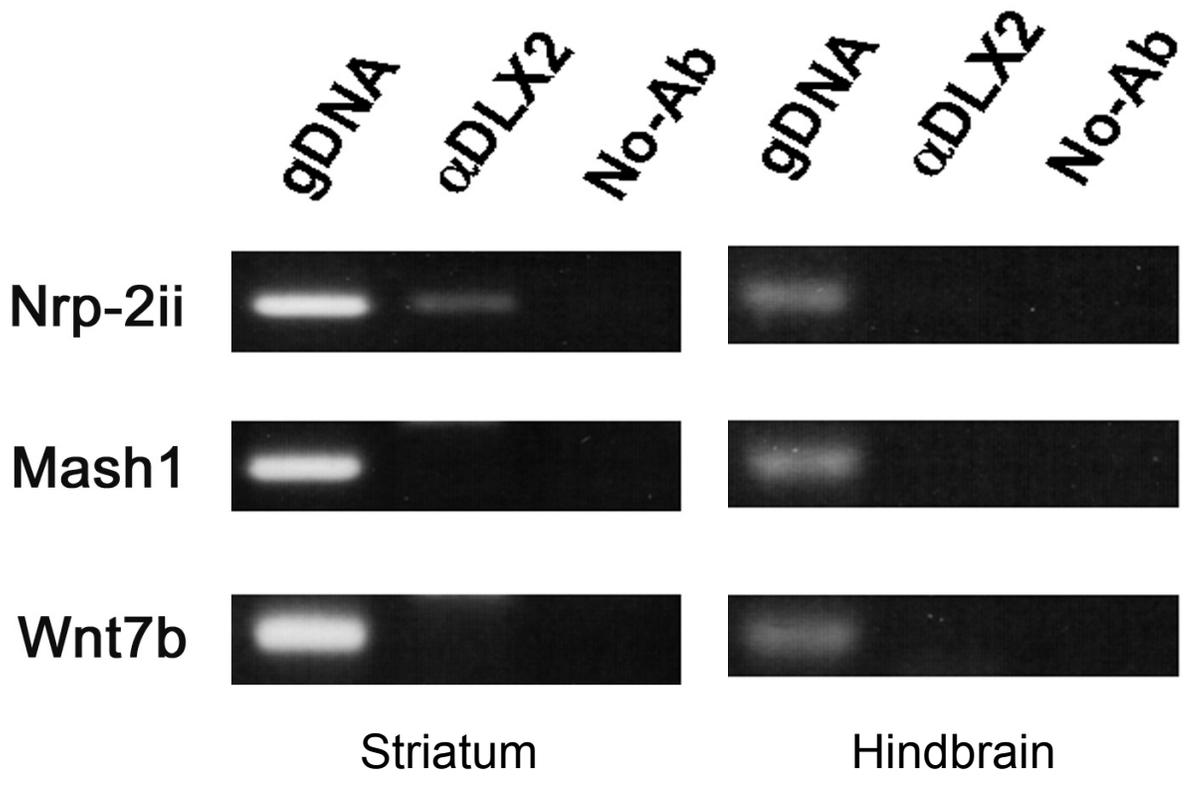
Appendix Figure 5. Neuropilin2 but neither *Mash*1 nor *Wnt*7b is a DLX homeoprotein target *in vivo*. Chromatin immunoprecipitation (ChIP) assays were performed on E13.5 mouse forebrain tissues using affinity-purified polyclonal DLX1 and DLX2 antibodies following cross-linking of protein-DNA complexes with 1% paraformaldehyde. Specific bands were evident for Neuropilin2 region ii (*Nrp*2ii), but not for regions of the *Mash*1 or *Wnt*7b promoters. Negative controls included performing ChIP without the addition of either primary antibody or the use of E13.5 hindbrain, tissue that does not express *Dlx* genes (right panels). Positive controls were mouse genomic DNA (gDNA). PCR bands were subcloned and confirmed by DNA sequencing.

A

E13.5

DLX1	*Nrp-2*	Combined

a

b

c

NCx

LGE

H

Str

MGE

PCx

POa

g

DLX2	*Nrp-2*	Combined

d

e

f

h

B

E16.5

DLX1	*Nrp-2*	Combined

a

b

c

NCx

H

LGE

MGE

Str

PCx

POa

g

DLX2	*Nrp-2*	Combined

d

e

f

h
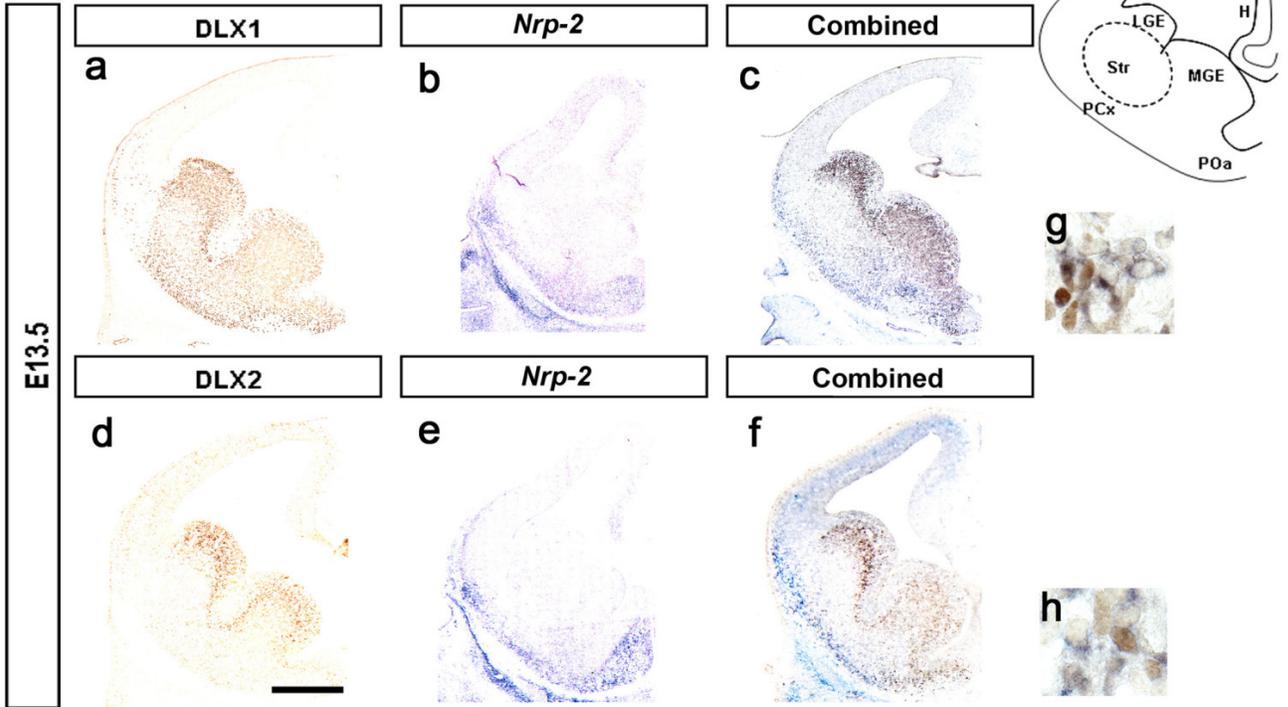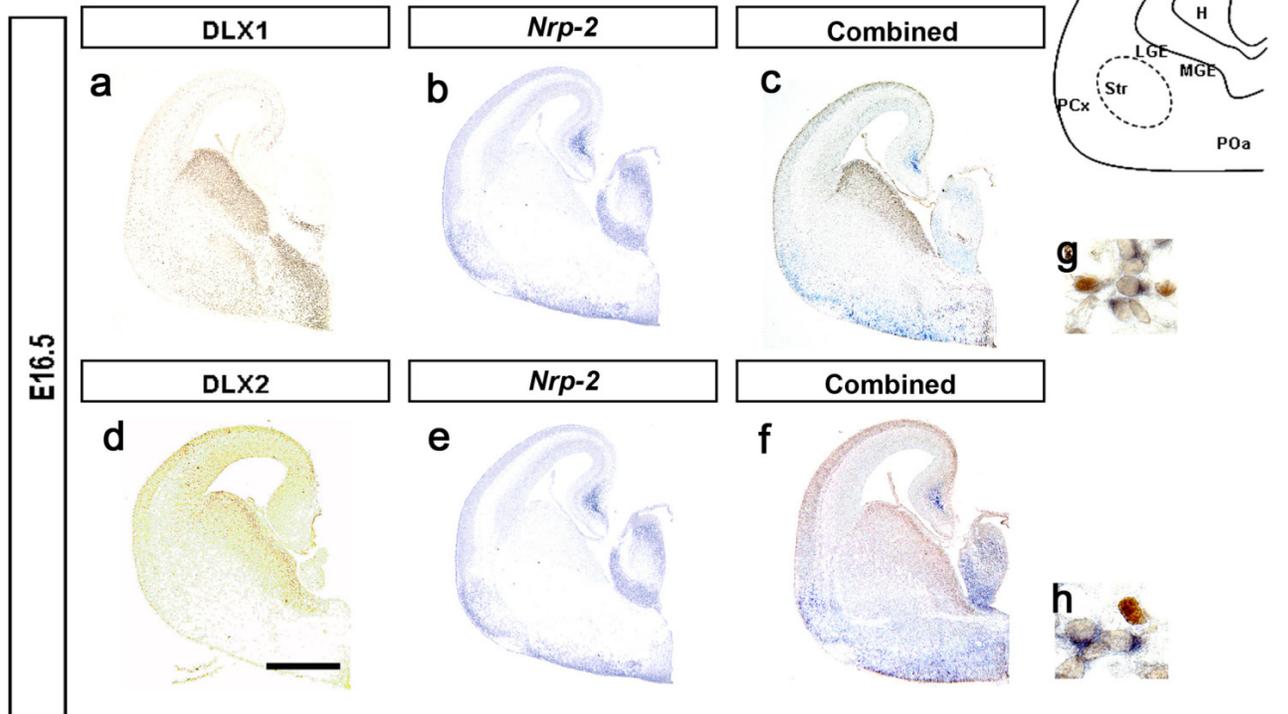
Appendix Figure 6. DLX1 or DLX2 and *Nrp*2 expression are complementary in the basal telencephalon of E13.5 (A) and E16.5 (B). Combined *in situ* hybridization for *Nrp*2 transcripts followed by immunohistochemistry for specific DLX proteins confirms the complementary expression patterns of DLX1 or DLX2 and *Nrp*2 in the basal ganglia at E13.5 (A *panels* c, f) and E18.5 (B *panels* c, f). Adjacent cryosections of E13.5 (A) and E16.5 (B) forebrain were either labelled with a specific riboprobe for *Nrp*2 (*panels* b,e), DLX1 antibody (*panels* a, d), or DLX2 antibody (*panels* b, e). The majority of DLX1/DLX2 positive cells are not co-expressed with *Nrp*2 positive cells (g, h). Scale bars, 200 μm for A, and 400 μm for B. [H, Hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; PCx, paleocortex; POa, anterior preoptic area; Str, striatum]. Inserts in the Merge column represent a 25x enlargement.

A

Dlx1/2+/- Nrp2+/- | *Dlx1/2-/- Nrp2+/-*

Mash1

a | b

c | d

B

Wnt7b

e | f

g | h

C

NRP1

i | j

E18.5

a,b,e,f

NCx | H

LGE

Str | MGE

Thal

c,d,g,h

AEP
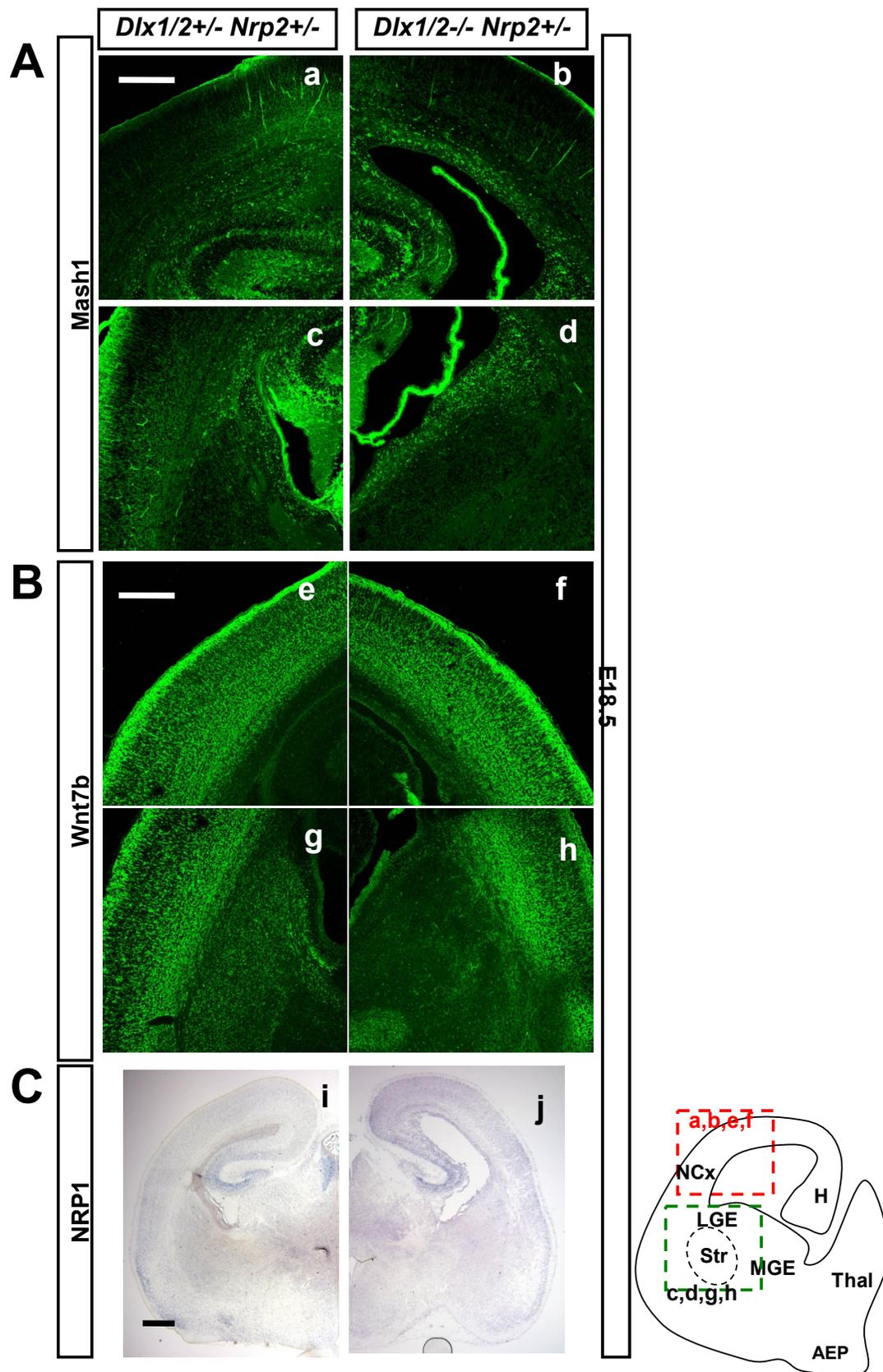
Appendix Figure 7. *Mash*1, *Wnt*7b, and *Neuropilin*1 are not downstream targets of *Dlx*1 and *Dlx*2. (A) At E18.5, Immunofluorescence experiments showed MASH1 expression primarily in the ventricular zone of neocortex and basal telencephalon of both wild-type (a, c) and double (b, d) mutants. (B) Immunofluorescence experiments showed WNT7b expression predominantly in all layers of the neocortex, except the ventricular zone of the wild-type (e, g) and double (f, h) mutant. (C) *In situ* hybridization experiments showed *Neuropilin*1 expression is in the hippocampus, as well as in mantle zone of the basal forebrain. When compared between wild-type (i) and the *Dlx*1/*Dlx*2 double (j) mutant, there is similar expression of *Nrp*1between the two genotypes (right panels compared to left panels). Coronal sections; scale bars, 500µm (A&B), 600µm (C). [H, Hippocampus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str, striatum; AEP: anterior entopeduncular area].

	Dlx1/2+/- Nrp2+/-	*Dlx1/2+/- Nrp2-/-*	*Dlx1/2-/- Nrp2+/-*	*Dlx1/2-/- Nrp2-/-*
Math2	a	b	c	d
Cux2	e	f	g	h
Tbr1	i	j	k	l
Lhx2	m	n	o	p
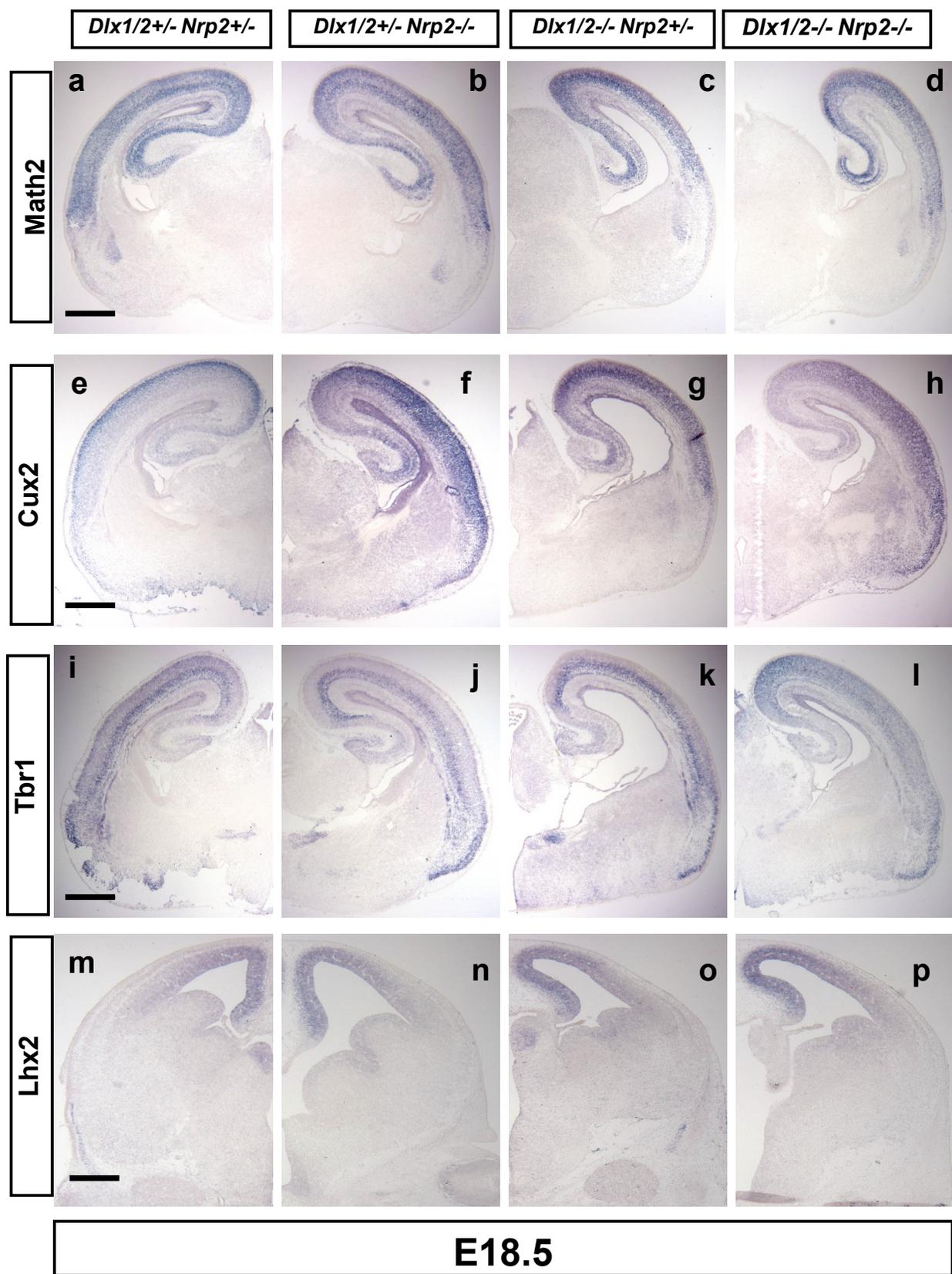
E18.5

Appendix Figure 8: Comparative analyses of cortical layer specification between four genotypes at E18.5 using *in situ* hybridization. At E18.5, expression of cortical neuronal markers *Math*2 (marker of cortical plate) (a,b,c,d), *Cux*2 (marker of layers II to IV) (e,f,g,h), and*Tbr*1 (marker of layer VI) (i,j,k,l) showed similarities in cortical lamination between all four genotypes. At E13.5, *in situ* hybridization studies showed no obvious difference in expression of *Lhx*2 (marker of ventricular zone) (m,n,o,p) in cortical lamination between all four genotypes. Coronal sections; scale bars, 500μm (a-l), 200μm (m-p). The experiment was done with the help of Tracie Parkinson, Eisenstat laboratory, Manitoba Institute of Cell Biology, University of Manitoba.

	Dlx1/2+/- Nrp2+/-	*Dlx1/2+/- Nrp2-/-*	*Dlx1/2-/- Nrp2+/-*	*Dlx1/2-/- Nrp2-/-*
Glutamate	a	b	c	d
MAP2	e	f	g	h
Msx	i	j	k	l
TrkB	m	n	o	p
Tbr1	q	r	s	t
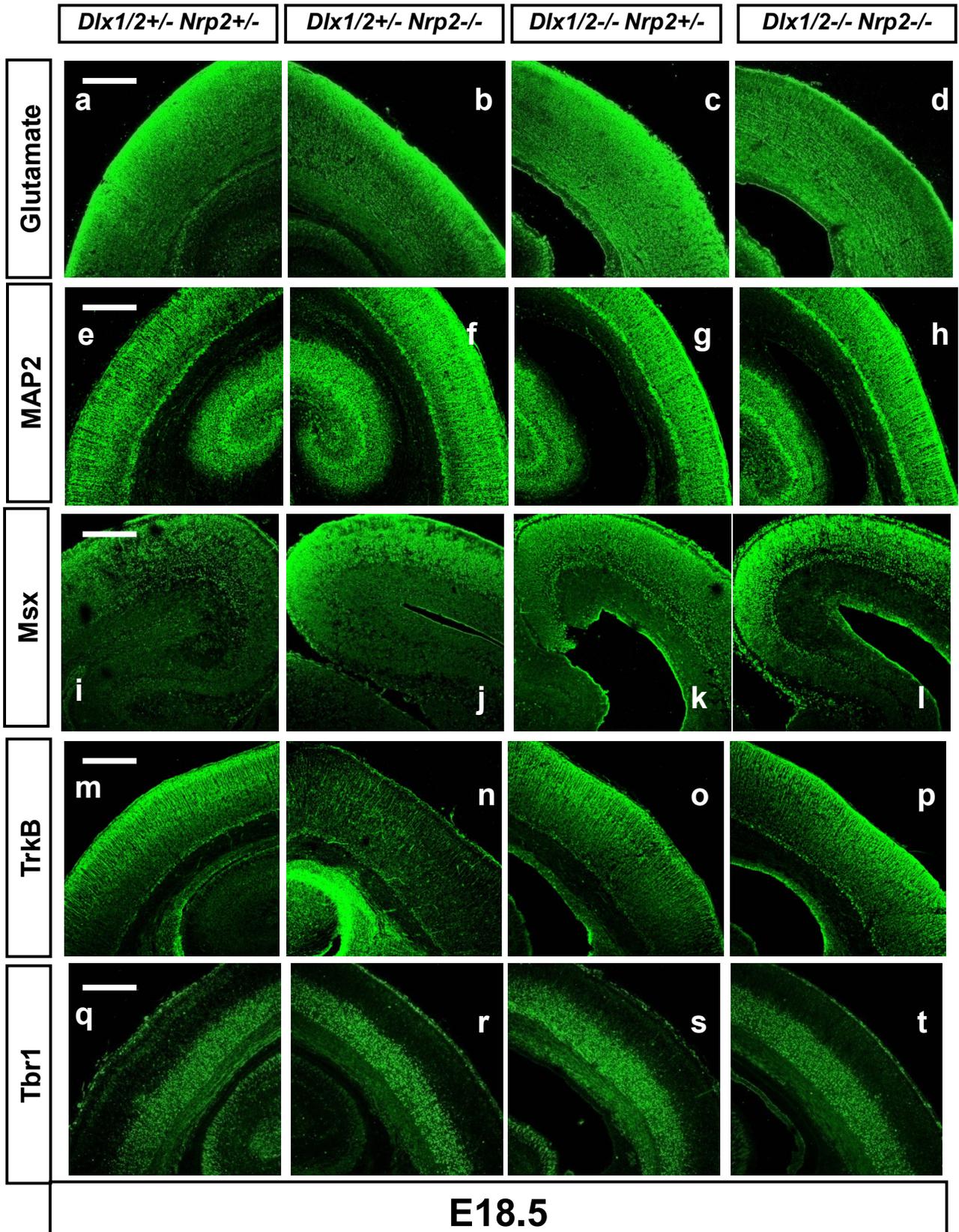
E18.5

Appendix Figure 9: Comparative analyses of cortical layer specification between four genotypes at E18.5 using immunofluorescence at E18.5. General expression of cortical neuronal markers: glutamate (marker of excitatory pyramidal neurons) (a,b,c,d), MAP2 (marker of neuronal cytoskeleton) (e,f,g,h), *Msx* (marker of a homeodomain transcription factor) (i,j,k,l), *Trk*B (marker of neuronal survival) (m,n,o,p), and *Tbr*1 (marker of layer VI) (q,r,s,t) showing unchanged patterns of expression between all four genotypes. Coronal sections; scale bars, 200μm.

E18.5

| *Dlx1/2+/- Nrp2+/-* | *Dlx1/2+/- Nrp2-/-* | *Dlx1/2-/- Nrp2+/-* | *Dlx1/2-/- Nrp2-/-* |

a   b   c   d

E18.5

2 days later

NCx

LGE

Str   MGE

H

Thal

AEP
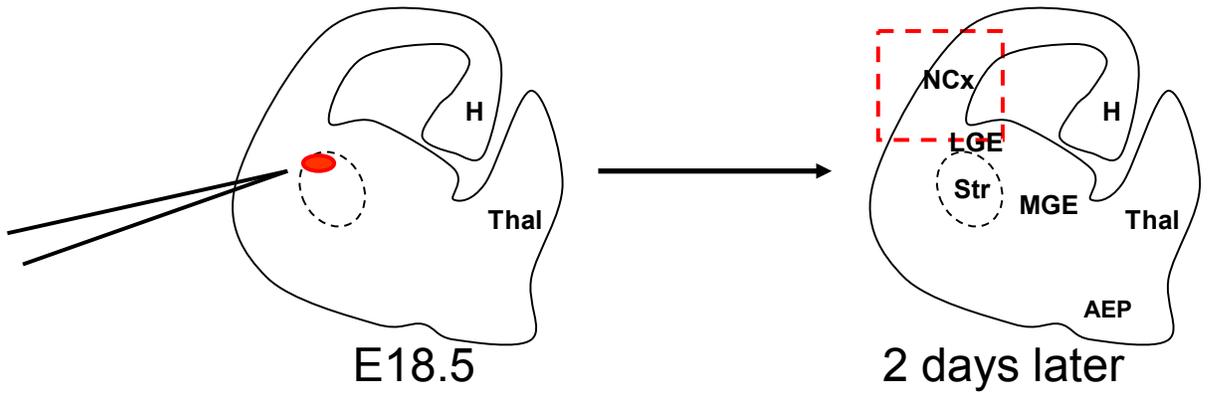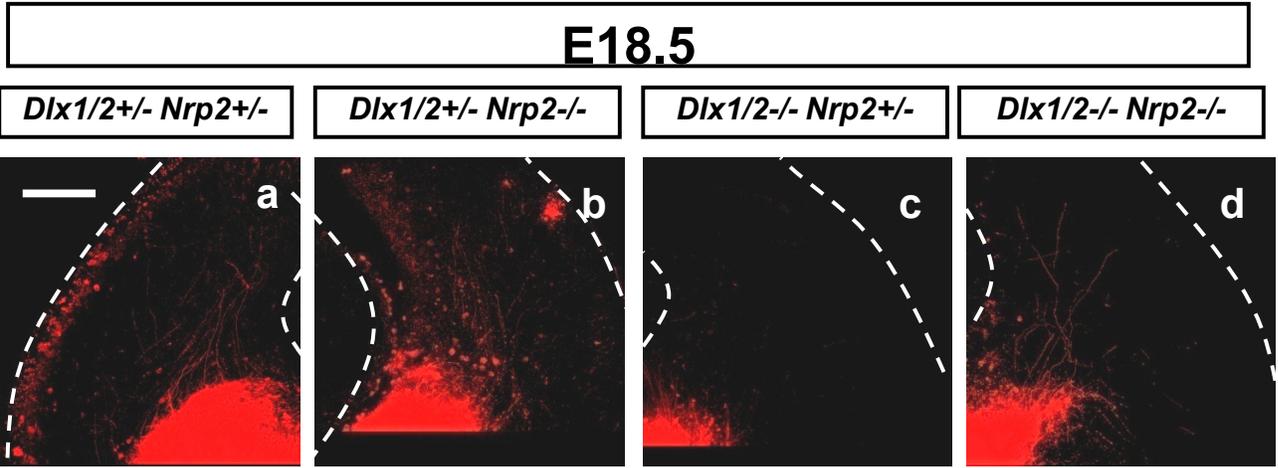
Appendix Figure 10. Cell migration from the LGE to the neocortex in E18.5 slice cultures of embryonic forebrain. These slices were prepared from E18.5 embryos. DiI was placed in the LGE of the slices which were then cultured for 48 hours. Many DiI-labelled cells and axonal processes are present in the neocortex of the wild-type (a) and single mutant (b). Analysis of the double mutant (c) shows a reduction of diI-labelled cells in the neocortex compared to the wild-type. In the triple mutant, few diI-labelled cells and axons are found in the neocortex. Coronal sections. Scale bar, 200 μm. [H, Hippocampus; NCx, neocortex; Str, striatum; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Thal, thalamus; AEP: anterior entopeduncular area].

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